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Design, synthesis and biological activity of selective and orally available TF/FVIIa complex inhibitors containing non-amidine P1 ligands

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Abstract—We found the novel selective and orally available non-amidine TF/FVIIa complex inhibitor **21e**, 4-({[(1*S*)-(aminocarbonyl)-3-methylbutyl]amino}carbonyl)-2′-({[4-(aminomethyl)phenyl]amino}carbonyl)-4′-(methylamino)biphenyl-2- carboxylic acid. The derivatives were synthesized by conversions of the isobutyl moiety and the introduction of alkylamino groups to 4′-position of the central phenyl ring of compounds **2a** and **2b** reported previously. Some compounds show increased in vitro anti-TF/FVIIa and PT prolongation activities. Among them, compound **21e** reached and sustained micromolar plasma concentration levels of up to 2 h after oral administration in mice. Moreover, compound **21e** did not prolong the bleeding time even at the highest dose level in cynomolgus monkeys, while PT was prolonged 3.7-fold increases at this dose.

1. Introduction

Tissue factor (TF) is an enzyme that functions in the first step of the blood coagulation cascade to initiate the extrinsic pathway by binding factor VIIa (FVIIa), which forms TF/FVIIa. In turn, TF/FVIIa activates factors X and IX to Xa and IXa, respectively, and ultimately cleaves fibrinogen to insoluble fibrin in the blood coagulation cascade.[1] Given that bleeding studies indicate that the inhibition of TF/FVIIa has the widest safety window with respect to therapeutic effectiveness and bleeding risk among anticoagulant approaches tested to date, such as thrombin and factor Xa (FXa),[2] the inhibition of TF/FVIIa has therefore become an attractive target for the development of novel anticoagulants. Several groups have reported the design and synthesis of novel TF/FVIIa inhibitors.[3] Many of these retain a highly basic amidine group bound in the S1 pocket of TF/FVIIa in the vicinity of Asp 189. It is generally accepted that inhibitors containing highly basic functions such as an amidine group are often poorly absorbed and/or are associated with undesirable side effects.[4] In addition, inhibitors which possess an amidine moiety are poor candidates for development to highly selective compounds because other serine protease enzymes such as thrombin, FXa, and trypsin have Asp 189 at the base of the S1 pocket.

Recently, we reported non-amidine TF/FVIIa inhibitors (**2a** and **2b**) produced by optimization of an amidine compound **1**[3a] at the substituents on the P1 phenyl portion of the S1 pocket. By further optimization of substituents on the central phenyl ring to the S2 pocket, a highly potent (IC_{50} = 0.69 μM) and selective (over 250-fold selectivity against FXa, thrombin, and trypsin) TF/FVIIa inhibitor **3** was discovered (Fig. 1).[5]

In an attempt to enhance the activity of this series, we report here our progress on two attractive modifications, **2a** and **2b**. On one hand, structural variation

Keywords: Thromboembolic disorders; Trypsin-like serine protease; The tissue factor/factor VIIa (TF/FVIIa) complex; Non-amidine inhibitor.
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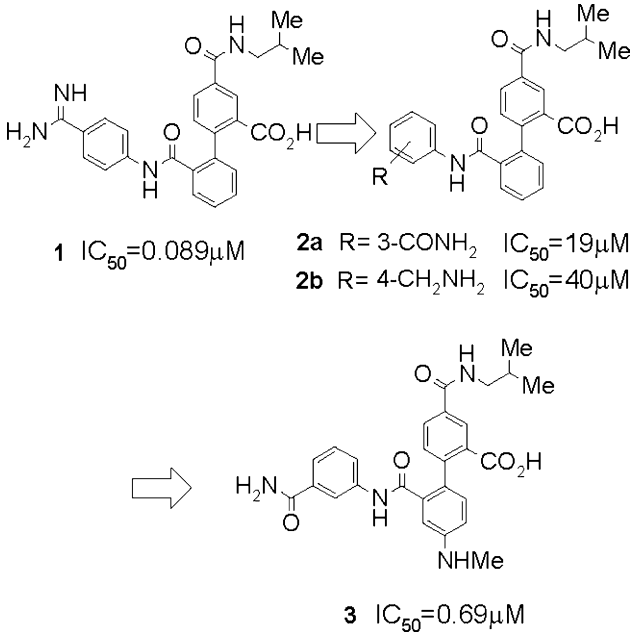
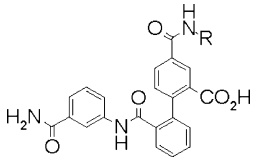
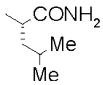
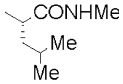
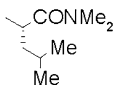


Figure 1. Optimization of the non-amidine inhibitors.

Table 1. In vitro inhibitory activities against TF/VIIa

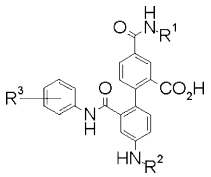


Compound	R	IC_{50}[a] (μM)
2a[b]		19
9a		19
9b		191
9c		8.5
9d		2.4
9e		4.2
9f		4.2
9g		3.2

[a] Human purified enzyme was used. IC_{50} values represent the averaged three determinations with the average standard error of the mean <10%.

[b] Taken from Ref. 5.

Table 2. In vitro inhibitory activities against TF/FVIIa



Compound	R^1	R^2	R^3	IC_{50}[a] (μM)
3[b]		Me	3-$CONH_2$	0.69
21a		Me	3-$CONH_2$	0.30
21b		Me	3-$CONH_2$	0.15
21c		Me	3-$CONH_2$	0.18
26a		Et	3-$CONH_2$	0.30
26b		*n*-Bu	3-$CONH_2$	2.2
26c		*i*-Pr	3-$CONH_2$	0.86
26d		*c*-Hex	3-$CONH_2$	91
21d		Me	4-CH_2NH_2	1.3
21e		Me	4-CH_2NH_2	0.59

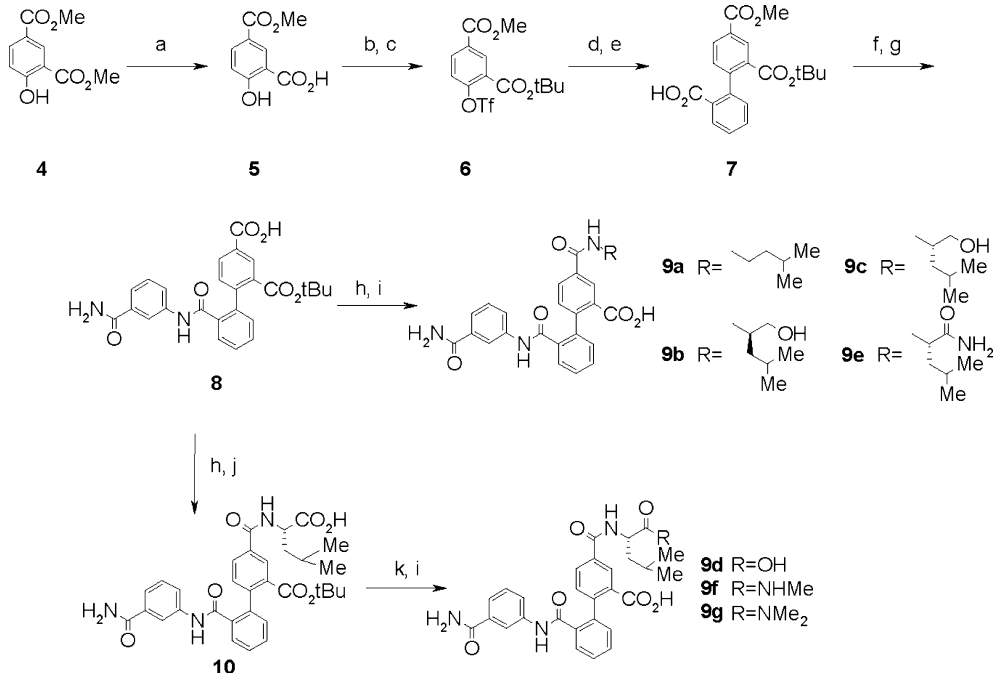
[a] Human purified enzyme was used. IC_{50} values represent the averaged three determinations with the average standard error of the mean <10%.

[b] Taken from Ref. 5.

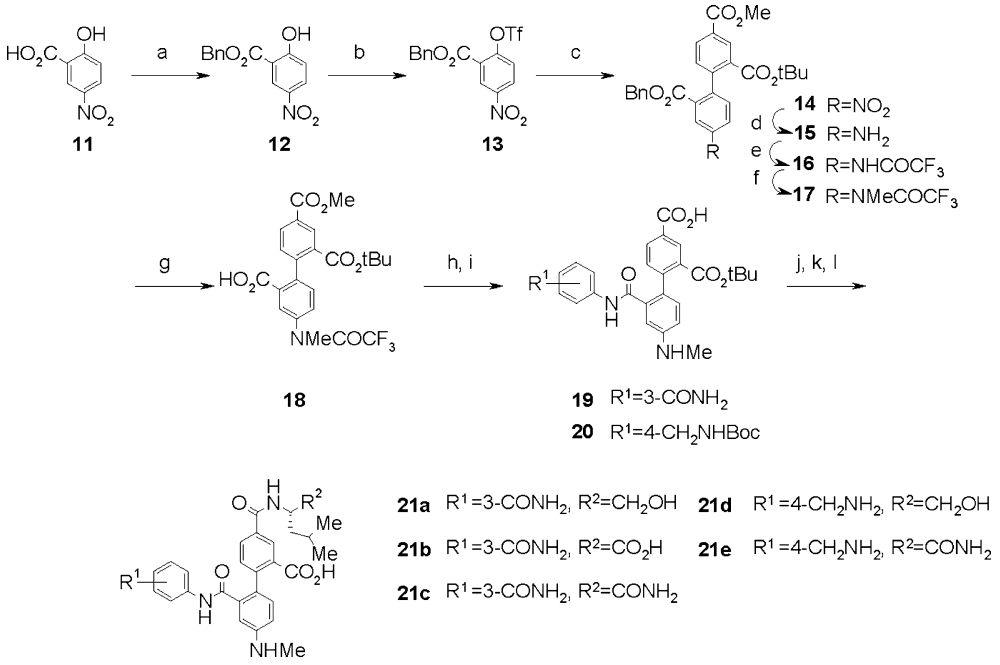
was brought about by modifying the iso-butyl group to novel synthetic variations such as **9a–g** (Table 1), and on the other hand we introduced a methylamino group at the 4′-position on the central phenyl ring and varied it by substitution with other alkylamino groups such as **21a–e** and **26a–d** (Table 2).

