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Novel fluorinated pseudopeptides as proteasome inhibitors

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ABSTRACT

We have designed novel small inhibitors of rabbit 20S proteasome using a trifluoromethyl-β-hydrazino acid scaffold. Structural variations influenced their inhibition of the three types of active sites. Proteasome inhibition at the micromolar level was selective, calpain I and cathepsin B were not inhibited.

The proteasome is a large, multisubunit, proteolytic complex that progressively degrades ubiquitinylated proteins to small peptides.[1] The proteasome is composed of a 20S catalytic core and two 19S regulatory caps which are responsible for the recognition, unfolding and translocation of protein substrates into the 20S catalytic core cavity. The eukaryotic 20S proteasome is formed by four stacked rings, and each of the two inner rings is composed of seven different β subunits.[2] The β1, β2 and β5 subunits contain the post-acid (PA), trypsin-like (T-L) and chymotrypsin-like (CT-L) active sites where peptide bonds are cleaved on the carboxyl side of acidic, basic and hydrophobic amino acid residues, respectively.[3] The ubiquitin–proteasome system is involved in the degradation of regulatory proteins that are crucial for many intracellular processes, including cell progression, apoptosis and NF-κB activation. Proteasome inhibitors cause selective apoptosis of malignant cells and are therefore a new class of antineoplastic agents.[4] Bortezomib (Velcade®) has been approved for treating incurable multiple myeloma and mantle lymphoma.[5] Carfilzomib (PR-171),[6] salinosporamide A (NPI-0052)[7] and CEP-18770[8] are in phases I and II clinical trials. Most proteasome inhibitors, such as epoxyketones, peptide aldehydes, peptide vinyl sulfones and peptide boronic acids, are covalent binding inhibitors because they bear a reactive group that forms a covalent bond with the catalytic O^{γ}-Thr in the three catalytic sites.[9–11] Non-covalent inhibitors should have smaller side effects in therapeutics because they should not have the inherent drawbacks often associated with reactive groups, such as a lack of specificity, excessive reactivity and instability.[12] However, they have been less extensively investigated. They include ritonavir,[13] aminobenzylstatine derivatives,[12] lipopeptides,[14] macrocyclic[15,16] or linear TMC-95 derivatives.[17] This report describes the first representatives of a new class of non-covalent 20S inhibitors based on a central fluorinated pseudopeptide.

Fluorine has become a fundamental tool in the development of drugs.[18–22] Trifluoromethylated compounds are particularly important, as shown by the number of CF_3-containing drugs and drug-candidates in clinical use or in development. The trifluoromethyl group is often used in medicinal chemistry to improve metabolic stability and/or biological activity.[23] It is hydrophobic, electron-rich, bulky and it can mimic functional groups such as methyl, isopropyl and phenyl. Consequently, incorporating a trifluoromethyl group into peptides and peptidomimetics can greatly alter their structural properties and thus their ability to interact with receptors or enzymes. The incorporation of a trifluoromethyl group into peptidomimetics to produce potent inhibitors of various enzymes has been extensively studied.[24]

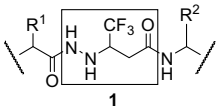

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Figure 1. New trifluoromethyl-β-hydrazino acid scaffold.

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As part of ongoing investigations into the design of proteasome inhibitors and the development of new trifluoromethyl peptidomimetics,[25–30] we have developed new trifluoromethyl inhibitors based on the trifluoromethyl-β-hydrazino acid scaffold **1** (Fig. 1).

