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Original article

Glutamine vinyl ester proteasome inhibitors selective for trypsin-like (β2) subunit

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Abstract

Here we report the study of a new series of peptide-based proteasome inhibitors with a vinyl ester moiety at C-terminal. The presence of Tic, a rigid analogue of phenylalanine, in the central portion of some derivatives is not favourable for the activity. The best analogue of the series shows a potent and selective inhibition for the β2 subunit and good enzymatic stability.

Keywords: Proteasome inhibitors; β2 selective inhibitors; Constrained residue; Vinyl ester tripeptides

1. Introduction

The 26S proteasome is a multicatalytic and multisubunit threonine protease complex (2.4 MDa) responsible for the degradation of normal and abnormal intracellular proteins and its composed of two subcomplexes, the first one 20S proteasome and the second two 19S regulatory particles, which cap the ends of the 20S core [1–4]. The substrates, marked for degradation by the binding of multiple molecules of ubiquitin (Ub), are recognized and bound to the 26S proteasome, unfolded, deubiquitinated and then transferred and degraded in the 20S catalytic chamber [5,6]. The 20S proteasome consists of four stacked rings each with seven distinct subunits, stacked one on top of each other, that are responsible for the proteolytic activity of the proteasome. The α-rings are outer, while the two inner β-rings form the central cavity of the cylinder and harbor the proteolytic sites. The proteasome utilizes in the catalytic site the side chain of an N-terminal threonine to perform the nucleophilic attack on the target bond. The peptidase activity can be assigned to the specific β subunits: β1 center cleaves peptide bonds preferentially on the carboxyl sides of acidic amino acid residues (peptidylglutamyl peptide hydrolyzing, PGPH), β2 after basic amino acid residues (trypsin-like activity, T-L), and β5 after hydrophobic residues (chymotrypsin-like activity, ChT-L) [7–9].

In the last two decades, the ubiquitin-proteasome system (UPS) has been recognized as an important component in numerous biological processes such as regulation of cell cycle progression, division, development and differentiation, apoptosis, cell proliferation, stress response, and modulation of the immune and inflammatory responses. The attractive idea to target the proteasomal pathway represents a new approach for the treatment of a range of pathologies such as cancer, inflammation, immune diseases and others [10–12].

Several class of inhibitors have been recognized and used to study the role of ubiquitin-proteasome pathway in cellular processes. In addition, proteasome inhibitors could be utilized as novel therapeutic agents to arrest tumor cell proliferation and as modulators of antigen presentation [13–15].

The most common inhibitors are short peptides linked to a C-terminal pharmacophore, able to interact with enzymatic

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catalytic threonine [16–24]. We have been interested in the development of peptide-based derivatives with selective activity towards the three catalytic sites and with good pharmacokinetics properties [25]. We recently report the identification of a new class of specific proteasome inhibitors selective for trypsin-like activity (β2 subunit) bearing a C-terminal leucine vinyl ester as pharmacophore that can function as substrate for catalytic threonine in Michael addition in the same way that has been suggested for the well-known peptide vinyl sulfone inhibitors. HMB-Val-Ser-Leu-VE is the most potent derivatives of the series with an $IC_{50} = 33$ nM. These compounds are non-toxic, and do not affect cell proliferation, but are able to modulate the generation of immunogenic peptides presented by MHC class I molecules [26]. Two small libraries of tripeptidic-based vinyl ester derivatives were synthesized and tested, starting with the HMB-Val-Gln-Leu-VE prototype. Investigations of P3 and P4 positions with a complete set of amino acid residues led to the identification of some derivatives that have showed remarkable selective inhibition of the β2 subunit. The 3-hydroxy-2-methylbenzoyl (HMB) moiety at the P4 position can be replaced with cyclic N-terminal substituent with different physicochemical properties or with aromatic or hydrophobic amino acid residues [27,28]. Some vinyl ester tripeptides have undergone X-ray crystallographic studies, with a conformational study of the molecules in the solid state, in vacuum and in a polar environment in order to define the inhibitor–enzyme interaction subsite pockets [29]. Through flexible alignment, our vinyl ester compounds have shown some conformational similarities with the cyclic inhibitors TMC95A (Fig. 1). The opportunity to display the molecules with a rigid conformation and good inhibitory activity could allow to obtain a lot of structural information and a target investigation about potency and selectivity.

