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^{19}F NMR monitoring of the eukaryotic 20S proteasome chymotrypsin-like activity: an investigative tool for studying allosteric regulation†‡

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The proteasome displays three distinct proteolytic activities. Currently, proteasome inhibitors are evaluated using specific fluorescent substrates for each of the individual active sites. However, the photophysical properties of the commonly used fluorophores are similar and thus, the simultaneous monitoring of the three proteolytic activities is not possible. We have developed a bimodal fluorescent fluorinated substrate as a novel tool to study the chymotrypsin-like (ChT-L) proteolytic activity and its regulation by inhibitors and by substrates of trypsin-like (T-L) and caspase-like sites (PA). We demonstrate that this substrate is reliable to evaluate the ChT-L inhibitory activity of new molecules either by fluorescence or ^{19}F NMR spectroscopy. We have found that the ChT-L activity is dramatically reduced in the presence of T-L and PA substrates. This work provides a proof of concept that the fluorinated substrate enables investigation of the allosteric regulation of the ChT-L activity.

Introduction

Recognized as the central enzyme of non-lysosomal protein degradation, the proteasome represents a main therapeutic target as it regulates a vast array of pathways during cell life and death, including cell-cycle progression. Consequently, selective inhibitors of the proteasome are promising drug candidates for treating diseases such as cancer. Bortezomib and Carfilzomib are clinically used for the treatment of incurable multiple myeloma (both compounds) and mantle lymphoma (Bortezomib).[1] Three distinct proteasome proteolytic activities are described as chymotrypsin-like (ChT-L), trypsin-like (T-L) and caspase-like or post-acid (PA) activities corresponding to the peptide cleavage on the carboxyl side of hydrophobic, basic and acidic amino acid residues, respectively.[2] Fluorescent substrates for each of the individual active sites have been designed and are commonly used to evaluate the inhibitory activity and the specificity of the proteasome inhibitors towards the three active sites. These substrates are peptides with a fluorophore capped at the C-terminus which gives a fluorescent response upon hydrolysis. However, commonly used fluorophores, 7-amino-4-methyl coumarin (AMC) or β-naphthylamide (β-NA), possess very similar photophysical properties (the wavelengths of excitation and emission are 360 and 460 nm for AMC and 340 and 405 nm for β-NA). AMC is usually preferred because β-NA is much less fluorescent than AMC and it is carcinogenic.[3b] Thus, the use of these fluorophores is a major lock for the simultaneous monitoring of the three proteolytic activities.[3,4] The current knowledge of the catalytic mechanism of proteasome is also essentially based on these fluorescent substrates. However, some previous studies suggest that an active site can allosterically activate or inhibit the others.[3–6] In particular, the ChT-L activity can be modulated in the presence of T-L or PA substrates (both substrates might induce a change in the activity and the conformation of 20S proteasome resulting from the binding of T-L and PA substrates at their specific site or at another site).[4,5,7]

The importance and significance of allostery in molecular therapies in general and in particular in the proteasome field must be more carefully examined and validated. However, in the absence of investigative tools, the study of allostery remains challenging.[8] A better understanding of proteasome regulation is expected to enable the development of more selective therapeutic agents. In particular, since inhibition of ChT-L activity has been the focus of antineoplastic agents, there is a crucial need for a simple and reliable method to study ChT-L proteolytic activity and to evaluate potential ChT-L proteasome inhibitors in the presence of one or both substrates with T-L and PA activities. The finding of fluorescent probes with a distinct and univocal wavelength has been scarcely described and is challenging.[4,5]

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For this purpose, we developed a new fluorine-containing substrate with ChT-L proteasome activity to evaluate univocally ChT-L proteasome inhibitors by ^{19}F NMR in the presence of PA or T-L substrates. The powerful and attractive role of the fluorine atom as a tag lies in the high sensitivity of ^{19}F NMR spectroscopy, the 100% natural abundance of the isotope ^{19}F and the lack of background signal (unlike ^{1}H and ^{13}C nuclei).[9,10] The FABS (Fluorine Atoms for Biochemical Screening) method uses ^{19}F NMR spectroscopy to detect the initial and enzymatically modified substrates. It has been used to screen inhibitors of a large panel of enzymes and to explore protein functions.[9,10] Moreover, ^{19}F NMR-based detection methods are known to be less subject to artefacts than fluorescence spectroscopy.[9,10]

Results and discussion

Design of the new fluorine-containing substrate with ChT-L proteasome activity

Requirements for our new substrate were as follows: a CF_3 group was introduced in order to enhance the NMR signal intensity (as demonstrated by Dalvit *et al.* with the 3-FABS method[9,11]); the CF_3 group was placed on a fluorescent core in order to offer a multimodal probe; the hydrolysis of the substrate must liberate the fluorinated fluorescent moiety with a ^{19}F chemical shift different from that of the substrate; a quantitative monitoring of the hydrolysis is essential to determine molecule inhibitory activities (IC_{50}).

