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SYNTHESIS AND BIOLOGICAL ACTIVITY OF P_2-P_4 AZAPEPTIDOMIMETIC P_1-ARGININAL AND P_1-KETOARGININAMIDE DERIVATIVES: A NOVEL CLASS OF SERINE PROTEASE INHIBITORS[1]

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Abstract: Molecular modeling and topographic considerations of the thrombin-specific sequences Boc-Asp-Pro-Arg-TS or Ac-d-Phe-Pro-Arg-TS (TS = transition state analog electrophilic center) and related scaffolds led to the design of novel P_2-P_4-azapeptidomimetic P_1-argininal and P_1-ketoargininamide derivatives (**3a-j**). The synthesis and biological activity of these potential serine protease inhibitors are presented.

The incorporation of azapeptide scaffolds into pharmaceutically interesting structures has been receiving increased attention. The recent report by Han and Janda[2] describing the azatide class of peptidomimetics, Fassler's[3] studies of azapeptides in the HIV-1 protease inhibitor area, as well as Boussard's[4] disclosure on azapeptides incorporating a proline residue has prompted us to report our own studies[5] in this area,[6] which focused on the design and synthesis of novel classes of antithrombotic drugs.

Thrombin, Factor VIIa, and Factor Xa are key members of the trypsin class of serine protease enzymes involved in the blood coagulation cascade. They play a vital role in the regulation of normal hemostasis and abnormal intravascular thrombus development.[7] Recent advances in the elucidation of the structure and function of human thrombin have led to an increased understanding of the pivotal role played by this multifunctional enzyme in the regulation of hemostatic processes as well as other activities related to the maintenance of vascular function.[8] Thrombotic vascular disease is a major cause of morbidity and mortality in the industrialized world. Therefore, the development of new classes of safe, efficacious antithrombotic drugs is currently an area of active research in many laboratories.[9]

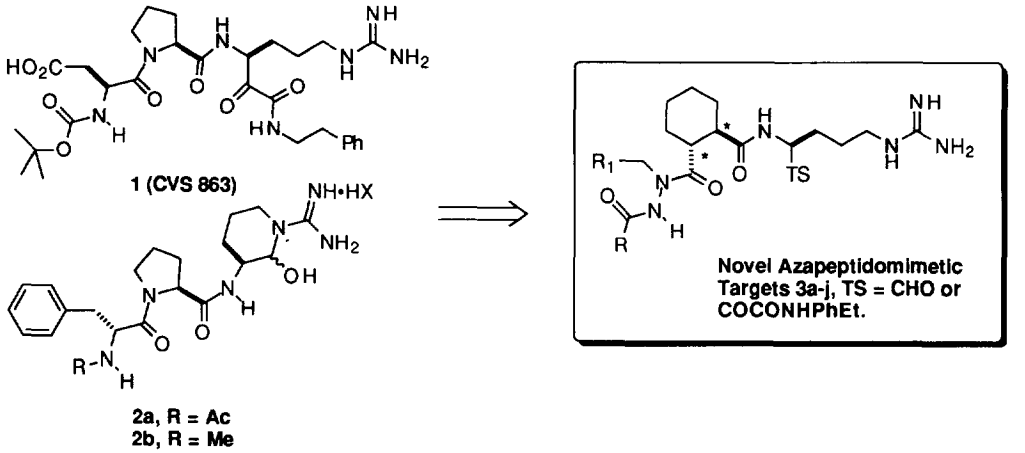


Figure 1. Structures of Prototypical Thrombin Inhibitors and Azapeptidomimetics **3a-j**.

In this context, we investigated the azapeptidomimetic derivatives **3a-j** as a novel class of direct serine protease inhibitors (Figure 1). The new targets incorporate an arginine-based aldehyde or ketoamide moiety as the electrophilic transition-state analog functionality at P_1.[9,10] Thrombin inhibitors possessing P_1-argininal groups have been found to express high levels of oral bioavailability.[11] Examination of the P_3 stereochemistry of the potent inhibitors **1** (CVS 863[9a]) and **2a,b** (d-Phe-Pro-Arg-H motif[9a,c,12a,b]) suggested that both D- and L-configurations could be accommodated in the thrombin active site under certain conditions. Further topographical insights were also obtained from the X-ray crystal structures of P-Pack[13] and related ketoargininamide inhibitors[9a,c,12c] bound into the thrombin active site.

