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Exploration of 4,4-disubstituted pyrrolidine-1,2-dicarboxamides as potent, orally active Factor Xa inhibitors with extended duration of action

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ABSTRACT

Aiming to improve upon previously disclosed Factor Xa inhibitors, a series of 4,4-disubstituted pyrrolidine-1,2-dicarboxamides were explored with the intent of increasing the projected human half-life versus **5** (projected human $t_{1/2}$ = 6 h). A stereospecific route to compounds containing a 4-aryl-4-hydroxypyrrolidine scaffold was developed, resulting in several compounds that demonstrated an increase in the half-life as well as an increase in the in vitro potency compared to **5**. Reported herein is the discovery of **26**, containing a (2*R*,4*S*)-4-hydroxy-4-(2,4-difluorophenyl)-pyrrolidine scaffold, which is a selective, orally bioavailable, efficacious Factor Xa inhibitor that appears suitable for a once-daily dosing (projected human $t_{1/2}$ = 23 h).

1. Introduction

In recent years there has been an intensive research effort to develop orally bioavailable antithrombotic agents. Such agents are expected to reduce cardiovascular complications and mortality associated with several life threatening conditions. Among these are disease states precipitated by abnormal coagulation and inappropriate thrombus formation within blood vessels such as myocardial infarction, unstable angina, stroke, pulmonary embolism, deep vein thrombosis and venous thromboembolism associated with surgery and cancers. This research has led to several compounds that have reached late stage clinical development.[1,2] These new agents must address the limitations associated with warfarin (Coumadin®) therapy. Regular coagulation monitoring is necessary to maintain safe and therapeutic plasma concentrations of warfarin, which has an inherent risk due to metabolism, drug–drug interactions and food effects. Thus safety and ease of use are of primary importance for a new antithrombotic agent.

Recently, direct thrombin inhibitors (DTI) in the form of prodrugs have advanced to the threshold of market launch, or have actually reached the market. Unfortunately, the promising DTI ximelagatran was withdrawn in 2005.[1,3] The decision was due to a significant risk of liver damage upon chronic use of the drug and an increased risk of heart attack.[4] Ximelagatran also showed some rebound thrombosis which has also been observed with other DTIs.[5] Another orally-active prodrug DTI, dabigatran etexilate (1, Fig. 1) is now in the market.[1,6]

Factor Xa (FXa) is a serine protease that acts at the interface of the intrinsic and extrinsic coagulation cascade and converts prothrombin to thrombin.[2] As further elaborated below, evidence indicates that FXa inhibition will have an enhanced risk-benefit margin compared to warfarin therapy as FXa is primarily responsible for clot-associated pro-coagulant activity.

Direct FXa inhibition has been demonstrated to disaggregate preformed platelet thrombi in vivo and produce long-term reduction of thrombus procoagulant activity (passivation) in animal models of thrombosis even with short-term FXa inhibition.[7] In contrast to the 'rebound thrombosis' observed shortly after termination of current antithrombin and antiplatelet therapies, direct

Abbreviations: FXa, Factor Xa; DTI, direct thrombin inhibitor; C_{max}, maximum plasma concentration; C_{min}, minimum plasma concentration; C_{max}/C_{min}, peak-to-trough plasma concentration ratio; PT, prothrombin time; 2 × PT, concentration required to give a doubling of the prothrombin time; PK/PD, pharmacokinetic/pharmacodynamic; AV, arteriovenous; IV, intravenous administration; PO, oral administration; QD, once daily dosing; ND, not determined; V_{dss}, volume of distribution at steady state; K_a, absorption rate constant; F, oral bioavailability; $t_{1/2}$, elimination half-life; CYP, cytochrome P450.

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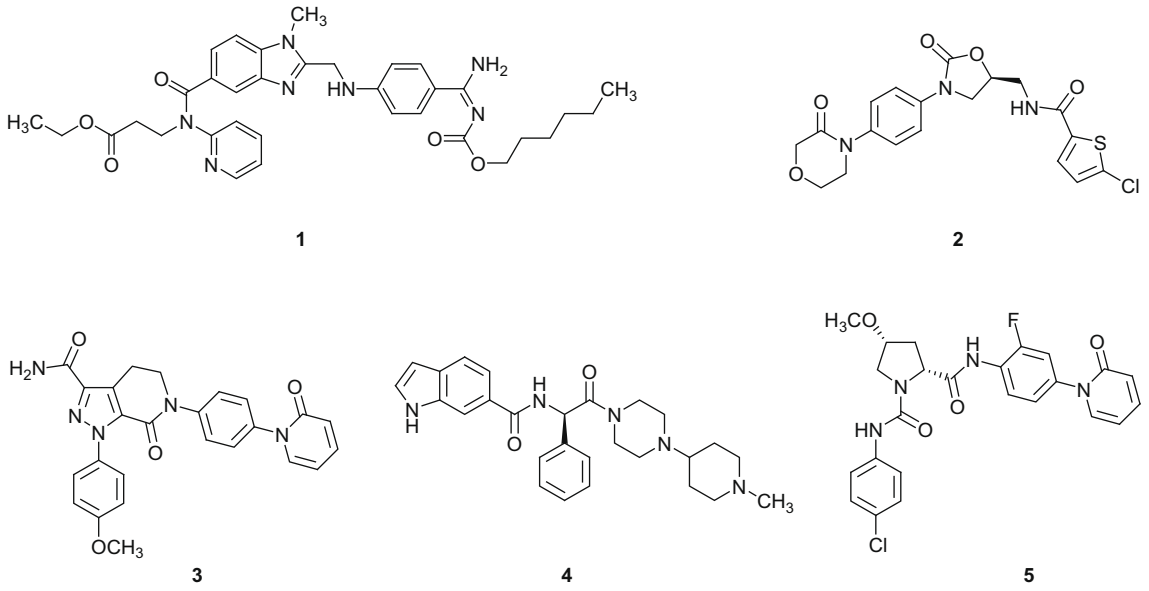


Figure 1. Structures of new oral anticoagulants in development: **1** = dabigatran etexilate, **2** = rivaroxaban, **3** = apixaban, **4** = LY-517717, **5** = eribaxaban.

FXa inhibition provides the potential for sustained antithrombotic prevention after therapy is terminated and may provide a unique advantage over currently available anticoagulants. Indirect inhibitors of FXa such as fondaparinux (Arixtra®) have demonstrated efficacy and safety which provides additional evidence that inhibitors of FXa may overcome current limitations of oral antithrombotic therapy.[1] The first generation of small molecule FXa inhibitors relied upon a benzamidine moiety for potency and consequently exhibited high clearance and lacked acceptable oral bioavailability.[2] Subsequently, poor pharmacokinetic and pharmacodynamic properties have been successfully addressed through the replacement of the benzamidine moiety with a neutral P1 element, as several FXa inhibitors are in late stage clinical trials. Prominent among these FXa inhibitors are rivaroxaban **2**,[8] apixaban **3**,[9] LY-517717 **4**[1] and eribaxaban **5**[10,11] as shown in Figure 1.

For an ideal anticoagulant therapy, the plasma concentration must remain within the therapeutic window to afford maximal protection without increasing the risk of bleeding. As the dose of an anticoagulant is increased, the incidence of clotting events decreases. However, a threshold of exposure exists where the risk of serious bleeding exceeds its benefit. Maintaining a plasma concentration within the therapeutic window may be affected by the peak-to-trough ratio (C_{max}/C_{min}) that is a function of the pharmacokinetic profile of the specific agent and its dosing regimen. Thus, the C_{max} of an anticoagulant could be higher and/or the C_{min} lower than ideal for the therapeutic window. Using some baseline assumptions (e.g., similar absorption rates), compounds with half-lives of 6 h, 12 h and 24 h have C_{max}/C_{min} ratios of 9.4, 3.0 and 1.8, respectively, when dosed every 24 h.[12] This effect of elimination half-life on C_{max}/C_{min} ratio for a once daily dosing regimen is presented graphically in Figure 2. This example demonstrates how a compound with extended half-life can minimize large fluctuations in drug plasma concentrations. An anticoagulant with a half-life appropriate for once daily dosing would lead to improved clinician confidence in dosing as well as enhanced convenience and patient compliance. Herein is described a number of 4,4-disubstituted pyrrolidine-1,2-dicarboxamide derivatives that were explored in an effort to develop a compound with a projected human half-life suitable for once daily dosing.

2. Molecular modeling and design

A co-crystal X-ray structure of Factor Xa protein and **5** was obtained in our laboratories (Fig. 3a).[10] It was observed that the C-4 hydrogen of the pyrrolidine ring was directed towards the unoccupied FXa active-site subpocket formed by the disulfide region (Cys191–Cys220) and the fully-extended side chains of residues Arg143, Glu147 and Gln192 of the enzyme. To probe this hydrophobic subpocket, possible replacements for the C-4 hydrogen, such as small alkyl groups and aryl rings, were modeled in the FXa active site and a select number of compounds were synthesized. It was hypothesized that further substitution at the C-4 site was not likely to adversely impact potency as the strong H-bond between Gly219 of FXa and the lone pairs of the 4-methoxy group or 4-hydroxy group would be preserved. Further details about the orientation of one of these compounds, the 4-hydroxy-4-phenyl analog **17** were revealed in a X-ray co-crystal structure (Fig. 3b). The crystal structure showed that the disulfide bridge region was still not fully occupied which suggested that space may still exist for additional substitution at the *ortho* position of the phenyl ring. A number of these analogs were subsequently synthesized. One of these analogs, the 2,4-difluorophenyl derivative **26,** was shown via X-ray crystallography, to bind to the enzyme with its *ortho* substituent occupying the disulfide region (Fig. 3c). Further discussion about the X-ray crystallography results are given in Section 4.