2. Chemistry

The synthesis of compounds (**9a–g**) is shown in Scheme 1. Dimethyl 4-hydroxyisophthalate **4** can be converted to the monomethyl ester **5** in pyridine under reflux conditions.[6] The monoacid **5** is protected by *tert*-butyl ester[7] and converted to the corresponding triflate **6** by treatment of trifluoromethanesulfonic anhydride. Suzuki cross-coupling reaction of **6** with (2-formylphenyl)boronic acid and oxidation of the resulting aldehyde afforded the acid **7**. Condensation of carboxylic acid **7**

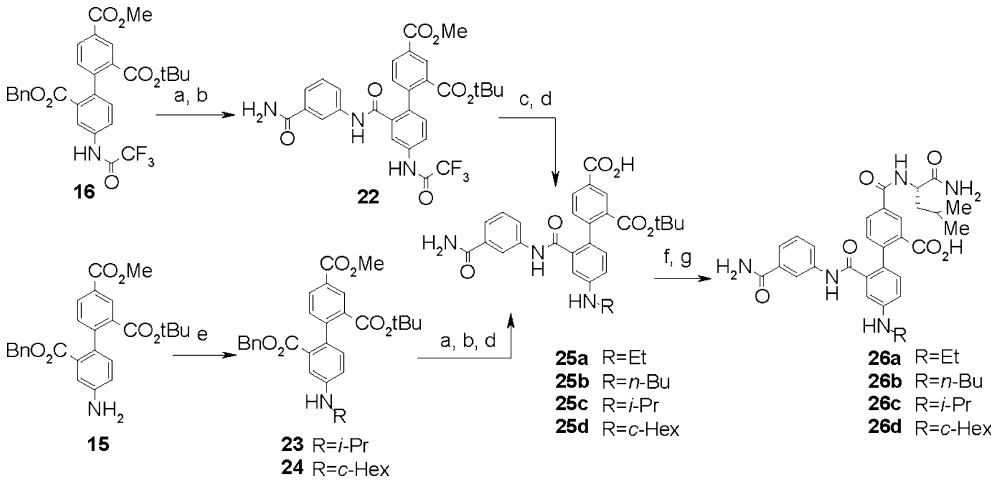


Scheme 1. Reagents and conditions: (a) pyridine; (b) H_2SO_4, $MgSO_4$, *tert*-butanol, CH_2Cl_2 (c) Tf_2O, pyridine, CH_2Cl_2; (d) (2-formylphenyl)boronic acid, $Pd(PPh_3)_4$, K_3PO_4, DMF; (e) $NaClO_2$ NaH_2PO_4 2-methyl-2-butene, *tert*-butanol, CH_3CN, H_2O; (f) 3-aminobenzamide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 1-hydroxybenzotriazole, DMF; (g) NaOH, MeOH; (h) amine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMF; (i) TFA, CH_2Cl_2; (j) H_2/Pd–C, MeOH; (k) amine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMF for **9f** and **9g**.



Scheme 2. Reagents and conditions: (a) BnBr, K_2CO_3, DMF; (b) Tf_2O, pyridine, CH_2Cl_2; (c) **6**, bis(pinacolato)diboron, $PdCl_2(PPh_3)$, AcOK, toluene, then **13**, $Pd(PPh_3)_4$, Na_2CO_3, toluene–H_2O; (d) Fe, AcOH; (e) TFAA, pyridine, CH_2Cl_2; (f) MeI, K_2CO_3 2-butanone; (g) H_2/Pd–C, MeOH; (h) $ArNH_2$ 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 1-hydroxybenzotriazole, DMF; (i) NaOH, MeOH; (j) amine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMF; (k) H_2/Pd–C, MeOH; for **21b**; (l) HCl, H_2O for **21a**, **21d**, and **21e**, TFA, CH_2Cl_2 for **21b** and **21c**.

with 3-aminobenzamide and hydrolysis of the methyl group under basic conditions gave the acid **8**. Condensation of carboxylic acid **8** with corresponding amine and deprotection of the *tert*-butyl group afforded the desired carboxylic acid (**9a**–**d**). The leucin derivatives (**9e**–**g**) were prepared from the intermediate **10**. After condensation



Scheme 3. Reagents and conditions: (a) H_2/Pd–C, MeOH; (b) 3-aminobenzamide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 1-hydroxybenzotriazole, DMF; (c) alkyl iodide, K_2CO_3, 2-butanone; (d) NaOH, MeOH; (e) acetone for **23** or cyclohexanone for **24**, $NaBH(OAc)_3$, AcOH; (f) 2-amino-4-methylpentanamide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, DMF; (g) TFA, CH_2Cl_2.

of **8** with leucin benzyl ester, deprotection of the benzyl group by catalytic hydrogenation afforded the acid **10**, which was converted to the desired products (**9e**–**g**) with similar methods to those described above.

The synthesis of compounds (**21a**–**e**) is shown in Scheme 2. One-pot Suzuki cross-coupling reaction between the triflate **6** and trifate **13**, which was synthesized in two steps from 2-hydroxy-5-nitrobenzoic acid **11**, provided the biaryl **14** in 74% yield.[8] The nitro group of **14** was converted to *N*-methyltrifluoroacetamide in three steps under standard conditions, followed by deprotection of the benzyl group of **17** to give the acid **18**. Condensation of **18** with corresponding anilines provided amides, followed by hydrolysis of both the methyl ester group and trifluoroacetyl moiety to give the corresponding acids (**19** and **20**). Condensation with several amines and deprotection of the *tert*-butyl group afforded the desired compounds (**21a**–**e**).

The synthesis of compounds (**26a**–**d**) is shown in Scheme 3. The ethyl and *n*-butyl analogues (**26a** and **26b**) were synthesized through the alkylation of **22**, which was prepared from **16** by similar methods to those described in Scheme 2. In the case of the isopropyl and cyclohexyl analogues (**26c** and **26d**), which have secondary alkyl group on nitrogen atom, due to low reactivity of corresponding alkyl halide, compounds were synthesized by the reductive alkylation of **15** with corresponding ketones and then converted to the desired products by the standard conditions described above.

3. Results and discussion

The prepared target compounds were evaluated by IC_{50} values for the inhibition of TF/FVIIa enzymatic activities using the chromogenic substrate S-2288.

In our previous study, molecular modeling of compound **2a** docked with TF/FVIIa[9] suggests that the isobutyl group lies in the pocket formed by Gln 40, Leu 41, Gln 143, Thr 151, Gly 193, and Lys 192,[5] which is known as the S1' site. In this pocket, there is a hydrophobic site (Gln 40 and Leu 41) and a hydrophilic one (Gln 143 and Lys 192). From this modeling, although the isobutyl group sits in the hydrophobic site, there is no interaction with the hydrophilic one. We hypothesized that inhibitors which could fill both the hydrophobic and hydrophilic pockets would have potentially enhanced in vitro potency.

As shown in Table 1, extending the carbon chain of compound **2a** yielded compound **9a**, which showed the same activity as **2a**. In order to confirm our hypothesis, we introduced hydrophilic functional groups at the 1-position of the 3-methylbutyl moiety of **9a**. At first, the relationship between the stereochemistry of this position and TF/FVIIa activity was investigated by the use of a hydroxymethyl group. Interestingly, each compound had different potencies among enantiomers (**9b** and **9c**), with the (*S*)-isomer **9c** approximately 20-fold more potent than (*R*)-isomer **9b** and the (*S*)-analogue **9c** 2-fold more potent than compound **9a** by the addition of hydrophilic group. Encouraged by this result, we next synthesized a number of *S*-form analogues which possessed hydrophilic groups and assayed their inhibitory activities (**9d**–**g**). The carboxylic acid analogue **9d** was about 8-fold more potent than the unsubstituted analogue **9a** and the amido analogues (**9e**–**g**) were equipotent compared to the carboxylic acid **9d**. We utilized molecular modeling of compound **9d** to understand the enhancement of potency conferred by introduction of the hydrophilic group.

The molecular modeling suggested that compound **9d** filled both hydrophobic and hydrophilic pockets, as we expected (Fig. 2). That is, the isobutyl group of the leucin moiety is in close proximity to the carbon-chain group of Gln 40, while, the carbonyl group of the carboxylic acid forms a hydrogen bond through accepting a hydrogen from the nitrogen atom of Gln 143. The reason why

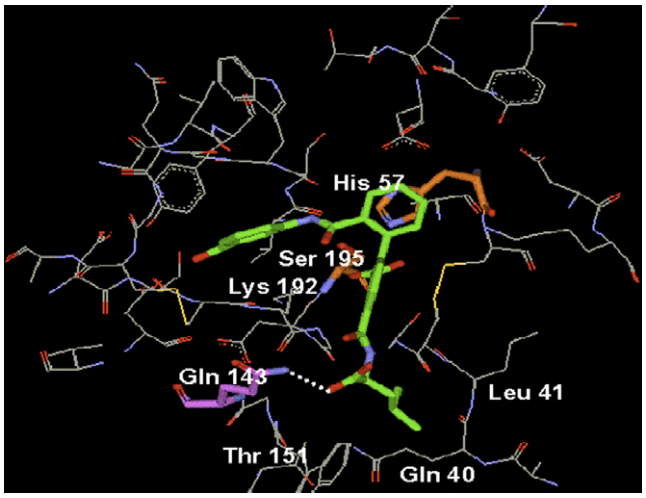


Figure 2. Docking model of **9d** in TF/FVIIa. Compound is shown in green. His 57 and Ser 195 are shown in orange. GIn 143 is shown in pink. Residue number of some key residues are displayed. The hydrogen bonds formed by the inhibitor are shown in dotted white line.

(*R*)-isomer **9b** shows relatively low activity compared to (*S*)-isomer **9c** is that these hydrophobic and hydrophilic interactions may not be possible with the enantiomer **9b**. Comparable activity relative to **9d** for amide analogues (**9e**–**g**) is that the carbonyl group of amido moieties engages in hydrogen-bonding interaction with Gln 143.

In our previous report, we described that compound **3** which was introduced a methylamino group at the 4′-position on the central phenyl ring of compound **2a** was more potent than compound **2a**,[5] presumably through an improved interaction with the S2 site on the enzyme. We also reported that not only the 3-aminocarbonylphenyl moiety but also the 4-aminomethylphenyl moiety worked as an alternative for the 4-amidinophenyl moiety in the S1 pocket (**2a** and **2b**).[5] We subsequently took advantage of these structure–activity relationships (SARs) for compounds **9c**–**e**.