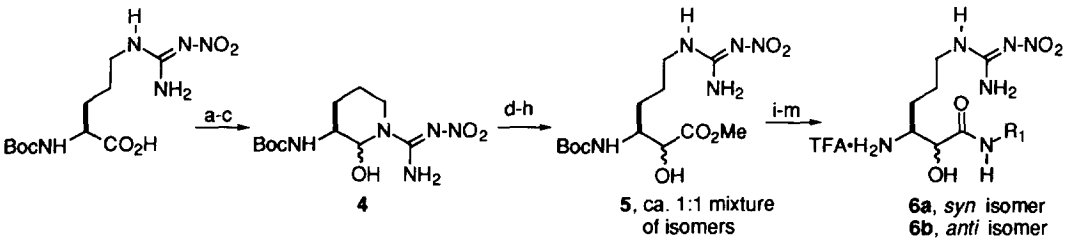
It was therefore of interest to replace this P_3-chiral center with an unusual type of azapeptide residue, whose trigonal N-alkyl substituted nitrogen atom adopts an orientation midway between D- and L-.[2,6] Appropriate N-substitutions were made to mimic phenylalanine, homophenylalanine, and aspartic ester side chains. The P_2 surrogate employed a relatively rigid *trans*-1,2-cyclohexanedicarbonyl system, which would provide a hydrophobic interaction with the 60-thrombin loop[11a,12b,c,13a] and also impart conformational constraints similar to those imposed by the proline backbone of **1** and **2a,b**. Hence, in this P_2-P_3 dipeptide surrogate we have removed one of the normal prolyl peptide bonds and replaced the natural P_3 chiral center with an appropriately N-substituted azapeptide residue.

By modeling, the new motif closely simulated a normal peptide backbone structure. The carbonyl and amide N-H functions are thought to provide essential hydrogen bond acceptor and donor elements necessary for high affinity antiparallel β-hydrogen bonding with the Gly 216 residue in the thrombin active site.[12c,13a,b] Such stabilizing interactions are very important in related classes of serine protease inhibitors.[11a] The absence of normal peptide bonds in this family could impart increased levels of metabolic stability, which in turn may afford drug candidates with useful pharmacological profiles.[14]

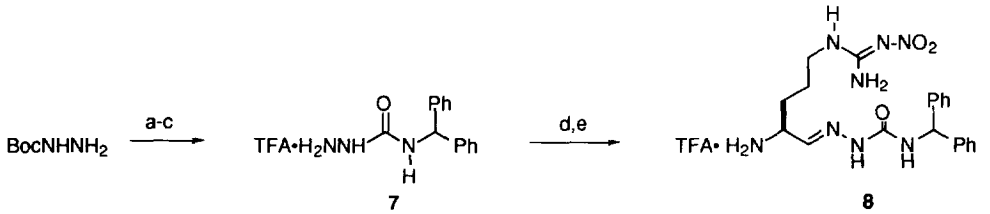
Chemistry

The synthesis of the P_1-ketoargininamide precursors are outlined in Scheme 1.[15] Starting with commercially available Boc-Arg(NO_2)-OH, mixed anhydride activation, formation of the Weinreb amide, low temperature $LiAlH_4$ reduction and careful acidic quenching afforded Boc-Arg(NO_2)-H, existing almost exclusively as the cyclol **4**.[16] Treatment of **4** with cyanide ion under classical cyanohydrin forming conditions, Pinner reaction, re-esterification and amino group reprotection delivered **5**, as a ca 1:1 mixture of α-hydroxyesters. Elaboration of **5** via standard amidation protocols, diastereomer separation, and acid-catalyzed Boc group deprotection provided good overall yields of β-amino-α-hydroxyamide salts **6a,b**. In addition to their role as ketoargininamide precursors, these substances also served as useful chiral handles, enabling diastereomer separation for some advanced intermediates which are discussed below.

