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Guanylpiperidine Peptidomimetics: Potent and Selective bis-Cation Inhibitors of Factor Xa

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Abstract—A novel series of rigid P_3-guanylpiperidine peptide mimics **3**–**14** was designed as potential factor Xa and prothrombinase inhibitors. Incorporation into a P_2-gly-P_1-argininal motif led to highly potent and selective inhibitors. The synthesis and biological activities of these derivatives are reported herein.

As a key member of the trypsin class of serine proteases involved in blood coagulation, factor Xa catalyzes the penultimate step in this biochemical cascade, the formation of the serine protease thrombin. Factor Xa mediated thrombin formation occurs following the assembly of the prothrombinase complex, which is composed of factor Xa and the non-enzymatic cofactor factor Va assembled on an appropriate phospholipid surface such as activated platelets.[1,2] A variety of small molecule synthetic scaffolds that selectively inhibit factor Xa as well as prothombinase activity are currently receiving considerable attention and are emerging as potential therapeutic agents for the prevention and treatment of thrombotic vascular disease.[3]

The incorporation of bis-cation binding elements in the design of new targets has recently emerged as an active area of investigation,[3] and is based upon the topography of the factor Xa active site.[4] Compounds **1** and **2**, discovered during the initial stages of work in this area,[5,6] are potent inhibitors of factor Xa (IC_{50}'s of 35 and 0.9 nM, respectively); however, they suffer from mediocre to poor selectivity profiles, especially against the key digestive enzyme trypsin.

The application of peptidomimetic inhibitors that contain rigid heterocyclic motifs is currently an area of active investigation. Conformationally constrained peptidomimetics can contribute a favorable entropic component to binding in the enzyme active site relative to their highly flexible peptide counterparts. Recent studies in our laboratories demonstrated that incorporation of a rigidified P_1-3-guanylpiperidinylalaninal arginine surrogate into the appropriate peptidyl or peptidomimetic template led to a potent and highly selective series of novel thrombin inhibitors.[7] The S_3 pocket in factor Xa features the cylinder-like orientation of the aromatic residues Trp^{215}, Tyr^{99}, and Phe^{174}, constituting a site that can accommodate critical cation-π interactions.[4] In this investigation, rigidified arginine surrogates of the P_3 *d*-arginine residue were utilized in the design of inhibitors of factor Xa to enhance this cation-π site interaction (Fig. 1). The synthesis and biological activity of this family of P_3 *d*-arginine mimics incorporating 3-guanylpiperidinylalanine, 4-guanylpiperidinylalanine and 4-guanylpiperidinylglycine moieties is described in this letter.

Chemistry

Synthetic route to inhibitors 3a and 3b[8]

N-α-Boc-*d*-3-pyridylalanine (Synthetech, Albany, OR) was converted to the di-protected 3-guanylpiperidine **15** in two steps by hydrogenation followed by guanylation. The rigid Arg mimic **15** was coupled to glycine ethyl ester and deprotected with HCl to produce the dipeptide

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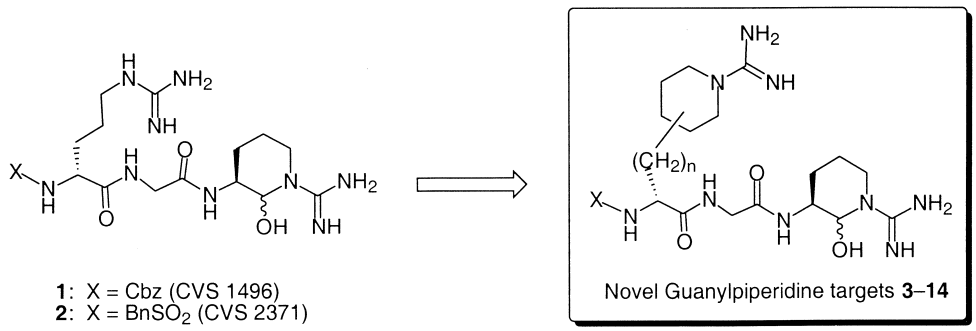


Figure 1. Strategy for the design of guanylpiperidine inhibitors **3**–**14**.

