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INVESTIGATION OF THE S_3 SITE OF THROMBIN: DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF 4-SUBSTITUTED 3-AMINO-2-PYRIDONES INCORPORATING P_1-ARGININALS

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Abstract: A novel scaffold for P_4–P_2 dipeptide mimics containing a rigid pyridone spacer was designed based on a virtual library strategy. Several selected nonpeptidic 4-aralkyl or 4-alkylpyridones incorporating a P_1-argininal sequence were prepared. The modeling studies, synthesis and biological activities of these unique pyridone derivatives are reported herein.

Thrombin, a member of the trypsin class of serine proteases involved in the coagulation cascade, is the primary enzymatic mediator of the coagulation response to vascular injury and regulates normal hemostasis and abnormal thrombus development.[1] Peptidomimetics which inhibit this key enzyme are emerging as potential therapeutic agents for the prevention and treatment of thrombotic vascular disease.[2]

Our recent work on orally active anticoagulants has led to a family of heterocycle-based thrombin inhibitors from which the novel lead candidates **1**, CVS 1578,[3] and **2**, CVS 1801,[4] have emerged. These P_3–P_2 heterocyclic moieties, which were appended onto a transition-state P_1-argininal motif, display exceptional potency on thrombin, while also exhibiting high selectivity against other key serine proteases. Pyridone-based transition-state peptidomimetics have also found utility as elastase inhibitors[5] and caspase-1 inhibitors.[6] Recently non-transition state protease inhibitors containing pyridone moieties have been described.[7]

The 4-position of the pyridone scaffold was developed as an alternate attachment point for tethering

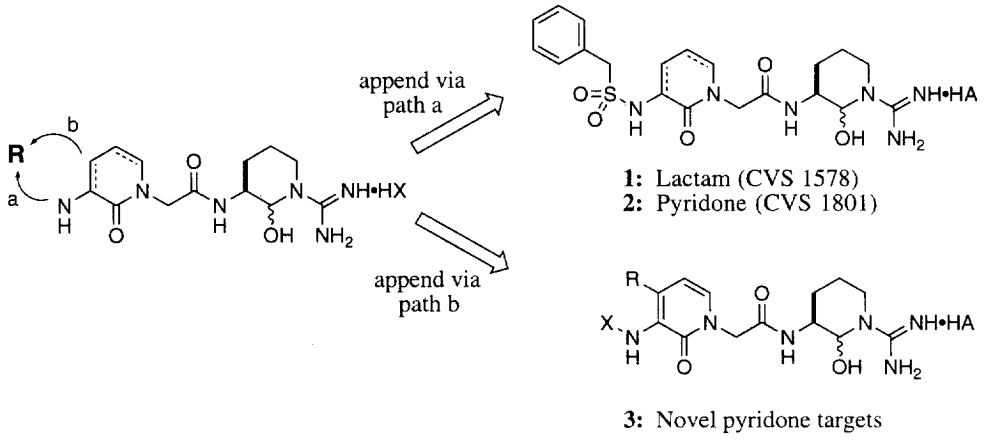

Figure 1. Strategy for the design of lactam **1**, pyridone **2** and 4-substituted-pyridones **3**.

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the P_4 aromatic ring into the S_3 site of thrombin (Figure 1). Moreover, these targets retain the P_1-argininal moiety, a functionality that often imparts useful levels of oral bioavailability to thrombin inhibitors.[3a,4] The molecular design, synthesis and biological activity of this novel family of peptide surrogates is described herein.

Molecular Modeling

A new series of potential inhibitors was designed via a virtual library strategy employing structural information from the X-ray crystal structure of **1** bound in the active site of thrombin.[3b] In this study the critical interactions provided by the P_4 benzyl group of **1** and **2** within the S_3 hydrophobic pocket of thrombin were probed. A virtual library of substituents was attached onto the 4-position of the pyridone ring of **2**, using a distance geometry program.[8] The substituents in our library consisted of a variety of functionalities of various sizes, polarities and hydrophobicities (see Figure 2). The structures of these potential ligands were individually idealized in extended conformations with *Insight II/Discover*™ (MSI Inc.) before they were used as input for distance geometry. Examination and docking of potential targets in the thrombin active site indicated that a two to three carbon chain would provide the optimal length for positioning hydrophobic substituents into the S_3 site, while maintaining the other energetically favorable interactions including that of the heterocyclic ring with the unique 60 loop of thrombin. Solutions from distance geometry calculations were structures with substituents that could fill the S_3 site without unfavorable van der Waals steric interactions, while maintaining the idealized bond lengths and angles within the specified limits that were 0.5 Å and 0.5 $Å^3$ for the distance and chiral errors, respectively.