Retro hydrazino-azapeptoids were recently described as covalent proteasome inhibitors with IC_{50}s of 50–500 μM,[31] whereas hydrazinopeptides inhibited the serine protease leukocyte elastase.[32] β-Hydrazino acids are peptidomimetic building blocks that have two nitrogen atoms. They can be considered to be analogs of β-amino acids in which the amine group has been replaced by a hydrazine. But while the β-amino acids are well documented,[33–35] almost nothing is known about these peptidomimetics. Obviously, these structures can mimic the typical secondary structure of native α-peptides, making them useful tools with which to design new protease inhibitors. To our knowledge, the synthesis of CF_3-β-hydrazino acid has not been reported. This scaffold was chosen because it has a trifluoromethyl group, which can greatly improve the acidity of the neighbouring hydrazine functional group and thus increase its hydrogen bond donor ability.[22] The non-covalent interactions of several peptidic and peptidomimetic proteasome inhibitors are mainly mediated by hydrogen bonds implicating in particular residues 21, 47 and 49 with the formation of an antiparallel β-sheet between the inhibitor and the amino acid residues of the binding pockets.[9] Occupancy of the S1 and S3 subsites appears to be essential for efficient inhibitor binding.[9,12] The elongation of the backbone may introduce flexibility, thus facilitating the putative interaction of substituents R^1 and R^2 with the enzyme S1 and S3 pockets. Compounds **2–8** (Fig. 2) were designed and synthesized. They have a phenylalanine amino acid at the C-terminal end whose phenyl group is assumed to occupy the S1 pocket. The N-terminus of our starting molecule **2** was inspired by aminobenzylstatine derivatives,[12] which interact with the S3 pocket and the phenoxysubstituted benzylic *N*-terminal group with the AS1 and AS2 accessory hydrophobic pockets. We introduced several structural variations in the pseudopeptide N-terminus in order to evaluate their influence on specific binding and affinity for the three types of active sites.

Compounds **2–8** were prepared as outlined in Scheme 1. The common intermediate **12** was obtained in four steps from ethyl 4,4,4-trifluorocrotonate **9**.[36] Michael addition of *tert*-butyl-carbazate **10** on **9** gave the N-protected trifluoromethyl-β-hydrazino ester **11** which was deprotected and coupled to the L-phenylalanine methyl ester. The cleavage of the Boc group of the hydrazine moiety gave compound **12**. This intermediate **12** was then used to obtain compounds **2–8** using standard solution phase coupling chemistry. A simple coupling with 3-phenoxyphenylacetic acid gave compound **3**. Coupling **12** with Fmoc-L-3,4-dimethoxyphenylalanine, cleavage of the Fmoc group, and coupling with 3-phenoxyphenylacetic acid gave compound **2**. Coupling **12** with *N*α-Boc-*N*ε-Z-L-lysine gave compound **6,** which was *N*ε-Z deprotected to give compound **7,** or *N*α-Boc deprotected to give compound **8**. Compound **8** was then coupled to 3-phenoxyphenylacetic acid to give compound **4** which was N-deprotected to provide product **5**. The coupling of N-protected trifluoromethyl-β-hydrazino acid (obtained from **11)** to L-phenylalanine methyl ester gave a 1:1 mixture of diastereoisomers that could not be separated by flash chromatography or by crystallisation. All the compounds progressed then as 1:1 diastereoisomer mixes (except for compound **2** which was obtained with a diastereiosomeric ratio of 2/3, probably because of the loss of one diastereiosomer during purification). The ratio was evaluated by NMR ^1H and ^{19}F.

The capacities of molecules **2–8** (diastereoisomeric mixtures) to inhibit the three activities of rabbit 20S proteasome were assayed using appropriate fluorogenic substrates (Fig. 3).[14,17,37] The aldehyde proteasome inhibitor MG132 (Z-LLL-H) was used as standard.[17]

Compound **2** inhibited the CT-L and PA activities of rabbit 20S proteasome (IC_{50} = 85 and 72 μM, respectively) (Table 1). Shortening the pseudopeptide by connecting directly the phenoxy benzyl moiety on the trifluoromethyl-β-hydrazino acid scaffold and eliminating the 3,4-dimethoxyphenylalanine totally removed the capacity of molecule **3** to inhibit all three active sites (Table 1). Replacing the 3,4-dimethoxyphenylalanine amino acid by the more hydrophilic lysine amino acid could facilitate the interaction of the

