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Design, synthesis and biological activity of novel peptidyl benzyl ketone FVIIa inhibitors

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ABSTRACT

Herein is described the synthesis of a novel class of peptidyl FVIIa inhibitors having a C-terminal benzyl ketone group. This class is designed to be potentially suitable as stabilization agents of liquid formulations of rFVIIa, which is a serine protease used for the treatment of hemophilia A and B inhibitor patients. A library of compounds was synthesized with different tripeptide sequences, N-terminals and D-amino acids in the P3 position. Cbz-D-Phe–Phe–Arg–bk (**33**) was found to be the best candidate with a potency of K_i = 8 μM and no substantial inhibition of related blood coagulation factors (thrombin and FXa). Computational studies revealed that **33** has a very stable binding conformation due to intramolecular hydrogen bonds, which cannot be formed with L-Phe in the P3 position. Nonpolar amino acids were found to be superior, probably due to a minimization of the cost of desolvation upon binding to FVIIa.

NovoSeven® (rFVIIa)[#] is a serine protease used for the treatment of hemophilia A and B inhibitor patients.[1] In our efforts to synthesize stabilization agents of liquid formulations of rFVIIa, we were seeking to develop a series of FVIIa inhibitors with a distinct set of properties not accounted for in previously reported FVIIa inhibitors.[2] An ideal inhibitor in our respect would be reversible and only medium potent (preferably low μM K_i) and with no substantial inhibition of related blood coagulation factors (e.g., FXa and thrombin). Potent FVIIa inhibitors would, on the other hand, be valuable for development of novel anticoagulants targeting the initial stage of the coagulation cascade. This would avoid prolonged bleedings as observed with traditional late-stage anticoagulants (thrombin inhibitors).[3] FVIIa inhibitors as stabilization agents should be nontoxic, exhibit high clearance in vivo, have a molecular weight of less than 700 g/mol to avoid an immune response and have a sufficient aqueous solubility. Ideally the synthetic route should be short and efficient and allow easy alternation of functional groups for a practical and fast structure-activity relationship analysis. Stabilization of protease formulations is not only important for FVIIa, but also in other applications such as for the development of liquid detergents.[4]

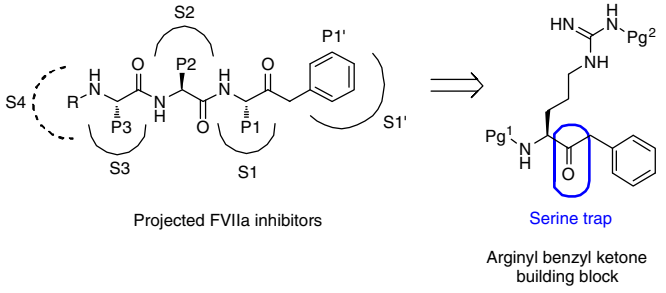

Figure 1. The projected FVIIa inhibitors consist of a tripeptide sequence with a benzyl ketone (bk) serine trap in the P1–P1' position. With arginine in the P1 position, the inhibitors can be synthesized from the building block shown to the right.

To meet our preferred inhibitor properties, we elected to explore a novel series of C-terminal modified peptides containing a benzyl ketone (bk) in the P1–P1' position (Fig. 1). Molecules containing a neutral electrophilic group (serine trap) in this position are potential competitive and covalently-bound inhibitors of serine proteases due to reaction with Ser195 in the active site.[5]

Abbreviations: bk, benzyl ketone; DIPEA, *N,N*-diisopropylethylamine; FXa, activated Factor X; HOAt, 1-hydroxy-7-azabenzotrizole; HPLC-CLND, high performance liquid chromatography–chemiluminescent nitrogen detection; MD, molecular dynamics; Pmc, 2,2,5,7,8-pentamethyl-chroman-6-ylsulfonyl; PS-SCL, positional-scanning synthetic combinatorial libraries; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophos-phonium hexafluorophosphate; rFVIIa, recombinant activated Factor VII; thr, thrombin; UPLC, ultra performance liquid chromatography.

