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α,β-Unsaturated *N*-Acylpyrrole Peptidyl Derivatives: New Proteasome Inhibitors

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Because of the encouraging results obtained using vinyl ester derivatives, we synthesized and tested a novel series of peptide-based proteasome inhibitors bearing a new pharmacophore unit at the C-terminal. *N*-Acylpyrrole moiety is a potential substrate for Michael addition by catalytic threonine. Several analogues have demonstrated a selective inhibition of the multicatalytic complex β1 subunits, the capacity to permeate cellular membrane, and good pharmacokinetics properties.

Introduction

The 26S proteasome, a multicatalytic and multisubunit threonine protease complex (2.4 MDa), is implicated in many biological processes, including the generation of antigenic peptides presented by major histocompatibility complex class I (MHC I) molecules and degradation of most cytosolic proteins in mammalian cells.[1−6] The 26S proteasome consists of a central 20S core containing three different active sites (β1, β2, and β5) and two 19S regulatory complexes. On the basis of the preferential cleavage of short fluorogenic peptidic substrates, the various activities of the proteasome have been assigned to individual subunits and classified as "chymotryptic-like" (β5), "tryptic-like' (β2)", and "peptidyl-glutamyl peptide hydrolyzing", PGPH[a] (β1).[7,8]

As previously mentioned, proteasome plays an essential role in protein turnover in living cells, and deregulation of the ubiquitin−proteasome pathway in humans causes several diseases, such as cancer and neurodegenerative, autoimmune, and metabolic disorders. Furthermore, proteasome inhibition has a negative influence on the stability of many proteins, especially those involved in cell cycle regulation. Indeed, most cells become sensitive to apoptosis after treatment with proteasome inhibitors.[9,10] Interestingly, tumor cells are usually more sensitive to proteasome inhibition than their normal counterparts. Indeed, healthy cells display cell-cycle arrest when treated with proteasome inhibitors but, unlike tumor cells, are not as susceptible to apoptosis.[11,12] Thus, selective inhibitors of catalytic proteasome subunits are appealing targets for drug development.[13]

Many inhibitors of the ubiquitin−proteasome pathway that are able to interact with the 20S catalytic core of the multicatalytic complex and that can readily enter cells and selectively inhibit this degradation pathway are currently available. These synthetic inhibitors possess a homogeneous structural profile; they are generally peptide-based compounds with a C-terminal pharmacophore function required for primary interaction with catalytic threonine on the enzyme. The peptide component seems to be important for determining specificity for the three catalytic sites by secondary interaction with the enzymatic pockets. Essentially, most of these inhibitors act on the chymotrypsin-like activity of the proteasome, although they do undergo partial inhibition. Recently, research has begun to address the development of molecules with specific activity for the proteasome subunits, and already considerable progress has been made, particularly by Crews et al. who, by varying the sequence at P2−P4 in epoxomicin, were able to generate specific inhibitors of the chymotrypsin-like and postacidic activities, although the physiological consequences of β1-subunit inhibition still remain to be clarified.[14,15] Likewise, new aldehyde compounds have shown an interesting selectivity for the trypsin-like activity, as have vinyl sulfone analogues, although the foremost proteasome inhibitor to date (bortezomib, Velcade), approved by the U.S. Food and Drug Administration as a prescription drug for the treatment of multiple myeloma, is selective for the chymotrypsin-like activity with a partial action on the β1 site.[16,17]

We recently reported the design and synthesis of small peptide molecules with selective inhibitory activity toward the three catalytic sites and good pharmacokinetic properties. In our early studies, we have identified new oligopeptide inhibitors with different C-terminal pharmacophore units: amide analogues, compounds with arecholine derivatives, and in particular, a class of compounds bearing a C-terminal vinyl ester selective and specific for the β2 activitity of the proteasome. The pharmacophoric vinyl ester group is able to interact with the catalytic Thr in the same way as the well-known vinyl sulfone analogues.[18] The best of these derivatives inhibit the β2 subunit in the nanomolar range, are nontoxic, do not affect cell proliferation, and are capable of modulating the generation of antigenic peptides linked by MHC class I molecules. N-Terminal elongation has also been performed on vinyl ester prototypes, and in another series we have cyclized the linear peptide portion: both the N-terminal elongated peptides and the cyclic analogues have been shown to selectively inhibit the β1 subunit.