As we expected, the methylamino analogues **21a**–**c** were shown to increase TF/FVIIa inhibitory potency about 15- to 30-fold compared to the 4′-unsubstituted analogues **9c**–**e**, and about 2- to 5-fold more potent than compound **3**, respectively. We then set out to study the effects of the alkyl group R^2 of **21c** on the nitrogen atom at the 4′-position of the central phenyl ring in an attempt to further improve S2 binding. However, as the size of the substituent R^2 increases, binding potency drops significantly, due to unfavorable steric interactions between the substituent and the residues of the S2 binding site (**26a-d**). It therefore appears that the aminomethyl substitution is optimal for binding to the S2 site. As for the S1 site, although 4-aminomethyl analogues (**21d** and **21e**) were about 4-fold less potent than that of the 3-aminocarbonyl ones (**21a** and **21c**), those compounds were found to inhibit TF/FVIIa in the micromolar or sub-micromolar range.

Table 3 shows a selectivity comparison of the selected compounds and the lead amidine analogue **1**. The inhibitors were also tested in standard clotting assays including prothrombin time (PT) and activated partial thromboplastin time (APTT) determinations, which were used as qualitative in vitro indicators of potential antithrombotic activity. From this it can be seen that the non-amidine compounds (**21b**, **21c**, and **21e**) show higher selectivity for TF/FVIIa over other serine proteases than amidine analogue **1**. The CT_2 value of PT for the compounds (**21b**, **21c**, and **21e**) is 20–47 μM, which is about 3- to 7-fold more potent than that of compound **3**. It should be noted that the compounds (**21b**, **21c**, and **21e**) do not double the APTT even at concentrations of 300 μM, as is expected for selective TF/FVIIa inhibitors, whereas the amidine analogue **1** did double APTT at a concentration of 4.3 μM.

Plasma levels following oral dosing of the compounds (**21b**, **21c**, and **21e**) were determined in pharmacokinetic studies in mice.[10] Inhibitor concentrations were determined at various time points based on human TF/FVIIa inhibitory activity, as assessed using an established ex vivo inhibition activity assay for FXa.[11] As shown in Table 3, the 3-aminocarbonyl analogue (**21c**) showed about the same plasma levels on oral administration as amidine analogue **1** even at lower oral dosing levels. On the other hand, the 4-aminomethyl analogue (**21e**)

Table 3. Selectivity profiles of TF/VIIa inhibitors

Compound	IC_{50}[a] (μM)				CT_2 (μM)		Inhibitor plasma[e] concentration (μg/ml): oral dosing in mice 0.5 h/2.0 h
	TF/FVIIa	FXa	Thrombin	Trypsin	PT[c]	APTT[d]	
1[b]	0.089	0.88	>200	4.8	4.2	4.3	0.30/0.16[f]
3[b]	0.69	>200	>200	>200	150	>300	NT[g]
21b	0.15	>200	>200	>200	33	>300	ND/ND[h]
21c	0.18	>200	>200	>200	20	>300	0.28/0.21
21e	0.59	>200	>200	>200	47	>300	1.8/2.9/2.3/0.13[i]

[a] Human purified enzyme was used. IC_{50} values represent the averaged three determinations with the average standard error of the mean <10%.
[b] Taken from Ref. 5.
[c] PT/CT_2, concentration of inhibitor required to double the prothrombin time in human plasma.
[d] $APTT/CT_2$, concentration of inhibitor required to double the activated partial thromboplastin time in human plasma.
[e] All doses were 100 mg/kg po ($n = 3$) unless otherwise indicated. Plasma concentrations determined by TF/FVIIa inhibition assay.
[f] 300 mg/kg po.
[g] Not tested.
[h] Not detected.
[i] 0.5 h/1.0 h/2.0 h/4.0 h.

Table 4. Effect of compound **21e** on ex vivo PT, APTT, and template bleeding time

	PT (s)	APTT (s)	Bleeding time (min)
Control ($n = 3$)	10.4 ± 0.4	28.9 ± 1.2	3.7 ± 0.2
Vehicle iv ($n = 2$)	10.6 ± 0.7	29.7 ± 0.1	3.8 ± 0.3
3 mg/kg iv ($n = 3$)	15.1 ± 2.6	29.1 ± 3.1	3.5 ± 0.3
10 mg/kg iv ($n = 3$)	24.5 ± 2.3*	32.2 ± 1.0	2.7 ± 0.2
30 mg/kg iv ($n = 3$)	38.4 ± 5.1**	35.0 ± 3.9	3.7 ± 0.4

Data are presented as means ± SEM.
Two-tailed Dunnett's multiple comparison test was used for evaluation of significance.
* $P < 0.05$.
** $P < 0.01$ compared with control.

attained and sustained micromolar concentration levels of up to 2 h.

With these encouraging results, **21e** was tested for bleeding risk in cynomolgus monkeys by measuring standard clotting assays (PT and APTT) and bleeding time. As shown in Table 4, **21e** had the expected effect of prolonging PT while not alerting APTT. PT was prolonged in a dose-dependent manner reaching 2.4- and 3.7-fold increases at 10 mg/kg iv and 30 mg/kg iv, respectively.[12] It is noteworthy that bleeding time never increased over baseline even at the highest dose level.

4. Conclusions

We have designed and synthesized non-amidine compounds as TF/FVIIa inhibitors. Among these compounds, the leucin amide derivatives (**21b**, **21c**, and **21e**) showed sub-micromolar potency in TF/FVIIa inhibitory activities and good selectivity against other serine protease such as FXa, thrombin, and trypsin. The CT_2 value of PT for compounds (**21b**, **21c**, and **21e**) is 20–47 μM, whereas the CT_2 value of APTT is over 300 μM, as is expected for selective TF/FVIIa inhibitors. The best compound **21e** showed higher plasma levels upon oral administration in mice compared to amidine analogue **1**. Moreover, the compound **21e** did not prolong the bleeding time even at the highest dose level in cynomolgus monkeys, while PT was prolonged 3.7-fold increases at this level.

5. Experimental

5.1. Chemistry

In general, reagents and solvents were used as purchased without further purification. Melting points were determined with a Yanaco MP-500D melting point apparatus and left uncorrected. ^{1}H NMR spectra were recorded on a JEOL JNM-LA300 or a JEOL JNM-EX400 spectrometer. Chemical shifts were expressed in δ (ppm) values with tetramethylsilane as an internal standard (in NMR description, s, singlet; d, doublet; t, triplet; m, multiplet and br, broad peak). Mass spectra were recorded on a JEOL JMS-LX2000 spectrometer. Elemental analyses were performed with a Yanaco MT-5 microanalyzer (C, H, N) and Yokogawa IC-7000S ion chromatographic analyzer (halogens) and were within ± 0.4% of theoretical values. Optical rotations were performed with a Horiba SEPA-200 optical rotation apparatus.

5.1.1. 2-Hydroxy-5-(methoxycarbonyl)benzoic acid (5). To a stirred solution pyridine (500 mL) was added dimethyl 4-hydroxyisophthalate **4** (36.0 g, 171 mmol), and the mixture was refluxed for 17 h. The mixture was concentrated in vacuo. The residue was acidified with 1 M HCl/H_2O (200 mL). The resulting precipitate was filtered, washed with H_2O, and dried in vacuo to give **5** (33.4 g, 100%) as a brown solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.84 (3H, s), 7.07 (1H, d, $J = 8.8$ Hz), 8.06 (1H, dd, $J = 2.2$ Hz, 8.8 Hz), 8.39 (1H, d, $J = 2.2$ Hz); FAB-MS (m/z): 197 $(M+H)^+$.

5.1.2. 3-*tert*-Butyl 1-methyl 4-{[(trifluoromethyl)sulfonyl]oxy}isophthalate (6). To a stirred solution of **5** (10.0 g, 51.0 mmol) and $MgSO_4$ (53.4 g, 444 mmol) in CH_2Cl_2 (500 mL) were added concd H_2SO_4 (5.9 mL, 111 mmol) and 2-methylpropan-2-ol (53.0 mL, 554 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was filtered and partitioned between $CHCl_3$ and 5% $NaHCO_3$ in H_2O and the organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give a colorless oil (9.91 g). To a solution of the compound obtained above (9.90 g) and pyridine (16.0 mL, 197 mmol) in CH_2Cl_2 (100 mL) was added trifluoromethanesulfonic anhydride (13.0 mL, 77.3 mmol), and the mixture was stirred at room temperature for 30 min. The mixture was partitioned between CH_2Cl_2 and H_2O, and the organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 9:1) to give **6** (13.3 g, 88%) as a colorless oil: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.58 (9H, s), 3.91 (3H, s), 7.72 (1H, d, $J = 8.6$ Hz), 8.30 (1H, dd, $J = 2.4$ Hz, 8.6 Hz), 8.43 (1H, d, $J = 2.4$ Hz); FAB-MS (m/z): 385 $(M+H)^+$.

5.1.3. 2′-(*tert*-Butoxycarbonyl)-4′-(methoxycarbonyl)biphenyl-2-carboxylic acid (7). To a stirred solution of **6** (7.00 g, 18.2 mmol), (2-formylphenyl)boronic acid (2.73 g, 18.2 mmol), and K_3PO_4 (5.80 g, 27.3 mmol) in DMF (70 mL) was added $Pd(PPh_3)_4$ (1.05 g, 0.911 mmol), and the mixture was stirred at 100 °C for 3 h. The mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 9:1) to give a colorless oil (4.19 g). To a solution of the resulting oil, $NaH_2PO_4 \cdot H_2O$ (1.47 g, 9.29 mmol), and 2-methyl-2-butene (6.5 mL, 61.4 mmol) in *t*-BuOH/H_2O/CH_3CN mixture (45 mL, 6:2:1 v/v) was added sodium chlorite (5.55 g, 80 wt %, 49.1 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give **7** (3.75 g, 58%) as a colorless amor-

phous powder: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.12 (9H, s), 3.91 (3H, s), 7.20 (1H, d, J = 7.3 Hz), 7.35 (1H, d, J = 7.9 Hz), 7.49–7.55 (1H, m), 7.59–7.65 (1H, m), 7.98 (1H, d, J = 7.7 Hz), 8.09 (1H, dd, J = 1.9 Hz, 7.9 Hz), 8.34 (1H, d, J = 1.9 Hz) ; FAB-MS (*m*/*z*): 357 $(M+H)^+$.