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Highly electrophilic groups such as boronic acids, trifluoromethyl ketones, aldehydes, α-keto carboxylates or α-keto heterocycles are known to afford very potent serine protease inhibitor.[6]

The benzyl ketone serine trap, which is less electrophilic, has only been reported once in connection with one particular thrombin inhibitor,[7] and therefore represents an unexplored class of potential FVIIa inhibitors. This group can be introduced directly[7] without the need of additional reduction/oxidation protection steps[8] or anchoring to a solid support,[9] which is required with more electrophilic serine traps. The benzyl group also gives the unique possibility to probe the S1' binding pocket by introduction of a variety of substituents—this is not possible or very limited with most of the other serine traps (e.g., aldehydes or α-keto heterocycles).

A peptide-based inhibitor was chosen because such compounds are easily degraded in vivo into nontoxic amino acids and because a peptide much better resembles the natural FVIIa substrate than a small molecule. Selection of the peptide sequences was based on a specificity profile (PS-SCL) for FVIIa,[10] suggesting that the optimal substrates were Tyr–Thr–Arg > Tyr–Leu–Arg > Phe–Leu–Arg > Phe–Phe–Arg. All of them have arginine at the P1 position because, that is, the only acceptable amino acid (besides of Lys) in that position. Introduction of D-amino acids in the P3 position is known from the literature to be beneficial for the potency.[8] However, the exact reason for this has not previously been investigated and we therefore decided to explore it further by introducing D-amino acids in the P3 position in some of the projected peptides.

It is not certain whether there is sufficient space in the active site of FVIIa to accommodate the benzyl ketone group. Therefore, we inspected visually the energy minimized H–Tyr–Thr–Arg–bk complexed with human FVIIa (PDB ID: 2PUQ). The study revealed sufficient space for the serine trap (Fig. 2). Interestingly, a cation–π-interaction was observed with Lys192, which is a nonconserved amino acid among the coagulation proteases.[11]

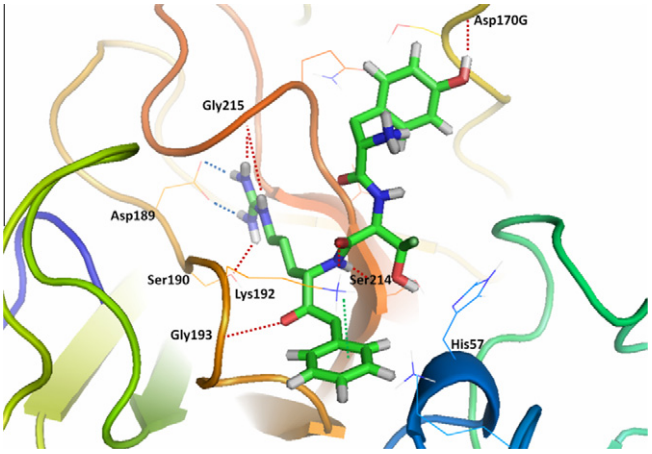


Figure 2. Energy minimization of H–Tyr–Thr–Arg–bk complexed with human FVIIa (2PUQ) revealed sufficient space for the benzyl ketone group and an interesting cation–π-interaction with the unique Lys192.

Initially, the arginyl benzyl ketone building block **3** was synthesized in good overall yield with no racemization from commercially available Boc–Arg(Pmc)-OH (**1**) via the Weinreb amide **2** (Scheme 1).[7,12] More functionalized benzyl groups would not be compatible with Grignard reagents, and the use of milder reactions would be required. Although development of such reactions has been part of our research,[13] we decided to limit the number of compounds by only considering an unsubstituted benzyl ketone serine trap.