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[a] Abbreviations: Boc, *tert*-butoxycarbonyl; ChT-L, chymotrypsin-like; Fmoc, fluorenylmethyloxycarbonyl; HATU, *O*-(7-azabenzotriazolyl)tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; PGPH, peptidylglutamyl peptide hydrolyzing; TFA, trifluoroacetic acid; T-L, trypsin-like; WSC, water-soluble carbodiimide (1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide); Z, benzyloxycarbonyl-*N*-hydroxysuccinimide; VAP, vinylacylpyrrole.

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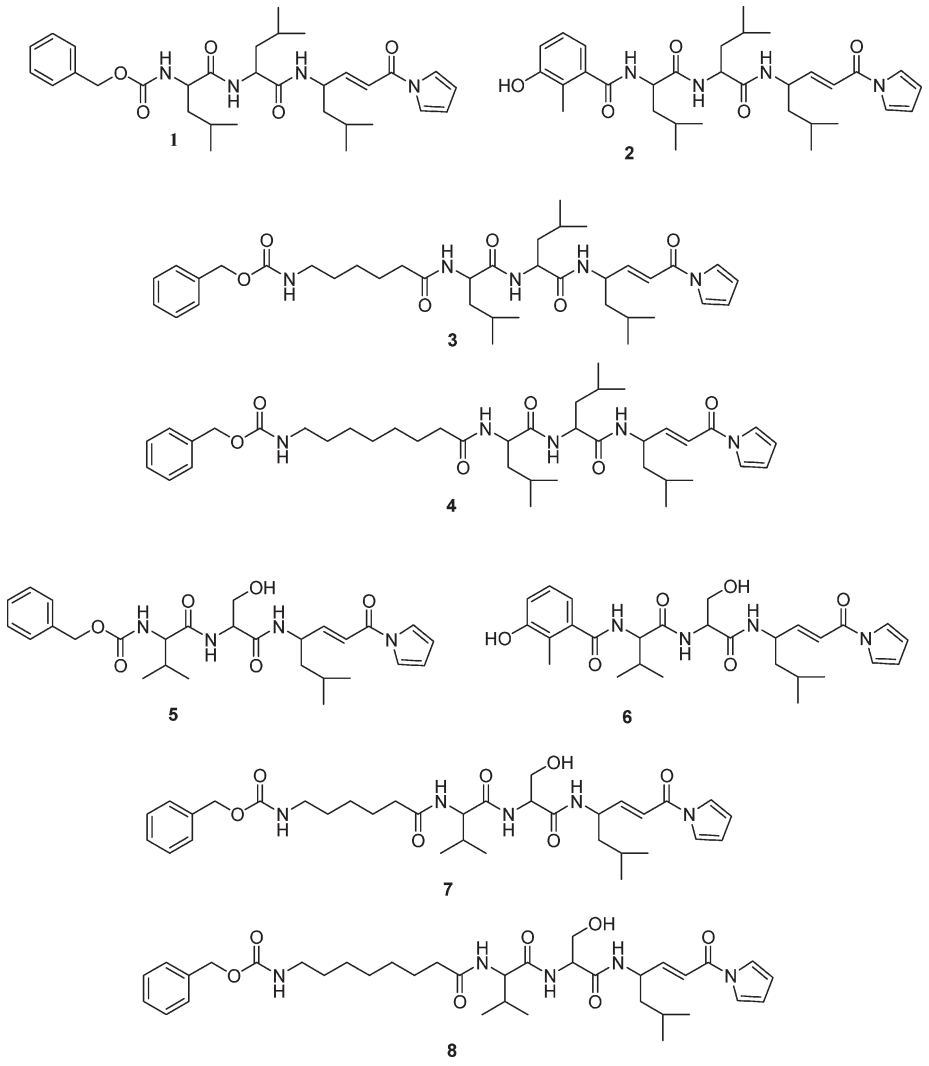


Figure 1. Structures of the novel pseudotripeptide inhibitors.

