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Design, Synthesis and Structure–Activity Relationship of a Series of Arginine Aldehyde Factor Xa Inhibitors. Part 1: Structures Based on the (D)-Arg-Gly-Arg Tripeptide Sequence

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Abstract—A series of arginine aldehyde inhibitors was designed as transition state (TS) analogues based on the known factor Xa specific substrate Cbz-D-Arg-Gly-Arg-pNA. $BnSO_2$-(D)Arg-Gly-Arg-H (**20**) was found to be the most potent and selective inhibitor of factor Xa and prothrombinase activity in this series.

Introduction

There is a continuing need for the development of novel anticoagulant agents for the treatment of cardiovascular diseases to replace existing therapy.[1–4] Direct and specific inhibition of the trypsin-like serine proteases in the coagulation cascade, especially thrombin, have been the focus of many efforts over the last decade to design novel anticoagulants. Thrombin occupies the final step in the conversion of fibrinogen to fibrin while also being the most potent platelet agonist and, therefore, plays a central role in both thrombosis as well as normal hemostasis. An attractive alternative to thrombin inhibition may be inhibition of the synthesis of thrombin itself. Prothrombin conversion is catalysed most efficiently by factor Xa in conjunction with factor Va in the prothrombinase complex which is primarily localized to activated platelet surfaces.[4]

We have utilized a transition state (TS) inhibitor approach to design specific, potent inhibitors of factor Xa recognizing that structural information found in good substrates of a target enzyme often afford potent TS inhibitors.[5] The relative rates of hydrolysis for two of the best known, commercially available, *para*-nitroanilide (pNA) factor Xa substrates are shown in Table 1. Both substrates reflect the natural preference of factor Xa for having an Arg-residue in P1 for the substrate primary specificity site and a Gly-residue in P2. The substrate S-2222 contains the negatively charged Glu in P3, as a well as an Ile residue in P4, reflecting the natural cleavage sequence found in prothrombin. Interestingly, S-2765, containing an unnatural D-Arg-residue in P3, has a higher relative rate of cleavage by factor Xa than S-2222 yet is the shorter tripeptide sequence. Both substrates show some 30- to 40-fold greater reactivity with factor Xa compared to thrombin, which is known to prefer a Pro-residue in P2. We therefore reasoned that the D-Arg-Gly-Arg sequence would be a good starting point for the preparation of TS analogues that might display both potent and specific inhibitory activity for factor Xa.

The use of arginine aldehydes as TS analogues inhibitors for serine proteases with trypsin-like specificity dates back to the work of Bajusz et al.[6] with their series of thrombin and trypsin inhibitors. More recently, Semple et al.,[7] have utilized argininals for potent and specific thrombin inhibitors. For preparation of argininals by solution phase, we utilized the reduction of an arginine lactam (Scheme 1a) as reported by Shuman et al.[8,9] The parent lactam **1** is readily prepared from Boc-Arg(Cbz)OH,[10] which is easily synthesized using Schotten–Baumann conditions employing Cbz-Cl and Boc-Arg. Coupling of the *N*-deprotected lactam with a suitably protected dipeptide gives the tripeptide lactams which can be reduced with LAH followed by careful removal of protecting groups by hydrogenation to afford the tripeptide argininals.[11]

Alternatively, multiple analogues of tripeptide argininals can also be prepared on solid phase utilizing a modification of the method of Webb et al.[12] (Scheme 1b).