16. Dipeptide **16** was capped with benzylsulfonyl chloride and then hydrolyzed to afford the free acid **17**. Intermediate **17** was coupled to nitroArg ethyl aminal[9] using standard peptide coupling techniques to produce the fully elaborated compound **18**. Conversion of compound **18** to targets **3a**/**3b** was easily accomplished by hydrogenolysis of the Cbz and nitro protecting groups followed by mild acidic hydrolysis of the aminal. The diastereomeric pair **3a** and **3b** was separated during RP HPLC purification (Scheme 1).

Synthetic route to inhibitors 4 and 5

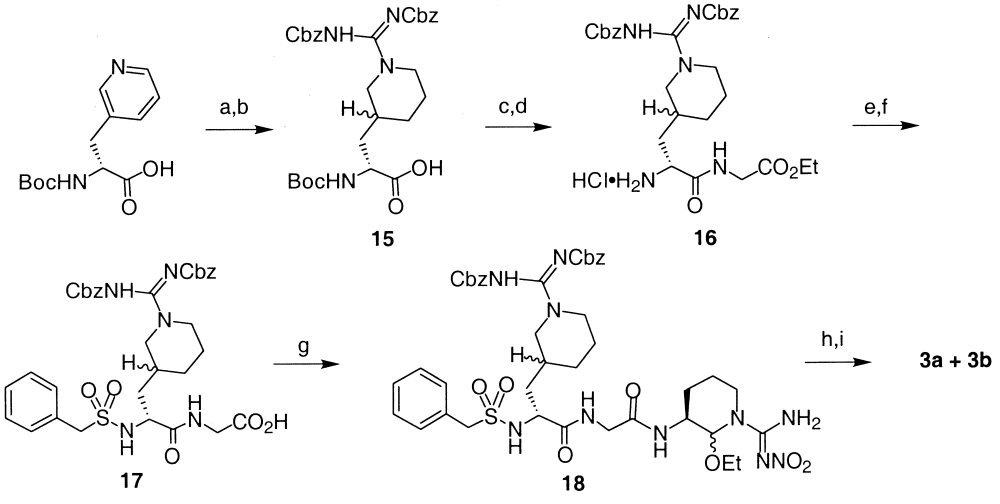
Intermediate **19** (Scheme 2) was prepared from compound **16** in four steps substituting 2-carbomethoxybenzylsulfonyl chloride for benzylsulfonyl chloride following the strategy used in Scheme 1 for inhibitors **3a** and **3b**. The methyl ester of compound **19** was subjected to lithium hydroxide hydrolysis to afford the free acid **20**. Intermediates **19** and **20** were each hydrolyzed to afford targets **4a**/**4b** and **5a**/**5b**, which were separated during the final RP HPLC purification.

Synthetic route to inhibitors 6–13

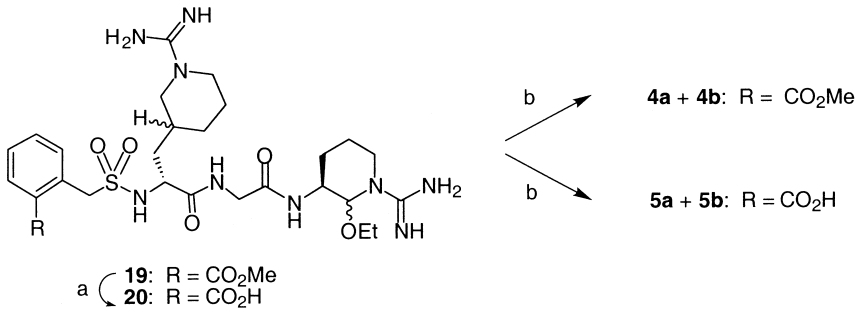
4-Guanylpiperidine targets **6**–**13** were prepared via protected dipeptide **22** in a similar manner to 3-guanylpiperidine targets **3**–**5**; however, the reaction sequence was changed to optimize yields (Scheme 3). In this case, compound **21** was prepared by coupling Boc-4-pyridylalanine to glycine methyl ester, and subsequent reduction to the piperidine and functionalization to the bis-Cbz-guanylpiperidine. To remove the Boc protection, compound **21** was then treated with HCl to deliver compound **22**, which was used to prepare targets **6**–**13** in a route analogous to the preparation of targets **3**–**5**.