All of the vinyl ester derivatives have also demonstrated good resistance to proteolysis in plasma and an ability to permeate cell membranes.[19−25]

Herein we report the synthesis and biological activity of a novel class of peptide-based derivatives containing central sequences derived from those yielding the best results in prior experiments (Leu-Leu-Leu and Val-Ser-Leu) and a new pharmacophore unit formed from an α,β-unsaturated *N*-acylpyrrole group on the terminal leucine. The new pharmacophore can function as a substrate of the γ-hydroxythreonine side chain in Michael addition (Figure 1) in a similar way to the vinyl ester function but more effectively. On the basis of previously data, a benzyloxycarbonyl group (Z) (**1**,**5**), a 3-hydroxy-2-methylbenzoic group (**2**,**6**), or a Z N-protected 8-aminooctanoic acid (**4**,**8**) was inserted at the N-terminal.

Results and Discussion

α,β-Unsaturated *N*-acylpyrrole peptidyl derivatives were synthesized following the strategy reported in Scheme 1 by a mixed solution−solid phase synthesis approach. N_α-Protected leucine−vinyl−acylpyrrole (Boc-Leu-VAP) was prepared from the corresponding aldehyde[26] by reaction with pyrrolylmethylenetriphenylphosphorane without racemization. The ylide was synthesized as described in the literature.[27] The N-terminal functionalized dipeptides were assembled by Fmoc solid phase strategy, and the final reaction with the C-terminal pharmacophore yielded the desired **1**−**8**. All products were purified by preparative RP-HPLC, and structural verification was achieved by mass spectrometry and NMR spectroscopy (Table 1S in Supporting Information).

Biological evaluation of the new pseudotripeptides was carried out to assess their inhibition of the trypsin-like, chymotrypsin-like, and postacidic activities of the proteasome. Their inhibitory capacity was assayed on proteasomes purified from lymphoblastoid cell lines (LCL), using fluorogenic substrates specific for the three main proteolytic activities of the enzyme complex.[28,29] All compounds were assayed at different concentrations (from 0.001 to 10 μM), and the IC_{50} values were obtained after 30 min of incubation (Table 1) and subsequently compared with the well-known proteasome inhibitors epoxomicin and MG132 (the values are reported as the average of three independent determinations). The results obtained show that these new *N*-acylpyrrole α,β-unsaturated peptide based derivatives possess a unique biological profile. In general, none of the compounds inhibited the chymotrypsin-like or the trypsin-like activities of the proteasomes isolated from LCL. Likewise, inhibition of the β2 subunit was relatively unpronounced, with IC_{50} of about 10 μM. Only **1**, **2**, and **8** showed a mild inhibitory capacity of the chymotryptic activity with IC_{50} slightly below 10 μM. The different biological results of the new

compounds regarding ChT-L activity seem to be independent of the N-terminal substituent, although more hydrophobic sequences appear to be preferred. Interestingly, the introduction of the new C-terminal pharmacophore unit produced an appreciable decrease in $\beta2$ and $\beta5$ activity toward the amino acid sequences that showed a favorable profile for the same catalytic tasks in previous series. However, selective inhibition of the postacidic activity of proteasomes was generally found to be higher than that reported for the well-known proteasome inhibitors epoxomicin and MG132. In particular, derivatives **2**, **4**, **7**, and **8** displayed postacidic inhibition IC_{50} in the fractional micromolar range. Compound **8** represents the most potent inhibitor of the series. A structure−activity analysis emphasized that the *N*-acylpyrrole group is a favorable substrate of the γ-hydroxythreonine side chain in the $\beta1$ subunit. The biological response to the presence at the N-terminal of the benzyloxycarbonyl group (Z) in **1** and **5** was found to be the lowest in terms of potency. Hence, a good balance between the hydrophilic/lypophilic characteristics of the amino acid chain and the N-terminal substituent seems to promote the interaction with the $\beta1$ subunit, thereby confirming that a bulky substituent at the N-terminal favors $\beta1$-specific inhibition. After 1 and 2 h of incubation, the inhibitory profile of the new compounds was preserved, although accompanied by progressive reduction of the IC_{50}, a profile that seems to indicate a reversible inhibition.

Selected inhibitors **2**, **4**, **7**, and **8** were assayed to evaluate their ability to permeate the cell membrane of LCLs. After cell treatment, proteasomes were purified and assayed for their proteolytic activity using specific substrate for T-L, ChT-L, and postacidic (PGPH) activities. The results obtained were comparable to those observed in the in vitro assay (Table 1).

