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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]

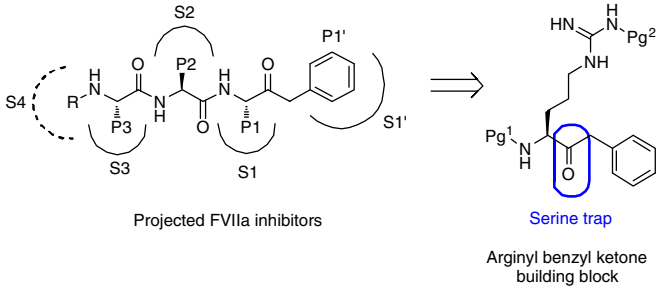

Figure 1. The projected FVIIa inhibitors consist of a tripeptide sequence with a benzyl ketone (bk) serine trap in the P1–P1' position. With arginine in the P1 position, the inhibitors can be synthesized from the building block shown to the right.

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]

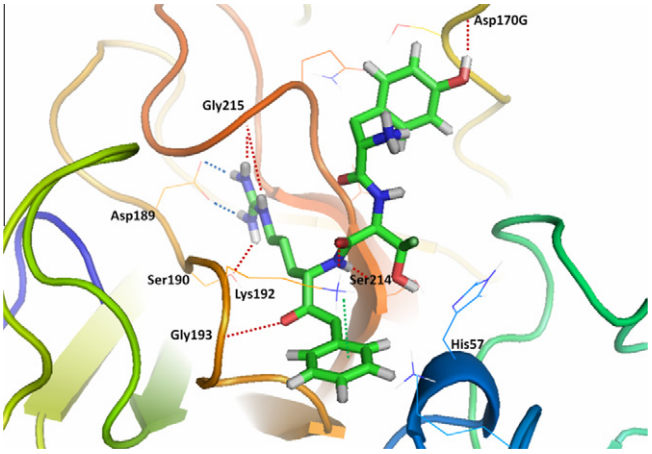


Figure 2. Energy minimization of H–Tyr–Thr–Arg–bk complexed with human FVIIa (2PUQ) revealed sufficient space for the benzyl ketone group and an interesting cation–π-interaction with the unique Lys192.

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.

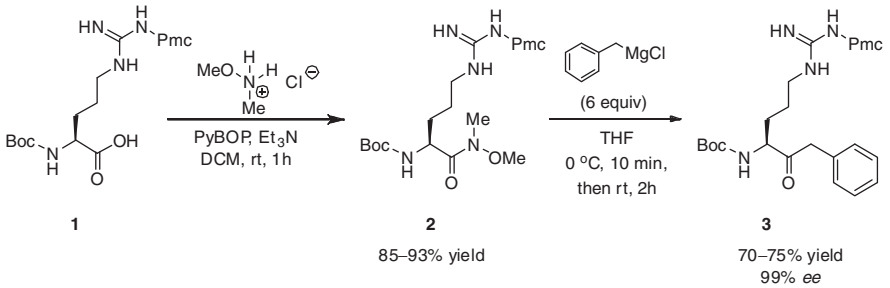
Three different dipeptides (**4**–**6**) were synthesized from the building block **3** using solution-phase EDC/HOAt-mediated synthesis followed by synthesis of seven tripeptides (**7**–**13**) by an analogous procedure (Scheme 2 and Table 1). Two peptides (**11** and **13**) have a D-Phe in the P3 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).

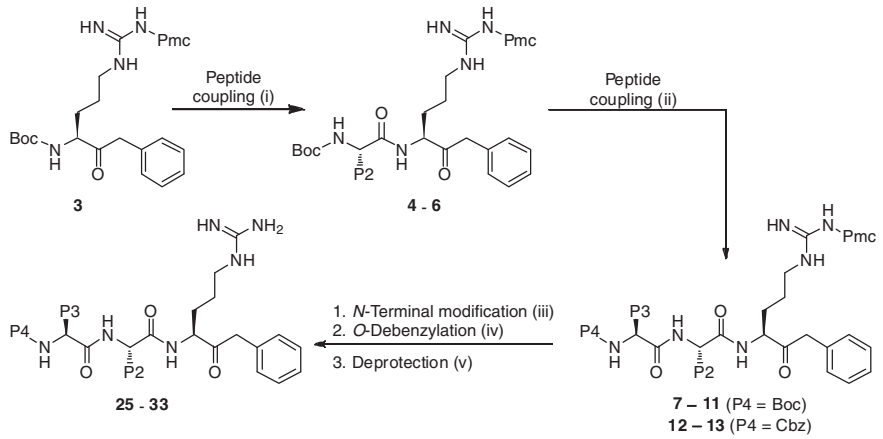
O-Debenzylation of Boc–Tyr(Bn)–Thr(Bn)–Arg(Pmc)–bk (**7**) and Ac–Tyr(Bn)–Thr(Bn)–Arg(Pmc)–bk (**14**) were successfully achieved with H_2 (80 bar) in MeOH + 10% AcOH (rt, 24 h) affording Boc–Tyr–Thr–Arg(Pmc)–bk (**21**) and Ac–Tyr–Thr–Arg(Pmc)–bk (**22**), respectively, in conversions >95%. Subjecting 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**.



