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# N-Terminal-prolonged vinyl ester-based peptides as selective proteasome β1 subunit inhibitors

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ABSTRACT

The synthesis and biological properties of vinyl ester peptide-based molecules bearing linear N-terminal amino acids are reported. Compounds were tested in vitro for their capacity to inhibit the chymotryptic-, tryptic-like, and post-acidic activities of the proteasome. Some analogues showed selective inhibition of post-acidic (PGPH) activity, which is attributed to the β1 subunit. Interestingly, active compounds demonstrated higher inhibitory activity toward 'standard' proteasomes than toward immunoproteasomes. The inhibitory potency was found to be related to the amino acidic sequence and to the length of the N-terminal residues. The new inhibitors demonstrated resistance to plasmatic proteases and a good capacity to permeate the cell membrane.



## 1. Introduction

Proteasomes are the primary sites of protein degradation in mammalian cells. These large (2.4 MDa) multi-subunit protease complexes perform ATP-dependent degradation of poly-ubiquitinated proteins, and are responsible for the majority of the non-lysosomal proteolysis which occurs in eukaryotic cells.[1] Their central component, the barrel-shaped proteolytic 20S proteasome core particle which consists of four seven-subunit rings, is capped at one or both ends by 19S regulatory particles.[2,3] 20S proteasomes belong to the family of N-terminal nucleophile hydrolases. They possess only three active proteolytic sites, all of which are located on the β subunits (β1, β2, β5) of each of the two middle rings. Each catalytic subunit possesses a characteristic substrate specificity;[4] the chymotrypsin-like site, ChT-L (β5), cleaves peptide bonds after hydrophobic residues, the trypsin-like site, Th-L (β2), after a basic residue, and the third site (β1) after an acidic residue (PGPH activity), preferentially aspartate residues demonstrating caspase-like activity.[5,6] The antiviral cytokine INF-γ induces transcription of the three additional β subunits (LMP2, MECL-1, and LMP7), which can replace their constitutive homologues (β1, β2, and β5) in a newly assembled proteasome.[7–10] The resulting immunoproteasomes show slightly different substrate specificities in vitro. In consequence, the immunoproteasome generates more peptides than the proteasome for major histocompatibility complex (MHC) class I antigen presentation.[11,12]

The functional integrity of proteasomes is required for a variety of cellular functions, such as metabolic adaptation, cell differentiation, cell cycle control, stress response, degradation of abnormal proteins, and generation of epitopes presented by MHC class I receptors.[13,14] Hence deregulation of the ubiquitin-proteasome protein degradation pathway in humans is implicated in several diseases, such as neurodegenerative, autoimmune, and metabolic disorders, in addition to cancer. The idea of targeting the proteasomal pathway represents an appealing new approach for treatment of these pathologies.

Indeed, inhibition of the proteasome influences the stability of many proteins, in particular those involved in cell cycle regulation: most of the cells treated with proteasome inhibitors become sensitive to apoptosis.[15,16] Thus, the development of new and potentially more active or selective proteasome inhibitors represents a stimulating approach for the treatment of many diseases.

In previous studies, we characterized a class of vinyl-ester-bearing proteasome inhibitors able to interact with catalytic threonine in the same way to that proposed for the well-known vinyl sulfone peptide. Moreover, these vinyl ester inhibitors showed good resistance to proteolysis and the ability to permeate the cell membrane.[17–23] Interestingly, some of these molecules demonstrated a selective inhibitory capacity for trypsin-like activity at nanomolar concentrations, were non-toxic, did not inhibit cell proliferation and were able to modulate the generation of antigenic peptides linked by MHC class I molecules.[18]