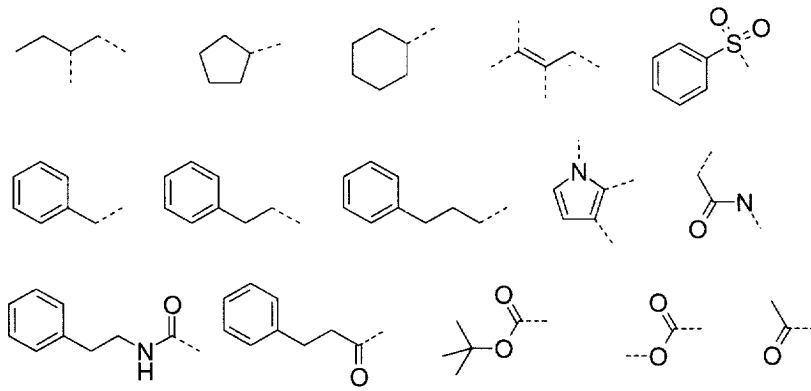


Figure 2. Selected examples of functionalities in the virtual library of substituents. Hashed lines denote attachment of moiety to the pyridone.

The distance geometry solution set of ligands was docked into the active site of thrombin and minimized using the conjugated gradient algorithm in *Insight II/Discover*™. For this process, the enzyme atoms were fixed while the inhibitor was free, except for the covalent bond from thrombin's Ser195-Oγ to the P_1-Arg-C1 atom. The minimization was performed using a 20 Å cutoff and until the RMS gradient was less than 0.01. Factors considered to be crucial in order to dock these completed structures into the active site of thrombin were: (i) the

β-sheet H-bonding interaction of Gly216's backbone O and NH to the NH of the amine and carbonyl of the pyridone, respectively, and (ii) the maintenance of the favorable hydrophobic packing of the pyridone ring under the unique "60 loop" of thrombin.

We concluded that a tether length of two to three sp^3 carbon atoms should be optimal for allowing a hydrophobic moiety to interact with the S_3 site, defined by residues Trp215, Ile174 and Leu99 of thrombin. Phenyl and cyclohexyl substituents appended from the 4-position of a pyridone framework were selected as candidates for synthesis (see Figure 3). The capping group of the amine, X, could be varied by traditional SAR approaches after the optimal 4-alkylpyridone structure was determined experimentally.

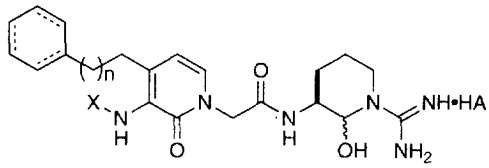
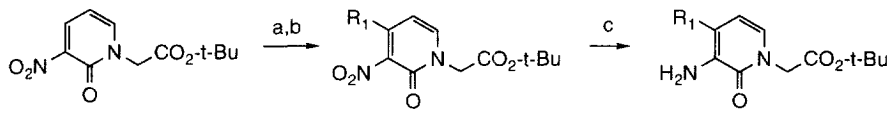


Figure 3. Hypothetically optimal pyridone targets **3a–g** (n=1,2).

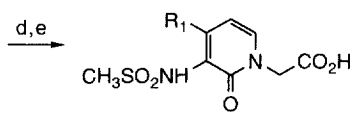
Chemistry[9]

Although the preparation of a Cbz protected 4-phenyl-3-amino-pyridone has been previously descibed,[5c] the procedure did not appear to be applicable to the preparation of our 4-alkylated-pyridone targets. Novel approaches for the synthesis of 4-substituted pyridones were employed. The intermediate P_4–P_2 peptide surrogates **6a–c** were synthesized as outlined in Scheme 1. Zinc-mediated 1,4-addition of a Grignard reagent, followed by reoxidation to the pyridone, proceeded in moderate yields for the cases shown, producing 4-alkylated nitropyridones **4a–c**. Reduction of the nitro group to form aminopyridones **5a–c**, followed by sulfonamide formation and cleavage of the t-butyl ester produced pyridone-acetic acid intermediates **6a–c**.