5.1.4. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-2-(*tert*-butoxycarbonyl)biphenyl-4-carboxylic acid (8). To a stirred solution of **7** (2.00 g, 5.61 mmol) and 3-aminobenzamide (4.30 g, 27.9 mmol) in DMF (20 mL) were added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC·HCl) (1.61 g, 8.42 mmol) and 1-hydroxybenzotriazole (HOBt) (1.14 g, 8.42 mmol), and the mixture was stirred at 60 °C for 12 h. The mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (AcOEt/hexane = 1:1) to give a colorless amorphous powder (2.29 g). To a stirred solution of the compound obtained above in MeOH (20 mL) was added 1 M NaOH/H_2O (5.3 mL, 5.3 mmol), and the mixture was stirred at room temperature for 12 h. The reaction mixture was acidified with 1 M HCl/H_2O (5.3 mL, 5.3 mmol). The resulting precipitate was filtered, washed with H_2O, and dried in vacuo to give **8** (1.87 g, 72%) as a colorless solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.19 (9H, s), 7.22–7.27 (1H, m), 7.27–7.34 (2H, m), 7.37 (1H, d, J = 7.9 Hz), 7.49–7.53 (1H, m), 7.53–7.64 (3H, m), 7.73–7.79 (1H, m), 7.88 (1H, br s), 8.01 (1H, br s), 8.04 (1H, dd, J = 2.0 Hz, 7.9 Hz), 8.31 (1H, d, J = 2.0 Hz), 10.16 (1H, s), 13.21 (1H, br s); FAB-MS (*m*/*z*): 461 $(M+H)^+$.

5.1.5. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-4-{[(3-methylbutyl)amino]carbonyl}biphenyl-2-carboxylic acid (9a). To a stirred solution of **8** (300 mg, 0.717 mmol) and 3-methylbutan-1-amine (125 mg, 1.43 mmol) in DMF (6 mL) was added WSC·HCl (206 mg, 1.07 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between AcOEt and H_2O, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 98:2) to give a colorless amorphous powder (321 mg). To a stirred solution of the compound obtained above (321 mg) in CH_2Cl_2 (6.0 mL) was added TFA (6.0 mL), and the mixture was stirred at room temperature for 4 h and concentrated in vacuo. A solution of the compound obtained above was washed with AcOEt and the resulting precipitate was filtered and dried in vacuo to give **9a** (99 mg, 29%) as a colorless solid: mp 137–138 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.90 (6H, d, J = 6.4 Hz), 1.38–1.46 (2H, m), 1.55–1.67 (1H, m), 3.24–3.33 (2H, m), 7.23 (1H, d, J = 6.8 Hz), 7.26–7.33 (3H, m), 7.48–7.57 (3H, m), 7.61 (1H, d, J = 8.3 Hz), 7.68 (1H, dd, J = 1.5 Hz, 7.4 Hz), 7.86 (1H, br s), 7.93 (1H, dd, J = 1.9 Hz, 7.9 Hz), 8.00 (1H, br s), 8.28 (1H, d, J = 1.9 Hz), 8.55–8.61 (1H, m), 10.20 (1H, br s), 12.88 (1H, br s); FAB-MS (*m*/*z*): 474 $(M+H)^+$; Anal. calcd for $C_{27}H_{27}N_3O_5 \cdot 0.6H_2O$: C, 66.96; H, 5.87; N, 8.68. Found: C, 66.78; H, 5.87; N, 8.51.

5.1.6. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-4-({[(1*R*)-(hydroxymethyl)-3-methylbutyl]amino}carbonyl)biphenyl-2-carboxylic acid (9b). Compound **9b** was synthesized from **8** and (2*R*)-amino-4-methylpentan-1-ol according to the same procedure as that for **9a**. Compound **9b** was obtained as a colorless solid (254 mg 70%): mp 134–135 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, d, J = 6.2 Hz), 0.89 (3H, d, J = 6.8 Hz), 1.28–1.52 (2H, m), 1.52–1.71 (1H, m), 3.20–3.50 (2H, m), 4.00–4.12 (1H, m), 4.69 (1H, br s), 7.17–7.40 (4H, m), 7.45–7.60 (3H, m), 7.60–7.70 (2H, m), 7.88 (1H, s), 7.91 (1H, dd, J = 2.0 Hz, 7.8 Hz), 8.04 (1H, s), 8.19 (1H, d, J = 7.8 Hz), 8.26 (1H, d, J = 2.0 Hz), 10.68 (1H, br s); FAB-MS (*m*/*z*): 504 $(M+H)^+$; Anal. Calcd for $C_{28}H_{29}N_3O_6 \cdot 1.2H_2O$: C, 64.04; H, 6.03; N, 8.00. Found: C, 64.06; H, 5.91; N, 8.21; $[\alpha]_D^{25}$ +23 ° (*c* 0.1, MeOH).

5.1.7. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-4-{[(1*S*)-hydroxymethyl)-3-methylbutyl]amino}carbonyl)biphenyl-2-carboxylic acid (9c). Compound **9c** was synthesized from **8** and (2*S*)-amino-4-methylpentan-1-ol according to the same procedure as that for **9a**. Compound **9c** was obtained as a colorless solid (141 mg 36%): mp 140–143 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.87 (3H, d, J = 6.4 Hz), 0.89 (3H, d, J = 6.9 Hz), 1.28–1.52 (2H, m), 1.52–1.71 (1H, m), 3.40 (2H, s), 3.98–4.14 (1H, m), 4.68 (1H, s), 7.17–7.40 (4H, m), 7.45–7.58 (3H, m), 7.58–7.70 (1H, m), 7.70–7.75 (1H, m), 7.87 (1H, s), 7.95 (1H, d, J = 7.9 Hz), 8.02 (1H, s), 8.20 (1H, d, J = 8.3 Hz), 8.29 (1H, s), 10.32 (1H, s), 12.90 (1H, br s); FAB-MS (*m*/*z*): 504 $(M+H)^+$; Anal. Calcd for $C_{28}H_{29}N_3O_6 \cdot 1.0H_2O \cdot 0.3A$cOEt: C, 64.00; H, 6.14; N, 7.67. Found: C, 63.93; H, 5.89; N, 7.48; $[\alpha]_D^{25}$ −23 ° (*c* 0.1, MeOH).

5.1.8. 4-({[(1*S*)-(Aminocarbonyl)-3-methylbutyl]amino}carbonyl)-2′-({[3-(aminocarbonyl)phenyl]amino}carbonyl)biphenyl-2-carboxylic acid (9e). Compound **9e** was synthesized from **8** and (2*S*)-2-amino-4-methylpentanamide according to the same procedure as that for **9a**. Compound **9e** was obtained as a colorless powder (95 mg 36%): ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (3H, d, J = 6.4 Hz), 0.88 (3H, d, J = 6.4 Hz), 1.43–1.54 (1H, m), 1.55–1.74 (2H, m), 4.33–4.44 (1H, m), 6.85–6.93 (2H, m), 6.94–7.01 (1H, m), 7.12–7.26 (3H, m), 7.28–7.38 (3H, m), 7.38–7.46 (2H, m), 7.52–7.58 (1H, m), 7.62 (1H, d, J = 7.8 Hz), 7.80 (1H, s), 8.00 (1H, s), 8.05 (1H, br s), 8.40 (1H, d, J = 7.3 Hz), 13.50 (1H, br s); FAB-MS (*m*/*z*): 517 $(M+H)^+$; HRMS (FAB) calcd for $C_{28}H_{28}N_4O_6$: 517.2087. Found: 517.2072; $[\alpha]_D^{25}$ −3° (*c* 0.1, MeOH).

5.1.9. (2*S*)-2-({[2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-2-(*tert*-butoxycarbonyl)biphenyl-4-yl]carbonyl}-mino)-4-methylpentanoic acid (10). To a stirred solution of **8** (0.400 g, 0.869 mmol), and benzyl (2*S*)-2-amino-4-methylpentanoate 4-methylbenzenesulfonate (0.683 g, 1.74 mmol) in DMF (4.0 mL) was added WSC·HCl (0.200 g, 1.04 mmol), and the mixture was stirred at

room temperature for 12 h. The mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 95:5) to give a colorless amorphous powder (0.200 g). To a stirred solution of the compound obtained above (0.190 g) in MeOH (2.0 mL) was added 10% Pd/C powder (19 mg), and the mixture was stirred under hydrogen atmosphere at room temperature for 4 h. The catalyst was filtrated on Celite and the filtrate was concentrated in vacuo to give **10** (0.154 g, 35%) as a colorless solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.88 (3H, d, J = 6.3 Hz), 0.91 (3H, d, J = 6.4 Hz), 1.53–1.62 (1H, m), 1.65–1.81 (2H, m), 4.38–4.47 (1H, m), 7.20–7.25 (1H, m), 7.27–7.37 (3H, m), 7.47–7.65 (3H, m), 7.71–7.78 (1H, m), 7.88 (1H, br s), 7.97 (1H, dd, J = 2.0 Hz, 8.0 Hz), 8.04 (1H, br s), 8.27 (1H, d, J = 2.0 Hz), 8.78 (1H, d, J = 7.7 Hz), 10.16 (1H, s); ESI-MS (m/z): 572 $(M-H)^-$.

5.1.10. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-4-[({3-methyl-(1*S*)-[(methylamino)carbonyl]butyl}amino)-carbonyl]biphenyl-2-carboxylic acid (9f). Compound **9f** was synthesized from **10** and methylamine·HCl according to the same procedure as that for **9a**. Compound **9f** was obtained as a colorless solid (198 mg, 77%): mp 157–158 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.86 (3H, d, J = 6.3 Hz), 0.89 (3H, d, J = 6.4 Hz), 1.48–1.75 (3H, m), 2.58 (3H, d, J = 4.9 Hz), 4.40–4.52 (1H, m), 7.23 (1H, dd, J = 1.5 Hz, 7.4 Hz), 7.26–7.35 (3H, m), 7.45–7.58 (3H, m), 7.63 (1H, d, J = 8.3 Hz), 7.69 (1H, dd, J = 1.5 Hz, 7.3 Hz), 7.82–7.93 (2H, m), 7.96–8.07 (2H, m), 8.36 (1H, d, J = 1.4 Hz), 8.66 (1H, d, J = 8.3 Hz), 10.28 (1H, s), 12.90 (1H, br s); FAB-MS (m/z): 531 $(M+H)^+$; Anal. Calcd for $C_{29}H_{30}N_4O_6 \cdot 1.0H_2O$: C, 63.49; H, 5.88; N, 10.21. Found: C, 63.32; H, 5.97; N, 10.08; $[\alpha]_D^{25}$ +18° (c 0.1, MeOH).