In order to fulfill all these criteria, the fluorophore AMC of Suc-LLVY-AMC, which is the commonly used specific substrate of the ChT-L site, was replaced by the fluorophore containing a CF_3-probe, 7-amino-4-trifluoromethylcoumarin (AFC, see Fig. 1). The use of such a multimodal probe has been very seldom reported; however, multimodal probes enable complementary experiments and can be further used in cellular systems and living bodies to perform fluorescence measurement or ^{19}F magnetic resonance imaging (MRI).[12–14] In particular, to our knowledge, AFC has only been used as a probe to detect an enzymatic process by fluorescence and ^{19}F NMR but not to quantify the enzymatic reaction and consequently evaluate the inhibitory activity of compounds on the enzyme activity.[12,13]

The substrate Suc-LLVY-AFC was easily synthesized in nine steps from the commercially available AFC by a series of deprotection and coupling reactions classically used in peptide chemistry (Scheme 1 and ESI‡).

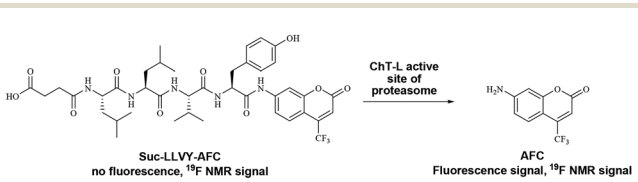
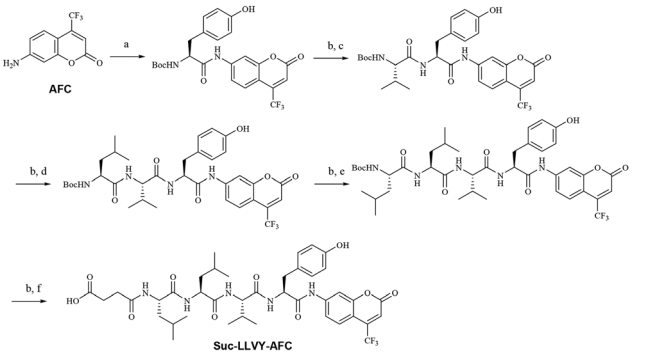


Fig. 1 Structure of the substrate Suc-LLVY-AFC and representation of the enzymatic reaction releasing the bimodal probe AFC.



Scheme 1 Synthesis of Suc-LLVY-AFC. (a) Boc-Tyr-OH, $POCl_3$, −15 °C, 15 min, 46%; (b) 4 M HCl, dioxane, rt, 2 h, quantitative; (c) Boc-Val-OH, HOBt, EDCI, DIPEA, DMF, rt, 16 h, 64%; (d) Boc-Leu-OH, HOBt, EDCI, DIPEA, DMF, rt, 16 h, 46%; (e) Boc-Leu-OH, HOBt, EDCI, DIPEA, DMF, rt, 16 h, 60%; (f) succinic anhydride, DIPEA, DMF, rt, 16 h, 56%.

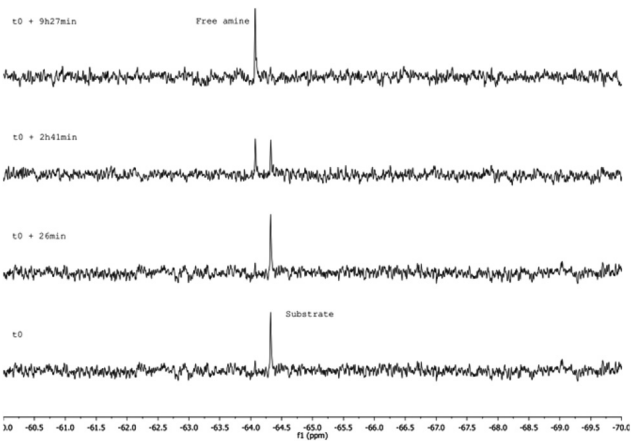


Fig. 2 ^{19}F NMR spectrum across 9h27 depicting the production of AFC (free amine) from Suc-LLVY-AFC (substrate, 30 μM) in the presence of rabbit 20S proteasome (6 nM).