Synthetic route to inhibitor 14

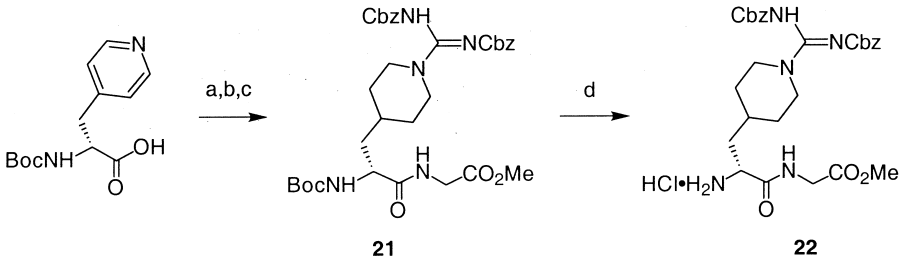
The preparation of the guanylpiperidylglycine intermediate **26** was accomplished in eight steps. The key step in the synthesis featured a modified Ugi reaction[10] between 4-pyridinecarboxaldehyde, formic acid, ethyl isocyanoacetate, and tetrapivaloyl-D-galactopyranosylamine following the Kunz protocol[11] and produced **23**



Scheme 1. Reagents and conditions: (a) H_2, PtO_2, EtOH, AcOH, H_2O, quant; (b) MeSC(NCbz)(NHCbz), DIEA, THF, Δ, 41%; (c) H-Gly-OEt, HCl salt, EDC, HOBt, DIEA, CH_3CN, 53%; (d) HCl, EtOH; (e) $BnSO_2Cl$, NMM, CH_3CN, 16–26% for two steps; (f) 1.0 N LiOH, EtOH; (g) EDC, HOBt, nitroargininal ethyl aminal•HCl, NMM, CH_3CN, 43–88% for two steps; (h) H_2, 10% Pd/C, EtOH, H_2O, HOAc; (i) 6.0 N HCl; preparative RP HPLC, 14% for **3a**, 24% for **3b**.



Scheme 2. Reagents and conditions: (a) 1.0 N LiOH, EtOH; (b) 6 N HCl; preparative RP HPLC purification, 35–55%.

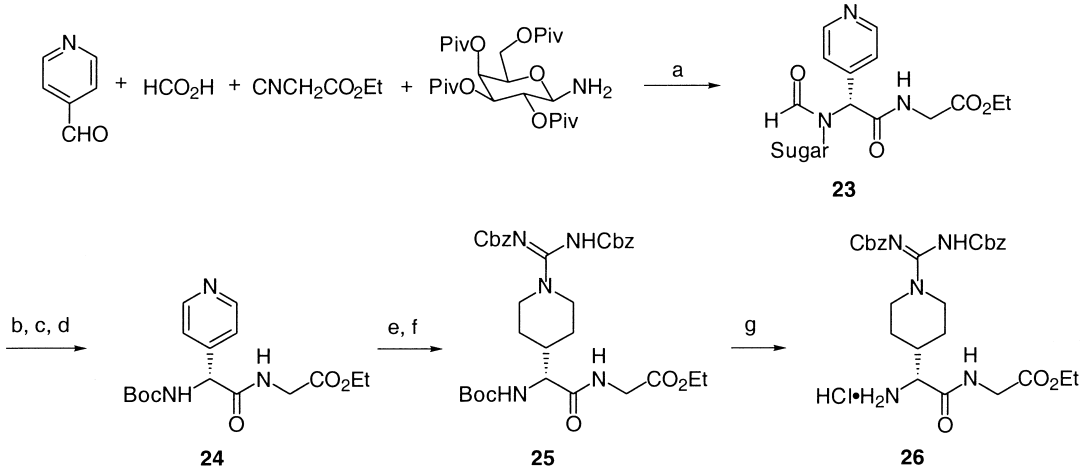


Scheme 3. Reagents and conditions: (a) H-Gly-OMe, HCl salt, EDC, HOBt, DIEA, CH_3CN, 97%; (b) H_2, PtO_2, EtOH, AcOH, H_2O, quant; (c) MeSC(NCbz)(NHCbz), DIEA, THF, Δ, 55%; (d) HCl, EtOAc, quant.