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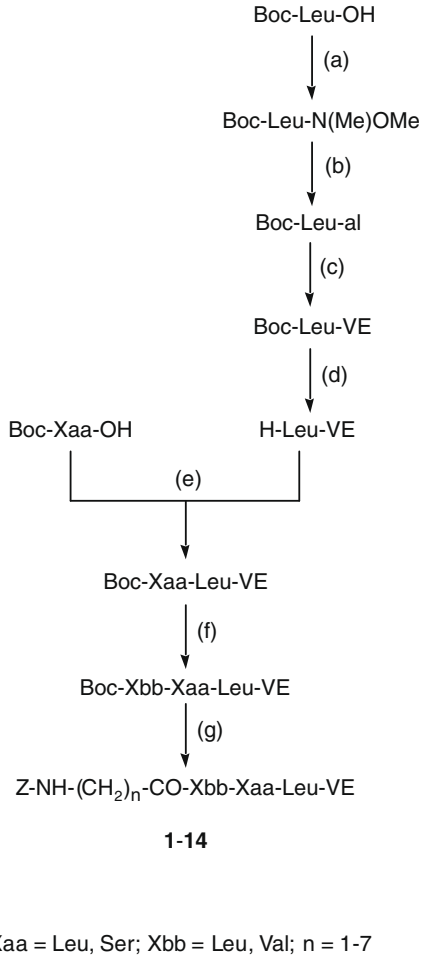
In this study we synthesized and tested new tripeptide-based derivatives carrying a C-terminal leucine vinyl ester (Leu-VE) as pharmacophore, and bearing the dipeptidic central sequences Leu-Leu or Val-Ser, which were chosen on the basis of previous studies.[18–23] The new compounds were functionalized at the N-terminal with linear amino acid molecules of different lengths (1–7 carbon atoms) with a benzyloxycarbonylic group (*Z*) on the terminal amine group.

## 2. Results and discussion

### 2.1. Chemistry

N-Prolonged vinyl ester pseudopeptides were synthesized by the classical solution method using C-terminal stepwise elongation, as reported in Scheme 1. Nα-Boc-protected leucine vinyl ester was prepared from the corresponding aldehyde without racemization using [(ethoxycarbonyl)methylidene]triphenylphosphorane.[24–26] WSC/HOBt was employed for the coupling steps, and Boc was removed by TFA treatment. All products were purified by preparative RP-HPLC, and structural verification was achieved by mass spectrometry and NMR spectroscopy. HPLC capacity factors ($K'$) and other physiochemical properties of compounds **1**–**14** are summarized in Table 1.




**Scheme 1.** Synthesis of N-prolonged vinyl ester pseudopeptides **1–14**. Reagents: (a) HN(Me)Ome, WSC, HOBt, NMM, DMF; (b) $LiAlH_4$, THF; (c) EtO–CO–CH=$PPh_3$, 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) *Z*-NH-$(CH_2)_n$COOH, WSC, HOBt, NMM, DMF.

### 2.2. Biological activity

Biological evaluation of the new vinyl ester derivatives was tested to assess inhibition of trypsin-like, chymotrypsin-like, and post-acidic activities of the proteasome. Their inhibitory capacities were assayed on proteasomes purified from lymphoblastoid cell lines (LCL) and freshly isolated human peripheral blood mononuclear cells (PBMC) using fluorogenic substrates specific for the three main proteolytic activities of the enzymatic complex.[27,28] All compounds were assayed at incremental concentrations (from 0.001 to 10 μM), and their $IC_{50}$ values, obtained after 30 min of incubation, are compared with those of the well-known proteasome inhibitors epoxomicin and MG132 in Table 2 (the values are reported as the average of three independent determinations).

Selected inhibitors **2–5** and **11**–**14** were assayed to evaluate their ability to permeate PBMC cell membranes. After cell treatment, proteasomes were purified and assayed for proteolytic activity as described above (Table 3). In addition, selected inhibitors were assayed at different concentrations for their capacity to inhibit cell growth (Fig. 1).

The metabolic stability of vinyl ester derivatives was evaluated by incubation in human plasma at 37 °C.[29] The degradation half-lives of the N-terminal-extended analogues, reported in Table 2, were determined as described in Section 4.