Enzymatic activity of the rabbit 20S proteasome on Suc-LLVY-AFC

The enzymatic activity of the rabbit 20S proteasome on Suc-LLVY-AFC was controlled by the concomitant decrease of the ^{19}F NMR substrate signal and increase of the ^{19}F NMR AFC signal (Fig. 2). Notably, although the fluoro group is far from the cleavage site, the released amine AFC displays a distinct chemical shift to Suc-LLVY-AFC (−64.09 and −64.34 ppm respectively). The Suc-LLVY-AFC signal disappeared totally in favour of the AFC signal in less than 10 hours (Fig. 2). The found K_m value of Suc-LLVY-AFC (20 μM, determined by fluorescence, see ESI‡) was similar to that of Suc-LLVY-AMC (30 μM[15]).

Inhibition of the ChT-L activity of rabbit 20S proteasome by hydrazino acid-based pseudopeptides

The Suc-LLVY-AFC substrate was then compared with the non-fluorinated substrate Suc-LLVY-AMC for its ability to be used to determine the inhibitory activity of molecules on the rabbit

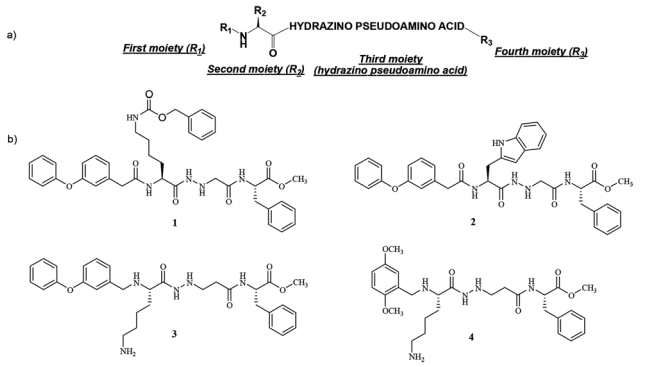


Fig. 3 (a) Schematic representation of hydrazino acid-based pseudopeptides. (b) Structures of molecules **1–4**.

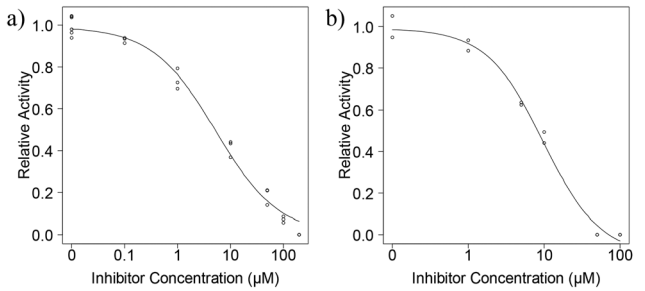


Fig. 4 Inhibition of the ChT-L activity of rabbit 20S proteasome by compound **1** at pH 7.5 and 37 °C, using the substrate Suc-LLVY-AFC. (a) Followed by fluorescence spectroscopy, λ_{ex} = 360 nm, λ_{em} = 480 nm; (b) followed by 3-FABS.

Table 1 Chymotrypsin-like (ChT-L) proteasome inhibition potency of molecules. IC_{50} (μM) at pH 7.5 and 37 °C, using the substrate Suc-LLVY-AFC

Compound	IC_{50} [a]	IC_{50} [b]
1	5.3 ± 2.2	9.7 ± 3.2
2	3.9 ± 0.6	8.5 ± 1.1
3	1.7 ± 0.3	3.1 ± 0.3
4	9.4 ± 1.4	10.5 ± 2.7

[a] Determined by fluorescence spectroscopy. [b] Determined by 3-FABS.