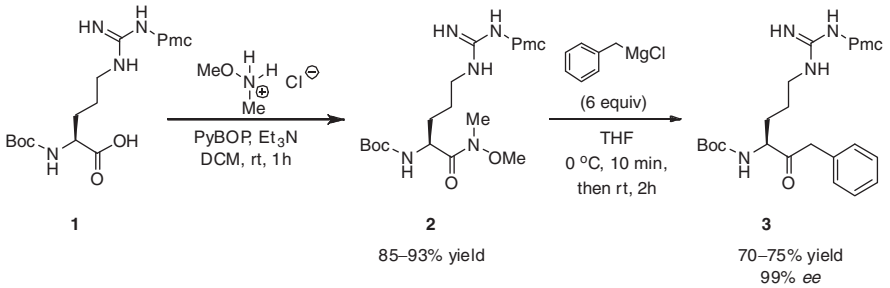
Three different dipeptides (**4**–**6**) were synthesized from the building block **3** using solution-phase EDC/HOAt-mediated synthesis followed by synthesis of seven tripeptides (**7**–**13**) by an analogous procedure (Scheme 2 and Table 1). Two peptides (**11** and **13**) have a D-Phe in the P3 position and two peptides (**12** and **13**) have an N-terminal Cbz-group. The Cbz-group was chosen as a directly incorporated N-terminal, which resembles an *N*-benzylsulfonyl group ($BnSO_2$–), that is, known from the literature for capping amino acids,[14] as well as used for N-terminals of serine protease inhibitors.[8]

The N-acetylated peptides Ac–Tyr(Bn)–Thr(Bn)–Arg(Pmc)–bk (**14**) and Ac–Phe–Phe–Arg(Pmc)–bk (**15**) were synthesized by selective N-terminal deprotection (20% TFA, DCM) followed by reaction with Ac_2O. Reaction with $BnSO_2Cl$ afforded the *N*-benzylsulfonylated peptides **16**–**20** representing all five sequences (Scheme 2 and Table 1).

O-Debenzylation of Boc–Tyr(Bn)–Thr(Bn)–Arg(Pmc)–bk (**7**) and Ac–Tyr(Bn)–Thr(Bn)–Arg(Pmc)–bk (**14**) were successfully achieved with H_2 (80 bar) in MeOH + 10% AcOH (rt, 24 h) affording Boc–Tyr–Thr–Arg(Pmc)–bk (**21**) and Ac–Tyr–Thr–Arg(Pmc)–bk (**22**), respectively, in conversions >95%. Subjecting the N-benzylsulfonylated peptides $BnSO_2$–Tyr(Bn)–Thr(Bn)–Arg(Pmc)–bk (**16**) and $BnSO_2$–Tyr(Bn)–Leu–Arg(Pmc)–bk (**17**) to the same reaction conditions unfortunately did not afford any traces of the desired products, $BnSO_2$–Tyr–Thr–Arg–bk (**23**) and $BnSO_2$–Tyr–Leu–Arg–bk (**24**), respectively. The reaction time was increased to several days, but only a mixture of the starting material and the tyrosine mono-O-debenzylated compounds were observed. Apparently, the *N*-benzylsulfonyl group causes catalyst poisoning. Further optimization was not pursued, and the synthesis of **23** and **24** was shelved.

Final Boc/Pmc-deprotection (95% TFA, DCM) followed by preparative HPLC purification and lyophilization afforded the final peptidyl benzyl ketones (**25**–**33**) in modest yield but in high purity (Table 2). Two of the final peptides, H–Tyr–Thr–Arg–bk (**25**) and H–Phe–Phe–Arg–bk (**28**) were not modified at the N-terminal.

The synthesized peptides were subjected to three competitive binding assays for screening of inhibitory activity against FVIIa, thrombin (thr) and FXa, respectively (Table 3). The solubility of the peptides in the assay buffer varied considerably. The tyrosine- and threonine-containing peptides **25** and **26** were highly soluble



Scheme 1. Synthesis of the arginyl benzyl ketone building block **3**.