## 3. Conclusions

The new vinyl ester derivatives were obtained from selected pseudo-tripeptide sequences (Leu-Leu-Leu and Val-Ser-Leu); they all present an N-terminal elongation of linear amino acid sequences carrying alkyl chains of increased length. All compounds were tested for their capacity to inhibit the three major activities of proteasomes isolated from lymphoblastoid cell lines (LCL) and PBMCs. The expression profile of the three catalytic subunits of proteasomes isolated from LCLs and PBMCs was obtained by western-blot analysis (data not shown), and this data demonstrated that, as expected, LCLs expressed a higher level of immunoproteasomes than PBMCs.

Inhibition data of all tested compounds are shown in Table 2. Surprisingly, each molecule presented a unique biological profile. In general, however, no compound significantly inhibited the chymotrypsin-like and trypsin-like activities, either of proteasomes isolated from PBMC or of immunoproteasomes isolated from LCL at concentrations up to 10 μM. Only compounds **3**, **4**, and **5** showed a mild inhibitory capacity toward tryptic activity, with $IC_{50}$ values a bit below a concentration of 10 μM. However, a potent and selective inhibition of the post-acidic activity of both types of proteasomes, in particular those purified from PBMCs, was observed. In fact, several derivatives demonstrated $IC_{50}$ values of post-acidic inhibition in a nanomolar range. Pseudopeptide **5**, with its tripeptide sequence of three leucine residues, elongated at the N-terminal with the *Z*-protected 6-amino esanoic acid, revealed itself to be the most active compound of the series, with an $IC_{50}$ value of 53 nM.

The more hydrophobic Leu-Leu-Leu sequence provided more potent inhibitors than the Val-Ser-Leu sequence. Furthermore, the length of the alkyl chain of the residue at the N-terminal was also shown to influence the activity.

Among derivatives **1**–**7**, the presence of 4, 5, or 6 methylene groups at the N-terminal seems to confer greater inhibitory activity, while among compounds **8**–**14**, inhibition of PBMC proteasomes was shown to increase with the increasing length of the alkyl chain, although other authors have obtained contrasting results. In our study, however, N-terminal elongation of vinyl sulfone inhibitors with the central structure L3VS produced potent but

**Table 1**
Analytical data and physiochemical properties of N-prolonged pseudopeptides **1–14**

| No. | Compound | HPLC | | $[\alpha]_D^{20}$[a] | Mp (°C) | M+H$^+$ |
|---|---|---|---|---|---|---|
| | | $K^1$ (a) | $K^1$ (b) | | | |
| **1** | Z-Gly-Leu-Leu-Leu-VE | 9.44 | 6.97 | +18.1 | 135–137 | 603.76 |
| **2** | Z-β-Ala-Leu-Leu-Leu-VE | 9.47 | 7.03 | +15.5 | 128–132 | 617.79 |
| **3** | Z-NH-$(CH_2)_3$-CO-Leu-Leu-Leu-VE | 9.59 | 7.15 | +13.7 | 125–128 | 631.82 |
| **4** | Z-NH-$(CH_2)_4$-CO-Leu-Leu-Leu-VE | 9.88 | 7.54 | +12.9 | 111–115 | 645.84 |
| **5** | Z-NH-$(CH_2)_5$-CO-Leu-Leu-Leu-VE | 9.13 | 7.68 | +12.1 | 121–123 | 659.86 |
| **6** | Z-NH-$(CH_2)_6$-CO-Leu-Leu-Leu-VE | 9.17 | 7.74 | +11.3 | 117–121 | 673.89 |
| **7** | Z-NH-$(CH_2)_7$-CO-Leu-Leu-Leu-VE | 9.26 | 7.77 | +9.9 | 116–119 | 687.92 |
| **8** | Z-Gly-Val-Ser-Leu-VE | 7.34 | 4.98 | +22.8 | 155–157 | 563.66 |
| **9** | Z-β-Ala-Val-Ser-Leu-VE | 7.39 | 5.12 | +22.4 | 154–159 | 577.68 |
| **10** | Z-NH-$(CH_2)_3$-CO-Val-Ser-Leu-VE | 7.57 | 5.18 | +21.7 | 147–151 | 591.71 |
| **11** | Z-NH-$(CH_2)_4$-CO-Val-Ser-Leu-VE | 7.94 | 5.22 | +21.0 | 146–148 | 605.73 |
| **12** | Z-NH-$(CH_2)_5$-CO-Val-Ser-Leu-VE | 8.11 | 5.49 | +18.5 | 139–142 | 619.76 |
| **13** | Z-NH-$(CH_2)_6$-CO-Val-Ser-Leu-VE | 8.23 | 5.55 | +17.6 | 130–132 | 633.79 |
| **14** | Z-NH-$(CH_2)_7$-CO-Val-Ser-Leu-VE | 8.40 | 5.78 | +17.4 | 127–130 | 647.81 |