3. Chemistry

The general synthetic route for the above 4,4-disubstituted pyrrolidine compounds is outlined in Scheme 1. Ketone **7** was obtained from commercially available *cis*-hydroxy-D-proline via protection as the *t*-butylcarbamate and oxidation of the alcohol with trichloroisocyanuric acid and catalytic TEMPO in dichloromethane.[13] The ketoacid **7** was treated with an excess of the appropriate Grignard reagent to produce the tertiary alcohol **8**. Use of the carboxylic acid intermediate was preferred over an ester as the resulting carboxylate anion prevented racemization at the proline α-carbon. Interestingly, the methyl and ethyl Grignard reagents resulted in an approximate 50:50 diastereomeric mixture of intermediate **8**, whereas aryl Grignard reagents proceeded stereoselectively to give the desired *cis*-hydroxyl product. When

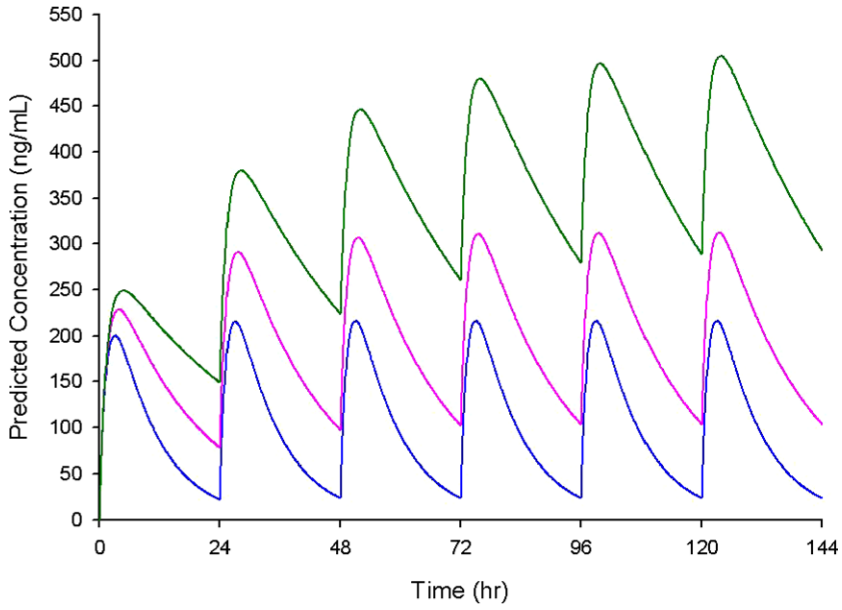


Figure 2. Simulated drug concentration versus time profiles illustrating the effect of elimination half-life on C_{max}/C_{min} ratio for a once daily dosing regimen (assuming V_{dss} = 1 L/kg, K_a = 0.7 h^{-1}, F = 100%). Blue line: $t_{1/2}$ = 6 h, C_{max}/C_{min} = 9.4. Magenta line: $t_{1/2}$ = 12 h, C_{max}/C_{min} = 3.0. Green line: $t_{1/2}$ = 24 h, C_{max}/C_{min} = 1.8.

R_1 was a small alkyl moiety, the mixture of diastereomers was coupled directly with the aniline or 2-aminopyridine derivative used as the moiety to bind in the S4 pocket (P4 binding moiety) using EEDQ.[14] The diastereomers were later separated by column chromatography after the final step. In the case of the ether analogs **12**, **15**, and **16**, intermediate **8** was alkylated with methyliodide or ethylbromide to give the corresponding methyl or ethyl ether. Selective alkylation was accomplished by forming the di-anion of **8** with excess sodium hydride in THF, followed by the addition of the alkylating agent. Only the alcohol reacts under these conditions, even with a large excess of alkylating agent. In the case of 4-aryl products, activation of the carboxylic acid with various coupling reagents resulted exclusively in intramolecular cyclization with the *cis*-4-hydroxy group to form lactone **9**.

Treatment of the aniline or 2-aminopyridine derivative used as the P4 binding moieties with trimethylaluminum in toluene followed by addition of lactone **9** with heating effected the ring opening to the desired product **10**.[15] While the use of these forcing conditions led to moderate yields of the needed intermediate, only the desired diastereomer was identified from the reaction mixture. Most of the by-products encountered were either decomposition by-products or unreacted lactone starting material. The final compounds were obtained by first removing the *t*-butylcarbamate with TFA in methylene chloride or by addition of **10** to freshly prepared HCl in methanol. Secondly, the deprotected amine was reacted with 4-chlorophenylisocyanate in methylene chloride with triethylamine, or in a biphasic mixture of sodium bicarbonate in water and dichloromethane to give the desired products **11–26**.

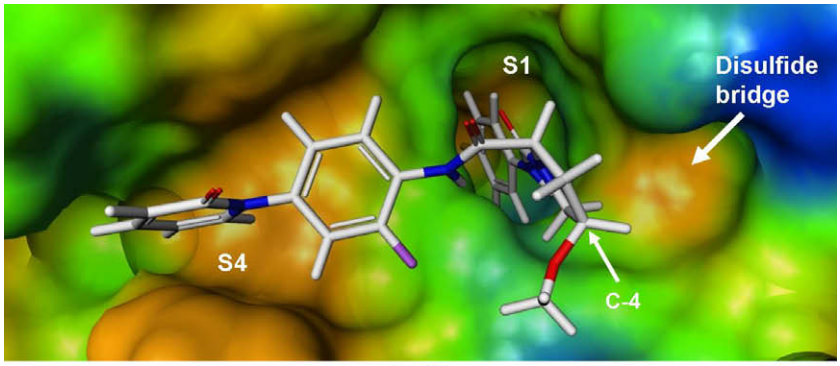
This initial route was suitable for small scale preparation of **26**. However, Grignard addition to the ketone and the opening of the lactone using trimethylaluminum posed potential safety issues on large scale. First, it is known that an *ortho*-halogen substituted aryl Grignard reagent can form unstable benzynes with unknown energetics.[16] Spectroscopic analysis and thermal stability studies were performed during the preparation of 2,4-difluorophenylmagnesium bromide. Although the benzyne related byproducts were observed at temperatures above room temperature (>23 °C), no such evidence of benzyne formation was detected when the Grignard reagent was formed below 0 °C. For this reason, large scale preparation of 2,4-difluorophenylmagnesium bromide was accomplished using transfer methodology utilizing isopropylmagnesium bromide with 2,4-difluorobromobenzene while keeping reaction temperatures below 0 °C.[17]

A second potential hazard of the large scale synthesis of **26** was the highly pyrophoric properties of trimethylaluminum. Direct opening of the lactone **9** (R_1 = 2,4-difluorophenyl) by heating with 1-(6-aminopyridin-3-yl)pyridin-2(1*H*)-one and without further activation was not accomplished. To increase the reactivity, potential bases to generate the corresponding anion of the aminopyridine P4 binding moiety were screened which revealed that anion formation with lithium hexamethyldisilazide in THF followed by the addition of lactone **9** gave the desired amide **10**. The compound obtained using these conditions was found to be identical by ^{1}H NMR and HPLC to **10** prepared using trimethylaluminum conditions.

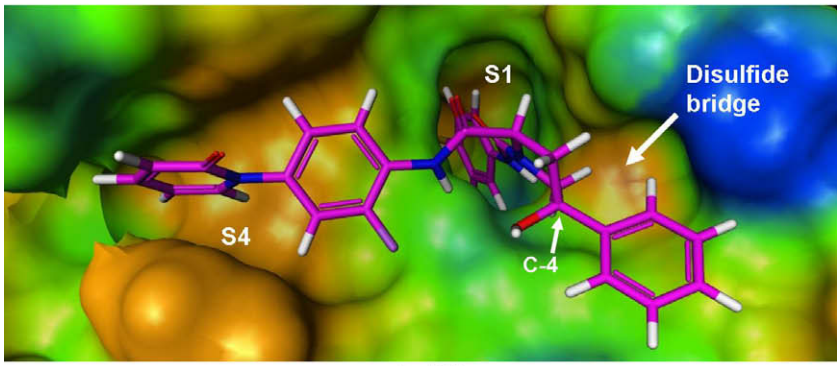
4. Results and discussion

A table of 4,4-disubstituted pyrrolidines is shown with the in vitro FXa binding affinity and half-life from rat IV cassette dosing studies (Table 1). In all cases the *cis*-hydroxy or *cis*-alkoxy derivatives are shown. As suggested by our molecular modeling results, replacement of the C-4 hydrogen with a methyl group, while maintaining a *cis*-4-hydroxy or *cis*-4-methoxy substituent did not adversely impact potency, as seen in **11** and **12**. These results also suggested that even with the addition of the methyl group at C-4, the lone pair of the oxygen atom was likely forming an H-bond with Gly219 similar to that observed with **5**. As would be expected based on lipophilicity, increasing the size of the R_1 group was observed to have an effect in the potency. However, it was noted that small changes in the size of the R_1 group (like methyl to ethyl as in **11** to **13** or **12** to **15**) did not yield noticeable changes in potency. Larger alterations in the size of R_1 (like ethyl to phenyl as in **14** to **17**) led to a modest (~2-fold) improvement of potency.