4a: R_1=Ph$(CH_2)_2$
4b: R_1=Chx$(CH_2)_2$
4c: R_1=Ph$(CH_2)_3$

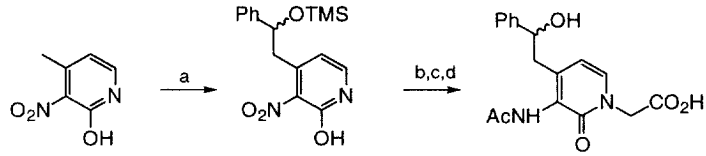
5a: R_1=Ph$(CH_2)_2$
5b: R_1=Chx$(CH_2)_2$
5c: R_1=Ph$(CH_2)_3$



6a: R_1=Ph$(CH_2)_2$
6b: R_1=Chx$(CH_2)_2$
6c: R_1=Ph$(CH_2)_3$

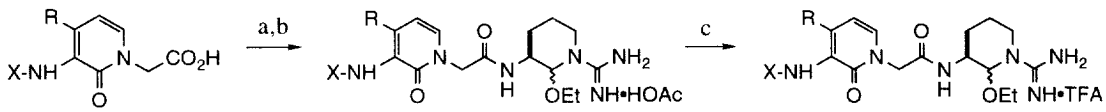
Scheme 1. *Reagents and conditions:* (a) 3 equiv R_1MgX, 2 equiv $ZnCl_2$, THF, 0 °C-rt; (b) 1.5 equiv Cs_2CO_3, 1.0 equiv $Pd(OAc)_2$, THF, 50°C, 33-41% for 2 steps; (c) H_2, Pd/C, MeOH, 80-97%; (d) CH_3SO_2Cl, collidine, THF, 17-57%; (e) TFA, CH_2Cl_2, 97-100%.

The intermediate P_4–P_2 peptide surrogate **8** was synthesized by the route outlined in Scheme 2. This pathway involved condensation of a stabilized carbanion with benzaldehyde. In our first attempt, treatment of 2-hydroxy-4-methyl-2-nitropyridine with two equivalents of lithium diisopropylamide, followed by reaction of the resultant dianion with benzaldehyde, led to a very poor yield of the desired adduct. Milder conditions were used in an improved one pot approach to prepare intermediate **7**: in situ formation of the transient trimethylsilyloxypyridine, carbanion generation, condensation with benzaldehyde, and finally trapping the intermediate hydroxyl with trimethylsilyl chloride. The trimethylsilyl protecting group was cleaved during the ester hydrolysis. Alkylation of the pyridyl nitrogen, reduction in the presence of acetic anhydride, and hydrolysis of the ethyl ester afforded intermediate **8**.



Scheme 2. *Reagents and conditions:* (a) NaH, THF, 0 °C; TMSCl, 0 °C-rt; $LiN(TMS)_2$, 0 °C; benzaldehyde, 0 °C-rt; TMSCl, 45%; (b) $LiN(TMS)_2$; $BrCH_2CO_2Et$, THF, 81%; (c) H_2, Pd/C, Ac_2O, EtOAc; (d) LiOH, 96% for 2 steps.

A convenient three step protocol was utilized for the efficient assembly of targets **3a–3d**: coupling of the 4-alkyl-pyridone-acetic acid synthons **6a–c** and **8**, to nitroArg ethyl aminal,[10] selective hydrogenolysis of the nitro group from the guanidine, and mild acidic hydrolysis to unmask the aldehyde. During hydrogenation, the hydroxyl group of **9d** was hydrogenolyzed to provide the desired phenethyl chain.



6a: R = $Ph(CH_2)_2$, X = Ms
6b: R = $Chx(CH_2)_2$, X = Ms
6c: R = $Ph(CH_2)_3$, X = Ms
8: R = $Ph[CH(OH)]CH_2$, X = Ac

9a: R = $Ph(CH_2)_2$, X = Ms
9b: R = $Chx(CH_2)_2$, X = Ms
9c: R = $Ph(CH_2)_3$, X = Ms
9d: R = $Ph[CH(OH)]CH_2$, X = Ac

3a: R = $Ph(CH_2)_2$, X = Ms
3b: R = $Chx(CH_2)_2$, X = Ms
3c: R = $Ph(CH_2)_3$, X = Ms
3d: R = $Ph(CH_2)_2$, X = Ac

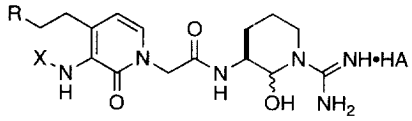
Scheme 3. *Reagents and conditions:* (a) EDC, HOBt, nitroargininal ethyl aminal, HCl salt, NMM, CH_3CN, 57-60%; (b) H_2, Pd/C, EtOH, HOAc, H_2O, 94-100%; (c) 6.0 N HCl; HPLC purification, 34-39%.