Scheme 2. Synthesis of peptidyl benzyl ketones. Reagents and conditions: (i) **3** to **4**–**6**: 20% TFA, DCM, rt, 30 min, then Boc-P2-OH, EDC-HCl, HOAt, DIPEA, DCM, rt, 1 h; (ii) **4**–**6** to **7**–**13**: 20% TFA, DCM, 0 °C, 4 h, then Boc/Cbz-P3-OH, EDC–HCl, HOAt, DIPEA, DCM, 0 °C, 1 h; (iii) **7**, **10** to **14**, **15**: 20% TFA, DCM, rt, 30 min, then Ac_2o, DIPEA, DCM, rt, 30 min, **7**–**11** to **16**–**20**: 20% TFA, DCM, rt, 30 min, then $BnSO_2Cl$, DIPEA, DCM, rt; (iv) **7**, **14** to **21**, **22**: Pd/C (10 wt %), MeOH, AcOH, (10%), H_2, (80 bar), rt, 24 h; (v) **10**, **12**, **13**, **15**, **18**–**33**: 95% TFA, DCM, rt, 30 min.

Table 1
Synthesis of protected peptidyl benzyl ketones, P1 = Arg

Entry	Peptide	P4[a]	P3[a]	P2[a]	Yield[b] (%)
1	**4**	—	Boc	Thr(Bn)	67
2	**5**	—	Boc	Leu	62
3	**6**	—	Boc	Phe	60
4	**7**	Boc	Tyr(Bn)	Thr(Bn)	62
5	**8**	Boc	Tyr(Bn)	Leu	39
6	**9**	Boc	Phe	Leu	29
7	**10**	Boc	Phe	Phe	54
8	**11**	Boc	D-Phe	Phe	27
9	**12**	Cbz	Phe	Phe	32
10	**13**	Cbz	D-Phe	Phe	27
11	**14**	Ac	Tyr(Bn)	Thr(Bn)	67
12	**15**	Ac	Phe	Phe	70
13	**16**	$BnSO_2$	Tyr(Bn)	Thr(Bn)	23
14	**17**	$BnSO_2$	Tyr(Bn)	Leu	>95[c]
15	**18**	$BnSO_2$	Phe	Leu	74
16	**19**	$BnSO_2$	Phe	Phe	32
17	**20**	$BnSO_2$	D-Phe	Phe	—[d]
18	**21**	Boc	Tyr	Thr	>95[c]
19	**22**	Ac	Tyr	Thr	>95[c]
20	**23**	$BnSO_2$	Tyr	Thr	—[d]
21	**24**	$BnSO_2$	Tyr	Leu	—[d]

[a] Notation corresponds to the respective amino acid side-chain.
[b] Purified yield.
[c] Conversion according to LC–MS analysis.
[d] No product was formed.

Table 2
Synthesis of final peptidyl benzyl ketones, P1 = Arg

Entry	Peptide	P4[a]	P3[a]	P2[a]	Yield[b] (%)	Purity[c] (%)
1	**25**	H	Tyr	Thr	35[d]	93
2	**26**	Ac	Tyr	Thr	14[d]	89
3	**27**	$BnSO_2$	Phe	Leu	30	97
4	**28**	H	Phe	Phe	49	95
5	**29**	Ac	Phe	Phe	57	96
6	**30**	$BnSO_2$	Phe	Phe	24[d]	95
7	**31**	$BnSO_2$	D-Phe	Phe	29[d]	95
8	**32**	Cbz	Phe	Phe	21	97
9	**33**	Cbz	D-Phe	Phe	22	99

[a] Notation corresponds to the respective amino acid side-chain
[b] Yield determined by HPLC–CLND and based on two steps.
[c] Determined by UPLC analysis (5 → 95% MeCN in H_2O, 0.05% TFA).
[d] Yield based on three steps, because N-terminal modification and/or deprotection step was used directly without purification.