On the basis of these evidences we have developed a new series of peptide-based molecules with the structure reported in Fig. 2.

Compounds **13**–**18** show a C-terminal glutamine vinyl ester while derivatives **25**–**28** bear the reference frame Leu-VE as pharmacophoric unit, that is, a potential substrates like a Michael acceptor for the catalytic threonine. This residue (data reported in literature) could confer a higher β2 subunit selectivity [30].

Compounds **13** and **14** have, respectively, the sequence Leu-Leu and Val-Ser as central dipeptidic portion by analogy with the most representative inhibitors of the previous series. We have obtained a major structural rigidity by putting in the center portion of the sequence the amino acid Tic (1,2,3,4-tetrahydro-isoquinolinic acid) [31]. This rigid analogue of phenylalanine was inserted in P3 position in the compounds **15**, **16**, **25** and **26** while in P2 position in the pseudopeptides **17**, **18**, **27** and **28**. All the derivatives of the series were functionalized in P4 position with a 2-methyl-3-hydroxybenzoyl group.

2. Chemistry

Vinyl ester tripeptides were synthesized by the classical solution method by means of C-terminal stepwise elongation.

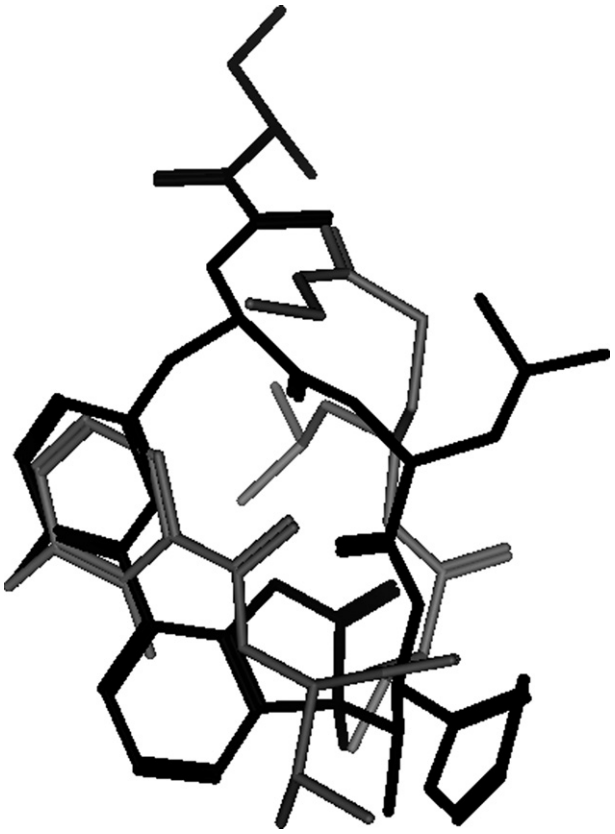


Fig. 1. Best superposition of HMB-Val-Ser-Leu-VE (light grey) and TMC95A (black) obtained by the flexible alignment procedure implemented in MOE (Molecular Operating Environment, Chemical Computing Group Inc., Version 2006.08). The TMC95A molecule was kept fixed in the conformation adopted in the proteasome binding site. The force field used was AMBER (W.D. Cornell, P. Cieplak, C.I. Bayly, I.R. Gould, K.M. Merz Jr., D.M. Ferguson, D.C. Spellmeyer, T. Fox, J.W. Caldwell, P.A. Kollman, A second generation force field for the simulation of proteins and nucleic acids, J. Am. Chem. Soc. 177 (1995) 5179–5197).