[a] *c* 1, MeOH.

**Table 2**
Inhibition of the proteolytic activities of proteasomes isolated from LCLs and PBMCs by the new vinyl ester derivatives

| No. | Compound | Isolated LCL enzyme $IC_{50}$[a] (μM) | | | Isolated PBMC enzyme $IC_{50}$[a] (μM) | | |
|---|---|---|---|---|---|---|---|
| | | T-L | ChT-L | PGHP | T-L | ChT-L | PGHP |
| | Epoxomycin | 0.284 | 0.005 | 4.560 | 0.758 | 0.009 | >10 |
| | MG132 | 1.077 | 0.002 | >10 | 0.911 | 0.023 | >10 |
| **1** | Z-Gly-Leu-Leu-Leu-VE | >10 | >10 | >10 | >10 | >10 | 0.545 |
| **2** | Z-β-Ala-Leu-Leu-Leu-VE | >10 | >10 | 2.340 | >10 | >10 | 0.213 |
| **3** | Z-NH-$(CH_2)_3$-CO-Leu-Leu-Leu-VE | >10 | >10 | 2.070 | 7.230 | >10 | 0.208 |
| **4** | Z-NH-$(CH_2)_4$-CO-Leu-Leu-Leu-VE | >10 | >10 | 0.870 | 6.050 | >10 | 0.078 |
| **5** | Z-NH-$(CH_2)_5$-CO-Leu-Leu-Leu-VE | >10 | >10 | 1.080 | 5.980 | >10 | 0.053 |
| **6** | Z-NH-$(CH_2)_6$-CO-Leu-Leu-Leu-VE | >10 | >10 | 1.670 | >10 | >10 | 0.165 |
| **7** | Z-NH-$(CH_2)_7$-CO-Leu-Leu-Leu-VE | >10 | >10 | 2.430 | >10 | >10 | 0.487 |
| **8** | Z-Gly-Val-Ser-Leu-VE | >10 | >10 | >10 | >10 | >10 | 2.890 |
| **9** | Z-β-Ala-Val-Ser-Leu-VE | >10 | >10 | >10 | >10 | >10 | 0.831 |
| **10** | Z-NH-$(CH_2)_3$-CO-Val-Ser-Leu-VE | >10 | >10 | 6.290 | >10 | >10 | 0.745 |
| **11** | Z-NH-$(CH_2)_4$-CO-Val-Ser-Leu-VE | >10 | >10 | 1.540 | >10 | >10 | 0.231 |
| **12** | Z-NH-$(CH_2)_5$-CO-Val-Ser-Leu-VE | >10 | >10 | 1.390 | >10 | >10 | 0.198 |
| **13** | Z-NH-$(CH_2)_6$-CO-Val-Ser-Leu-VE | >10 | >10 | 2.710 | >10 | >10 | 0.186 |
| **14** | Z-NH-$(CH_2)_7$-CO-Val-Ser-Leu-VE | >10 | >10 | 5.640 | >10 | >10 | 0.175 |