20S proteasome ChT-L activity. For that purpose, we employed the α-hydrazino acid-based molecule **1**, previously reported by our group.[15] Molecule **1** is one element of a library of new pseudopeptides where a natural α-amino acid is replaced by an α- or a β-hydrazino acid (Fig. 3).[15,16]

The inhibition of the ChT-L activity of rabbit 20S proteasome by compound **1** was followed by fluorescence spectroscopy and by 3-FABS using the substrate Suc-LLVY-AFC (Fig. 4). The IC_{50} of **1** determined by 3-FABS was similar to that determined by fluorescence spectroscopy (9.7 ± 3.2 μM and 5.3 ± 2.2 μM respectively, Table 1). These two values of IC_{50} were similar to the value obtained previously by fluorescence spectroscopy using the classical Suc-LLVY-AMC substrate (1.1 ± 0.1 μM).[15]

After having proved that Suc-LLVY-AFC is a good reference substrate, we took advantage of this novel easy method to evaluate new ChT-L proteasome non-covalent inhibitors. While most of the described proteasome inhibitors interact covalently with the active site of the enzyme, non-covalent bonding inhibitors are being studied to a lesser extent. They might be of particular interest because they are devoid of reactive electrophilic function prone to nucleophilic attack and, thus, they might induce lower side effects and improved bioavailability.[17,18] Three new α- and β-hydrazino acid-based pseudopeptides **2**, **3**, **4** were prepared (Fig. 3b). In compound **2**, a tryptophan residue replaced the lysine residue previously described in molecule **1**.[15] In the N-terminal part of pseudopeptides **3** and **4**, 3-phenoxyphenylmethyl and 3,4-dimethoxyphenylmethyl replaced respectively the 3-phenoxyphenylacetyl and 3,4-dimethoxyphenylacetyl groups in previously described inhibitors.[15]

The synthesis of compounds **2–4** was undertaken by a series of classical deprotection and coupling reactions (see ESI‡).

The inhibition of the ChT-L activity of the rabbit 20S proteasome by molecules **2–4** was investigated by fluorescence spectroscopy and by 3-FABS using the substrate Suc-LLVY-AFC. Again, results obtained with the two methods were very similar (Table 1). Molecules **2–4** inhibited the ChT-L activity with IC_{50} values up to the micromolar range. The inhibitory potency of **2** was not affected by the replacement of the lysine residue by tryptophane. Similarly for compounds **3** and **4**, increasing the flexibility of the N-terminal part of pseudopeptides by introducing 3-phenoxyphenylmethyl and 3,4-dimethoxyphenylmethyl, afforded similar and good inhibitory activities (the 3-phenoxyphenylacetyl and 3,4-dimethoxyphenylacetyl analogous compounds exhibited IC_{50} values of 1.4 and 8.8 μM[15]).

Monitoring of the ChT-L activity in the presence of PA and T-L substrates

We then employed Suc-LLVY-AFC (40 μM) to simultaneously monitor the ChT-L activity in the presence of fluorescent non-fluorinated substrates commonly used for PA (Z-LLE-AMC, 100 μM) and T-L (Boc-LRR-AMC, 50 μM) activities. A concentration of about twice the K_m of Z-LLE-AMC was used. However, due to a low solubility of Boc-LRR-AMC a lower concentration was used (50 μM). The presence of both substrates causes no background ^{19}F NMR signal. The ChT-L activity decreased dramatically upon exposure to both PA and T-L substrates (Fig. 5 and 6). We observed 69 and 75% of inhibition of the ChT-L activity by Z-LLE-AMC (100 μM) and Boc-LRR-AMC (50 μM) respectively (Fig. 5 and 6). The hypothesis is that the binding of these substrates to their respective specific active sites might induce a change in the conformation of 20S proteasome conformation and therefore induce a change in the ChT-L proteolytic activity. These results confirmed the previously reported negative effect of PA substrate on ChT-L activity.[4,5,7] However, the allosteric effect of T-L substrate on

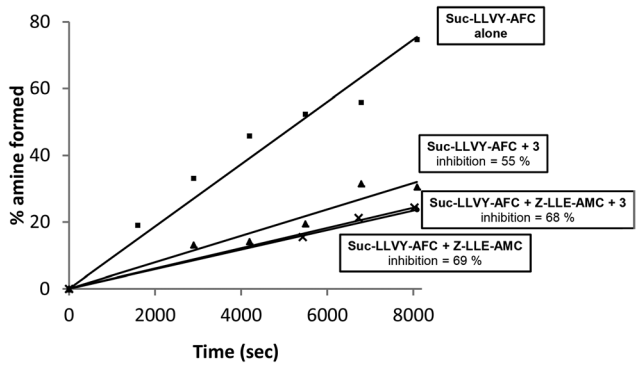


Fig. 5 Monitoring of the ChT-L activity as assessed with Suc-LLVY-AFC alone (40 μM, ■), in the presence of the PA substrate (100 μM, Z-LLE-AMC, ●), in the presence of inhibitor **3** (3 μM, ▲), or in the simultaneous presence of the PA substrate (100 μM, Z-LLE-AMC) and inhibitor **3** (3 μM, X).