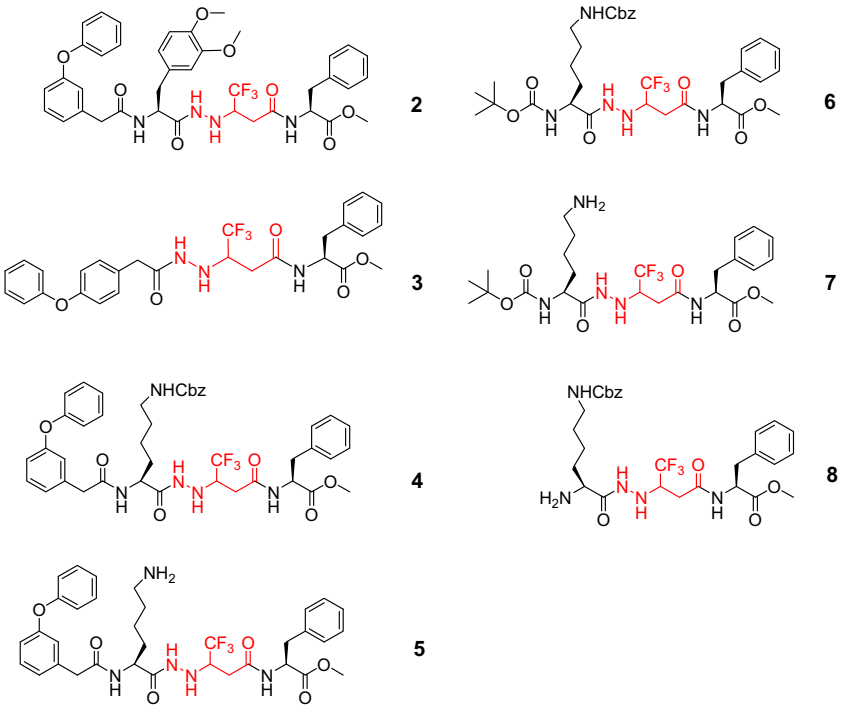
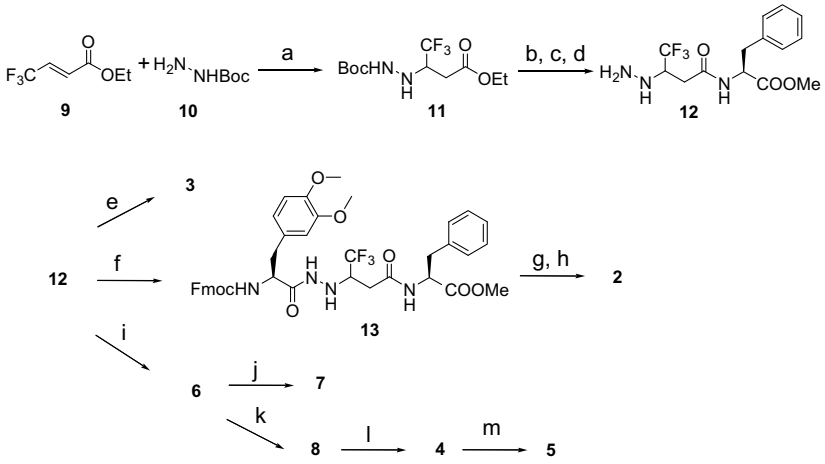


Figure 2. Synthesized trifluoromethyl-β-hydrazino compounds **2–8**.



Scheme 1. Synthesis of compounds **2**–**8**. Reagents and conditions: (a) MeOH, 70 °C, 94%; (b) 2 N aq NaOH, THF/MeOH, rt, 98%; (c) L-phenylalanine methyl ester hydrochloride, HBTU, HOBt, DIPEA, DCM/DMF, rt, 91%; (d) TFA, DCM, rt, 100%; (e) 3-phenoxyphenylacetic acid, HBTU, HOBt, 2,4,6-collidine, rt, 42%. (f) Fmoc-L-3,4-dimethoxyphenylalanine, HBTU, HOBt, 2,4,6-collidine, rt, 22%; (g) 10% piperidine/DMF, rt, 84%; (h) 3-phenoxyphenylacetic acid, HBTU, HOBt, 2,4,6-collidine, rt, 41%; (i) Nα-Boc-Nε-Z-L-lysine, HBTU, HOBt, DIPEA, DMF, rt, 57%; (j) H_2, 20% Pd/C, MeOH, rt then citric acid monohydrate, rt, 51%; (k) TFA, DCM, rt, 100%; (l) 3-phenoxyphenylacetic acid, HBTU, HOBt, 2,4,6-collidine, rt, 71%; (m) H_2, 20% Pd/C, MeOH, rt then TFA, rt, 100%.

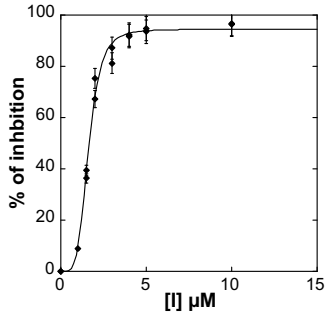


Figure 3. Inhibition of CT-L activity of rabbit 20S proteasome by compound **5** at pH 7.5 and 37 °C. The experimental data were fitted to equation% inhibition = $100[\mathbf{5}]^{nH}/(IC_{50}{}^{nH} + [\mathbf{5}]^{nH})$ with nH = 4.5.