Compounds **13**–**18** bearing a glutamine vinyl ester moiety at C-terminal were synthesized following the strategy reported in Scheme 1. The N_{γ}-trityl-glutamine vinyl ester was prepared from the corresponding aldehyde by reaction with [(ethoxycarbonyl)methylidene]triphenylphosphorane without racemization [32,33]. WSC/HOBt were employed for the coupling steps, and Boc and Trt were removed by TFA treatment. Synthesis of compounds **25**–**28** with leucine vinyl ester at C-terminal follows the procedure describe above. Introduction of 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (Tic) in P2 or P3 position in compounds **15**–**18** and **25**–**28**, is possible without modifying standard acylation protocols. All products were purified by preparative RP-HPLC, and structural verification was achieved by mass spectrometry and NMR spectroscopy. HPLC capacity factors (K^1) and other physicochemical properties of compounds **13**–**18** and **25**–**28** are summarized in Table 1.

3. Biological activity

We tested the inhibitory capacity of the new vinyl ester derivatives on purified 20S proteasome using fluorogenic

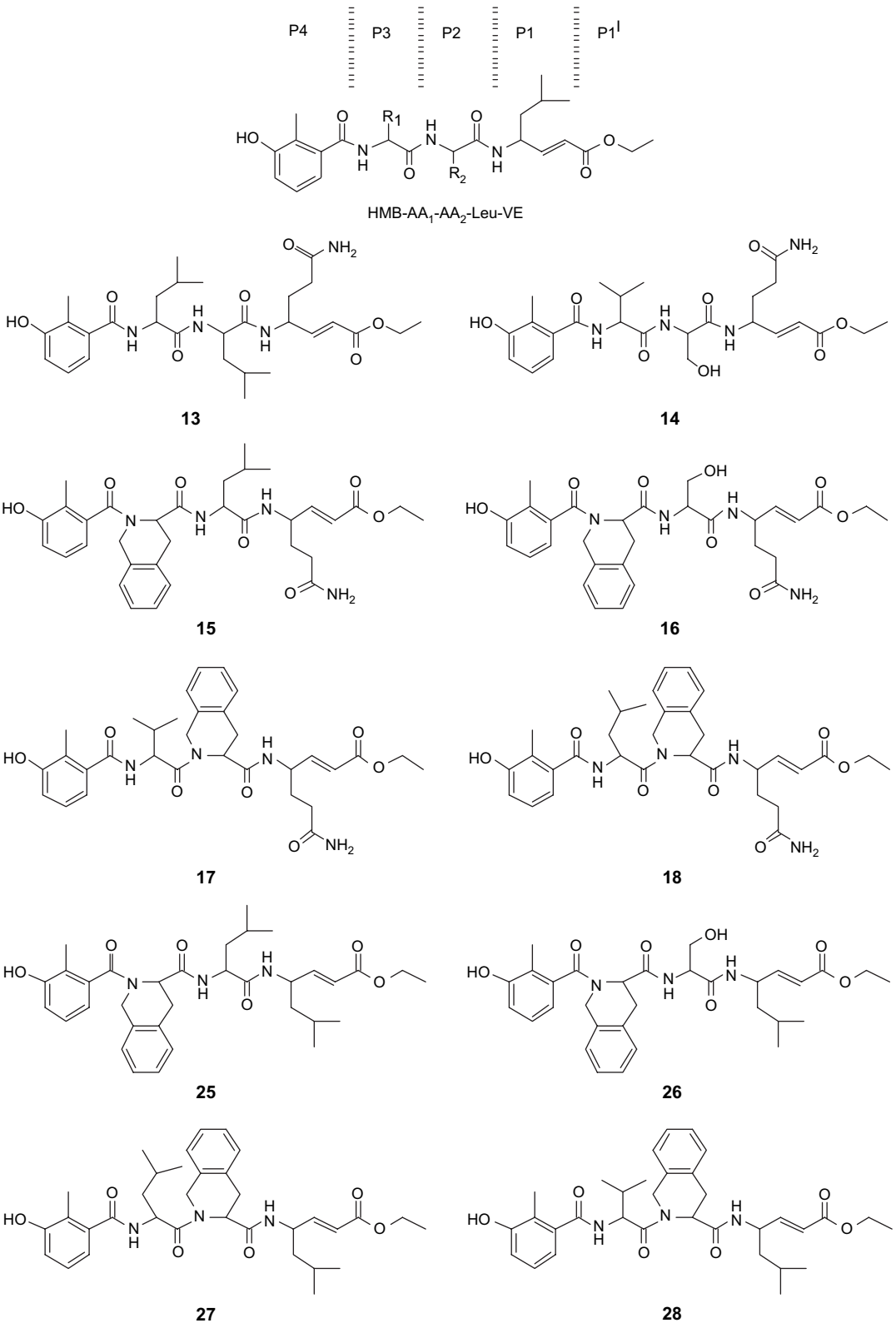
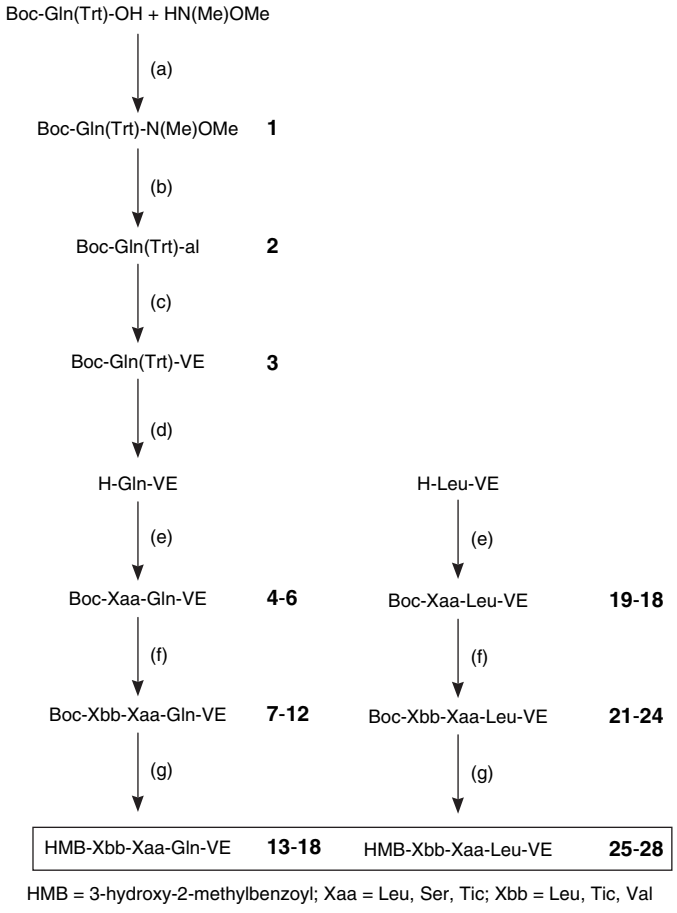