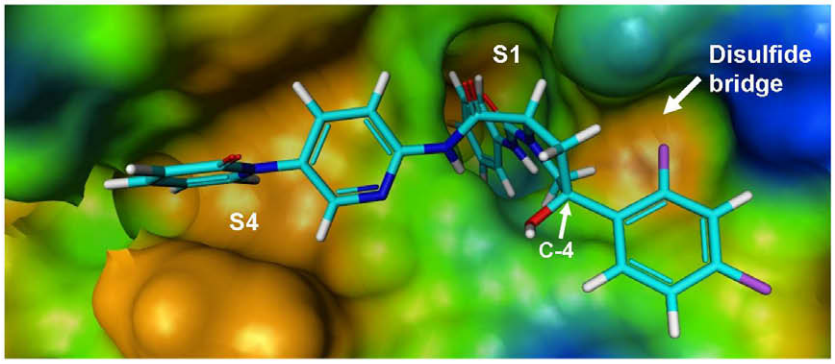
The anticoagulant activity of several compounds was evaluated via prolongation of fibrin clot formation in human plasma as measured by the doubling of prothrombin time (2 × PT) and the results are shown in Table 1. Several compounds achieved doubling of PT at concentrations of <1 μM. As previously observed by other groups,[18] the anticoagulant activity seemed to be correlated with



3a (**5**)



3b (**17**)



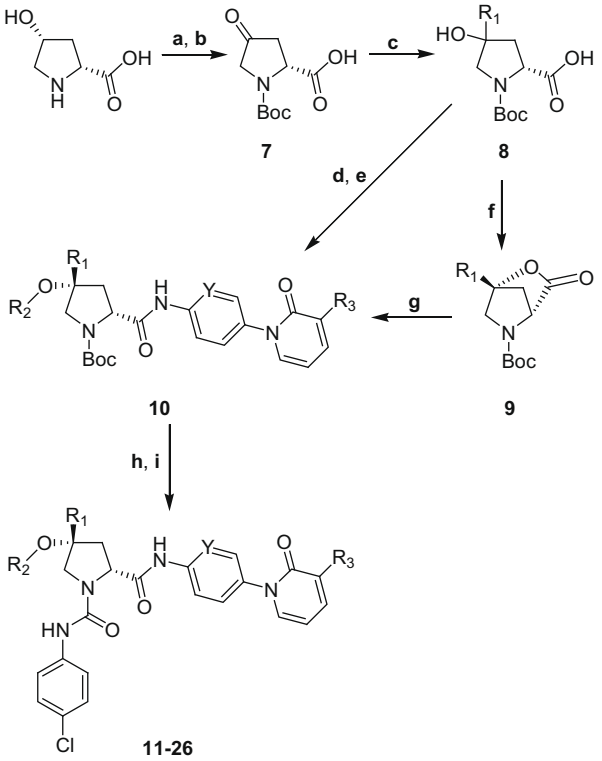
3c (**26**)

Figure 3. Co-crystal X-ray structures of FXa protein and **5** (white), **17** (magenta), and **26** (cyano) in the active site with a resolution of 2.3, 1.9, and 2.05 Å, respectively. Enzyme shown with Connolly surface (probe radius 1.4 Å). Surface color represents hydrophobicity, ranging from orange (lipophilic) to blue (hydrophilic).

the lipophilicity (as measured by clog*D* at pH 7.4) of the new compounds. It was observed that more lipophilic compounds like **17** (clog*D* = 2.34) required FXa inhibitory potency <0.1 nM to achieve anticoagulant activity of <1 μM.

At first glance, replacing the C-4 hydrogen with other more lipophilic groups appeared to positively impact the PK profile. For example, comparing **5** with **13**, an encouraging increase in half-life was achieved by replacing the C-4 hydrogen with an ethyl group. However the effect of C-4 substitution on half-life is not straightforward since the rat IV cassette half-life of **13** was surprisingly long when compared to **14** and **17**. Unfortunately, this apparently long half-life for **13** was not validated in a definitive rat single dose IV PK study ($t_{1/2}$ = 2 h). While **17** had a shorter half life than **13** ($t_{1/2}$ = 4 h versus 5.2 h, respectively) the half-life of **17** ($t_{1/2}$ = 4 h) in single dosed rat was approximately twice that of **5** ($t_{1/2}$ = 2 h).[10] Due to the increase in both half-life and potency, the 4-hydroxy-4-aryl substitution pattern became the focus of further research.

As seen from **6** (Table 1), replacing the 4-fluorophenyl moiety with 2-aminopyridine while maintaining the same C-4 substitution as in **5** caused a 10-fold reduction in binding potency (IC_{50} = 4.60 nM versus 0.57 nM, respectively). However, the discovery of **17**, with a 7-fold increase in potency and increase in half-life compared to **5**, enabled the combination of the 4-hydroxy-4-phenylpyrrolidine core with the 2-aminopyridine P4 binding moiety. This combination surprisingly resulted in an inhibitor with subnanomolar potency (**18**, Table 2). While **18** had a shorter rat half life than **17**, the 2-aminopyridine replacement in **18** resulted in a decrease in clearance and an increase in the volume of distribution in rat as compared to **5** (6.73 mL/min/kg and 1490 mL/kg vs 12 mL/min/kg and 1100 mL/kg, respectively). In addition, **18** was measured to have a rat oral bioavailability of 47%. As a result, the 2-aminopyridine P4 binding moiety of **18** became a focus of the discovery effort due to the opportunity to achieve a reduction in molecular weight and lipophilicity.



Scheme 1. General route to 4,4-disubstituted pyrrolidine FXa inhibitors. Reagents and conditions: (a) *cis*-hydroxy-D-proline, Boc_2O, 1 M NaOH, THF, 0 °C to room temperature, 81%; (b) trichloroisocyanuric acid, TEMPO, CH_2Cl_2, 0 °C to room temperature, 97%; (c) R_1MgBr, THF, for R_1 = Ph, 53%; (d) 1-(4-amino-3-fluorophenyl)pyridin-2(1*H*)-one, 1-(4-amino-3-fluorophenyl)-3-methylpyridin-2(1*H*)-one, or 1-(6-aminopyridin-3-yl)pyridin-2(1*H*)-one,[10] EEDQ, Et_3N, $CHCl_3$, reflux; (e) R_2X, NaH, THF; (f) BOP, Et_3N, DMF, for R_1 = Ph, quantitative yield; (g) P4 amine (see step d), trimethylaluminum, toluene, 0 °C to 100 °C, for R_1 = Ph, 49%; (h) TFA, CH_2Cl_2 or HCl, MeOH; (i) 4-chlorophenylisocyanate, saturated sodium bicarbonate, CH_2Cl_2 or Et_3N, CH_2Cl_2.

Surprisingly, it was found that while **18** had an increase in rat half-life compared to **5**, the half-life of **18** in dog was actually lower (2.9 h for **18** vs 4.9 h for **5**). The decrease in dog half-life was hypothesized to be a result of the added phenyl ring providing a site of metabolism. Small ring substituents, such as methyl or halogens, were targeted in an attempt to hinder possible metabolism (Table 2). First, methyl-substituted phenyl rings were investigated. Accordingly, the 3-methyl **20** showed a decrease in both potency and half-life. Both the 4-methyl **19** and 2-methyl **21** had increased in vitro potency with decreased half-life. The methyl group itself could be a site of metabolism, therefore the 2-trifluoromethyl analog **22** was examined. This change did not show a significant increase in half-life. A further gain in potency was achieved with the 2-chloro substituent incorporated in **23** (IC_{50} = 0.02 nM). An X-ray co-crystal structure later confirmed this chloro was binding in the disulfide bridge region as suggested by the original modeling studies. Compound **23** had a rat half-life similar to that of **5**, however the half-life in the dog was less than 1 h. Several fluoro-substituted phenyl analogs were examined. Similar to **19**, the 4-fluoro group of **24** increased potency but not half-life. Retaining the 4-fluoro group and incorporating a 3-fluoro group in **25** showed increased rat half-life relative to **24**, but not to the extent needed for predicted QD dosing. Finally, the 2,4-difluoro analog **26** demonstrated an increase in rat half-life (3.3 h) and a substantial increase in the dog half-life (7.3 h) as determined by IV cassette studies (12.6 h in single dose dog experiments, Table 3). In addition, the 2,4-difluorophenyl ring was found to increase the potency 5-fold (FXa IC_{50} = 0.10 nM) versus the unsubstituted phenyl **18**. The rat and dog oral bioavailabilities for **26** were 19% and 24%, respectively (Table 3). Clearance was low in both rat and dog with volumes of distribution slightly greater than the volume of total body water suggesting moderate tissue distribution. Utilizing allometric scaling calculations,[19] **26** was estimated to have a human half-life of 23 h, and human oral bioavailability was predicted to be 23%. In addition, **26** was measured to have >10,000-fold selectivity based upon K_i determinations against a number of related serine proteases (activated protein C, plasmin, thrombin, tissue plasminogen activator, and trypsin). Furthermore, **26** showed considerably weak inhibition of four of the major drug-metabolizing CYPs at 3 μM (CYP1A2: 0%; CYP2C9: 9.53%; CYP2D6: 0.5%; CYP3A4: 3.5%).[20]

The increased FXa inhibitory potency obtained with the monofluorinated **24** and di-fluorinated **25** and **26** led us to review the potential formation of polar interactions between these compounds and the protein (fluorine–protein interactions).[21] The X-ray crystal structure of **26** was used to look for this type of interactions. Although, it has been recognized that distinct fluorophilic environments in proteins include the multipolar C–F···C=O, C–F···H–N and C–F···H–Cα interactions with peptide bonds, particularly those in hydrophobic environments, the C-2 fluorine atom of **26** is located away from the backbone atoms of the enzyme (>3.9 Å distance), pointing into the side chain amide residue of Gln192 (F···N distance: 3.6 Å). Interaction between C–F and side-chain amide residues of Gln and Asn have been observed.[22] The guanidinium group of Arg have also been reported to interact with C–F.[22] Interestingly, the C-2 fluorine atom of **26** is also located in the vicinity of the guanidinium group of Arg143 (distance between F and the central C of the guanidinium group: 4.1 Å). On the basis of this analysis, it appears that introduction of the 2,4-difluorophenyl group into the lipophilic subpocket may lead to the formation of a number of fluorine–protein interactions with the side chains of residues Arg143 and Gln192 that ultimately had a favorable effect on the binding affinity.