in excellent yield (Scheme 4). HPLC analysis of crude **23** indicated a 4:1 diastereomeric ratio at the newly created pyridylglycine α-methine center, with the desired α-(*R*)-stereochemistry predominating. Silica gel flash chromatogaphic purification readily effected diastereomer separation and provided pure α-(*R*)-**23**. Simultaneous hydrolysis of the *N*-formyl, ester and sugar moieties, followed by re-esterification and α-*N*-protection with Boc_2O afforded compound **24**. Platinum-catalyzed hydrogenation of the pyridyl moiety proceeded smoothly to provide the expected piperidine intermediate, which in turn was converted to the bis-Cbz-guanylpiperidine **25**. Acid-catalyzed cleavage of the α-*N*-Boc protecting group delivered advanced intermediate **26**, which was elaborated to the final target **14** via a protocol analogous to that described for the preparation of targets **3a** and **3b** above.

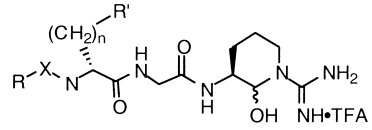
Results and Discussion

Fifteen bis-cation arginals **3**–**14** were prepared and evaluated for their ability to inhibit four relevant serine proteases (Table 1). In accordance with our design strategies, highly potent factor Xa inhibitors were discovered that were considerably more trypsin-selective than their corresponding non-rigid counterparts, vide supra. They also displayed favorable selectivity profiles against the fibrinolytic protease plasmin. Within the 3-guanylpiperidine class, the diastereomeric pairs **3**, **4** and



Scheme 4. Reagents and conditions: (a) $ZnCl_2$, Et_2O, THF, −30° to 0 °C, 96%, 81% α-(*R*)-isomer; (b) EtOH, HCl; H_2O; (c) EtOH, HCl; 80% for two steps; (d) Boc_2O, Et_3N, DMAP, dioxane, 23%; (e) H_2, PtO_2, EtOH, H_2O, HOAc; (f) MeSC(NCbz)(NHCbz), THF, 41% for two steps; (g) EtOH, HCl, quant.

Table 1. In vitro IC_{50} values (nM) of inhibitors **3**–**14** against human factor Xa, thrombin, plasmin and trypsin[a]



Compound	R	X	*n*	R′	Factor Xa	Thrombin	Plasmin	Trypsin
3a	Bn	SO_2	1	3-Guanylpiperidine	1.2	>2500	919	120
3b	Bn	SO_2	1	3-Guanylpiperidine	0.92	>2500	726	31
4a	(2′-CO_2Me)Bn	SO_2	1	3-Guanylpiperidine	1.08	>2500	894	124
4b	(2′-CO_2Me)Bn	SO_2	1	3-Guanylpiperidine	1.37	1100	1950	15
5a	(2′-CO_2H)Bn	SO_2	1	3-Guanylpiperidine	2.1	>2500	2500	240
5b	(2′-CO_2H)Bn	SO_2	1	3-Guanylpiperidine	4.2	352	>2500	33
6	Bn	SO_2	1	4-Guanylpiperidine	0.83	>2500	869	169
7	(2′-CO_2Me)Bn	SO_2	1	4-Guanylpiperidine	0.94	>2500	965	147
8	(3′-CO_2Me)Bn	SO_2	1	4-Guanylpiperidine	2.2	>2500	387	145
9	(2′-CO_2H)Bn	SO_2	1	4-Guanylpiperidine	5.7	>2500	2500	447
10	(3′-CO_2H)Bn	SO_2	1	4-Guanylpiperidine	4.3	>2500	231	211
11	(2′-Tetrazole)Bn	SO_2	1	4-Guanylpiperidine	2.6	>2500	872	537
12	(4′-CO_2Et)phenethyl	O(CO)	1	4-Guanylpiperidine	16[b]	>2500	1440	158
13	Me	O(CO)	1	4-Guanylpiperidine	23[b]	>2500	>2500	412
14	Bn	SO_2	0	4-Guanylpiperidine	1.1	>2500	678	25
Reference standards								
1	Bn	O(CO)		d-Arg	34.9	>2500	>2500	115
2	Bn	SO_2		d-Arg	0.9	>2500	1190	75