Fig. 2. Structure of the prototype and new vinyl ester pseudotripeptides inhibitors.

substrate, specific for the three major proteolytic activities of the enzymatic complex [34]. All the compounds were assayed at different concentrations (from 0.001 to 10 μM) for their capacity to inhibit the in vitro trypsin-like (T-L) activity, chymotrypsin-like (ChT-L) and post-acidic (PGPH) activities of the proteasome purified from lymphoblastoid cell lines. IC_{50} values against proteasome subsite activities obtained after 30 min of incubation are reported in Table 2. Similar IC_{50} were obtained after 3 h of incubation.



Scheme 1. Synthesis of the vinyl ester derivatives **13**–**18** and **25**–**28**. Reagents: (a) WSC, HOBt, NMM, DMF; (b) $LiAlH_4$, THF; (c) EtO-CO-CH=PPh3, toluene; (d) TFA; (e) Boc-Xaa-OH, WSC, HOBt, NMM, DMF; (f) (1) TFA; (2) Boc-Xbb-OH, WSC, HOBt, NMM, DMF; (g) (1) TFA; (2) HMB-OH, WSC, HOBt, NMM, DMF.

The cell membrane permeation of the most representative compounds **13**, **14**, **25** and **26** was tested in live cells. After cell treatment, proteasomes were purified and assayed for proteolytic activity as described above.

The enzymatic stability of inhibitors was determined by incubation at 37 °C in human plasma [35]. The degradation half-lives of the derivatives were determined as described in experimental protocols.

4. Results and discussion

The data obtained from enzyme inhibition tests underlined that the glutamine vinyl ester could be a substrate able to interact with catalytic threonine. In comparison to the prototype sequence bearing the leucine vinyl ester, the pharmacophore substitution did not yield interesting results in terms of potency and selectivity (Table 2).