The synthesis of the P_1-argininal precursors is depicted in Scheme 2. By an efficient three-step protocol, *t*-butyl carbazate was elaborated into semicarbazide derivative **7**. Condensation with hemiaminal **4** followed by acidic cleavage of the α-Boc protecting group delivered the stable ω-nitroargininal-diphenylmethylsemicarbazone salt **8**.[17]

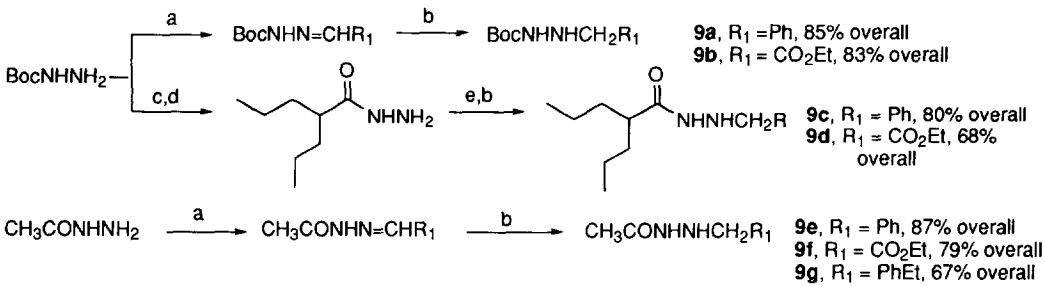


Scheme 1. Synthesis of Ketoargininamide Synthons **6a,b**. *Reagents and conditions:* (a) i-BuOCOCl, NMP, -5° C; (b) MeONHMe, -5° C,1.5 h, 88%; (c) $LiAlH_4$, THF, -70° C to 0° C, 2.5 h; $KHSO_4$, H_2O, -70° C to -30° C, 74%; (d) KCN, $KHCO_3$, THF, H_2O, rt; (e) MeOH, HCl (gas), 0° C to rt; (f) 6 N HCl, rt; (g) MeOH, HCl (gas), reflux; (h) Boc_2O, $NaHCO_3$, THF, H_2O, 0° C to rt, 53% overall for steps (d-h); (i) NaOH, MeOH, H_2O, 0° C to rt; (j) Dowex H^+, 85-93%; (k) $PhEtNH_2$, BOP, NMM, DMF, rt; (l) Flash Column isomer sep'n., 92%; (m) TFA, CH_2Cl_2, 0° C to rt, 91-97%.



Scheme 2. Synthesis of P_1-Argininal Synthon **8**. *Reagents and conditions:* (a) CDI, DMF,rt, 30 min; (b) Ph_2CHNH_2, rt, 1 h, 85%; (c)TFA, CH_2Cl_2, 0° C to rt, 80%; (d) **4**, NaOAc, EtOH, H_2O, reflux, 90%; (e) TFA, CH_2Cl_2, 0° C to rt, 1 hr, ~95%.

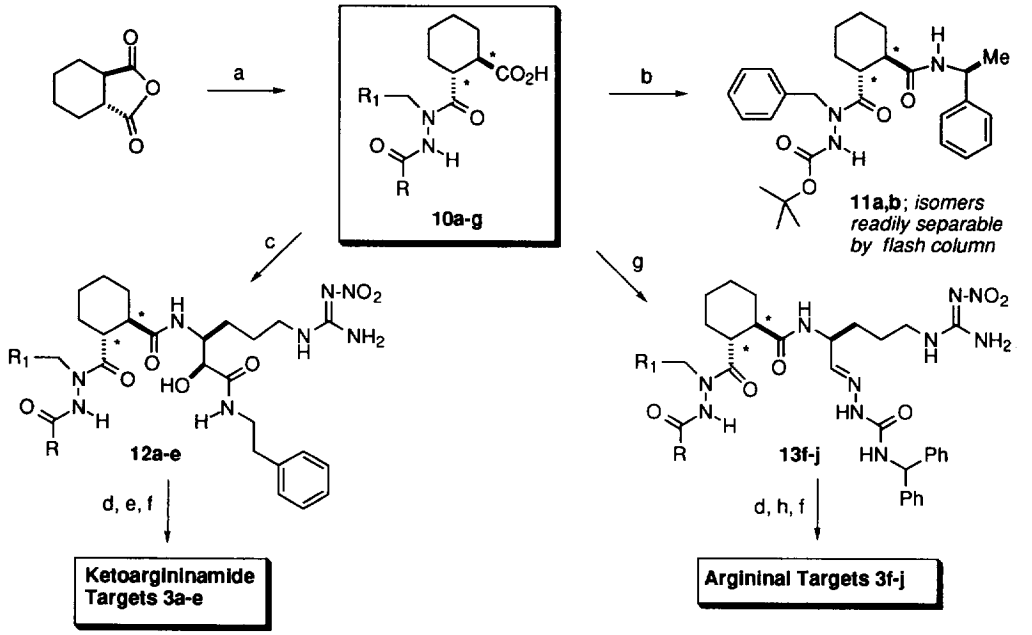
The preparation of the P_3-P_4 azapeptide mimic synthons are outlined in Scheme 3. Based on our modeling considerations for new thrombin inhibitors derived from reference compounds **1** and **2**, we focused on the synthesis of aza-mimics of aspartic acid (**9b,d,f**), phenylalanine (**9a,c,e**) and homophenylalanine (**9g**). Thus, condensation of an appropriately substituted acylhydrazine derivative with an aldehyde or ethyl glyoxalate followed by careful hydrogenation delivered good overall yields of N-acyl-N'-(substituted)-alkylhydrazines. **9a-g**.[6]