Results and Discussion

Seven 4-alkyl-pyridone argininals were prepared and evaluated for their ability to inhibit various serine proteases (Table 1).[11] Potent and selective inhibitors of thrombin were discovered. The most active compounds in the series contain the phenethyl side chain. The methanesulfonamide moiety appears to be critical for optimum binding. The NH at the 3-position of the pyridone as well as the pyridone carbonyl are involved in an anti-parallel β-sheet interaction with Gly 216 of thrombin. The sulfonamide NH of **3a** appears to be a preferred

hydrogen bond donor relative to the corresponding amide NH of **3d**. Steric interactions of the sulfonamide moiety with the phenethyl substituent may alter the lowest energy conformations of compounds **3e–3g**, leading to compounds with sub-optimal fits in the thrombin active site. The most active compound in this new array of inhibitors, **3a**, is not as potent as the lead compound **2** against thrombin. The attachment of the benzylsulfonamide of **2** provides an superior fit into the S_3 pocket. Nonetheless, the potency of **3a** against thrombin in conjunction with its selectivity towards factor Xa is notable.

Table 1. In Vitro IC_{50} Values (nM) of Heterocyclic Peptide Mimics Against Thrombin, Factor Xa, Plasmin, and Trypsin[a]



compound	X	R	thrombin	factor Xa	plasmin	trypsin
1			6.2	2500	>2500	791
2			0.505	20.9	>2500	26.2
3a	CH_3SO_2	Ph	7.95	>2500	>2500	135
3b	CH_3SO_2	cyclohexyl	256	2500	>2500	257
3c	CH_3SO_2	$PhCH_2$	203	>2500	>2500	479
3d	CH_3CO	Ph	472	>2500	>2500	1220
3e	$PhSO_2$	Ph	89.1	2360	>2500	188
3f	CH_3NHSO_2	Ph	50.5	2500	250–2500	103
3g	$CF_3CH_2SO_2$	Ph	194	>2500	>2500	1220

[a]Concentration of compounds **1, 2, 3a–g** necessary to inhibit human enzyme (thrombin, Factor Xa, plasmin, and trypsin) cleavage of the chromogenic substrates described in ref 3a by 50%.

Conclusion

A novel series of 4-substituted-pyridone dipeptide surrogates was appended onto a reactive P_1-argininal resulting in the discovery of potent and selective thrombin inhibitors. The design of novel inhibitors was guided by generation and docking of a virtual library of various alkylated pyridones in the active site of thrombin. Of these new compounds, **3a** expressed superior in vitro potency vs thrombin, with desirable selectivity towards other trypsin-like serine proteases. The sulfonamide/heterocycle motif provides a useful scaffold from which many new systems have emerged.

Acknowledgment: The X-ray crystallographic work of **1** in the active site of thrombin was performed by K. Håkanson and A. Tulinsky (Dept. of Chemistry, MSU). Thanks to S. Anderson and P. R. Bergum for the technical support in performing the in vitro assays. Thanks to S. H. Carpenter for technical support in the preparation of Boc-N^g-nitro-L-argininal ethyl aminal.

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8. Conventional distance geometry methodology was modified in two fundamental ways to improve docking performance. First, the origin for the metric matrix calculation was assigned randomly to an atom center. With this modification, not only were the structures that were produced more randomized, but the method is faster than the conventional center of mass approach. Additionally, the concept of elastic constraints was implemented to strongly constrain user-selected atoms during the 4-dimensional structure refinement, with the upper bound of such constraints successively relaxed on each iteration of the subsequent 3-dimensional refinement if the corresponding interatomic distance violated the constraint. Without explicitly forcing a solution, this modification enables a bias to be included in the docking of two molecules or enables a directional preference in the growth of a fragment from a fixed structural motif.
9. All new compounds were characterized by full spectroscopic (NMR, IR, low resolution MS) data. Yields refer to spectroscopically and chromatographically homogeneous (≥95% by ^{1}H NMR, HPLC, TLC) materials.
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11. Compounds **3e–g** were prepared using the same general procedure of Schemes 1 and 3, substituting the relevant sulfonyl chloride or sulfamoyl chloride for methanesulfonyl chloride in the reaction of **5a**.