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

**Table 3**
In vivo proteasome inhibition and enzymatic stability of selected analogues **2–5** and **11–14**

| No. | Compound | Cellular inhibition PBMC $IC_{50}$[a] (μM) | | | Half-life (min) plasma |
|---|---|---|---|---|---|
| | | T-L | ChT-L | PGHP | |
| **2** | Z-β-Ala-Leu-Leu-Leu-VE | >10 | >10 | 0.320 | >360 |
| **3** | Z-NH-$(CH_2)_3$-CO-Leu-Leu-Leu-VE | >10 | >10 | 0.330 | >360 |
| **4** | Z-NH-$(CH_2)_4$-CO-Leu-Leu-Leu-VE | 9.540 | >10 | 0.128 | >360 |
| **5** | Z-NH-$(CH_2)_5$-CO-Leu-Leu-Leu-VE | 8.860 | >10 | 0.086 | >360 |
| **11** | Z-NH-$(CH_2)_4$-CO-Val-Ser-Leu-VE | > 10 | >10 | 0.450 | >360 |
| **12** | Z-NH-$(CH_2)_5$-CO-Val-Ser-Leu-VE | >10 | >10 | 0.410 | >360 |
| **13** | Z-NH-$(CH_2)_6$-CO-Val-Ser-Leu-VE | >10 | >10 | 0.237 | >360 |
| **14** | Z-NH-$(CH_2)_7$-CO-Val-Ser-Leu-VE | >10 | >10 | 0.219 | >360 |

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

non-selective analogues of the multicatalytic complex subunits.[30,31]

In summary, this new series of N-terminal-elongated vinyl ester derivatives, designed on the basis of previous results, yielded several potent analogues selective for the β1 subunit. Data reported in Table 3 demonstrate that our compounds posses favorable plasma stability. The most potent derivatives were tested for their capacity to permeate cellular membrane, and in vivo inhibition of PBMCs produced values slightly below but comparable to the in vitro data.

Since proteasomes play a key role in cell viability and proliferation, we evaluated the pro-apoptotic and anti-proliferative activities of two selected compounds on a lymphoblastoid cell line and on Burkitt's lymphoma cells. To this end, the two cell lines were treated for 3 days with various concentrations (from 0.1 to 10 μM) of compounds **5** and **14**, whose cellular effects were evaluated by trypan blue exclusion and cell counting. No toxicity was observed, while inhibition of cell proliferation was detected on both cell lines treated with the highest concentration (10 μM) of