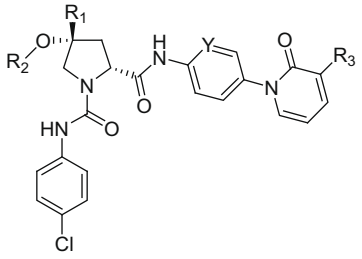
The in vivo antithrombotic effect of **26** was evaluated in a rabbit arteriovenous (AV) shunt model of thrombosis. The model employed is a modification of the method of Wong et al.[23] with thrombus weight used as the primary endpoint of this efficacy study. The antithrombotic effect is expressed as the percent reduction of thrombus weight. The calculation is simply the difference in thrombus weight between the baseline thrombus weight and treated subject (i.e., the thrombus weight from the first shunt) divided by the baseline thrombus weight. The percent reductions in thrombus weight were 38 ± 12%, 74 ± 8%, and 93 ± 1% at the doses of 1, 3, and 10 μg/kg/min via intravenous administration, respectively. The relationship between plasma concentrations and thrombus mass was modeled and **26** was determined to have an EC_{50} = 118 nM. Subsequent studies following oral administration at doses of 10 and 30 mg/kg produced a dose-dependent reduction in thrombus weight of 56 ± 6% and 84 ± 6% respectively. These results indicated that **26** produced a reliable dose-dependent antithrombotic effect in the rabbit A–V shunt model of thrombosis by both intravenous and oral administration.

The species-dependent inhibition of **26** towards purified FXa was in agreement with the results previously reported for **5**.[10] Inhibition of human FXa (IC_{50} = 0.211 nM) by **26** was similar to rabbit (IC_{50} = 0.108 nM) but greater than dog (IC_{50} = 0.887 nM) and rat (IC_{50} = 1.32 nM). These results provided further support for the use of rabbit as an appropriate animal model for the pharmacological characterization of this inhibitor.

5. Conclusions

To improve upon previously discovered orally-active, direct FXa inhibitors, we designed **26**, containing a (2*R*,4*S*)-4-

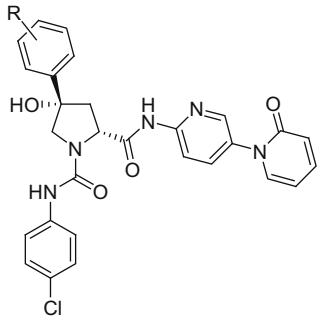
Table 1
In vitro FXa binding, half-life, and 2 × PT data



Compound	R_1	R_2	R_3	Y	FXa IC_{50}[a] (nM)	Rat $t_{1/2}$[b] (h)	2 × PT (μM)	clog D[d]
5	H	Me	H	C–F	0.57	2.6 (2.0)[c]	0.58	1.04
6	H	Me	H	N	4.60	2.0	0.55	0.89
11	Me	H	Me	C–F	0.54	1.6	1.08	1.53
12	Me	Me	H	C–F	0.31	1.7	N.D.	1.58
13	Et	H	Me	C–F	0.50	5.2	1.39	2.06
14	Et	H	H	C–F	0.18	2.4	0.50	1.47
15	Et	Me	H	C–F	0.22	1.7	N.D.	2.11
16	Et	Et	H	C–F	0.05	1.4	N.D.	2.64
17	Ph	H	H	C–F	0.08	4.0[c]	0.73	2.34

[a] IC_{50} values are means of multiple measurements ($n \geqslant 2$) according to Ref. 10.
[b] Determined by rat IV cassette studies unless noted otherwise.
[c] Determined by rat IV singleton studies. Values for **5** were taken from Ref. 10.
[d] clog D values were calculated using ACD/Log D Suite, v. 9.03, Advanced Chemistry Development, Inc: Toronto, Ontario, Canada.

Table 2
In vitro FXa binding, and PK parameters



Compound	R	FXa IC_{50}[a] (nM)	$t_{1/2}$[b] (h)	Cl[b] (mL/min/kg)	V_{dss}[b] (mL/kg)	clog D[e]
5		0.57	2.0[c] (4.9)[d]	12[c]	1100[c]	1.04
18	H	0.50	2.9[c] (2.9)	6.73[c]	1490[c]	2.19
19	4-Me	0.18	1.8	10.3	1580	2.65
20	3-Me	1.20	1.4	6.78.	438	2.65
21	2-Me	0.11	1.3	5.12	468	2.65
22	2-CF_3	0.34	1.9 (1.0)	2.89	393	2.76
23	2-Cl	0.02	2.9 (0.8)	4.16	923	2.79
24	4-F	0.16	1.8	9.44	1970	2.24
25	3,4-Di-F	0.08	2.1 (2.1)	13.1	1600	2.21
26	2,4-Di-F	0.10	3.3 (7.3)	7.64	1810	2.33

[a] IC_{50} values are means of multiple measurements ($n \geqslant 2$) according to Ref. 10.
[b] Determined by rat IV cassette unless otherwise noted, values in parentheses under $t_{1/2}$ are results of dog IV cassette studies.
[c] Determined by rat IV singleton studies.
[d] Determined by dog IV singleton studies. Values for **5** were taken from Ref. 10.
[e] clog D values were calculated using ACD/Log D Suite, v. 9.03, Advanced Chemistry Development, Inc: Toronto, Ontario, Canada.

hydroxy-4-(2,4-difluorophenyl)-pyrrolidine scaffold, that showed a marked increase in half-life in both rats and dogs when compared to our previously described orally efficacious **5**.[10] The predicted human half-life of **26** (23 h) modeled for once-daily dosing with an estimated peak-to-trough ratio of two. In the rabbit AV shunt model of thrombosis, both IV and oral administration of **26** produced a dose-dependent reduction in thrombus weight.

6. Experimental

6.1. General chemistry

All chemicals, reagents and solvents were purchased from commercial sources (e.g., Aldrich Chemical Co., Inc., Milwaukee, WI; Mallinckrodt Baker, Inc., Paris, KY, etc.) where available and used without further purification. All final compounds were

Table 3
Pharmacokinetic parameters of **26** following single-dose administration in male animals

Species	Dose (mg/kg)	Route	C_{max} (ng/mL)	T_{max} (h)	AUC_{inf} (ng*h/mL)	Cl (mL/min/kg)	V_{dss} (L/kg)	$t_{1/2}$ (h)	F[d] (%)
Rat[a]	0.5 ($N = 2$)	IV	—	—	978	9.5	2.4	3.3	—
Rat	3[c] ($N = 3$)	PO	236	1.7	1010	—	—	—	19
Dog[b]	0.5 ($N = 3$)	IV	—	—	4870	2.1	2.0	12.6	—
Dog	3[c] ($N = 2$)	PO	397	2.0	6450	—	—	—	24

[a] Rat species = Sprague Dawley.
[b] Dog species = Beagle.
[c] Dosed as a suspension of the final crystalline form.
[d] F = Absolute oral (PO) bioavailability.

characterized by proton nuclear magnetic spectroscopy (^{1}H NMR) with a 400 MHz Varian spectrometer and mass spectrometry (MS) using atmospheric pressure chemical ionization (APCI) or electron scatter (ES) ionization sources. All final compounds were determined to be consistent with the proposed structure by ^{1}H NMR and MS. Microanalyses were performed by Quantitative Technologies Inc. and were within 0.4% of the calculated values. All final compounds were purified by silica gel flash chromatography. A typical eluting system consisted of an ethyl acetate (100%)–ethyl acetate/methanol (90%:10%) gradient. All final compounds were greater than 95% pure as determined by analytical reverse-phase HPLC (Vydac 218TP54 C18 column; Mobile phase A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile). The synthesis for compounds **5** and **6** has been previously published.[10] The synthesis of **14**, **15**, and **18** exemplifies the typical conditions utilized for the preparation of **11**–**26** and is detailed below. For intermediates **8–10**, a single compound number represents several distinct intermediates containing different R_1 and R_2 groups. Where this occurs, the R groups are defined (e.g., **8,** R_1 = ethyl). Alternative conditions for the large scale preparation of intermediates **8** (R_1 = 2,4-difluorophenyl) and **10** (R_1 = 2,4-difluorophenyl, R_2 = H) are described following the general conditions.

6.1.1. (*R*)-1-(*tert*-Butoxycarbonyl)-4-oxopyrrolidine-2-carboxylic acid (7)

6.1.1.1. Step 1: (2*R*,4*R*)-1-(*tert*-butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic acid. A suspension of *cis*-hydroxy-D-proline (30.0 g, 229 mmol) in 300 mL THF was cooled to 0 °C, added 1 M NaOH (275 mL, 275 mmol) followed by the portionwise addition of di-*tert*-butyl-dicarbonate (49.9 g, 229 mmol). The cooling bath was removed and the resulting solution was stirred at ambient temperature overnight. The volume was reduced in vacuo, acidified with 1 M HCl, then extracted twice with ethyl acetate. Washed combined organics with brine, dried with anhydrous magnesium sulfate, filtered and concentrated to yield (2*R*,4*R*)-1-(*tert*-butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic acid as an oil that solidifies over time. (42.6 g, 81%) ^{1}H NMR (DMSO-d_6, δ) 4.11–4.24 (m, 1H), 4.02–4.11 (m, 1H), 3.39–3.54 (m, 1H), 3.02–3.14 (m, 1H), 2.19–2.37 (m, 1H), 1.72–1.84 (m, 1H), 1.26–1.41 (m, 9H); MS: APCI (AP−): 230.1 $(M-H)^+$.