Generally, the new sequences confirm the capacity to inhibit trypsin activity while only in few cases we have seen a β5 subunit inhibition at a concentration less than 10 μM; all the analogues are not active against post-acidic subsite.

P1 substitutions are critical on the catalytic subunits inhibition. The simple substitution of the Leu-VE with the Gln-VE in P1 position of the prototype sequence determines an insignificant decrease of the activity on the β2 subunit. Most impressive results are the conformational noise that derives from the presence of the Tic amino acid in the core of the structure.

The most potent and selective inhibitor of the series, compound **14**, preserves structural characteristics related to the prototype. In the new compound the position P1 is substituted with a propyl amide chain.

The presence of the constrained analogue of the phenylalanine in the central portion of the sequence is not favourable for the activity; this conformational constraint is really negative in compounds that carrying Tic in P2 position compared to P3 position. The pseudotripeptides **17**, **18**, **27** and **28** have showed a β2 subunit inhibition less than the other derivatives ($IC_{50} > 1$ μM).

The most active compounds **13**, **14**, **25** and **26** were evaluated on the basis of their ability to permeate cellular membrane; data obtained confirm the in vitro activity. IC_{50} values of inhibition are from 0.06 to 0.2 μM for the trypsin-like activity while it is >10 μM for the chymotrypsin-like activity (Table 2). These derivatives have shown the in vivo capacity to inhibit the enzymatic complex.

Table 1
Analytical data and physicochemical properties of vinyl ester tripeptides

No	Compound	HPLC		m.p. (°C)	$[\alpha]_D^{20a}$	$M+H^+$
		K^I(a)	K^I(b)			
	HMB-Val-Ser-Leu-VE					
13	HMB-Leu-Leu-Gln-VE	7.10	5.68	178–181	−25.5	560.7
14	HMB-Val-Ser-Gln-VE	6.15	4.98	202–207	−15.9	520.6
15	HMB-Tic-Leu-Gln-VE	7.32	5.95	183–186	−18.8	607.5
16	HMB-Tic-Ser-Gln-VE	6.23	5.02	198–200	−30.4	581.4
17	HMB-Val-Tic-Gln-VE	7.15	5.77	156–159	−23.7	593.4
18	HMB-Leu-Tic-Gln-VE	7.82	6.45	177–180	−20.9	607.5
25	HMB-Tic-Leu-Leu-VE	8.43	6.98	154–158	−36.4	592.5
26	HMB-Tic-Ser-Leu-VE	7.11	5.66	191–195	−37.2	566.4
27	HMB-Leu-Tic-Leu-VE	8.51	7.13	170–172	−16.9	592.5
28	HMB-Val-Tic-Leu-VE	8.22	6.48	173–176	−25.1	578.4

[a] c = 1, MeOH.

Table 2
Subsites of proteasome inhibition and enzymatic stability of new vinyl ester derivatives

No	Compound	IC_{50} (μM)[a]					Half-life of plasma (min)
		Isolated enzyme			In vivo inhibition		
		T-L	ChT-L	PGHP	T-L	ChT-L	
	HMB-Val-Ser-Leu-VE	0.033	>10	>10	0.050	>10	>360
13	HMB-Leu-Leu-Gln-VE	0.145	8.410	>10	0.180	>10	>360
14	HMB-Val-Ser-Gln-VE	0.056	>10	>10	0.067	>10	>360
15	HMB-Tic-Leu-Gln-VE	0.435	>10	>10			>360
16	HMB-Tic-Ser-Gln-VE	0.349	>10	>10			>360
17	HMB-Val-Tic-Gln-VE	2.340	>10	>10			>360
18	HMB-Leu-Tic-Gln-VE	5.890	>10	>10			>360
25	HMB-Tic-Leu-Leu-VE	0.157	6.890	>10	0.196	>10	>360
26	HMB-Tic-Ser-Leu-VE	0.098	7.360	>10	0.119	>10	>360
27	HMB-Leu-Tic-Leu-VE	>10	>10	>10			>360
28	HMB-Val-Tic-Leu-VE	8.510	>10	>10			>360

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

Stability to enzymatic hydrolysis of the vinyl ester derivatives was evaluated towards human plasma. All the compounds of the series have underlined a high resistance to the proteases with an half-lives upper to 6 h (Table 2).