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Table 1. Second order rate: kcat/km[a]

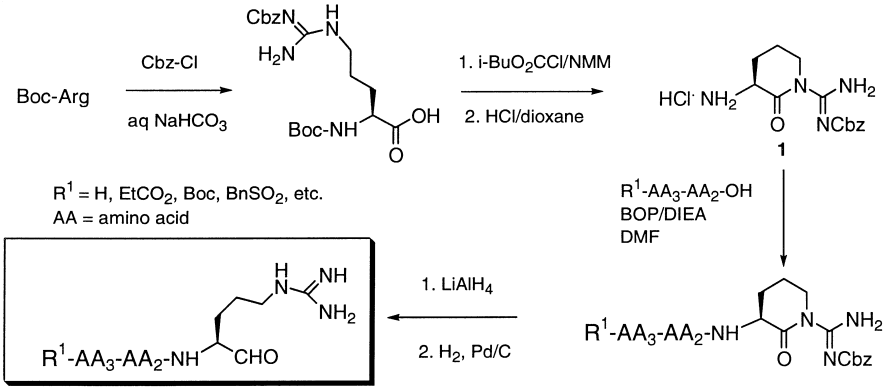
Substrate	Substrate number[b]	Factor Xa (bovine)	Thrombin
Bz-Ile-Glu-Gly-Arg-pNA	S-2222	0.35	0.018
Cbz-D-Arg-Gly-Arg-pNA	S-2765	2.27	0.106

[a]Rates are expressed in terms of L/μmols.
[b]Numbering is that used by the manufacturer: Chromogenix. S-2222 is supplied as a 50% mixture of the Glu(γ-OMe) and the acid although the methyl ester is some 30% less active.

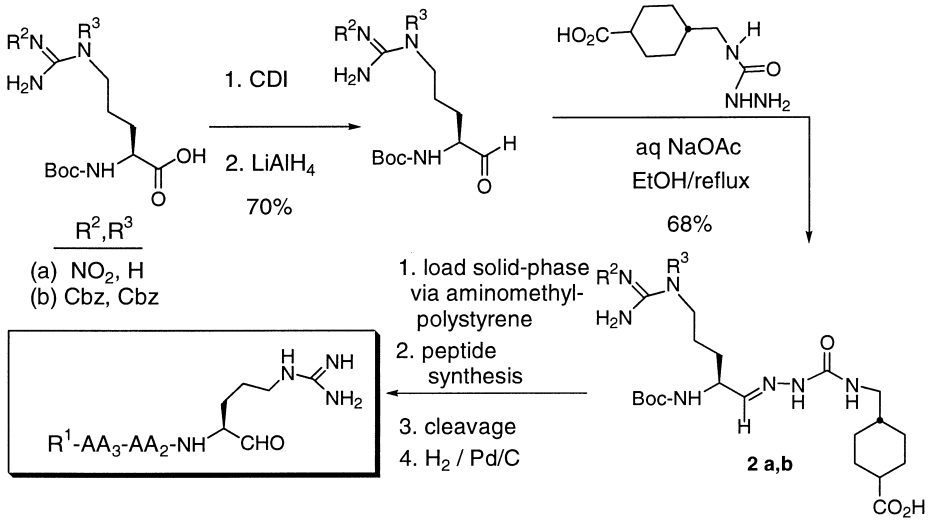
Following their procedure, Boc-Arg(NO_2)-semicarbazone linker **2a** (R^2, $R^3 = NO_2$, H) was synthesized and loaded onto resin, followed by Boc-deprotection and amino acid coupling using standard coupling techniques. Cleavage from the resin was achieved by an exchange reaction with formaldehyde. However, final removal of the nitro group from the Arg(NO_2) residue via reductive methods is often complicated by reduction of the aldehyde moiety. Arginine bis-Cbz-protection provides a more reductively labile protecting group giving consistently better yields of pure tripeptide aldehydes. Thus, Boc-Arg($Cbz)_2$-linker **2b** (R^2, R^3 = Cbz) was prepared in a similar fashion and utilized as shown in Scheme 1b.