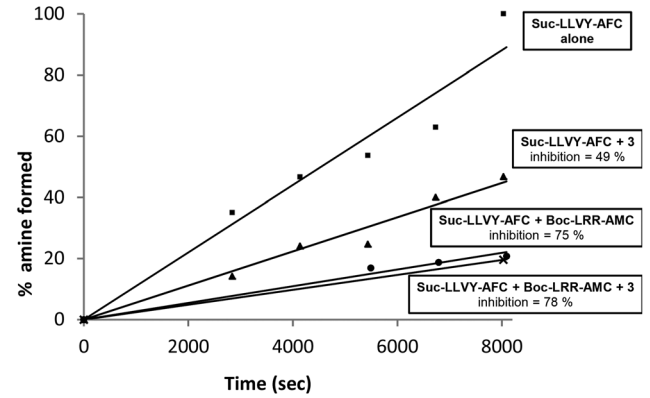


Fig. 6 Monitoring of the ChT-L activity as assessed with Suc-LLVY-AFC alone (40 μM, ■), in the presence the T-L substrate (50 μM, Boc-LRR-AMC, ●), in the presence of inhibitor 3 (3 μM, ▲), or in the simultaneous presence of the TL substrate (50 μM, Boc-LRR-AMC) and inhibitor **3** (3 μM, X).

the ChT-L activity has been less studied in the literature and results have been divergent. Indeed, an increased ChT-L activity[4] or its inhibition[5] in the presence of T-L substrates have been reported. Kisselev and Goldberg indicated that commercially available substrates for assaying T-L activity such as Boc-LRR-AMC might lack specificity at low concentrations because the K_m of the 26S proteasome for these substrates is high (>0.5 mM).[3] Thus, the decrease of the ChT-L activity by this T-L substrate might not be due to an allosteric effect but rather to its lack of specificity.

Monitoring of the ChT-L activity inhibition by pseudopeptides in the presence of PA and T-L substrates

To our knowledge, the evaluation of ChT-L inhibitors in the presence of both ChT-L and PA or T-L substrates has not been previously reported. Thus, we evaluated the inhibition of the ChT-L activity by the pseudopeptide **3** in the presence of a PA and a T-L substrate. At 3 μM, compound **3** inhibited about 50% of the ChT-L activity, while in the simultaneous presence of PA substrate (100 μM, Z-LLE-AMC) or of T-L substrate (50 μM, Boc-LRR-AMC), the ChT-L activity was further decreased (68 and 78% of inhibition respectively, Fig. 5 and 6). Surprisingly, in both cases, no synergic effect could be observed and the inhibition of the ChT-L activity was not increased in the case of the simultaneous presence of the inhibitor **3** and the PA or T-L substrates compared to the case where the PA or T-L substrates were added in the absence of **3**. The results seem to indicate that the allosteric effect is superior to the inhibitory effect of **3**. The superiority of the allosteric effect to the inhibitor activity should be further clarified. This may lead to a difference between the activity of some inhibitors determined *in vitro* with one specific ChT-L substrate, with thereafter the activity observed in cells in which natural PA and T-L substrates are present.