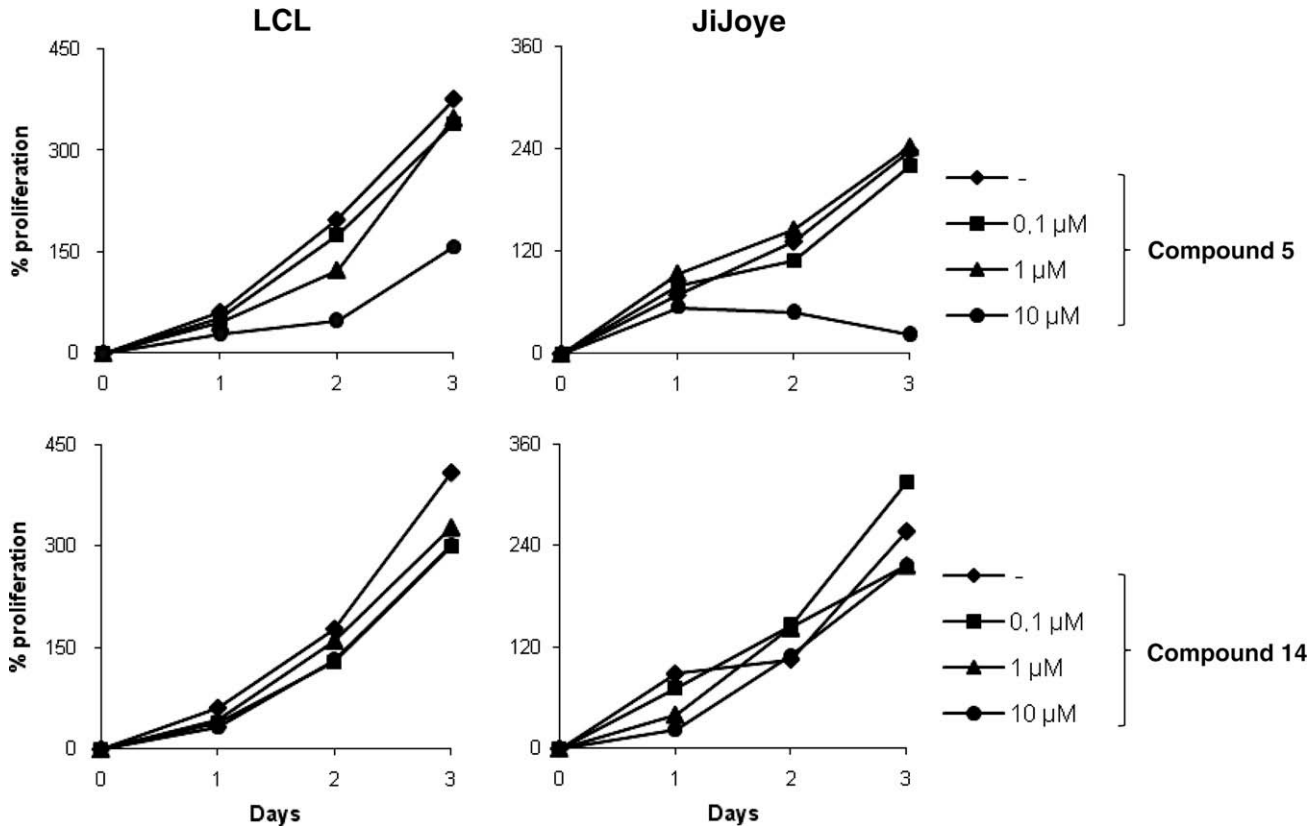



**Figure 1.** Effect of compounds **5** and **14** on cell proliferation. LCL and JiJoye cells were cultured for three days in the presence or absence of the indicated concentrations of compounds **5** and **14**. Results are expressed as % proliferation. The means of three independent experiments performed in duplicate are shown.

compound **5** (Fig. 1). This suggests that inhibitors of the post-acidic activity of proteasomes neither induce cell death nor greatly inhibit cell proliferation.

The development and application of proteasome inhibitors have attracted the attention of biomedical sciences in recent years, as active and selective inhibitors for the three subunits of the multicatalytic complex can represent potential therapeutic instruments for several pathologies, especially in the oncological field. Potent and selective inhibitors of the β1 subunit are not well known, and the prevalent biological role of the post-acidic activity of the proteolytic complex remains to be clarified. However, the possibility of obtaining molecules with this biological profile could be useful in investigation of functions mediated by the β1 subunit. In addition, these compounds may be used in association with other inhibitors with different selectivity and specificity.

## 4. Experimental protocols

### 4.1. General

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

Crude products 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 30 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 the time adopted for elution of the compounds. HPLC analysis was performed using a Beckman System Gold with a Hypersil BDS C18 column (5 μm; 4.6 × 250 mm). Analytical determination and capacity factor ($K'$) of the peptides were assayed via 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 90% B for 25 min and (b) from 30% to 100% B for 25 min. No pseudopeptide showed more than 1% impurity when monitored at 220 and 254 nm.