Results and Discussion

Initially, because of synthetic accessibility, we prepared the N-terminal Boc-derivatized tripeptide analogues, rather than the Cbz-protected analogues, and identified Boc-D-Arg-Gly-Arg-H (**3**) as a very potent inhibitor of factor Xa with $IC_{50} = 50$ nM.[13] This analogue also displayed a desirable selectivity versus thrombin (Table 2). Curiously, this inhibitor gave poor activity toward the prothrombinase complex (abbreviated IIase).[14–16] Eight analogues were prepared in this initial series of P3 modifications (Table 2), which illustrates a stringent requirement at P3 for both residue configuration and the highly basic guanidino group. For example, replacement of P3 with L-Arg (**4**) results in a 40-fold loss of inhibitory activity while replacement with D-Lys (**5**) reduced activity by a factor of 200. The shorter and less basic sidechain residue, D-Orn (**6**), also loses significant inhibitory activity. Substitution of the basic guanidine functionality of the Arg sidechain with the urea-containing residue D-citruline (D-Cit, **7**) or with the amidine containing residue D-Orn((-C=NH)–CH_3) **8**, diminishes activity compared to the Boc-D-Arg-Gly-Arg-H compound. Either the dual hydrogen bond donating ability of guanidine is critical or a cation-π interaction,



Scheme 1a. Solution-phase synthesis via arginine lactam.



Scheme 1b. Modified solid-phase synthesis.

Table 2. Tripeptide argininals with P3 modifications

Analogue	Boc-P3-Gly-Arg-H	IC_{50}s (μM)			
		Xa	IIa	IIase	IIa/Xa
3	-DArg-	0.050	180	4.0	3600
4	-(L)Arg-	2.0	79	29	40
5	-DLys-	10	>100	68	>10
6	-DOrn-	12	>500	65	42
7	-DCit-	3.0	>100	12	33
8	-DOrn((C=NH)-CH_3)-	1.0	88	10	88
9	-DHar-	0.10	400	0.17	4000
10	-DHar$(Me)_4$-	3.0	>100	12	30

as originally proposed by Johnson,[17] may explain our results. Along those lines, D-homoarginine (D-Har, **9**) retains activity although the tetra-methylated version,[18] D-Har$(Me)_4$ (**10**), loses significant binding.

A fairly stringent SAR is also noted for P2 amino acid residue replacements (Table 3). Substitution with Ala (**11**) results in a 3-fold loss of inhibitory activity to factor Xa as well as a significant loss of thrombin selectivity. Whereas, a D-Ala substitution **12** results in a somewhat greater loss of activity, insertion of two methyl groups at the alpha carbon as found in the Aib-containing analogue **13** affords a 10-fold reduction in activity. The β-Ala residue containing analogue **14** displayed significantly reduced potency compared to the other analogues. Not unexpectedly, based on substrate specificity, substitution of P2 with proline **15** increases thrombin inhibitory activity (decreased IIa/Xa selectivity), but did not decrease factor Xa inhibitory activity. Again, configuration is extremely important at P2 since the D-Pro analogue **16** is >1000-fold less active than the L-Pro analogue. The α-napthylalanine substituted analogue **17** at P2, which has been shown to be very advantageous for inhibitors described by Corvas[19] was still active (IC_{50} = 0.14 μM) toward factor Xa in our series but provided no additional advantage. Unfortunately, none of the P2 substitutions resulted in higher activity toward factor Xa over that provided by our initial analogue Boc-D-Arg-Gly-Arg-H.

Finally, substitutions in the P4 position were explored and shown to have a marked effect on factor Xa as well as prothrombinase activity (Table 4). Of interest, removal of the Boc group afforded analogue **18**, which showed a 3- to 4-fold decreased inhibitory activity

Table 3. Tripeptide argininals with P2 modifications

Analogue	Boc-D-Arg-P2-Arg-H	IC_{50}s (μM)			
		Xa	IIa	IIase	IIa/Xa
3	-Gly-	0.050	>100	4	>2000
11	-Ala-	0.167	5.0	0.081	30
12	-DAla-	0.230	>10	0.800	>40
13	-Aib-	0.550	>500	2.0	>900
14	-βAla-	11	>500	14	45
15	-Pro-	0.046	0.20	0.021	4
16	-D-Pro-	60	>500	440	8
17	-L-αNapAla-	0.14	42	0.34	300