Table 3
Inhibition of blood coagulation serine proteases

Entry	Peptide	IC_{50}[a] (μM)			K_i[b] (μM)
		FVIIa	Thr	FXa	FVIIa
1	**25**	>1340	>1340	>1340	—
2	**26**	>1340	>1340	1078	—
3	**27**	>580	>580	>580	—
4	**28**	>1480	>1480	>1480	–
5	**29**	>400	>400	>400	—
6	**30**	26	>590	>590	43 ± 1.9
7	**31**	640	>640	>640	—
8	**32**	109	>650	508	89 ± 2.8
9	**33**	16	560	440	8 ± 2.4

[a] IC_{50}-value determined by a competitive binding assay against FVIIa, thrombin (thr) or FXa, respectively.
[b] Determined by linear fitting of $(v_0/v_n) - 1$ versus $[I]$, where $K_i = 1/\text{slope}$.

in the buffer, whereas the N-benzylsulfonylated peptides **27**, **30** and **31** and in particular the Cbz-modified peptides **32** and **33** were less soluble. However concentrations of at least 400 μM were obtained for all compounds, which do not restrict them to be used as formulation aids.

The most potent FVIIa inhibitor was found to be Cbz-D-Phe–Phe–Arg–bk (**33**) with an $IC_{50} = 16$ and $K_i = 8$ μM (Table 3). The inhibitor has a 35- and 28-fold selectivity over thrombin and FXa, respectively. Two less potent inhibitors were found to be $BnSO_2$–Phe–Phe–Arg–bk (**30**) with $K_i = 43$ μM and Cbz–Phe–Phe–Arg–bk (**32**) with $K_i = 89$ μM. Inhibitor **30** possesses at least a 23-fold selectivity against both thrombin and FXa, whereas **32** is almost equally potent against all three coagulation factors. The sequence was not reported as being the most active by the FVIIa PS-SCL,[10] but it is in accordance with results obtained by Parlow and South.[8]

Molecular dynamics (MD) simulations using GROMACS ver. 3.3.3 were employed to analyze the interaction between FVIIa and the two most potent inhibitors; Cbz-D-Phe–Phe–Arg–bk (**33**, $K_i = 8$ μM) and the weaker L-diastereomer **32** ($K_i = 89$ μM), which could possibly explain the importance of the P3 stereo configuration in this case. MD cannot describe the bond formation between Ser195 and the inhibitor, but it is assumed that the interactions with the rest of the binding pocket will be approximately the same in the pre-reactive complex as in the covalent complex. The simulations of **33** and **32** in the active site of FVIIa were started ($t = 0$ ns)