6.1.1.2. Step 2: (*R*)-1-(*tert*-butoxycarbonyl)-4-oxopyrrolidine-2-carboxylic acid (7). (2*R*,4*R*)-1-(*tert*-Butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic acid (19.1 g, 82.6 mmol) was then dissolved in 400 mL dichloromethane, added trichloroisocyanuric acid (19.2 g, 82.6 mmol), then cooled the suspension to 0 °C. Added TEMPO (0.65 g, 4.1 mmol) and continued stirring at 0 °C for 30 min. The cooling bath was removed and the solution was stirred at ambient temperature for 2.5 h. Added 200 mL water and stirred for 15 min. The organics were removed in vacuo, diluted with 300 mL ethyl acetate, then filtered through a plug of Celite, washing with ethyl acetate. The filtrate was acidified with 40 mL of 1M HCl, separated layers, washed organics with water (4 × 200 mL), brine (100 mL), dried with magnesium sulfate, filtered, and concentrated to yield **7** as a white solid (18.4 g, 97%): ^{1}H NMR (DMSO-d_6, δ) 11.10 (s, 1H), 4.48 (t, J = 8.42 Hz, 1H), 3.68–3.85 (m, 1H), 3.56–3.67 (m, 1H), 2.96–3.15 (m, 1H), 2.46–2.51 (m, 1H), 1.35 (m, 9H); MS: APCI (AP−): 228.1 $(M-H)^+$.

6.1.2. 1-(*tert*-Butoxycarbonyl)-4-ethyl-4-hydroxypyrrolidine-2-carboxylic acid (8, R_1 = ethyl)

A solution of **7** (6.3 g, 28 mmol) in 100 mL anhydrous THF under an argon atmosphere was first cooled to −78 °C, then added a 1 M solution of ethylmagnesium bromide (60 mL, 60 mmol) in THF dropwise over 30 min. Stirred the solution at −78 °C for 1 h, then removed cooling bath and stirred at ambient temperature overnight. The solution was quenched with 30 mL of satd ammonium chloride. Acidified the solution with 1.2 M HCl, extracted with ethyl acetate, dried combined organics with anhydrous magnesium sulfate, filtered and concentrated in vacuo. The resulting dark oil was purified by column chromatography eluting with a gradient of $CHCl_3$–MeOH–AcOH (98:2:2 to 92:6:2). Combined and concentrated desired fractions to yield **8** (R_1 = ethyl) as an approximate 50:50 mixture of diastereomers. MS: APCI (AP−): 258.2 $(M-H)^+$.

6.1.3. Large scale preparation of (2*R*,4*S*)-1-(*tert*-butoxy carbonyl)-4-(2,4-difluorophenyl)-4-hydroxypyrrolidine-2-carboxylic acid 8 (R_1 = 2,4-difluorophenyl)

To a flask equipped with a mechanical stirrer, nitrogen inlet, and thermocouple was added 2 M isopropyl magnesium bromide in THF (497 mL, 996 mmol) followed by THF (100 mL). The solution was cooled to −26 °C and 2,4-difluorobromobenzene in THF (180 mL) was added dropwise maintaining an internal temperature below −14 °C over approximately 30 min. The solution was stirred for 1 h warming to −7 °C at which point **7** (95 g, 414 mmol) was charged as a solid in portions while maintaining an internal temperature below 0 °C. After complete addition the reaction was stirred at 0 °C for an additional hour then cooled to −30 °C. To the reaction was added 3 N HCl (240 mL). The aqueous layer was extracted with THF (250 mL), the organic layers combined and condensed to an off white solid which was carried on without purification.

6.1.4. *tert*-Butyl 4-ethyl-2-((2-fluoro-4-(2-oxopyridin-1(2*H*)-yl)phenyl)carbamoyl)-4-hydroxypyrrolidine-1-carboxylate (10, R_1 = ethyl, R_2 = H, R_3 = H, Y = C–F)

The diastereomeric mixture **8** (R_1 = ethyl, 0.52 g, 1.9 mmol) was dissolved in 10 mL chloroform, followed by the addition of 1-(4-amino-3-fluorophenyl)pyridin-2(1*H*)-one[10] (0.39 g, 1.9 mmol), triethylamine (0.8 mL, 5.7 mmol), and EEDQ (0.71 g, 2.9 mmol). The solution was refluxed overnight. The solution was cooled to ambient temperature, diluted with ethyl acetate, washed with 1.2 M HCl, satd sodium bicarbonate, brine, dried with anhydrous magnesium sulfate, filtered and concentrated in vacuo. Purified resulting material by column chromatography (0–10% MeOH in ethyl acetate). Combined and concentrated desired fractions to yield **10** (R_1 = ethyl, R_2 = H, R_3 = H, Y = C–F) as an approximate 50:50 mixture of diastereomers. (0.41 g, 47%) MS: APCI (AP−): 458.1 $(M-H)^+$.

6.1.5. (2*R*,4*R*)-N^1-(4-Chlorophenyl)-4-ethyl-N^2-(2-fluoro-4-(2-oxopyridin-1(2*H*)-yl)phenyl)-4-hydroxypyrrolidine-1,2-dicarboxamide (14)

To **10** (R_1 = ethyl, R_2 = H,, R_3 = H, Y = C–F, 0.45 g, 1.0 mmol) in 20 mL DCM was added 10 mL TFA and stirred at ambient temperature for 1.5 h. The solution was concentrated in vacuo. Redissolved in 30 mL DCM, cooled to 0 °C, added triethylamine (0.70 mL, 5.1 mmol) slowly, followed by 4-chlorophenylisocyanate (0.16 g, 1.0 mmol) which was predissolved in 3 mL DCM. The cooling bath was removed and the solution was stirred at ambient temperature for 1 h. The solution was concentrated in vacuo, redissolved in 150 mL ethyl acetate, washed with water, 10% citric acid, satd sodium bicarbonate, brine, dried with anhydrous magnesium sulfate, filtered and concentrated. Purified on a silica gel column eluting with gradient of 0–10% MeOH in ethyl acetate. Combined and concentrated pure fractions to yield **14** (0.21 g, 43%): ^{1}H NMR (DMSO-d_6, δ) 9.84 (s, 1H), 8.47 (s, 1H), 8.08 (t, J = 8.66 Hz, 1H), 7.59–7.64 (m, 1H), 7.52–7.57 (m, 2H), 7.45–7.51 (m, 1H), 7.42 (dd, J = 11.59, 2.32 Hz, 1H), 7.23–7.29 (m, 2H), 7.20 (dd, J = 7.81, 2.20 Hz, 1H), 6.41–6.48 (m, 1H), 6.25–6.33 (m, 1H), 5.09 (s, 1H), 4.56 (dd, J = 9.39, 3.29 Hz, 1H), 3.62 (d, J = 9.76 Hz, 1H), 3.43 (d, J = 10.00 Hz, 1H), 2.17–2.27 (m, 1H), 1.98 (dd, J = 13.18, 2.93 Hz, 1H), 1.59 (q, J = 7.32 Hz, 2H), 0.93 (t, 3H); MS: APCI (AP+): 499.1, 501.1 $(M+H)^+$, (AP−): 497.1, 499.1 $(M-H)^+$. Anal. Calcd for $C_{25}H_{24}ClFN_4O_4 \cdot 0.20H_2O$: C, 60.18; H, 4.85; N, 11.23. Found: C, 59.83; H, 4.70; H, 11.08.

6.1.6. (2*R*,4*S*)-1-(*tert*-Butoxycarbonyl)-4-hydroxy-4-phenylpyrrolidine-2-carboxylic acid (8, R_1 = phenyl, R_2 = H)

A solution of **7** (2.90 g, 12.7 mmol) in 30 mL anhydrous THF was added dropwise to a 1.0 M solution of phenylmagnesium bromide in tetrahydrofuran (35.0 mL, 35.0 mmol) at 0 °C. After addition was complete the solution was stirred at 0 °C for another 5 min, then quenched with 30 mL of satd ammonium chloride. The organics were removed in vacuo. Partitioned between ethyl acetate and 1.2 M HCl, washed organics with brine, dried with anhydrous magnesium sulfate, then filtered. The organics were concentrated to a volume of 50 mL, then slowly added hexanes to the stirring solution. A total of 150 mL hexanes were added. The resulting solid was filtered off, washing with hexanes to yield **8** (R_1 = phenyl) as a single diastereomer (2.07 g, 53%): ^{1}H NMR (DMSO-d_6, δ) 7.44 (d, J = 7.56 Hz, 2H), 7.27–7.36 (m, 2H), 7.23 (t, J = 6.83 Hz, 1H), 5.48 (s, 1H), 4.20–4.35 (m, 1H), 3.47–3.65 (m, 2H), 2.54–2.63 (m, 1H), 2.16–2.28 (m, 1H), 1.33–1.41 (m, 9H); MS: APCI (AP−): 306.2 $(M-H)^+$.