[a]Concentration of compounds **1**–**14** necessary to inhibit human enzymes (factor Xa, factor IIa, plasmin and trypsin) by the cleavage of the chromogenic substrates described in ref 13 by 50%.
[b]Estimated K_i for classical fast inhibition.[14]

5 demonstrated similar activities against factor Xa, but displayed different selectivity profiles. Targets **3a**, **4a** and **5a** appear to have better trypsin selectivity. The in vitro results for the 3-guanylpiperidinylalanine, 4-guanylpiperidinylalanine and 4-guanylpiperidinylglycine scaffolds indicate that high potency is retained in all three spatial permutations, suggesting that they indeed serve as efficient arginine surrogates. Furthermore, the mode of presentation of the P_3-cation to the S_4 binding pocket appears to have little effect on the relative in vitro activity within this group of analogues. In general, P_4-sulfonamide-containing targets display an increased potency against factor Xa when compared to the P_4-carbamate containing targets.

The assembly of the prothrombinase complex results in specific structural and kinetic changes in factor Xa, resulting in a 100 000-fold improvement in the catalytic efficiency of prothrombin activation by factor Xa.[12] The potency of our inhibitors was measured against the natural substrate prothrombin by the prothrombinase complex (Table 2). In most of the analogues examined, inhibitor potency against the prothrombinase complex was measured by using IC_{50} values and was 2- to 4-fold higher than the corresponding results using the amidolytic substrate with the uncomplexed factor Xa. Significantly, compounds **12** and **13**, both containing N-terminal carbamate caps, display different kinetic profiles for factor Xa and prothrombinase. These compounds display classical kinetics when inhibiting factor Xa, and slow-tight binding kinetics when inhibiting prothrombinase.[15] It may be inferred that for compounds **12** and **13** the aldehyde carbonyl does not interact covalently with the catalytic triad when complexed to free factor Xa, but does when complexed to the prothrombinase active site.

In vivo pharmacokinetic studies on bis-cations indicated substantial blood pressure/MAP effects during iv dosing that are attenuated by the presence of P_4-ester, P_4-acid or P_4-tetrazole functions. The iv dosing (5 mg/kg) of compound **6** produced a profound and prolonged MAP effect. This effect was attenuated in the dosing of **7**, and non-existent in the dosing of **9**.

Table 2. In vitro IC_{50} values (nM) of inhibitors against factor Xa and the prothrombinase complex (Ptase)

Compound	Factor Xa[a]	Ptase[b]	Ratio Ptase/FXa
3a	1.2	4.2	2.0
3b	0.92	3.3	3.6
6	0.83	3.1	3.7
9	5.3	12	2.3
10	16[c]	14	0.9
11	23[c]	69	3.0
Reference standards			
1a	35	7.9	0.23
1b	0.9	3.4	3.8

[a]Concentration of compounds necessary to inhibit the cleavage of the chromogenic substrate by human enzyme factor Xa described in ref 13 by 50%.
[b]Concentration of compounds necessary to inhibit the activation of human prothrombin in a preformed complex of factor Xa, factor Va and phospholipid vesicles as described in ref 16 by 50%.
[c]Estimated K_i for classical fast inhibition.[14]

Conclusion

A series of guanylpiperidine amino acids was designed and appended onto a P_1-argininal moiety resulting in the discovery of a novel class of highly potent and selective factor Xa inhibitors. From this work, compounds **6**, **7**, **9** and **11** emerged as leading candidates that combined both high in vitro potency versus factor Xa with desirable selectivity profiles towards other trypsin-like serine proteases. These scaffolds may find utility in the design and synthesis of new classes of drug targets, especially protease inhibitors which incorporate arginine-like residues, and may lead to inhibitors with improved levels of activity and/or selectivity.

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