5. Conclusions

We designed, synthesized and tested as proteasome inhibitors new tripeptidic-based derivatives bearing a vinyl ester C-terminal pharmacophoric unit as substrate of the catalytic γ-hydroxy threonine side chain in Michael addition. With the aim to obtain rigid molecules useful for close structural studies, we have inserted the Tic residue in the prototype sequence. Biological results of the new constrained compounds are not so interesting in terms of inhibition of the trypsin-like activity of the proteasome. The compounds that differ from the prototype only for the residue bearing the vinyl ester function in the C-terminal position are the most active and selective.

Considering that these molecules show good cell membrane permeation and resistance to proteolysis, we can use this new series of compounds as the base for the development of rigid structures able to show both potency and selectivity of the catalytic subunits of the proteasome.

6. Experimental protocols

6.1. General

Amino acids, amino acid derivatives and chemicals were purchased from Bachem, Novabiochem and Fluka (Switzerland).

Crude vinyl ester tripeptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 × 4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0 to 100% of solvent B (60%, v/v, acetonitrile in 0.1% TFA), 30 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 × 250 mm). Analytical determination and capacity factor (K^I) 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 for 25 min and (b) from 30 to 90% B for 25 min. All pseudopeptides showed less than 1% impurity when monitored at 220 and 254 nm.

The molecular weight of the compounds was determined by electrospray ionization (ESI) (MICROMASS ZMD 2000) and the values are expressed as $M + H^+$. TLC was performed in precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: (c) AcOEt/n-hexane (1:1, v/v), (d) CH_2Cl_2/methanol (9.5:0.5, v/v), (e) CH_2Cl_2/methanol (9:1, v/v), (f) CH_2Cl_2/methanol/toluene (17:2:1, v/v/v). Ninhydrin (1%) or chlorine iodine spray reagents were employed to detect the peptides. Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined by a Perkin–Elmer 141 polarimeter with a 10-cm water-jacketed cell. ^{1}H NMR spectroscopy was obtained on a Bruker AC 200 spectrometer.

6.2. Chemistry

6.2.1. Boc-Gln(Trt)-VE (*3*)

To a solution of the Boc-Gln(Trt)-al (3 mmol) in toluene (15 mL) was added [(ethoxycarbonyl)methylidene]triphenylphosphorane (5 mmol). The reaction mixture was stirred for 12 h at room temperature. The residue obtained after evaporation of the solvent was purified by flash chromatography (silica gel, CH_2Cl_2/MeOH 20:1). $[\alpha]_D^{20} = -41.5$ ($c = 1$, MeOH). ^{1}H NMR ($CDCl_3$): δ 1.08 (t, 3H), 1.33 (s, 9H), 1.82 (q, 2H), 2.18 (t, 2H), 4.08 (q, 2H), 4.29 (m, 1H), 5.85 (d, $J = 16.3$, 1H), 6.95 (dd, $J = 16.4$, 1H), 7.06–7.20 (m, 15H), 8.02 (s, 2H). HPLC: $K^I(a) = 5.90$. MS: $M + H^+ = 543.7$. Anal. Calcd. for $C_{33}H_{38}N_2O_5$: C, 73.04; H, 7.06; N, 5.16; O, 14.74. Found: C, 73.28; H, 7.11; N, 5.23.

6.2.2. H-Leu-VE

Leucine vinyl ester was prepared as described in Ref. [26].

6.2.3. General synthetic procedures

6.2.3.1. TFA deprotection. Boc was removed by treating vinyl ester intermediates with aqueous 90% TFA (1:10, w/v) for 30–40 min. After evaporation, the residue was worked up as described, triturated with Et_2O, centrifugated and the resulting solid was collected and dried.

6.2.3.2. Coupling with WSC/HOBt. The deprotected α-amine intermediate (1 mmol), NMM (1 mmol), WSC (1 mmol) and HOBt (1 mmol) were added to a solution of carboxylic component (1 mmol) in DMF (3 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 18 h at rt; then the solution was diluted with AcOEt (80 mL) and washed consecutively with 0.1 N HCl, brine, $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue was treated with Et_2O and the resulting solid separated by centrifugation.