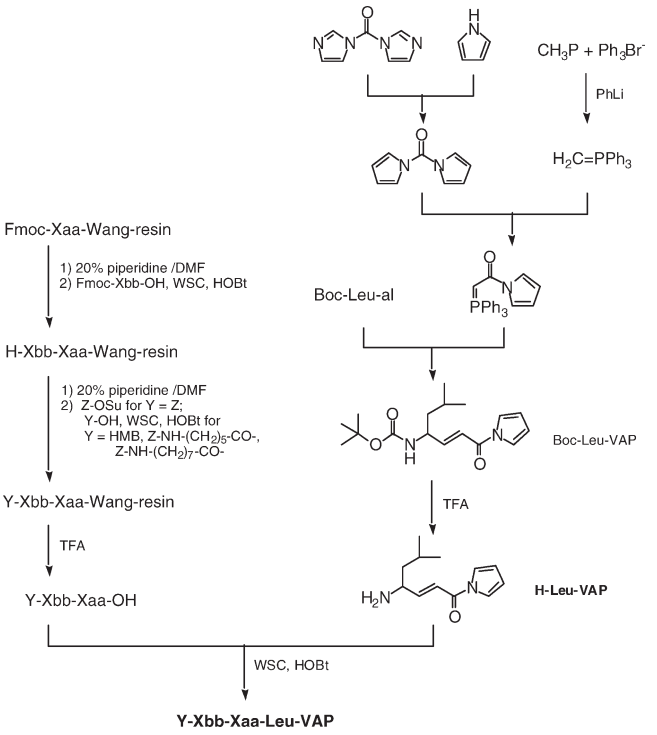
The susceptibility of these selected vinylacylpyrrole derivatives to enzymatic hydrolysis was determined by incubation at 37 °C in culture medium (RPMI) in the presence of 10% fetal calf serum and in human plasma.[30] The pseudopeptides show great stability in the cell culture medium and strong enzymatic resistance to human plasma proteases.

The degradation half-lives of the N-terminal-extended analogues shown in Table 1 were determined as described in the experimental protocols.

Since proteasomes play a key role in cell viability and proliferation, we evaluated the proapoptotic and antiproliferative activities of two selected compounds on a lymphoblastoid cell line and on Burkitt's lymphoma cells. The two cell lines were treated with different concentrations (from 0.1 to 10 μM) of **2** and **8** for 3 days, and the cellular effects of the compounds were evaluated by Trypan blue exclusion and cell counts. Both pseudotripeptides failed to induce cell death and to inhibit cell proliferation (data not shown).

The eight molecules are superimposed (Figure 1S in Supporting Information), using the "flexible alignment" procedure implemented in the MOE system of programs [MOE 2000.10 version, Chemical Computing Group Inc., 2008.2009.10]. The van der Waals surface of the least (**6**) and the most active (**8**) molecule is represented by pink and green dots, respectively. One of the critical points determining the ligand−proteasome interaction appears to be the molecular size. Actually, the residues delimiting the $\beta1$-binding pocket delineate a quite elongated active site, spanning a distance of about 18 Å, where the Thr1 residue is located in the middle.[31] To obtain a deeper insight into the structural aspects of the inhibitor−enzyme interaction mode, **8** was chosen to perform a docking simulation using a proteasome X-ray structure retrieved from the literature.[32] In Figure 2, a schematic diagram of the inhibitor−protein interactions is shown; because of its great conformational freedom, the molecule can easily accommodate itself in the binding site

Scheme 1. Synthesis of **1**−**8**[a]



[a] Xaa = Leu, Ser. Xbb = Leu, Val. Y = Z, HMB, Z-NH-(CH2)5-CO-, Z-NH-(CH2)7-CO.