Table 1
IC_{50} (μM) or % inhibition at 100 μM of rabbit 20S proteasome at pH 7.5 and 37 °C. *x*, activation factor. Values are means of three experiments. CT-L, chymotrypsin-like activity; PA, post-acid activity; T-L, trypsin-like activity; ni, non-inhibitor

Compound	CT-L	PA	T-L
2	85 ± 15	72 ± 0.7	*x*4
3	*x*2	ni	ni
4	ni	200	ni
5	1.6 ± 0.1	2.7 ± 0.1	8.4 ± 1.3
6	ni	ni	*x*3
7	32 ± 2	6 ± 0.5	30%
8	5.9 ± 0.5	ni	4.4 ± 1.2

εNH_2 group with the aspartic residues in the S3 pocket of the T-L active site.[16,17] It should also increase the solubility of the compound. Indeed, molecule **5** inhibited CT-L (IC_{50} = 1.6 μM) and PA (IC_{50} = 2.7 μM) activities and also T-L activity (IC_{50} = 8.4 μM), whereas protecting the lysine group eliminated inhibitory power (CT-L and T-L) or decreased it (PA, factor = 100) (molecule **4**). The phenoxysubstituted benzylic *N*-terminal group was not essential for inhibition since molecule **7** was a moderate inhibitor (compare compound **5**). The free N-terminus was favorable (IC_{50} = 5.9 μM, CT-L) and (IC_{50} = 4.4μM, T-L), although the εNH_2 group was protected (molecule **8** compared to molecule **7**). Again, a positive charge on the lysine lateral chain or N-terminus stimulated binding to the T-L active site. But, neither lysosomal cathepsin B nor cytosolic calpain I was inhibited by compounds **5**, **7** and **8**.[38] We used a cell-based chemiluminescent assay[39] to show that compound **5** inhibited the CT-L activity in human HeLa cells (20% inhibition at 50 μM after incubation for 1.5 h).

In conclusion, we have identified a series of fluorinated pseudopeptides that incorporate a trifluoromethyl-β-hydrazino acid scaffold, as a new class of proteasome inhibitors. These new fluorinated pseudopeptides are very easy to synthesize. A limited SAR around the fluorinated scaffold resulted in the discovery of compounds having differential inhibitory capacities for CT-L, PA and T-L in micromolar range without effect on challenging proteolytic enzymes such as calpain and cathepsin B. These encouraging results have led us to further optimize the lead compounds **5**, **7** and **8** using molecular modeling and continuing biological evaluation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.012.

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37. CT-L, PA and T-L activities were determined by monitoring the hydrolysis of Suc-LLVY-AMC, Z-LLE-βNA and Boc-LRR-AMC, respectively, for 45 min at 37 °C in the absence (control) or presence of test compounds (0.1–200 μM). The buffers (pH 7.5) were 20 mM Tris, 1 mM DTT, 10% glycerol, 0.02% (w/v) SDS for CT-L and PA activities, and 20 mM Tris, 1 mM DTT, 10% glycerol for T-L activity. The IC_{50} values (inhibitor concentrations giving 50% inhibition) were obained by plotting the percent inhibition against inhibitor concentration to equation:% inhibition = $100[I]/(IC_{50}{}^{nH} + [I]^{nH})$, or equation% inhibition = $100[I]^{nH}/(IC_{50}{}^{nH} + [I]^{nH})$ where nH is the Hill number. The K_m values of the fluorogenic substrates in our experimental conditions were: 30 ± 5 μM (Suc-LLVY-AMC), 77 ± 4 μM (Z-LLE-βNA) and 26 ± 6 μM (Boc-LRR-AMC).
38. Calpain I and cathepsin B, from Calbiochem, USA, were assayed at 37 °C using Suc-LLVY-AMC in 50 mM Tris–HCl (pH 7.2), 10 mM DTT, 2 mM $CaCl_2$ and ZRR-AMC in 352 mM KH_2PO_4 (pH 6), 48 mM $NaHPO_4$, 1 mM EDTA, 1 mM DTT, respectively.
39. Proteasome Glo Cell-Based Assay (Promega) using MG-132 as a standard.