*6.2.4. ^{1}H NMR data of compounds **13**, **14**, **25** and **26***

HMB-Leu-Leu-Gln-VE (**13**). ^{1}H NMR ($CDCl_3$): δ 1.02 (s, 12H), 1.27 (t, 3H), 1.75–1.84 (m, 8H), 2.15 (t, 2H), 4.15 (q, 2H), 4.35–4.51 (m, 3H), 5.07 (s, 1H), 5.92 (d, $J = 16.2$, 1H), 6.09 (s, 2H), 6.88 (dd, $J = 16.4$, 1H), 7.02–7.24 (m, 3H), 7.89 (s, 3H).

HMB-Val-Ser-Gln-VE (**14**). ^{1}H NMR ($CDCl_3$): δ 1.07 (d, 6H), 1.29 (t, 3H), 1.72 (m, 2H), 2.09 (s, 1H), 2.18 (t, 2H), 2.37 (s, 3H), 2.55 (m, 1H), 4.11 (q, 2H), 4.19–4.25 (m, 3H), 4.39–4.50 (m, 2H), 5.04 (s, 2H), 5.82 (d, $J = 16.0$, 1H), 6.06 (s, 2H), 6.84 (dd, $J = 16.2$, 1H), 7.11–7.33 (m, 3H), 8.02 (s, 3H).

HMB-Tic-Leu-Leu-VE (**25**). ^{1}H NMR ($CDCl_3$): δ 0.99 (s, 12H), 1.25 (t, 3H), 1.56–1.82 (m, 6H), 2.30 (s, 3H), 2.9–3.2 (m, 2H), 4.03 (q, 2H), 4.21 (m, 1H), 4.42–4.55 (m, 3H), 4.89 (m, 1H), 5.02 (s, 1H), 5.95 (d, $J = 16.3$, 1H), 6.89 (dd, $J = 16.1$, 1H), 7.00–7.13 (m, 7H), 8.03 (s, 2H).

HMB-Tic-Ser-Leu-VE (**26**). ^{1}H NMR ($CDCl_3$): δ 1.01 (s, 6H), 1.30 (t, 3H), 1.49 (m, 2H), 1.79 (m, 1H), 2.03 (s, 1H), 2.34 (s, 3H), 2.96–3.17 (m, 2H), 3.87 (m, 2H), 4.11 (m, 1H), 4.19 (q, 2H), 4.27–4.51 (m, 4H), 4.84 (m, 1H), 5.12 (s, 1H), 5.92 (d, $J = 16.3$, 1H), 6.85 (d, $J = 16.3$, 1H), 6.89–7.10 (m, 7H), 8.01 (s, 2H).

6.3. Purification of proteasomes

Partially purified proteasomes were obtained from lymphoblastoid cell lines, untreated or treated for 12 h at 37 °C with the inhibitors, as previously described [36]. A subsequent purification was carried out by affinity chromatography (mAb α-subunit, Affinity). Fractions containing proteasomes were combined and dialyzed against 25 mM Tris–HCl, pH 7.5. Protein concentration was determined using BCA protocol (Pierce, Rockford, IL, U.S.A).

6.4. Proteasome inhibition assays

Suc-LLVY-AMC, Boc-LRR-AMC and Ac-YVAD-AMC (Sigma) were used to measure chymotrypsin-like, trypsin-like and post-acidic proteasome activities, respectively. Substrates were incubated at 37 °C for 30 min with proteasomes, untreated or pretreated with 0.001–10 μM of test compounds, in activity buffer. 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 expressed as IC_{50}. The data were plotted as percentage control (the ratio of percentage conversion in the presence and 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.

6.5. Enzymatic stability assays

The degradation kinetics of new vinyl ester tripeptides 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 up to 360 min. The incubation was terminated by addition of ethanol (0.2 mL), the mixture poured at 21 °C, and after centrifugation (5000 rpm for 10 min) aliquots (20 μL) of the clear supernatant were injected into 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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