5.1.11. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-4-{[((1*S*)-carboxy-3-methylbutyl)amino]carbonyl}biphenyl-2-carboxylic acid (9d). Compound **9d** was synthesized from **10** according to the same procedure as that for **9a** without condensation. Compound **9d** was obtained as a colorless solid (237 mg, 78%): mp 155–157 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.88 (3H, d, J = 6.4 Hz), 0.92 (3H, d, J = 6.3 Hz), 1.50–1.63 (1H, m), 1.63–1.86 (2H, m), 4.38–4.52 (1H, m), 7.23 (1H, d, J = 6.8 Hz), 7.26–7.35 (3H, m), 7.45–7.57 (3H, m), 7.61 (1H, d, J = 7.4 Hz), 7.69 (1H, d, J = 5.9 Hz), 7.87 (1H, s), 7.97 (1H, d, J = 7.8 Hz), 8.04 (1H, s), 8.32 (1H, s), 8.80 (1H, d, J = 7.8 Hz), 10.44 (1H, s), 12.70 (1H, br s); FAB-MS (m/z): 518 $(M+H)^+$; Anal. Calcd for $C_{28}H_{27}N_3O_7 \cdot 1.2H_2O$: C, 62.38; H, 5.50; N, 7.79. Found: C, 62.39; H, 5.51; N, 7.64; $[\alpha]_D^{25}$ +7° (c 0.1, MeOH).

5.1.12. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-4-[({(1*S*)-[(dimethylamino)carbonyl]-3-methylbutyl}amino)carbonyl]biphenyl-2-carboxylic acid (9g). Compound **9g** was synthesized from **10** and dimethylamine·HCl according to the same procedure as that for **9a**. Compound **9g** was obtained as a colorless solid (237 mg, 81%): mp 152–153 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.92 (6H, d, J = 6.3 Hz), 1.35–1.46 (1H, m), 1.59–1.77 (2H, m), 2.84 (3H, s), 3.08 (3H, s), 4.88–5.00 (1H, m), 7.23 (1H, dd, J = 1.5 Hz, 7.4 Hz), 7.26–7.35 (3H, m), 7.45–7.57 (3H, m), 7.62 (1H, d, J = 8.3 Hz), 7.69 (1H, dd, J = 1.4 Hz, 7.4 Hz), 7.87 (1H, s), 7.98 (1H, dd, J = 1.5 Hz, 8.1 Hz), 8.02 (1H, s), 8.34 (1H, d, J = 1.4 Hz), 8.78 (1H, d, J = 8.3 Hz), 10.27 (1H, s), 12.89 (1H, br s); FAB-MS (m/z): 545 $(M+H)^+$; Anal. Calcd for $C_{30}H_{32}N_4O_6 \cdot 1.2H_2O$: C, 63.64; H, 6.12; N, 9.90. Found: C, 63.59; H, 6.07; N, 9.66; $[\alpha]_D^{25}$ +19° (c 0.1, MeOH).

5.1.13. Benzyl 2-hydroxy-5-nitrobenzoate (12). To a stirred solution of 2-hydroxy-5-nitrobenzoic acid**11** (49.8 g, 271 mmol) and $KHCO_3$ (32.5 g, 325 mmol) in DMF (270 mL) was added benzyl bromide (38.6 mL, 325 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between AcOEt and 5% $NaHCO_3$ in H_2O and the organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give **12** (68.6 g, 93%) as a yellow solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 5.40 (2H, s), 7.19 (1H, d, J = 9.2 Hz), 7.33–7.47 (3H, m), 7.47–7.55 (2H, m), 8.33 (1H, dd, J = 2.9 Hz, 9.2 Hz), 8.54 (1H, d, J = 2.9 Hz), 11.61 (1H, br s); FAB-MS (m/z): 272 $(M-H)^-$.

5.1.14. Benzyl 5-nitro-2-{[(trifluoromethyl)sulfonyl]oxy}-benzoate (13). To a stirred solution of **12** (14.9 g, 54.5 mmol) and pyridine (8.8 mL, 109 mmol) in CH_2Cl_2 (300 mL) was added trifluoromethanesulfonic anhydride (13.8 mL, 82.0 mmol), and the mixture was stirred at room temperature for 30 min. The mixture was partitioned between CH_2Cl_2 and H_2O, and the organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 9:1) to give **13** (11.2 g, 51%) as a colorless solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 5.44 (2H, s), 7.35–7.53 (5H, m), 7.92 (1H, d, J = 9.2 Hz), 8.63 (1H, dd, J = 2.8 Hz, 9.2 Hz), 8.73 (1H, d, J = 2.8 Hz).

5.1.15. 2′-Benzyl 2-*tert*-butyl 4-methyl 4′-nitrobiphenyl-2,2′,4-tricarboxylate (14). To a stirred solution of **6** (1.00 g, 2.60 mmol), bis(pinacolato)diboron (726 mg, 2.86 mmol), PPh_3 (41 mg, 0.156 mmol), and $PdCl_2(PPh_3)_2$ (55 mg, 0.078 mmol), in toluene (30 ml) was added AcOK (306 mg, 3.12 mmol) at room temperature under an argon atmosphere. The mixture was refluxed overnight, and then **13** (1.05 mg, 2.60 mmol), $Pd(PPh_3)_4$ (150 mg, 0.13 mmol), and 2 mol dm^{-3}aq Na_2CO_3 (6.5 ml) were added to the reaction mixture. The solution was refluxed overnight. The mixture was extracted with AcOEt and the organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 9:1) to give **14** (951 mg, 74%) as a colorless solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.15 (9H, s), 3.93 (3H, s), 5.05 (2H, s), 7.04–7.11 (2H, m), 7.20–7.30 (3H, m), 7.33 (1H, d, J = 8.1 Hz), 7.56 (1H, d, J = 8.4 Hz), 8.05 (1H, dd, J = 1.5 Hz, 8.1 Hz), 8.28 (1H, d, J = 1.5 Hz), 8.44 (1H, dd, J = 2.3 Hz,

8.4 Hz), 8.72 (1H, d, J = 2.3 Hz); FAB-MS (m/z): 491 $(M-H)^-$.

5.1.16. 2′-Benzyl 2-*tert*-butyl 4-methyl 4′-aminobiphenyl-2,2′,4-tricarboxylate (15). To a stirred solution of **14** (8.96 g, 18.2 mmol) in AcOH (90 mL) was added Fe (5.08 g, 91.0 mmol), and the mixture was stirred at 60 °C for 1 h. The mixture was filtered and partitioned between AcOEt and 1 M NaOH/H_2O, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 9:1) to give **15** (6.02 g, 72%) as a yellow oil: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.17 (9H, s), 3.90 (3H, s), 4.94 (2H, s), 5.51 (2H, br s), 6.76–6.88 (2H, m), 7.02–7.10 (2H, m), 7.20–7.28 (5H, m), 7.93 (1H, d, J = 8.0 Hz), 8.11 (1H, s); FAB-MS (m/z): 460 $(M-H)^-$.

5.1.17. 2′-Benzyl 2-*tert*-butyl 4-methyl 4′-[(trifluoroacetyl)amino]biphenyl-2,2′,4-tricarboxylate (16). To a stirred solution of **15** (6.00 g, 13.0 mmol) and pyridine (1.3 mL, 15.6 mmol) in CH_2Cl_2 (160 mL) was added TFAA (2.4 mL, 16.9 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between AcOEt and 5% $NaHCO_3$ in H_2O and extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 2:1) to give **16** (5.08 g, 70%) as colorless solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.15 (9 H, s), 3.92 (3H, s), 5.01 (2H, s), 7.04–7.11 (2H, m), 7.21–7.34 (5H, m), 7.96–8.04 (2H, m), 8.22 (1H, d, J = 1.6 Hz), 8.35 (1H, d, J = 2.0 Hz), 11.57 (1H, s); FAB-MS (m/z): 558 $(M+H)^+$.

5.1.18. 2′-Benzyl 2-*tert*-butyl 4-methyl 4′-[methyl(trifluoroacetyl)amino]biphenyl-2,2′,4-tricarboxylate (17). To a stirred solution of **16** (5.07 g, 9.09 mmol) and K_2CO_3 (2.51 g, 18.2 mmol) in 2-butanone (100 mL) was added MeI (1.7 mL, 27.3 mmol), and the mixture was stirred at 60 °C for 4 h. After cooling, the mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 3:1) to give **17** (4.33 g, 83%) as a colorless oil: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.13 (9 H, s), 3.33 (3H, s), 3.92 (3H, s), 4.99 (2H, s), 7.01–7.08 (2H, m), 7.18–7.32 (4H, m), 7.37 (1H, d, J = 8.0 Hz), 7.77 (1H, d, J = 8.0 Hz), 7.99 (1H, d, J = 7.9 Hz), 8.10 (1H, s), 8.20 (1H, s); FAB-MS (m/z): 572 $(M+H)^+$.

5.1.19. 2′-(*tert*-Butoxycarbonyl)-4′-(methoxycarbonyl)-4-[methyl(trifluoroacetyl)amino]biphenyl-2-carboxylic acid (18). To a stirred solution of **17** (4.32 g, 7.56 mmol) in MeOH (100 mL) was added 10% Pd/C powder (500 mg), and the mixture was stirred in a hydrogen atmosphere at room temperature for 12 h. The catalyst was filtrated on Celite and the filtrate was concentrated in vacuo to give **18** (3.53 g, 97%) as a colorless solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.16 (9H, s), 3.57 (3H, s), 7.33–7.36 (2H, m), 7.72 (1H, d, J = 7.9 Hz), 8.02 (1H, br s), 8.10 (1H, dd, J = 1.8 Hz, 7.9 Hz), 8.36 (1H, d, J = 1.8 Hz); FAB-MS (m/z): 480 $(M-H)^-$.