Scheme 3. Preparation of P_3-P_4 Azapeptide Precursor Hydrazine Derivatives **9a-g**. *Reagents and conditions:* (a) R_1CHO, EtOH, rt; (b) H_2, Pd/C, THF, 25-30 psi; (c) $(C_3H_7)_2CHCOCl$, Et_3N, 0° C to rt, ~quant.; (d) TFA, CH_2Cl_2, 0° C to RT, 91%; (e) R_1CHO, Et_3N, THF, $MgSO_4$, rt, 90-95%.

Scheme 4 outlines the coupling and elaboration of the azapeptidomimetic P_2-P_3-P_4 moieties. During the course of our work, we contemplated the use of a chiral *trans*-cyclohexyl half-ester intermediate, in principal available by chemoenzymatic hydrolysis (PLE or subtilisin) of a racemic trans-dialkylester. After appropriate

coupling reactions such a compound would allow us to secure advanced intermediates with the desired (*R,R*)-stereochemistry at P_2. However, perusal of the existing literature methods revealed that only material of low optical purity (ca 45-59%) would be obtained via the esterase protocol.[18a,b] Coupled with the tedious nature of a classical quinine resolution,[18c-e] we proceeded as follows. Facile reaction of *trans*-1,2-cyclohexanedicarboxylic anhydride with acylhydrazine derivatives **9a-g** generated the carboxylic acid-hydrazide intermediates **10a-g** in excellent yields. In order to investigate the feasibility of isomer separation at the P_2 moiety, we coupled **10a** with (S)-α-methylbenzylamine (α-MBA) and found the resultant diastereomers **11a,b** to be readily separable by silica gel flash chromatography. This encouraging result suggested that late-stage intermediates containing chiral P_1-ketoargininamide/argininal moieties might also be separable by chromatography.



Scheme 4. Coupling and Elaboration of Azapeptide Intermediates. *Reagents and conditions:* (a) **9a-g**, Et_2O or THF, rt to reflux, 88-91%; (b) for R= *t*-BuO, R_1 = Ph: (*S*)-α-MBA, EDC, HOBt, NMM,THF, rt, 90%; (c) **6a** or **6b**, BOP, NMM, DMF, rt, 62-68%, product **12a-e** from **6a** shown; (d) H_2, Pd/C, EtOH, H_2O, HOAc or 0.5 N HCl, 15-30 psi, ~quant; (e) EDC, DCAA, DMSO, Toluene, ~5° to rt, ~30-50%; (f) RP-HPLC; (g) **8**, BOP, NMM, DMF, rt or EDC, HOBt, NMM, DMF, rt, 50-69%; (h) CH_2O, TFA, rt, pH 1 buffer, 25-40%.

BOP-mediated coupling of **6a,b** (product **12a-e** from **6a** shown) with **10a,b,d,e** afforded a series of advanced intermediates **12a,b,d,e** in 62-68% yield. Except for the cases where R = *t*-BuO or CH_3 and R_1= CO_2Et, the P_2 isomers were inseparable by flash chromatography. Therefore, all targets shown are racemic at the *trans*-cyclohexyl residue as indicated with an asterisk. P_1-Nitro-group deblocking via catalytic hydrogenation at low pressure followed by a Moffatt oxidation afforded the ketoargininamide targets **3a-e** in modest overall yields whose R and R_1 groups are defined in Table 1. Interestingly, target **3d** resulted from a transesterification process during prolonged hydrogenolysis of precursor **12b** in a methanolic milieu. BOP or EDC-mediated couplings of protected argininal precursor **8** with carboxylic acid **10a,b,e-g** afforded the corresponding advanced intermediates **13f-j.** In these systems, the P_2 isomers were inseparable by HPLC or flash

chromatography. P_1-Nitro-group deblocking via catalytic hydrogenation at low pressure followed by acid-catalyzed hydrolysis/semicarbazone exchange delivered the argininal targets **3f-j**, whose R and R_1 groups are also defined in Table 1, in fair overall yields.