Table 4. Tripeptide argininals with P4 modifications

Analogue	P4-DArg-Gly-Arg-H	IC_{50}s (μM)			
		Xa	IIa	IIase	IIa/Xa
3	Boc-	0.050	180	4	3600
18	H-	0.190	55	0.828	290
19	EtOCO-	0.030	>100	0.135	>3300
20	$BnSO_2$-	0.015	50	0.012	3300
21	$PhEtSO_2$-	0.028	64	0.038	2300
22	$NapEtSO_2$-	0.027	39	0.020	1500
23	PhEtCO-	0.200	75	2	375
24	BnNHCO-	0.220	170	0.18	770
25	2-NapOAc-	0.550	>100	0.69	180
26	$HO_2C(CH_2)_2CO$-	0.224	>100	2	450
27	$HO_2C(CH_2)CO$-	0.100	>100	0.50	1000
28	HO_2CCO-	0.056	95	0.63	1900
29	$BnSO_2$-DArg-Sar-Arg-H	0.020	2	0.058	100

toward factor Xa, but a 4-fold increase in activity toward the prothrombinase complex. Likewise, the ethylcarbamoyl analogue **19** displayed a 30-fold increase in binding to prothrombinase, while thrombin selectivity was retained. Even more striking is the activity of the benzylsulphonamide analogue ($BnSO_2$, **20**) which displayed an IC_{50} value of 15 nM toward factor Xa and equivalent activity to prothrombinase. Additional analogues such as the $PhEtSO_2$-containing analogue **21**, and the $\alpha NapEtSO_2$-containing analogue **22**, all maintain potent activity to both factor Xa and prothrombinase. Of significance, replacement of the sulphonamide linkage of inhibitor **21** with an amide linkage, as found in the PhEtCO-containing analogue **23**, displayed almost a 10-fold decrease in factor Xa inhibition and a 50-fold drop in prothrombinase inhibitory activity. Both the urea linked analogue **24** and the 2-naphthoxyacetyl-containing analogue **25** lose significant activity. The last series of P4 changes were synthesized to investigate the known natural preference of a negatively charged residue (i.e. Asp/Glu) albeit in P3. As the chain length decreases within the succinic to oxalic-containing analogues, inhibitory activity to factor Xa becomes equivalent to analogue **3**. Finally, combining the best P4 residue, $BnSO_2$, with a Sar-residue at P2 (analogue **29**) again reinforced our earlier observations that a P2 Gly-residue is critically required to maintain high factor Xa versus thrombin selectivity.

Conclusion

Starting with the substrate sequence, Cbz-D-Arg-Gly-Arg, we have designed a series of TS analogues of factor Xa containing the argininal functionality. Initially Boc-D-Arg-Gly-Arg-H (**3**) was found to be a very potent factor Xa inhibitor as well as an extremely selective inhibitor with respect to thrombin.[20] Replacement of both the P3 and P2 residues uncovers a very narrow SAR suggesting an extremely specific interaction with the enzyme. The best P4 replacements are the sulfonamide containing analogues ($BnSO_2$- group affords the most potent compound of this series, **20**) which are also effective inhibitors of the prothrombinase complex. Although the P4 site can tolerate larger sulfonamides,

linkages that utilize amide or urea functionality are substantially less active. The acidity of the N–H of this linkage may be important in its interaction with this enzyme.[21–23] Concerning the interaction of the D-arginine residue, recent X-ray crystallographic data[24] for the binding of the small molecule DX-9065a to factor Xa, as well as for trypsin, suggests that the P3 D-arginine sidechain may also bind into the 'aryl-binding' (S4) pocket. This would leave the sulphonamide moiety available to interact with the backbone residues which comprise the antiparallel β-sheet.

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