Table 1. Inhibition of Proteasome Subunits and Enzymatic Stability of α,β-Unsatured *N*-Acylpyrrole Derivatives

		isolated enzyme LCL, IC_{50} (μM)[a]			in vivo inhibition LCL, IC_{50} (μM)[a]			
	compd	T-L	ChT-L	PGHP	T-L	ChT-L	PGHP	half-life (min) plasma
	epoxomycin	0.284	0.005	4.560				
	MG132	1.077	0.002	> 10				
1	Z-Leu-Leu-Leu-VAP	> 10	7.890	0.091				
2	HMB-Leu-Leu-Leu-VAP	> 10	3.630	0.065	> 10	5.600	0.135	> 360
3	Z-NH-$(CH_2)_5$-Leu-Leu-Leu-VAP	> 10	> 10	0.155				
4	Z-NH-$(CH_2)_7$-Leu-Leu-Leu-VAP	> 10	> 10	0.062	> 10	> 10	0.116	> 360
5	Z-Val-Ser-Leu-VAP	> 10	> 10	0.122				
6	HMB-Val-Ser-Leu-VAP	> 10	> 10	0.178				
7	Z-NH-$(CH_2)_5$-Val-Ser-Leu-VAP	> 10	> 10	0.097	> 10	> 10	0.147	> 360
8	Z-NH-$(CH_2)_7$-Val-Ser-Leu-VAP	> 10	5.080	0.036	> 10	> 10	0.058	> 360

[a] The values reported are the average of three independent determinations.

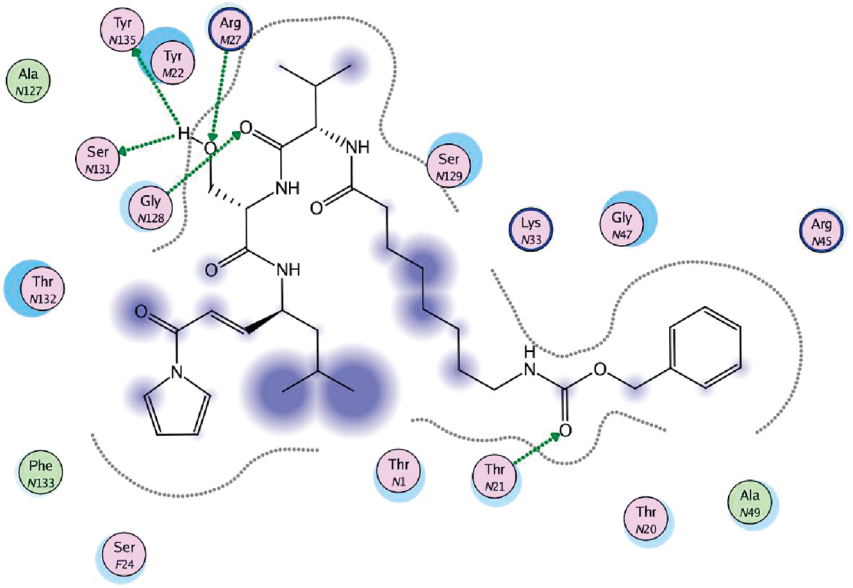


Figure 2. Schematic view of the ligand **8**−proteasome interactions. Hydrogen bonds are drawn as dotted lines. Polar and apolar residues of the binding site are represented as pink and green circles, respectively.

matching also the pocket dimensions. In contrast, **6**, whose equilibrium geometry is shown in Figure 2S in Supporting Information, is quite rigid, suggesting that the alchilic chain, present in the most active molecules, can play an important role in enabling the inhibitors to adapt to the binding site. A second docking simulation has been performed in order to better understand the selectivity of molecule **8** toward the β1 catalytic site. The structure used is the 2-spiro-lactacystin/proteasome complex,[33] in view of the fact that the inhibition properties of the spiro-lactone and of molecule **8** toward the β5 subunit are strictly comparable. The results are graphically displayed in Figure 3S in Supporting Information where the docked molecule is shown superimposed to the crystallographic ligand and surrounded by the interacting residues. It is interesting to note that the middle part of molecule **8** can mimic the disposition of N/O atoms of the smaller lactacystin molecule, a fact that could reasonably explain the similar biological behavior. In contrast, the terminal *N*-acylpyrrole moiety, which in the previous simulation appears to be involved in hydrophobic contacts with the surrounding residues, here is no longer implied in any interaction, actually pointing toward the inner part of the proteasome channel. These findings appear to support the idea that the C-teminal fragment plays an important role in the definition of the β1−β5 selectivity.