6.1.7. (1*R*,4*S*)-*tert*-Butyl 6-oxo-4-phenyl-5-oxa-2-aza-bicyclo[2.2.1]heptane-2-carboxylate (9, R_1 = phenyl)

To a solution of **8** (R_1 = phenyl, 0.50 g, 1.6 mmol) in 10 mL DMF was added triethylamine (0.34 mL, 2.4 mmol) and BOP (0.79 g, 1.8 mmol). Stirred at ambient temperature overnight. The solution was diluted with ethyl acetate, washed with 1 M HCl, sat. sodium bicarbonate, brine, dried with anhydrous magnesium sulfate, filtered and concentrated in vacuo to yield **9** (R_1 = phenyl) as a sticky oil (0.47 g, quant. yield): ^{1}H NMR (DMSO-d_6, δ) 7.49–7.60 (m, 2H), 7.39–7.49 (m, 3H), 4.63 (s, 1H), 3.76 (d, J = 10.49 Hz, 1H), 3.59 (dd, J = 10.49, 1.71 Hz, 1H), 2.41–2.62 (m, 2H), 1.41 (s, 9H).

6.1.8. (2*R*,4*S*)-*tert*-Butyl 4-hydroxy-2-((5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)carbamoyl)-4-phenylpyrrolidine-1-carboxylate (10, R_1 = phenyl, R_2 = H, R_3 = H, Y = N)

1-(6-Aminopyridin-3-yl)pyridin-2(1H)-one[10] (4.00 g, 21.3 mmol) was suspended in 75 mL anhydrous toluene under an argon atmosphere, cooled to 0 °C, then added 2.0 M trimethylaluminum in toluene (10.7 mL, 21.3 mmol) slowly. After addition the cooling bath was removed and stirred at ambient temperature for 10 minutes. Added **9** (R_1 = phenyl, R_2 = H, 2.47 g, 8.54 mmol) and heated solution to 100 °C for 5 h. The solution was cooled to ambient temperature then carefully poured into 200 mL ice water. Partitioned between 200 mL ethyl acetate and 100 mL 1 M HCl, separated layers, washed organics with 1 M HCl (2 × 200 mL), re-extracted aqueous layers with 150 mL ethyl acetate. Combined organic extracts, washed with brine, dried with anhydrous magnesium sulfate, filtered and concentrated in vacuo. Purified crude material by column chromatography (gradient = 0–10% MeOH in ethyl acetate) Combined and concentrated pure fractions to yield **10** (R_1 = phenyl, R_2 = H, R_3 = H, Y = N) as an off-white solid (2.0 g, 49%): ^{1}H NMR (DMSO-d_6, δ) 10.24–10.55 (m, 1H), 8.33 (s, 1H), 8.17 (d, J = 8.79 Hz, 1H), 7.80–7.95 (m, 1H), 7.61–7.71 (m, J = 5.62 Hz, 1H), 7.41–7.54 (m, 3H), 7.32 (t, J = 7.45 Hz, 2H), 7.23 (t, J = 7.32 Hz, 1H), 6.45 (d, J = 8.79 Hz, 1H), 6.29 (t, J = 7.32 Hz, 1H), 5.83–6.00 (m, 1H), 4.39–4.55 (m, 1H), 3.58–3.70 (m, 2H), 2.69 (dd, J = 13.43, 9.77 Hz, 1H), 2.21 (dd, J = 13.43, 3.42 Hz, 1H), 1.23–1.39 (m, 9H); MS: APCI (AP+): 477.2 $(M+H)^+$, (AP−): 475.2 $(M-H)^+$.

6.1.9. Large scale preparation of (2*R*,4*S*)-*tert*-butyl 4-(2,4-difluorophenyl)-4-hydroxy-2-((5-(2-oxopyridin-1(2*H*)-yl)pyridin2-yl)carbamoyl)pyrrolidine-1-carboxylate 10 (R_1 = 2,4-difluorophenyl, R_2 = H, R_3 = H, Y = N)

1-(6-Aminopyridin-3-yl)pyridin-2(1H)-one (23 g, 120 mmol) was charged to a 250 mL three-necked, round bottom flask equipped with a mechanical stirrer, thermocouple, and nitrogen inlet. To the flask was added anhydrous THF (140 mL) and stirring begun. The resulting grayish slurry was cooled to an internal temperature of 5 °C and a solution of 1 M lithium hexamethyldisilazide in THF (120 mL, 120 mmol) was added at a rate to keep the internal temperature below 10 °C. The resulting slurry was stirred for 25 min warming to 15 °C. The reaction was cooled to −2 °C and **9** (R_1 = 2,4-difluorophenyl, 20 g, 61 mmol) was added as a solid in 3 equiv portions keeping the internal temperature below 3 °C. The mixture was stirred for 1 h at which point the reaction was quenched with 100 mL of 3 N HCl keeping below 18 °C during the addition. The reaction was diluted with 185 mL of methyl *t*-butyl ether and the organic layer washed successively with 2 × 60 mL 1N HCl, 60 mL saturated aqueous sodium bicarbonate, and 60 mL saturated aqueous sodium chloride. The aqueous layers were extracted with a mixture of 110 mL methyl *t*-butyl ether/35 mL THF. The organic layers were combined and dried over anhydrous magnesium sulfate and condensed in vacuo to yield **10** (R_1 = 2,4-difluorophenyl, R_2 = H, R_3 = H, Y = N) as a tan solid (32 g, 102%). This product was used without further purification.

6.1.10. (2*R*,4*S*)-N^1-(4-Chlorophenyl)-4-hydroxy-N^2-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)- 4-phenylpyrrolidine-1,2-dicarboxamide (18)

A fresh solution of HCl in methanol was prepared by the addition of acetyl chloride (1.0 mL, 14.5 mmol) to 10 mL anhydrous methanol at 0 °C under an argon atmosphere. Removed cooling bath and stirred at ambient temperature for 5 min. This solution was then transferred via cannula to a flask containing **10** (R_1 = phenyl, R_2 = H, R_3 = H, Y = N, 0.46 g, 0.97 mmol). Stirred reaction at ambient temperature for 3.5 h. The solution was concentrated in vacuo. The resulting solid was used directly in the next step. MS: APCI (AP+): 377.2 $(M+H)^+$, (AP−): 375.2 $(M-H)^+$. The solid (0.97 mmol) was dissolved in 25 mL DCM and 10 mL satd sodium bicarbonate followed by the addition of 4-chlorophenylisocyanate (0.15 g, 0.97 mmol). The biphasic mixture was stirred at ambient temperature for 2 h. Diluted the mixture with 100 mL DCM, separated layers, then dried organics with anhydrous magnesium sulfate, filtered and concentrated in vacuo. Purified material by column chromatography (gradient = 0–10% MeOH in ethyl acetate). The pure

fraction were combined and concentrated in vacuo to yield **18** as a white solid (0.20 g, 39%): ^{1}H NMR (DMSO-d_6, δ) 10.33 (s, 1H), 8.55 (s, 1H), 8.37 (d, J = 3.17 Hz, 1H), 8.23 (d, J = 8.78 Hz, 1H), 7.91 (dd, J = 8.91, 2.81 Hz, 1H), 7.67–7.73 (m, 1H), 7.55–7.62 (m, 4H), 7.48–7.56 (m, 1H), 7.35–7.43 (m, 2H), 7.24–7.34 (m, 3H), 6.50 (dd, J = 9.39, 2.07 Hz, 1H), 6.28–6.40 (m, 1H), 5.95 (s, 1H), 4.73 (dd, J = 9.64, 2.56 Hz, 1H), 3.98 (d, J = 10.00 Hz, 1H), 3.86 (d, J = 10.00 Hz, 1H), 2.77 (dd, J = 13.05, 9.88 Hz, 1H), 2.27–2.38 (m, 1H). Anal. calcd for $C_{28}H_{24}ClN_5O_4{\cdot}0.65H_2O$: C, 62.08; H, 4.71; N, 12.93. Found: C, 62.00; H, 4.59; N, 12.63.

6.1.11. (2*R*,4*R*)-*N*^{1}-(4-Chlorophenyl)-4-ethyl-*N*^{2}-(2-fluoro-4-(2-oxopyridin-1(2*H*)-yl)phenyl)-4-methoxypyrrolidine-1,2-dicarboxamide (15)

The 50:50 diastereomeric mixture **8** (R_1 = ethyl, 0.50 g, 1.9 mmol) was dissolved in 10 mL anhydrous THF under an argon atmosphere, cooled to 0 °C, then added sodium hydride (60% in mineral oil, 0.19 g, 4.8 mmol) in portions. Removed cooling bath and stirred at ambient temperature for 15 min. Added methyliodide (0.24 mL, 3.9 mmol) in one portion. Stirred the reaction at ambient temperature overnight. Some starting material was observed by MS. Recooled solution to 0 °C, added methyliodide (0.25 mL, 4.0 mmol) followed by sodium hydride (0.1 g, 2.5 mmol). Stirred at ambient temperature overnight. The solution was carefully quenched with 1.2 M HCl, extracted with ethyl acetate, dried with anhydrous magnesium sulfate, filtered and concentrated in vacuo to yield a dark oil that was carried on without purification. MS: APCI (AP−): 272.1 $(M-H)^+$. This material was further elaborated to **15** in a similar manner as **14** (0.11 g, 24%): ^{1}H NMR (DMSO-d_6, δ) 9.54 (s, 1H), 8.51 (s, 1H), 8.00 (t, J = 8.66 Hz, 1H), 7.64 (dd, J = 7.08, 1.71 Hz, 1H), 7.55–7.60 (m, 2H), 7.47–7.54 (m, 1H), 7.44 (dd, J = 11.59, 2.32 Hz, 1H), 7.26–7.33 (m, 2H), 7.17–7.25 (m, 1H), 6.47 (d, J = 9.27 Hz, 1H), 6.27–6.35 (m, 1H), 4.58 (dd, J = 9.39, 3.05 Hz, 1H), 3.77 (d, J = 10.25 Hz, 1H), 3.44 (d, J = 10.49 Hz, 1H), 3.10 (s, 3H), 2.30 (dd, J = 13.66, 2.93 Hz, 1H), 2.09–2.19 (m, 1H), 1.57–1.79 (m, 2H), 0.88 (t, J = 7.44 Hz, 3H); MS: APCI (AP+): 513.1, 515.1 $(M+H)^+$, (AP−): 511.1,513.1 $(M-H)^+$. Anal. Calcd for $C_{26}H_{26}ClFN_4O_4{\cdot}0.17H_2O$: C, 60.52; H, 5.14; N, 10.86. Found: C, 60.17; H, 5.06; N, 10.60.