Conclusions

In conclusion, we have found a novel bimodal substrate to monitor the ChT-L activity of the 20S proteasome. We replaced the fluorophore AMC of the commonly used ChT-L substrate Suc-LLVY-AMC by the fluorophore containing the CF_3-probe, 7-amino-4-trifluoromethylcoumarin (AFC). We confirmed that this new substrate Suc-LLVY-AFC is reliable to evaluate the ChT-L inhibitory activity of molecules either by fluorescence or by the 3-FABS method by ^{19}F NMR spectroscopy giving results similar to those obtained solely by fluorescence with the commonly used substrate Suc-LLVY-AMC. We lifted a major lock existing with the actual fluorescent substrates that totally prevent the simultaneous monitoring of the three proteolytic activities, and demonstrated that Suc-LLVY-AFC is a relevant tool to simultaneously and quantitatively monitor the ChT-L activity in the presence of commonly used fluorescent PA and T-L substrates. Our results confirmed the influence of these PA and T-L substrates on the ChT-L activity. Indeed, we observed a dramatic decrease of the ChT-L activity upon exposure to both PA and T-L substrates, in the absence and presence of an inhibitor. Such a new fluorinated reference substrate will be helpful to further study the catalytic mechanism of proteasome and opens the prospect of deeper investigation of the allosteric regulation of ChT-L activity. This ^{19}F NMR approach is very simple due to the easy access to the fluorinated substrate and to the high sensitivity of the fluorine chemical shifts to local environment changes. It can be conducted with routine NMR equipment. This approach can also be extended to studies in living cells.

Experimental

Proteasome activity assays (IC_{50} determination by fluorescence)

ChT-L activity of purified 20S rabbit proteasome (Boston-BioChem) was determined by monitoring the hydrolysis of the

Suc-Leu-Leu-Val-Tyr-AFC substrate. Proteasome (0.3 nM final concentration) was incubated in 96-well plates (200 μL final volume) in the absence (control) or presence of various concentrations of inhibitors (0.1–100 μM, stock solutions in DMSO) in the following buffer: 20 mM Tris-HCl, 1 mM DTT, 0.02% (w/v) SDS, and 10% glycerol, pH 7.4. After 15 min of incubation at 37 °C, the substrate was added (2 μL of a 5 mM DMSO solution, 50 μM final concentration) and the rate of hydrolysis was monitored with a Fluostar Optima (BMG Labtech) microtiter plate reader by recording the fluorescence of the hydrolyzed AFC group (excitation filter: 360 nm; emission filter: 480 nm). The final DMSO concentration was kept constant at 2% (v/v) and each inhibitor was tested 3 times in triplicate. The initial linear portion of the curves (60–300 min) gave access to V_o and V_i values: the slopes of the reaction progress curves respectively in the absence or presence of inhibitors (V_o was considered to be 100% of the proteasome activity, while V_i < 100% were considered as inhibition events). These data were used to calculate the IC_{50} values (inhibitor concentration causing 50% decrease in proteasome activity) by fitting the experimental data to the equation: % inhibition = 100 $(1 - V_i/V_o)$. Dose–response curves were fitted to the data point by nonlinear regression with a 4-parameter log-logistic model as implemented in the drc package (v2.3-7)[19] within the R software environment (v3.0.1).[20] IC_{50} and the corresponding confidence intervals were determined with the same software using the delta method.

Proteasome activity assays (IC_{50} determination by ^{19}F NMR experiments)

All NMR experiments were performed at 37 °C using a Bruker Avance 400 MHz NMR spectrometer operating at a ^{19}F Larmor frequency of 376 MHz with a 5 mm inverse dual probe head (^1H–^{19}F). The ^{19}F-NMR spectra were recorded using a pulse sequence of proton decoupling with a spectral width of 12 019 Hz, an acquisition time of 1 s and a relaxation delay of 4 s. The spectra were analyzed with TOPSPIN 2.1 (Bruker). The enzymatic assays were performed with 400 μL sample volume in NMR tubes containing an insert filled with D_2O. Proteasome (20 nM final concentration) was pre-incubated for 15 min at 37 °C in the absence (control) or presence of various concentrations of inhibitors (0.1–100 μM, stock solutions in DMSO) in the buffer (20 mM Tris-HCl, 1 mM DTT, 0.02% (w/v) SDS, and 10% glycerol, pH 7.4) before the addition of the substrate (50 μM final concentration, stock solutions in DMSO). The final DMSO concentration was kept constant at 2% (v/v) and each inhibitor was tested twice. The rate of substrate hydrolysis by the proteasome was monitored in real-time in the NMR spectrometer at 37 °C by measuring every 22 min for 132 min (6 spectra) the integration of the ^{19}F-NMR signal of the release AFC. These curves gave access to V_o (control) and V_i (different inhibitor concentration) values, used to calculate the IC_{50} values (inhibitor concentration causing 50% decrease in proteasome activity) by fitting the experimental data to the equation: % inhibition = 100 $(1 - V_i/V_o)$ in the same way as fluorescence.

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