Biological Activity

The biological activity of the targets **3a-j** along with the standards **1** and **2** is shown in Table 1. The in vitro assays were carried out using a range of important human serine protease enzymes, including thrombin (FIIa), factor VIIa (FVIIa), factor Xa (FXa), plasmin and trypsin.[19] All new targets were selective against FXa and plasmin. The acyl-azaPhe-*trans*-Chx-ketoargininamide mimetics **3a,c** expressed modest levels of activity on thrombin. The related P_1-argininal/ketoargininamides incorporating P_3-azaAsp(OEt) or P_3-aza-homoPhe moieties were less active on thrombin. Compound **3c** also demonstrated modest activity on FVIIa and was the most active trypsin inhibitor of the series. Targets **3a,c,i** were amongst the more active trypsin inhibitors. These compounds were less active than the standards **1** and **2.** Possible reasons for the decreased activity may include the presence of the racemic *trans*-cyclohexyl P_2-residue, higher barriers to rotation for the alkylated amides versus the α-carbons of the amino acid templates, the intermediate orientation of the N-alkyl group versus D- and L-amino acids, or a less than ideal match of the P_2 cyclohexane dicarboxylate to proline.

Table 1. In vitro IC_{50} values (μM) of azapeptidomimetics **3a-j** and reference standards **1** and **2** against a range of important serine proteases.[a,b]

Compound #	R	R_1	P_1 T.S.[c]	FIIa	FVIIa	FXa[d]	Plasmin	Trypsin[e]
3a	*t*-BuO	Ph	K.A.	0.88	-	Inact.	Inact.	0.25 (h)
3b	*t*-BuO	CO_2Et	K.A.	>2.5	~25	Inact.	Inact.	0.25-2.5
3c	CH_3	Ph	K.A.	3.2	8.5	Inact.	>2.5	0.108
3d	*t*-BuO	CO_2Me	K.A.	Inact.	18	Inact.	Inact.	1.31
3e	$(C_3H_7)_2CH$	CO_2Et	K.A.	13.2	-	Inact.	Inact.	22.2 (h)
3f	*t*-BuO	Ph	Ald.	>2.5	>25	Inact.	Inact.	>2.5
3g	*t*-BuO	CO_2Et	Ald.	>2.5	22	Inact.	Inact.	0.25-2.5
3h	CH_3	Ph	Ald.	Inact.	>25	Inact.	Inact.	>2.5
3i	CH_3	CO_2Et	Ald.	22.1	>25	Inact.	Inact.	0.59
3j	CH_3	$PhCH_2CH_2$	Ald.	12.8	>25	Inact.	Inact.	5.7 (h)
1	*reference*	*compounds*	K.A.	0.0023	0.706	0.698	2.71	0.00076
2a	*see*	*Figure 1*	Ald.	0.284	~4.3	>2.5	>2.5	0.156

[a]Concentration of inhibitors **1**, **2a**, and **3a-j** necessary to inhibit thrombin (FIIa), FVIIa, FXa, plasmin, and trypsin cleavage of the chromogenic substrates described in reference 11 by 50%. [b]All new target compounds were characterized by ^{1}H-NMR, RP-HPLC, low/high resolution mass spectroscopy. [c]P_1 transition state analog functionality, where K. A. denotes phenethylketoamide and Ald. denotes argininal. [d]Inact. denotes inactive at IC_{50} ≥ 50 μM. [e]Bovine trypsin unless value is followed by (h), signifying human trypsin.

Conclusion

We have developed convenient, new, synthetic methodology for the synthesis of a novel azapeptidomimetic array that was incorporated into a series of potential serine protease inhibitors. Ten targets were evaluated on a variety of important human serine protease enzymes. Six of these compounds expressed interesting levels of biological activity, albeit of a lower magnitude than the highly potent reference standards **1** and **2**. The ketoargininamide Boc-azaPhe-*trans*-Chx-Arg-CONH(2-PhEt), **3a**, was the most biologically interesting thrombin inhibitor. In general, P_1-ketoargininamide transition state analogs were more potent

inhibitors than the corresponding argininal derivatives. The incorporation of these azapeptides into other pharmaceutically relevant target molecules may be of interest.

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References and Notes

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19. In vitro enzyme assays were performed according to the protocols described in reference 11.

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