6.1.12. (2*R*,4*R*)-*N*^{1}-(4-Chlorophenyl)-*N*^{2}-(2-fluoro-4-(3-methyl-2-oxopyridin-1(2*H*)-yl)phenyl)-4-hydroxy-4-methylpyrrolidine-1,2-dicarboxamide (11)

This compound was synthesized in a similar manner to **14**. ^{1}H NMR (DMSO-d_6, δ) 9.85 (s, 1H), 8.46 (s, 1H), 8.06 (t, J = 8.78 Hz, 1H), 7.54 (d, 2H), 7.35–7.47 (m, 3H), 7.25 (d, J = 9.0 Hz, 2H), 7.18 (m, 1H), 6.21 (t, J = 6.83 Hz, 1H), 5.28 (s, 1H), 4.55 (dd, J = 9.03, 4.15 Hz, 1H), 3.61 (d, J = 9.52 Hz, 1H), 3.43 (d, J = 9.52 Hz, 1H), 2.20–2.25 (m, 1H), 2.03 (m, 4H), 1.31 (s, 3H); MS: APCI (AP+): 499.1,501.1 $(M+H)^+$, (AP−): 497.1,499.1 $(M-H)^+$. Anal. Calcd for $C_{25}H_{24}ClFN_4O_4{\cdot}0.14H_2O$: C, 59.88; H, 4.88; N, 11.17. Found: C, 59.85; H, 4.84; N, 11.09.

6.1.13. (2*R*,4*R*)-*N*^{1}-(4-Chlorophenyl)-*N*^{2}-(2-fluoro-4-(2-oxopyridin-1(2*H*)-yl)phenyl)-4-methoxy-4-methylpyrrolidine-1,2-dicarboxamide (12)

This compound was synthesized in a similar manner to **14**. ^{1}H NMR (DMSO-d_6, δ) 9.57 (s, 1H), 8.49 (s, 1H), 7.98 (t, J = 8.54 Hz, 1H), 7.61 (m, 1H), 7.55 (d, J = 9.02 Hz, 2H), 7.40–7.50 (m, 2H), 7.27 (d, J = 9.03 Hz, 2H), 7.18 (m, 1H), 6.45 (m, 1H), 6.28 (t, J = 5.37 Hz, 1H), 4.56 (m, 1H), 3.61 (d, J = 10.25 Hz, 1H), 3.45 (d, J = 10.00 Hz, 1H), 3.29 (s, 3H), 2.50 (m, 1H), 2.25 (m, 1H), 1.31 (s, 3H); MS: APCI (AP+): 499.1, 501.1 $(M+H)^+$, (AP−): 497.1,499.1 $(M-H)^+$. Anal. Calcd for $C_{25}H_{24}ClFN_4O_4{\cdot}0.32H_2O$: C, 59.49; H, 4.92; N, 11.10. Found: C, 59.10; H, 4.85; N, 10.96.

6.1.14. (2*R*,4*R*)-*N*^{1}-(4-Chlorophenyl)-4-ethyl-*N*^{2}-(2-fluoro-4-(3-methyl-2-oxopyridin-1(2*H*)-yl)phenyl)-4-hydroxypyrrolidine-1,2-dicarboxamide (13)

This compound was synthesized in a similar manner to **14** (0.090 g, 22%): ^{1}H NMR (DMSO-d_6, δ) 9.85 (s, 1H), 8.49 (s, 1H), 8.10 (t, J = 8.66 Hz, 1H), 7.57 (d, 2H), 7.35–7.52 (m, 3H), 7.28 (d, J = 8.78 Hz, 2H), 7.21 (d, J = 9.03 Hz, 1H), 6.23 (t, J = 6.83 Hz, 1H), 5.12 (s, 1H), 4.58 (dd, J = 9.64, 3.05 Hz, 1H), 3.64 (d, J = 9.27 Hz, 1H), 3.45 (d, J = 10.00 Hz, 1H), 2.18–2.29 (m, 1H), 2.03 (s, 3H), 1.95–2.01 (m, 1H), 1.61 (q, J = 7.40 Hz, 2H), 0.95 (t, 3H); MS: APCI (AP+): 513.1,515.1 $(M+H)^+$, (AP-): 511.1,513.1 $(M-H)^+$. Anal. Calcd for $C_{26}H_{26}ClFN_4O_4{\cdot}1.85H_2O$: C, 57.16; H, 5.48; N, 10.26. Found: C, 56.77; H, 5.43; N, 10.19.

6.1.15. (2*R*,4*R*)-*N*^{1}-(4-Chlorophenyl)-4-ethoxy-4-ethyl-*N*^{2}-(2-fluoro-4-(2-oxopyridin-1(2*H*)-yl)phenyl)pyrrolidine-1,2-dicarboxamide (16)

This compound was synthesized in a similar manner to **14**. ^{1}H NMR (DMSO-d_6, δ) 9.50 (s, 1H), 8.51 (s, 1H), 8.03 (t, J = 8.66 Hz, 1H), 7.59–7.64 (m, 1H), 7.52–7.58 (m, 2H), 7.45–7.51 (m, 1H), 7.42 (dd, J = 11.59, 2.32 Hz, 1H), 7.25–7.31 (m, 2H), 7.17–7.23 (m, 1H), 6.45 (dd, J = 9.27, 2.20 Hz, 1H), 6.24–6.31 (m, 1H), 4.57 (dd, J = 9.27, 2.93 Hz, 1H), 3.73 (d, J = 10.49 Hz, 1H), 3.42 (d, J = 10.49 Hz, 1H), 2.31 (dd, J = 13.05, 2.56 Hz, 1H), 2.11 (dd, J = 13.30, 9.39 Hz, 1H), 1.67–1.75 (m, 2H), 1.56–1.65 (m, 2H), 0.97 (t, J = 6.83 Hz, 3H), 0.87 (t, J = 7.44 Hz, 3H); MS: APCI (AP+): 527.2,529.2 $(M+H)^+$, (AP−): 525.2,527.2 $(M-H)^+$; HRMS ($[M+H]^+$) for $C_{27}H_{28}ClFN_4O_4$: calcd 527.1856; found 527.1852.

6.1.16. (2*R*,4*S*)-*N*^{1}-(4-Chlorophenyl)-*N*^{2}-(2-fluoro-4-(2-oxopyridin-1(2*H*)-yl)phenyl)-4-hydroxy-4-phenylpyrrolidine-1,2-dicarboxamide (17)

This compound was synthesized in a similar manner to **18**. ^{1}H NMR (DMSO-d_6, δ) 9.88 (d, J = 1.22 Hz, 1H), 8.54 (s, 1H), 8.16 (t, J = 8.66 Hz, 1H), 7.65 (dd, J = 7.20, 1.83 Hz, 1H), 7.54–7.61 (m, 4H), 7.43–7.55 (m, 2H), 7.40 (t, J = 7.56 Hz, 2H), 7.26–7.34 (m, 3H), 7.21–7.26 (m, 1H), 6.48 (d, J = 8.78 Hz, 1H), 6.27–6.35 (m, 1H), 4.73 (dd, J = 9.64, 2.81 Hz, 1H), 3.92–4.00 (m, 1H), 3.85 (d, J = 10.00 Hz, 1H), 2.76 (dd, J = 12.93, 9.76 Hz, 1H), 2.35 (dd, J = 12.93, 2.20 Hz, 1H); MS: APCI (AP+): 547.1,549.0 $(M+H)^+$, (AP-): 545.0,547.0 $(M-H)^+$. Anal. Calcd $C_{29}H_{24}ClFN_4O_4{\cdot}0.39$ ethyl acetate: C, 63.14, H, 4.70; N, 9.64. Found: C, 62.74, H, 4.45; N, 9.62.

6.1.17. (2*R*,4*S*)-*N*^{1}-(4-Chlorophenyl)-4-hydroxy-*N*^{2}-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)-4-*p*-tolylpyrrolidine-1,2-dicarboxamide (19)

This compound was synthesized in a similar manner to **18** (0.13 g, 37%): ^{1}H NMR ($CDCl_3$, δ) 10.58 (br s, 1H), 8.40 (d, 1H), 8.20 (d, 1H), 7.70 (d, 1H), 7.40–7.12 (m, 9H), 7.00 (br s, 1H), 6.62 (d, 1H), 6.30 (t, 1H), 5.28 (d, 1H), 4.84 (d, 1H), 3.92 (d, 1H), 3.78 (d, 1H), 2.79–2.60 (m, 2H), 2.34 (s, 3H); MS (ES^+): 544.04 $(M+H)^+$. Anal. calcd for $C_{29}H_{26}ClFN_5O_4{\cdot}0.5H_2O$: C, 62.99, H, 4.92, N, 12.66. Found: C, 63.04, H, 4.67; N, 12.28.