5.1.20. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-2-(*tert*- butoxycarbonyl)-4′-(methylamino)biphenyl-4-carboxylic acid (19). To a stirred solution of **18** (3.20 g, 6.65 mmol) and 3-aminobenzamide (3.17 g, 19.9 mmol) in DMF (60 mL) were added WSC·HCl (1.91 g, 9.98 mmol) and HOBt (1.35 g, 9.98 mmol), and the mixture was stirred at 60 °C for 12 h. The mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 98:2) to give a colorless amorphous powder (4.34 g). To a stirred solution of the compound obtained above (4.34 g) in MeOH (80 mL) was added 1 M NaOH/H_2O (16.6 mL, 16.6 mmol), and the mixture was refluxed for 2 h. The reaction mixture was acidified with 1 M HCl/H_2O (16.6 mL, 16.6 mmol). The resulting precipitate was filtered, washed with H_2O, and dried in vacuo to give **19** (3.25 g, 100%) as a yellow solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.28 (9 H, s), 2.78 (3H, s), 6.71 (1H, dd, J = 2.0 Hz, 8.4 Hz), 6.85 (1H, d, J = 2.0 Hz), 6.94 (1H, d, J = 8.4 Hz), 7.26–7.35 (3H, m), 7.50 (1H, d, J = 7.7 Hz), 7.60 (1H, d, J = 8.2 Hz), 7.87 (1H, br s), 7.95 (1H, dd, J = 1.3 Hz, 7.3 Hz), 8.00 (1H, br s), 8.20 (1H, d, J = 1.3 Hz), 9.98 (1H, s); FAB-MS (m/z): 490 $(M+H)^+$.

5.1.21. 2-(*tert*-Butoxycarbonyl)-2′-{[(4-{[(*tert*- butoxycarbonyl)amino]methyl}phenyl)amino]carbonyl}-4′-(methylamino)biphenyl-4-carboxylic acid (20). Compound **20** was synthesized from **18** and *tert*-butyl (4-aminobenzyl)carbamate according to the same procedure as that for **19**. Compound **20** was obtained as a colorless solid that was used directly in the next step.

5.1.22. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-4-({[(1*S*)-(hydroxymethyl)-3-methylbutyl]amino}carbonyl)-4′-(methylamino)biphenyl-2-carboxylic acid (21a). To a stirred solution of **19** (300 mg, 0.613 mmol) and (2*S*)-amino-4-methylpentan-1-ol (86 mg, 0.734 mmol) in DMF (6 mL) was added WSC·HCl (342 mg, 0.736 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 98:2) to give a colorless amorphous powder (275 mg). To a stirred solution of the compound obtained above (275 mg) in H_2O (2.0 mL) was added concd HCl aq (2.0 mL), and the mixture was stirred at room temperature for 5 h. The reaction mixture was concentrated in vacuo to give **21a** (240 mg, 64%) as a colorless amorphous powder: ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.86 (3H, d, J = 6.3 Hz), 0.88 (3H, d, J = 6.9 Hz), 1.36 (1H, ddd, J = 4.4 Hz, 9.3 Hz, 13.7 Hz), 1.45 (1H, ddd, J = 4.8 Hz, 8.8 Hz, 13.7 Hz), 1.52–1.68 (1H, m), 2.87 (3H, s), 3.33

(1H, dd, J = 5.8 Hz, 10.7 Hz), 3.41 (1H, dd, J = 5.8 Hz, 10.7 Hz), 3.99–4.12 (1H, m), 6.95–7.24 (3H, m), 7.24–7.40 (3H,m), 7.52 (1H, d, J = 7.8 Hz), 7.61 (1H, d, J = 7.8 Hz), 7.89 (1H, br s), 7.93 (1H, d, J = 7.8 Hz), 8.00 (1H, s), 8.18 (1H, d, J = 8.8 Hz), 8.24 (1H, s), 10.21 (1H, s); FAB-MS (m/z): 533 $(M+H)^+$; Anal. Calcd for $C_{29}H_{32}N_4O_6 \cdot 1.0HCl \cdot 2.5H_2O$: C, 56.72; H, 6.24; N, 9.12; Cl, 5.77. Found: C, 56.96; H, 6.14; N, 8.85; Cl, 6.01; $[\alpha]_D^{25}$ −7°(c 0.1, MeOH).

5.1.23. 4-({[(1*S*)-(Aminocarbonyl)-3-methylbutyl]amino}-carbonyl)-2′-({[3-(aminocarbonyl)phenyl]amino}carbonyl)-4′-(methylamino)biphenyl-2-carboxylic acid (21c). To a stirred solution of **19** (400 mg, 0.817 mmol) and (2*S*)-amino-4-methylpentanamide·HCl (681 mg, 4.09 mmol) in DMF (8.0 mL) were added WSC·HCl (235 mg, 1.23 mmol) and NEt_3 (0.57 mL, 4.09 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between AcOEt and H_2O, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 97:3) to give a pale yellow solid (340 mg). To a stirred solution of the compound obtained above (325 mg) in CH_2Cl_2 (5.0 mL) was added TFA (6.0 mL), and the mixture was stirred at room temperature for 12 h. The reaction mixture was concentrated in vacuo. To a stirred solution of the compound obtained above in AcOEt (10 mL) was added 4 M HCl/AcOEt (2.5 mL, 10 mmol), and the resulting precipitate was filtered, washed with AcOEt, and dried in vacuo to give **21c** (248 mg, 55%) as a colorless solid: mp 181–184 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.86 (3H, d, J = 6.4 Hz), 0.89 (3H, d, J = 6.4 Hz), 1.50–1.55 (1H, m), 1.61–1.72 (2H, m), 2.87 (3H, s), 4.40–4.46 (1H, m), 6.96 (1H, br s), 7.05–7.16 (3H, m), 7.28–7.33 (3H, m), 7.42 (1H, br s), 7.51 (1H, d, J = 7.8 Hz), 7.62 (1H, d, J = 7.9 Hz), 7.90 (1H, br s), 7.96–8.00 (2H, m), 8.30 (1H, d, J = 2.0 Hz), 8.59 (1H, d, J = 8.3 Hz), 10.22 (1H, br s); FAB-MS (m/z): 546 $(M+H)^+$ Anal. Calcd for $C_{29}H_{31}N_5O_6 \cdot 1.0HCl \cdot 2.0H_2O \cdot 0.5AcOEt \cdot 0.1TFA$: C, 55.64; H, 6.00; N, 10.40; Cl, 5.26. Found: C, 55.96; H, 6.02; N, 10.67; Cl, 5.03; $[\alpha]_D^{25}$ +3° (c 0.1, MeOH).

5.1.24. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-4-{[((1*S*)-carboxy-3-methylbutyl)amino]carbonyl}-4′-(methylamino)biphenyl-2- carboxylic acid (21b). Compound **21b** was synthesized from **19** and benzyl (2*S*)-amino-4-methylpentanoate·TsOH according to the same procedure as that for **21c** with deprotection of the benzyl group in the presence of Pd/C under a hydrogen atmosphere. Compound **21b** was obtained as a colorless solid (295 mg, 59%): mp 170–173 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.87 (3H, d, J = 6.4 Hz), 0.91 (3H, d, J = 6.4 Hz), 1.54–1.80 (3H, m), 2.88 (3H, s), 4.41–4.47 (1H, m), 7.05–7.35 (6H, m), 7.52 (1H, d, J = 7.8 Hz), 7.62 (1H, d, J = 8.3 Hz), 7.90 (1H, br s), 7.95 (1H, dd, J = 1.5 Hz, 7.8 Hz), 8.01 (1H, br s), 8.29 (1H, d, J = 1.5 Hz), 8.79 (1H, d, J = 8.3 Hz), 10.24 (1H, br s); FAB-MS (m/z): 547 $(M+H)^+$; Anal. Calcd for $C_{29}H_{30}N_4O_7 \cdot 1.0HCl \cdot 2.0H_2O \cdot 0.25AcOEt \cdot 0.1TFA$: C, 55.59; H, 5.73; N, 8.59; Cl, 5.43. Found: C, 55.55; H, 5.81; N, 8.70; Cl, 5.65; $[\alpha]_D^{25}$ +4° (c 0.1, MeOH).

5.1.25. 2′-({[4-(Aminomethyl)phenyl]amino}carbonyl)-4-({[(1*S*)-(hydroxymethyl)-3-methylbutyl]amino}carbonyl)-4′-(methylamino)biphenyl- 2-carboxylic acid (21d). Compound **21d** was synthesized from **20** and (2*S*)-amino-4-methylpentan-1-ol according to the same procedure as that for **21a**. Compound **21d** was obtained as a colorless amorphous powder (130 mg, 45%): ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.86 (3H, d, J = 6.8 Hz), 0.89 (3H, d, J = 6.3 Hz), 1.36 (1H, ddd, J = 3.9 Hz, 8.7 Hz, 13.7 Hz), 1.46 (1H, ddd, J = 4.4 Hz, 9.8 Hz, 13.7 Hz), 1.53–1.67 (1H, m), 2.85 (3H, s), 3.33 (1H, dd, J = 5.8 Hz, 10.8 Hz), 3.41 (1H, dd, J = 5.9 Hz, 10.8 Hz), 3.87–3.96 (2H, br), 4.00–4.11 (1H, m), 7.03 (1H, br s), 7.06–7.20 (2H, m), 7.27 (1H, d, J = 8.3 Hz), 7.35 (2H, d, J = 8.3 Hz), 7.50 (2H, d, J = 8.3 Hz), 7.93 (1H, dd, J = 1.9 Hz, 8.3 Hz), 8.22 (1H, d, J = 8.3 Hz), 8.34 (1H, d, J = 1.9 Hz), 8.31 (3H, br s), 10.13 (1H, s); FAB-MS (m/z): 519 $(M+H)^+$; Anal. Calcd for $C_{29}H_{32}N_4O_6 \cdot 2.1HCl \cdot 2.9H_2O$: C, 53.80; H, 6.52; N, 8.65; Cl, 11.55. Found: C, 54.00; H, 6.40; N, 8.67; Cl, 11.46; $[\alpha]_D^{25}$ −10.4°(c 0.67, MeOH).