Conclusions

In this work we have designed, synthesized, and defined the biological profile of a new series of α,β-unsaturated *N*-acylpyrrolic, peptide-based proteasome inhibitors. The best derivatives of this series interact selectively for the β1 catalytic site of the proteasome and display good pharmacokinetic properties. Thus, this new class of inhibitors may represent a useful tool for elucidating the mechanisms underlying antigen production by the ubiquitin−proteasome system and may have future applications in new therapeutic protocols aimed at increasing the generation and presentation of poorly expressed CTL epitopes.

Experimental Section

General. Amino acids, amino acid derivatives, resins, and chemicals were purchased from Bachem, Novabiochem, or Fluka (Switzerland). Crude pseudopeptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm assembly column C18 (30 cm × 4 cm, 300 Å, 15 μm spherical particle size column). The column was perfused at a flow rate of 50 mL/min with a mobile phase containing solvent A (10% v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0 to 50% of solvent B (60% v/v, acetonitrile in 0.1% TFA) in 25 min was adopted for the elution of compounds. HPLC analysis was performed by a Beckman System Gold with a Hypersil BDS C18 column (5 μm; 4.6 mm × 250 mm). Analytical determination and capacity factor (K') of the peptides were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at flow rates of 1 mL/min using the following linear gradients: (a) from 0% to 100% B in 25 min and (b) from 10% to 70% B in 25 min. All pseudopeptides showed less than 1% impurities when monitored at 220 and 254 nm. Molecular weights of compounds were determined by a ESI Micromass, ZMD2000 mass spectrometer. ^{1}H NMR spectroscopy was obtained on a Bruker AC 200 spectrometer. The purity of tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Chemistry Department, University of Ferrara, with a Yanagimoto MT-5 CHN recorder elemental analyzer. All tested compounds possess a purity of at least 95% of the theoretical values.

Proteasome Subunit Inhibition Assays. Partially purified proteasomes were obtained from lymphoblastoid cell lines, untreated or treated for 12 h at 37 °C with the inhibitors, as previously described.[34]

Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-AMC (Sigma) were used to measure chymotrypsin-like, trypsin-like, and postacidic proteasome activities, respectively. Substrates were incubated at 37 °C with proteasomes, untreated or pretreated with 0.001−10 μM test compounds, in activity buffer for 30 min. Fluorescence was determined by a fluorimeter (Spectrafluor Plus, Tecan, Salzburg, Austria) using an excitation of 360 nm and emission of 465 nm. Activity was evaluated in fluorescence units, and the inhibitory activity of the compounds is expressed as IC_{50}. The data were plotted as percentage control (the ratio of percentage conversion in the presence or absence of inhibitor) vs inhibitor concentration and fitted with the equation $Y = 100/[1 + (X/IC_{50})^A]$, where IC_{50} is the inhibitor concentration at 50% inhibition and A is the slope of the inhibition curve.

Growth Inhibition Assays. The cells were seeded in duplicate in a 24-well plate at a density of 50 × 10^3 cells/mL in medium

containing 10% FCS. A lymphoblastoid cell line (LCL) and Burkitt's lymphoma JiJoye cells were cultured in medium alone or in medium containing **5** or **14** at different concentrations (from 0.1 to 10 μM), and cell viability and proliferation were evaluated by Trypan blue exclusion and cell counting for 3 days.

Enzymatic Stability Assays. The stability of selected new vinyl ester inhibitors under proteases degradation was studied in human plasma. Test compounds were incubated with plasma (0.6 mL) in a total volume of 1.5 mL of 10 mM Tris-HCl buffer, pH 7.5. Incubation was performed at 37 °C for 360 min. The incubation was terminated by addition of ethanol (0.2 mL), the mixture was poured at 21 °C, and after centrifugation (5000 rpm for 10 min) aliquots (20 μL) of the clear supernatant were injected into an RP-HPLC column. HPLC was performed as described in analytical determinations. The degradation half-life ($T_{1/2}$) was obtained by a least-squares linear regression analysis of a plot of the logarithmic inhibitor concentration versus time, using a minimum of five points.

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Supporting Information Available: General synthetic procedures, ^{1}H NMR of inhibitors **2** and **8**, Table 1S listing analytical data of **1**−**8**, experimental procedure for molecular modeling studies, and Figures 1S, 2S, and 3S from molecular modeling studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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