6.1.18. (2*R*,4*S*)-*N*^{1}-(4-Chlorophenyl)-4-hydroxy-*N*^{2}-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)-4-*m*-tolylpyrrolidine-1,2-dicarboxamide (20)

This compound was synthesized in a similar manner to **18** (0.21 g, 35%): ^{1}H NMR ($CDCl_3$, δ) 10.50 (br s, 1H), 8.35 (d, 1H), 8.26 (s, 1H), 7.73 (m, 1H), 7.44 (m, 1H), 7.35–7.11 (m, 9H), 6.90 (br s, 1H), 6.63 (d, 1H), 6.28 (m, 1H), 5.24 (s, 1H, OH), 4.91 (d, 1H), 3.90 (d, 1H), 3.79 (d, 1H), 2.70 (m, 2H), 2.35 (s, 3H); MS (ES^+): 544.01 $(M+H)^+$. Anal. Calcd for $C_{29}H_{26}ClN_5O_4$: C, 64.03, H, 4.82, N, 12.87. Found: C, 63.58, H, 4.89, N, 12.60.

6.1.19. (2*R*,4*S*)-N^1-(4-Chlorophenyl)-4-hydroxy-N^2-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)-4-*o*-tolylpyrrolidine-1,2-dicarboxamide (21)

This compound was synthesized in a similar manner to **18** (0.148 g, 49%): ^{1}H NMR ($CDCl_3$, δ) 8.35 (m, 2H), 7.84 (dd, 1H), 7.62 (m, 2H), 7.45 (m, 3H), 7.20 (m, 4H), 6.63 (dd, 1H), 6.48 (ddd, 1H), 4.76 (dd, 1H), 4.32 (dd, 1H), 3.91 (d, 1H), 2.97 (dd, 1H), 2.74 (d, 1H); MS (ES^+): 544 $(M+H)^+$. Anal. Calcd for $C_{29}H_{26}N_5O_4Cl{\cdot}0.6CHCl_3$: C, 57.74, H, 4.36, N, 11.37. Found: C, 57.88, H, 4.13, N, 11.34.

6.1.20. (2*R*,4*S*)-N^1-(4-Chlorophenyl)-4-hydroxy-N^2-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)-4-(2-(trifluoromethyl) phenyl)pyrrolidine-1,2-dicarboxamide (22)

This compound was synthesized in a similar manner to **18** (0.215 g, 39%): ^{1}H NMR ($CDCl_3$, δ) 10.40 (br s, 1H), 8.35 (d, 1H), 8.31 (d, 1H), 7.82 (d, 1H), 7.75 (dd, 1H), 7.71 (d, 1H), 7.57 (t, 1H), 7.47 (t, 1H), 7.45–7.18 (m, 6H), 6.74 (br s, 1H), 6.64 (d, 1H), 6.27 (t, 1H), 4.97 (d, 1H), 4.91 (s, 1H, OH), 4.12 (d, 1H), 3.89 (d, 1H), 2.99 (d, 1H), 2.85 (dd, 1H); MS (ES^+): 562.12 $(M+H)^+$. Anal. Calcd for $C_{29}H_{23}ClF_3N_5O_4$: C, 58.25, H, 3.88, N, 11.71. Found: C, 57.90, H, 3.69, N, 11.50.

6.1.21. (2*R*,4*S*)-4-(2-Chlorophenyl)-N^1-(4-chlorophenyl)-4-hydroxy-N^2-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)pyrrolidine-1,2-dicarboxamide (23)

This compound was synthesized in a similar manner to **18** (0.348 g, 40%): ^{1}H NMR (CD_3OD, δ 8.35 (m, 2H), 7.85 (dd, 1H), 7.75 (dd, 1H), 7.60 (m, 2H), 7.45 (m, 4H), 7.34 (m, 2H), 7.22 (m, 2H), 6.62 (dd, 1H), 6.47 (ddd, 1H), 4.90 (m, 1H), 4.28 (d, 1H), 4.15 (d, 1H), 3.28 (dd, 1H), 2.60 (d, 1H); MS (ES^+): 564 $(M+H)^+$. Anal. Calcd for $C_{28}H_{23}N_5O_4Cl_2{\cdot}0.7CHCl_3$: C, 53.19, H, 3.69, N, 10.80. Found: C, 52.92, H, 3.43, N, 10.61.

6.1.22. (2*R*,4*S*)-N^1-(4-Chlorophenyl)-4-(4-fluorophenyl)-4-hydroxy-N^2-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)pyrrolidine-1,2-dicarboxamide (24)

This compound was synthesized in a similar manner to **18**. ^{1}H NMR (DMSO-d_6, δ) 10.35 (s, 1H), 8.56 (s, 1H), 8.37 (d, J = 2.69 Hz, 1H), 8.22 (d, J = 8.79 Hz, 1H), 7.87–7.94 (m, J = 8.91, 2.56 Hz, 1H), 7.66–7.73 (m, J = 6.72, 2.08 Hz, 1H), 7.48–7.64 (m, 6H), 7.17–7.33 (m, 5H), 6.50 (d, J = 8.79 Hz, 1H), 6.35 (t, J = 7.45 Hz, 1H), 6.02 (s, 1H), 4.72 (d, J = 9.52 Hz, 1H), 3.96 (d, J = 10.01 Hz, 1H), 3.84 (d, J = 9.77 Hz, 1H), 2.70–2.81 (m, 1H), 2.25–2.36 (m, J = 13.43, 1.71 Hz, 1H); MS: APCI (AP+): 548.0, 551.0 $(M+H)^+$, (AP−): 546.0, 548.0 $(M-H)^+$. Anal. Calcd for $C_{28}H_{23}ClFN_5O_4{\cdot}0.23H_2O$: C, 60.91, H, 4.28, N, 12.68. Found: C, 60.52, H, 4.13, N, 12.46.

6.1.23. (2*R*,4*S*)-N^1-(4-Chlorophenyl)-4-(3,4-difluorophenyl)-4-hydroxy-N^2-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)pyrrolidine-1,2-dicarboxamide (25)

This compound was synthesized in a similar manner to **18** (0.35 g, 26%): ^{1}H NMR (DMSO-d_6, δ) 10.35 (s, 1H), 8.55 (s, 1H), 8.37 (d, 1H), 8.22 (d, J = 9.28 Hz, 1H), 7.91 (dd, J = 8.79, 2.69 Hz, 1H), 7.70 (dd, J = 6.84, 1.95 Hz, 1H), 7.39–7.66 (m, 6H), 7.26–7.32 (m, 2H), 6.46–6.54 (m, J = 9.28 Hz, 1H), 6.32–6.37 (m, 1H), 6.12 (s, 1H), 4.74 (dd, J = 9.65, 2.56 Hz, 1H), 3.81–3.98 (m, 2H), 2.76 (dd, J = 12.70, 9.77 Hz, 1H), 2.28–2.36 (m, 1H); MS: APCI (AP+): 566.0, 568.0 $(M+H)^+$, (AP-): 564.0, 565.0 $(M-H)^+$. Anal. Calcd for $C_{28}H_{22}ClF_2N_5O_4{\cdot}0.38H_2O$: C, 58.71, H, 4.01, N, 12.23. Found: C, 58.67, H, 3.66, N, 12.01.

6.1.24. (2*R*,4*S*)-N^1-(4-Chlorophenyl)-4-(2,4-difluorophenyl)-4-hydroxy-N^2-(5-(2-oxopyridin-1(2*H*)-yl)pyridin-2-yl)pyrrolidine-1,2-dicarboxamide (26)

This compound was synthesized in a similar manner to **18**. ^{1}H NMR (DMSO-d_6, δ) 10.3 (s, 1H), 8.5 (s, 1H), 8.3 (d, J = 2.3 Hz, 1H), 8.2 (d, J = 9.0 Hz, 1H), 7.9 (dd, J = 8.9, 2.6 Hz, 1H), 7.6 (dd, J = 6.9, 1.5 Hz, 1H), 7.5 (d, J = 9.0 Hz, 2H), 7.5 (dd, 1H), 7.2 (d, J = 9.0 Hz, 2H), 7.2 (d, J = 9.0 Hz, 1H), 7.1 (m, 1H), 6.4 (d, J = 9.2 Hz, 1H), 6.0 (s, 1H), 6.3 (m, 1H), 4.7 (dd, J = 9.0, 1.4 Hz, 1H), 3.9 (m, 2H), 2.8 (m, 1H), 2.4 (d, J = 14.0 Hz, 1H); MS: APCI (AP+): 566.0, 568.0 $(M+H)^+$. Anal. Calcd for $C_{28}H_{22}ClF_2N_5O_4{\cdot}0.40H_2O$: C, 58.67, H, 4.01, N, 12.22. Found: C, 58.29, H, 3.89, N, 12.12.

6.2. Enzyme inhibition assays

All enzyme inhibition assays (FXa, thrombin, trypsin, activated protein C, plasmin, and tissue plasminogen activator) were performed as previously described.[10]

6.3. X-ray crystallography

X-ray diffraction data from the crystals were collected at the Advanced Photon Source facility on beamline 17ID. Intensity data were measured at about −180 °C. One X-ray data set was collected for each crystal. Auto-indexing and processing of the measured intensity data were carried out with the HKL2000 software package.[24] The intensity data-collection statistics are summarized in Supplementary data Table 1 (see Supplementary data). The crystal structures of FXa complexes were solved by molecular replacement using MOLREP[25] with the FXa protein coordinates (PDB code 2PHB) as the search model. The molecular replacement solution was further optimized by rigid-body, coordinates and *B*-value minimization using REFMAC.[26] Calculated (2Fo–Fc) and (Fo–Fc) electron density maps were utilized for interactive fitting of protein structures into electron density using the COOT software program.[27] Placement of the ligands into electron density maps was carried out with X-LIGAND[28] implemented in QUANTA (Accelrys Inc.). The coordinates and structure factors may be found in the Protein Data Bank under ID code 2W3K for **17** and 2W3I for **26**.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.01.063.

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