5.1.26. 4-({[(1*S*)-(Aminocarbonyl)-3-methylbutyl]amino}-carbonyl)-2′-({[4-(aminomethyl)phenyl]amino}carbonyl)-4′-(methylamino)biphenyl-2-carboxylic acid (21e). Compound **21e** was synthesized from **20** and (2*S*)-amino-4-methylpentanamide according to the same procedure as that for **21a** except for the purification by ODS gel column chromatography (0.001 M HCl aq/CH_3CN = 10:3). Compound **21e** was obtained as a colorless amorphous powder (180 mg, 57%): ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.86 (3H, d, J = 6.4 Hz), 0.90 (3H, d, J = 6.9 Hz), 1.45–1.59 (1H, m), 1.59–1.83 (2H, m), 2.92 (3H, s), 3.85–3.97 (2H, m), 4.40–4.50 (1H, m), 6.97 (1H, br s), 7.20–7.28 (1H, m), 7.28–7.40 (3H, m), 7.40–7.55 (3H, m), 8.02 (1H, dd, J = 2.0 Hz, 7.9 Hz), 8.33 (1H, d, J = 2.0 Hz), 8.39 (2H, br s), 8.82 (1H, d, J = 8.3 Hz), 10.23 (1H, s); FAB-MS (m/z): 532 $(M+H)^+$; Anal. Calcd for $C_{29}H_{33}N_5O_5 \cdot 2.7HCl \cdot 2.5H_2O$: C, 51.60; H, 6.08; N, 10.37; Cl, 14.18. Found: C, 51.68; H, 6.23; N, 10.41; Cl, 14.17; $[\alpha]_D^{25}$ +15° (c 0.1, MeOH).

5.1.27. 2-*tert*-Butyl 4-methyl 2′-({[3-(aminocarbonyl)-phenyl]amino}carbonyl)-4′-[(trifluoroacetyl)amino]biphenyl-2,4-dicarboxylate (22). To a stirred solution of **16** (585 mg, 1.05 mmol) in MeOH (10 mL) was added 10% Pd/C powder (60 mg), and the mixture was stirred under a hydrogen atmosphere at room temperature for 12 h. The catalyst was filtrered on Celite and the filtrate was concentrated in vacuo to give a colorless solid (477 mg). To a stirred solution of the solid obtained above (457 mg) and 3-aminobenzamide (311 mg, 1.96 mmol) in DMF (10 mL) were added WSC·HCl (281 mg, 1.47 mmol) and HOBt (199 mg, 1.47 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was

purified by silica gel column chromatography ($CHCl_3$/MeOH = 98:2) to give **22** (423 mg, 72%) as a pale yellow solid: ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.23 (9H, s), 3.88 (3H, s), 7.28–7.36 (3H, m), 7.42 (1H, d, J = 7.9 Hz), 7.52 (1H, d, J = 7.5 Hz), 7.59 (1H, d, J = 7.9 Hz), 7.85–7.92 (2H, m), 7.97–8.04 (2H, m), 8.08 (1H, d, J = 8.0 Hz), 8.33 (1H, s), 10.24 (1H, s), 11.57 (1H, s); ESI-MS (m/z): 586 $(M+H)^+$.

5.1.28. 2′-Benzyl 2-*tert*-butyl 4-methyl 4′-(isopropylamino)biphenyl-2,2′,4-tricarboxylate (23). To a stirred solution of **15** (500 mg, 1.08 mmol) and acetone (0.8 mL, 10.8 mmol) in AcOH (5.0 mL) was added $NaBH(OAc)_3$ (458 mg, 2.16 mmol), and the mixture was stirred at room temperature for 24 h. The mixture was partitioned between AcOEt and 5% $NaHCO_3$ in H_2O and extracted with AcOEt, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 9:1) to give **23** (496 mg, 91%) as a yellow oil: ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.15 (6H, d, J = 6.2 Hz), 1.16 (9H, s), 3.54–3.67 (1H, m), 3.90 (3H, s), 4.95 (2H, s), 5.89 (1H, d, J = 8.0 Hz), 6.79 (1H, dd, J = 2.4 Hz, 8.4 Hz), 6.91 (1H, d, J = 8.4 Hz), 7.01–7.07 (2H, m), 7.18–7.29 (5H, m), 7.93 (1H, dd, J = 1.8 Hz, 8.0 Hz), 8.11 (1H, d, J = 1.8 Hz); ESI-MS (m/z): 504 $(M+H)^+$.

5.1.29. 2′-Benzyl 2-*tert*-butyl 4-methyl 4′-(cyclohexylamino)biphenyl-2,2′,4-tricarboxylate (24). Compound **24** was synthesized from **15** and cyclohexanone according to the same procedure as that for **23**. Compound **24** was obtained as a yellow oil (313 mg, 89%): ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14–1.38 (14H, m), 1.55–1.78 (3H, m), 1.89–2.00 (2H, m), 3.90 (3H, s), 4.95 (3H, s), 5.92 (1H, d, J = 7.9 Hz), 6.79 (1H, dd, J = 2.2 Hz, 8.4 Hz), 6.89 (1H, d, J = 8.3 Hz), 7.01–7.07 (2H, m), 7.18–7.26 (5H, m), 7.90–7.96 (1H, m), 8.12 (1H, br s); ESI-MS (m/z): 544 $(M+H)^+$.

5.1.30. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-2-(*tert*-butoxycarbonyl)-4′-(ethylamino)biphenyl-4-carboxylic acid (25a). To a stirred solution of **22** (200 mg, 0.342 mmol) and K_2CO_3 (52 mg, 0.376 mmol) in 2-butanone (5.0 mL) was added EtI (0.060 mL, 0.746 mmol), and the mixture was stirred at 60 °C for 2 days. After cooling, the mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 99:1) to give a pale yellow solid (162 mg). To a stirred solution of the compound obtained above (147 mg) in MeOH (5.0 mL) was added 1 M NaOH/H_2O (0.70 mL, 0.70 mmol), and the mixture was refluxed for 2 h. The reaction mixture was acidified with 1 M HCl/H_2O (0.70 mL, 0.70 mmol). The resulting precipitate was filtered, washed with H_2O, and dried in vacuo to give **25a** (119 mg, 77%) as a pale yellow solid: ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14–1.24 (3H, m), 1.27 (9H, s), 3.16 (2H, q, J = 7.0 Hz), 3.67 (1H, br s), 6.75 (1H, d, J = 8.3 Hz), 6.89 (1H, s), 6.94 (1H, d, J = 8.3 Hz), 7.26–7.35 (3H, m), 7.50 (1H, d, J = 7.9 Hz), 7.59 (1H, d, J = 8.0 Hz), 7.87 (1H, s), 7.95 (1H, dd, J = 1.9 Hz, 7.9 Hz), 8.00 (1H, s), 8.20 (1H, d, J = 1.9 Hz), 9.98 (1H, s); ESI-MS (m/z): 502 $(M-H)^-$.

5.1.31. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-2-(*tert*-butoxycarbonyl)-4′-(butylamino)biphenyl-4-carboxylic acid (25b). Compound **25b** was synthesized from **22** and 1-iodobutane according to the same procedure as that for **25a**. Compound **25b** was obtained as a yellow solid (152 mg, 67%): ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.17 (3H, t, J = 7.1 Hz), 1.27 (9H, s), 1.33–1.49 (2H, m), 1.49–1.63 (2H, m), 3.07–3.15 (2H, m), 6.73 (1H, d, J = 7.7 Hz), 6.85–6.95 (2H, m), 7.26–7.35 (3H, m), 7.49 (1H, d, J = 7.0 Hz), 7.58 (1H, d, J = 7.9 Hz), 7.86 (1H, s), 7.95 (1H, d, J = 7.9 Hz), 8.00 (1H, s), 8.19 (1H, s), 9.96 (1H, s); ESI-MS (m/z): 530 $(M-H)^-$.

5.1.32. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-2-(*tert*-butoxycarbonyl)-4′-(isopropylamino)biphenyl-4-carboxylic acid (25c). To a stirred solution of **23** (486 mg, 0.965 mmol) in MeOH (10 mL) was added 10% Pd/C powder (50 mg), and the mixture was stirred under a hydrogen atmosphere at room temperature for 12 h. The catalyst was filtered on Celite and the filtrate was concentrated in vacuo to give a yellow solid (394 mg). To a stirred solution of the compound obtained above (389 mg) and 3-aminobenzamide (449 mg, 2.82 mmol) in DMF (5.0 mL) were added WSC·HCl (270 mg, 1.41 mmol) and HOBt (191 mg, 1.41 mmol), and the mixture was stirred at room temperature for 12 h. The mixture was partitioned between AcOEt and H_2O and extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 99:1) to give a yellow solid (442 mg). To a stirred solution of the compound obtained above (422 mg) in MeOH (5.0 mL) was added 1 M NaOH/H_2O (1.0 mL, 1.0 mmol), and the mixture was refluxed for 2 h. The reaction mixture was acidified with 1 M HCl/H_2O (1.0 mL, 1.0 mmol). The resulting precipitate was filtered, washed with H_2O, and dried in vacuo to give **25c** (347 mg, 69%) as a pale yellow solid: ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.18 (6H, d, J = 6.1 Hz), 1.27 (9H, s), 3.60–3.72 (1H, m), 5.81 (1H, br s), 6.71 (1H, d, J = 8.3 Hz), 6.85 (1H, s), 6.91 (1H, d, J = 8.3 Hz), 7.26–7.36 (3H, m), 7.49 (1H, d, J = 7.7 Hz), 7.58 (1H, d, J = 8.2 Hz), 7.86 (1H, s), 7.95 (1H, d, J = 7.9 Hz), 8.01 (1H, s), 8.19 (1H, s), 9.97 (1H, s); ESI-MS (m/z): 516 $(M-H)^-$.

5.1.33. 2′-({[3-(Aminocarbonyl)phenyl]amino}carbonyl)-2-(*tert*-butoxycarbonyl)-4′-(cyclohexylamino)biphenyl-4-carboxylic acid (25d). Compound **25d** was synthesized from **24** according to the same procedure as that for **25c**. Compound **25d** was obtained as a pale yellow solid (224 mg, 85%): ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.21–1.40 (14H, m), 1.55–1.78 (3H, m), 1.91–2.00 (2H, m),

6.72 (1H, d, J = 7.3 Hz), 6.85–6.94 (2H, m), 7.25–7.36 (3H, m), 7.49 (1H, d, J = 7.9 Hz), 7.58 (1H, d, J = 8.0 Hz), 7.86 (1H, s), 7.94 (1H, d, J = 8.0 Hz), 8.00 (1H, s), 8.19 (1H, s), 9.96 (1H, s); ESI-MS (m/z): 556 $(M-H)^-$.