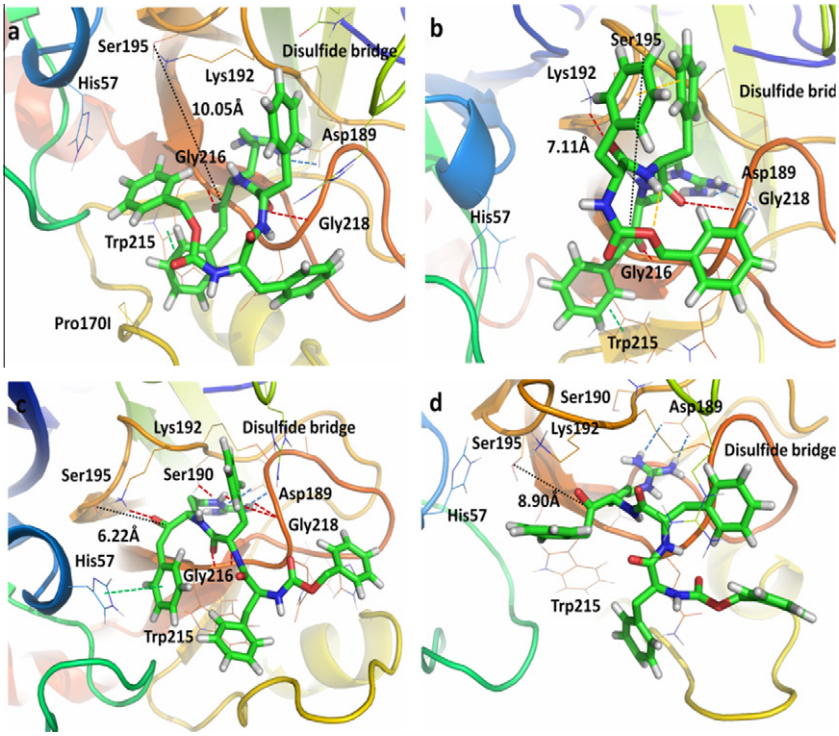


Figure 3. Conformations of peptides in the FVIIa active site during MD simulations. (a) Cbz-D-Phe–Phe–Arg–bk (**33**) at $t = 0$; (b) **33** at $t = 18$ ns; (c) Cbz–Phe–Phe–Arg–bk (**32**) at $t = 0$ ns. d) **32** at $t = 18$ ns. Intermolecular interactions: H-bond (red), salt-bridge (blue), π-stacking (gray). Intramolecular interactions (orange).

from their minimized docking poses (Fig. 3a and c). In the initial complexes both inhibitors form the well-known salt-bridge to Asp189, and they also both form hydrogen bonds to backbone Gly216 and Gly218. Inhibitor **33** has a π-stacking interaction with Trp215, whereas **32** is π-stacking with His57 and has two additional hydrogen bonds to Lys192 and Ser190.

During the simulations ($t = 18$ ns) an additional hydrogen bond was formed to the unique Lys192 along with some very favorable intramolecular interactions (Fig. 3b and d). An intramolecular hydrogen bond between the Cbz oxygen and the P2 amide *N*-H and an intramoleular π-stacking interaction between the phenyl groups of P2 and P3 were found. These conformational changes result in bringing the benzyl ketone group in closer proximity to Ser195 (from 10.05 in the beginning to 7.11 Å after 18 ns, c.f. Fig. 3a and b), thus enabling the reaction between these two groups. In contrast, the diastereomeric peptide **32** is unable to form the same intramolecular interactions due to steric restrictions. During the MD simulation, **32** lost all its interactions with FVIIa, except for the salt-bridge to Asp189, and the benzyl ketone group moves further away from Ser195 (from 6.22 to 8.90 Å, Fig. 3c and d). In this particular case, a D-configuration in the P3 position was found to be important. However, it does not represent a general trend since $BnSO_2$–Phe–Phe–Arg–bk (**30**) was found to be a medium potent FVIIa inhibitor ($K_i = 43$ μM), whereas $BnSO_2$-D-Phe–Phe–Arg–bk (**31**) was inactive. Since the Cbz oxygen was found to participate in the favorable intramolecular interactions in **33**, the N-terminal group must have a significant influence on which P3 stereoisomer is the most favorable.

The fact that all active inhibitors have a large, hydrophobic N-terminal, and furthermore are composed of nonpolar amino acids, suggests that desolvation is also an important factor for binding. The polar peptides, for example, H–Tyr–Thr–Arg–bk (**25**), will naturally have to pay a higher cost of desolvation upon binding to FVIIa, which might explain why **25** is an inactive compound. Cbz-D-Phe–Phe–Arg–bk (**33**) has a very stable binding conformation, where the cost of desolvation is minimized, since there are fewer interactions with water that need to be replaced upon binding. It will also reduce the loss in degrees of freedom and, correspondingly, the loss in entropy if these intramolecular interactions are already present in the unbound state of the peptide.

In conclusion, a novel class of medium potent FVIIa peptidyl inhibitors was developed with Cbz-D-Phe–Phe–Arg–bk (**33**) being the most active ($K_i = 8$ μM). This peptide represents a good candidate for the future development of stabilization agents for liquid formulations of rFVIIa. The usage of an N-terminal Cbz-group as a structural equivalent to $BnSO_2$– has not previously been reported, and is synthetically superior due to its easy introduction. The influence of the D-configuration at the P3 position was investigated and found to contribute to a stable binding conformation of Cbz-D-Phe–Phe–Arg–bk (**33**) due to formation of intramolecular hydrogen bonds. However, a D-amino acid in the P3 position is not tantamount to a potent inhibitor since the overall intramolecular interactions have to be taken into account, in particular the N-terminal group. A minimized cost of desolvation upon binding to FVIIa might also explain the activity of the inhibitor compared to the polar sequences.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedures, characterization of compounds **2**–**21** and **25**–**33** data from the competitive binding assays and the computational studies) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.025.

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