5.1.34. 4-({[(1*S*)-(Aminocarbonyl)-3-methylbutyl]amino}-carbonyl)-2′-({[3-(aminocarbonyl)phenyl]amino}carbonyl)-4′-(ethylamino)biphenyl-2-carboxylic acid (26a). Compound **26a** was synthesized from **25a** and (2*S*)-amino-4-methylpentanamide according to the same procedure as that for **21c**. Compound **26a** was obtained as a pale yellow solid (34 mg, 27%): mp 178–180 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (3H, d, J = 6.4 Hz), 0.89 (3H, d, J = 6.4 Hz), 1.22 (3H, t, J = 6.8 Hz), 1.47–1.57 (1H, m), 1.57–1.73 (2H, m), 3.24 (2H, q, J = 6.8 Hz), 4.36–4.50 (1H, m), 6.84–7.15 (4H, m), 7.25–7.34 (3H, m), 7.40 (1H, s), 7.50 (1H, d, J = 7.8 Hz), 7.61 (1H, d, J = 9.2 Hz), 7.88 (1H, s), 7.96 (1H, dd, J = 1.9 Hz, 7.8 Hz), 8.00 (1H, s), 8.28 (1H, d, J = 1.9 Hz), 8.57 (1H, d, J = 7.8 Hz), 10.17 (1H, s); FAB-MS (m/z): 560 $(M+H)^+$; HRMS (FAB) calcd for $C_{30}H_{33}N_5O_6$: 558.2353. Found: 558.2359; $[\alpha]_D^{25}$ +9° (c 0.1, MeOH).

5.1.35. 4-({[(1*S*)-(Aminocarbonyl)-3-methylbutyl]amino}-carbonyl)-2′-({[3-(aminocarbonyl)phenyl]amino}carbonyl)-4′-(*n*-butylamino)biphenyl-2-carboxylic acid (26b). Compound **26b** was synthesized from **25b** and (2*S*)-amino-4-methylpentanamide according to the same procedure as that for **21c**. Compound **26b** was obtained as a colorless solid (51 mg, 33%): mp 166–169 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (3H, d, J = 6.4 Hz), 0.89 (3H, d, J = 6.4 Hz), 0.94 (3H, t, J = 7.3 Hz), 1.36–1.47 (2H, m), 1.47–1.56 (1H, m), 1.56–1.75 (4H, m), 3.10–3.22 (2H, m), 4.38–4.48 (1H, m), 6.95 (2H, br s), 7.00–7.13 (2H, m), 7.25–7.35 (3H, m), 7.40 (1H, s), 7.50 (1H, d, J = 7.8 Hz), 7.61 (1H, d, J = 8.3 Hz), 7.88 (1H, s), 7.96 (1H, dd, J = 1.5 Hz, 7.8 Hz), 7.99 (1H, s), 8.28 (1H, d, J = 1.5 Hz), 8.56 (1H, d, J = 7.8 Hz), 10.16 (1H, s); FAB-MS (m/z): 588 $(M+H)^+$; Anal. Calcd for $C_{32}H_{37}N_5O_6$·0.9HCl·3.3-H_2O·0.8TFA: C, 52.33; H, 5.92; N, 9.08; Cl, 4.14. Found: C, 52.10; H, 5.79; N, 9.41; Cl, 4.07; $[\alpha]_D^{25}$ −14° (c 0.1, MeOH).

5.1.36. 4-({[(1*S*)-(Aminocarbonyl)-3-methylbutyl]amino}-carbonyl)-2′-({[3-(aminocarbonyl)phenyl]amino}carbonyl)-4′-(isopropylamino)biphenyl-2-carboxylic acid (26c). Compound **26c** was synthesized from **25c** and (2*S*)-amino-4-methylpentanamide according to the same procedure as that for **21c**. Compound **26c** was obtained as a colorless solid (158 mg, 78%): mp 172–175 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.86 (3H, d, J = 6.4 Hz), 0.89 (3H, d, J = 6.3 Hz), 1.26 (6H, d), 1.46–1.58 (1H, m), 1.58–1.74 (2H, m), 3.67–3.80 (1H, m), 3.39–4.48 (1H, m), 6.95 (1H, s), 7.00–7.25 (3H, m), 7.28–7.35 (3H, m), 7.41 (1H, s), 7.52 (1H, d, J = 7.8 Hz), 7.61 (1H, d, J = 7.8 Hz), 7.89 (1H, s), 7.96–8.01 (2H, m), 8.31 (1H, s), 8.58 (1H, d, J = 7.8 Hz), 10.20 (1H, s); FAB-MS (m/z): 574 $(M+H)^+$; Anal. Calcd for $C_{31}H_{35}N_5O_6$·0.85HCl·2.2-H_2O·0.2AcOEt·0.15TFA: C, 56.78; H, 6.23; N, 10.31; Cl, 4.44. Found: C, 56.38; H, 6.14; N, 10.48; Cl, 4.83; $[\alpha]_D^{25}$ −5° (c 0.1, MeOH).

5.1.37. 4-({[(1*S*)-(Aminocarbonyl)-3-methylbutyl]amino}-carbonyl)-2′-({[3-(aminocarbonyl)phenyl]amino}carbonyl)-4′-(cyclohexylamino)biphenyl-2-carboxylic acid (26d). Compound **26d** was synthesized from **25d** and (2*S*)-amino-4-methylpentanamide according to the same procedure as that for **21c**. Compound **26d** was obtained as a colorless solid (42 mg, 17%): mp 180–183 °C (AcOEt); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.86 (3H, d, J = 6.3 Hz), 0.89 (3H, d, J = 6.4 Hz), 1.13–1.30 (5H, m), 1.46–1.80 (6H, m), 1.93–2.01 (2H, m), 3.38 (1H, br s), 4.40–4.46 (1H, m), 6.92–7.17 (4H, m), 7.29–7.33 (3H, m), 7.40 (1H, br s), 7.50 (1H, d, J = 7.8 Hz), 7.60 (1H, d, J = 8.3 Hz), 7.88 (1H, br s), 7.95–7.99 (2H, m), 8.28 (1H, br s), 8.56 (1H, d, J = 8.3 Hz), 10.16 (1H, br s); FAB-MS (m/z): 614 $(M+H)^+$; Anal. Calcd for $C_{34}H_{39}N_5O_6$·0.95HCl·2.0H_2O·0.1AcOEt·0.05TFA: C, 59.29; H, 6.46; N, 10.02; Cl, 4.82. Found: C, 59.11; H, 6.55; N, 10.25; Cl, 4.66; $[\alpha]_D^{25}$ +8° (c 0.1, MeOH).

5.2. Docking study

Docking simulation was carried out with the GOLD program (CCDC, Cambridge, UK) with the active site of TF/FVIIa complex (PDB code: 1DAN). After docking, energy minimization was performed based on the MMFF94s force field using MOE 2004.03 (Chemical Computing Group Inc, Montreal, CA).

5.3. Biology

5.3.1. Chromogenic assay. The hydrolysis rates of synthetic substrates were assayed by continuously measuring absorbance at 405 nm at 37 °C with a microplate spectrophotometer (Spectramax 340PC, Molecular Devices Co., California, USA). Reaction mixtures (40 μL) were prepared in 96-well plates containing chromogenic substrate and an inhibitor in either 20 mM HEPES, 0.01% BSA, 5 mM $CaCl_2$, and 0.15 M NaCl, pH 7.4. Reactions were initiated with 10-μL portions of the enzyme solution. Enzymes and substrates were used as follows: human TF/human FVIIa and S-2288; human factor Xa and S-2222; human thrombin and S-2238; and human trypsin and S-2222. The concentration of an inhibitor required to inhibit enzyme activity by 50% (IC_{50}) was calculated from concentration–response curves in which the logit transformation of residual activity was plotted against the logarithm of inhibitor concentration.

5.3.2. Plasma clotting time assay. Citrated human blood samples were collected in accordance with the requirements of Astellas Research Ethics Committee. Platelet-poor plasma was prepared by centrifugation at 3000 rpm for 10 min and stored at −40 °C until use. Plasma clotting times were measured using a KC10A coagulometer (Amelung Co., Lehbringsweg, Germany) at 37 °C. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using HemosIL™ RecombiPlasTin and HemosIL™ SynthASil (Instrumentation Laboratory Company, Lexington,

MA, USA), respectively. Coagulation times for each test sample were compared with coagulation times measured using 4% DMSO in water as a control. The concentration required to double clotting time (CT_2) was estimated from each individual concentration–response curve. Each measurement was performed three times and is represented as the mean value.

5.3.3. Plasma concentrations derived from anti-TF/FVIIa activity in mice ex vivo studies. Male ICR mice weighing 23–43 g were fasted overnight. Inhibitors were dissolved or suspended in 0.5% methylcellulose aqueous solution and administered to the mice orally at 100 mg/kg using a gastric tube. After oral administration of the inhibitor, blood was withdrawn from the inferior vena cava in the presence of a 1/10th volume of 3.8% sodium citrate, then centrifuged to obtain platelet-poor plasma. The inhibitors in the plasma were extracted with AcOEt.. After measured the TF/FVIIa inhibitory activity in the extracts by chromogenic assay as described above, the concentration of an inhibitor required to inhibit the corresponding enzyme activity was calculated from each individual concentration–response curve. Each measurement was performed three times and represented as the mean value.

5.3.4. Ex vivo coagulation assays and template bleeding time in cynomolgus monkeys. Male cynomolgus monkeys weighing 4.9–5.6 kg were used. A dosing solution of the test drug (HCl salt of **21e** or vehicle) was cumulatively administered into the saphenous vein of non-fasted animals at an interval of 10 min under ketamine and pentobarbital anesthesia. After 1-min infusion of each dose, template bleeding time was measured and citrated blood was collected from the femoral vein to measure PT and APTT. A template bleeding device (Simplate, Organon Teknika, Tokyo) was placed on the plantaleft forearm skin and triggered. Blood flowing from the incision was gently wiped away with filter paper every 30 s. Bleeding time was measured as time elapsed until bleeding stopped.

5.3.5. Statistical analyses. Statistical analyses for plasma clotting time assay in cynomolgus monkeys was performed using Dunnett multiple comparison test compared with the vehicle group. A p value of less than 0.05 was considered significant.

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10. Because of the presence of a species difference among human, mice and cynomolgus monkeys (PT/CT_2 = 47 μM (human), >300 μM (mice), and 42 μM (cynomolgus monkeys), respectively), we planned to investigate PK profile based on inhibitory activity against human TF/FVIIa after oral administration in mice and then investigate the bleeding risk in cynomolgus monkeys by measuring the standard clotting assays (PT and APTT) and bleeding time. Bioavailability of compound **21e** calculated by ex vivo study in mice is about 10%.
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