The molecular weights of the compounds were determined by electrospray ionization (ESI) (MICROMASS ZMD 2000), and the values are expressed as $[MH]^+$. TLC was performed on pre-coated plates of silica gel F254 (Merck, Darmstadt, Germany), exploiting 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), and (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 using a Bruker AC 200 spectrometer.

### 4.2. Chemistry

#### 4.2.1. H-Leu-VE

The leucine vinyl ester was synthesized as described in Ref. 18.

#### 4.2.2. General synthetic procedures

**4.2.2.1. TFA deprotection.** Boc was removed by treating intermediates with aqueous 90% TFA (1:10, w/v) for 30–40 min. After evaporation, the residue was triturated with $Et_2O$, centrifuged, and the resulting solid was collected and dried.

**4.2.2.2. Coupling with WSC/HOBt.** Aminic component (1 mmol), NMM (1 mmol), WSC (1 mmol), and HOBt (1 mmol) were added

to a solution of acylating agent (1 mmol) in DMF (3 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 12 h at room temperature; the solution was then diluted with AcOEt (50 mL) and washed consecutively with HCl 0.1 N, $NaHCO_3$, and brine. The organic phase was dried ($MgSO_4$), filtered, and evaporated to dryness. The residue was then treated with $Et_2O$ and the resulting solid separated by centrifugation.

#### 4.2.3. $^1$H NMR data

**4.2.3.1. *Z*-Gly-Leu-Leu-Leu-VE (1).** $^1$H NMR ($CDCl_3$): $\delta$ 0.97–1.02 (m, 18H); 1.32 (t, 3H); 1.53 (m, 2H); 1.72–1.86 (m, 6H); 3.80 (s, 2H); 4.15–4.24 (m, 3H); 4.48–4.56 (m, 2H); 5.31 (s, 2H); 5.92 (d, $J$ = 16.4, 1H); 6.99 (dd, $J$ = 16.5, 1H); 7.20–7.27 (m, 5H); 7.66 (br s, 3H). MS: M+H$^+$ = 603.76.

**4.2.3.2. *Z*-β-Ala-Leu-Leu-Leu-VE (2).** $^1$H NMR ($CDCl_3$): $\delta$ 0.98–1.01 (m, 18H); 1.31 (t, 3H); 1.54 (m, 2H); 1.70–1.83 (m, 6H); 2.44 (t, 2H); 3.30 (t, 2H); 4.13–4.21 (m, 3H); 4.46–4.53 (m, 2H); 5.29 (s, 2H); 5.93 (d, $J$ = 16.5, 1H); 7.01 (dd, $J$ = 16.5, 1H); 7.18–7.24 (m, 5H); 7.70 (br s, 3H). MS: M+H$^+$ = 617.79.

**4.2.3.3. *Z*-NH-$(CH_2)_3$-CO-Leu-Leu-Leu-VE (3).** $^1$H NMR ($CDCl_3$): $\delta$ 1.00–1.04 (m, 18H); 1.27 (t, 3H); 1.52 (m, 2H); 1.72–1.85 (m, 8H); 2.41 (t, 2H); 2.99 (t, 2H); 4.10–4.18 (m, 3H); 4.41–4.49 (m, 2H); 5.22 (s, 2H); 5.88 (d, $J$ = 16.4, 1H); 6.98 (dd, $J$ = 16.3, 1H); 7.13–7.20 (m, 5H); 7.65 (br s, 3H). MS: M+H$^+$ = 631.82.

**4.2.3.4. *Z*-NH-$(CH_2)_4$-CO-Leu-Leu-Leu-VE (4).** $^1$H NMR ($CDCl_3$): $\delta$ 0.99–1.02 (m, 18H); 1.25 (t, 3H); 1.53–1.59 (m, 4H); 1.69–1.80 (m, 8H); 2.39 (t, 2H); 3.02 (t, 2H); 4.12–4.19 (m, 3H); 4.38–4.48 (m, 2H); 5.27 (s, 2H); 5.93 (d, $J$ = 16.7, 1H); 7.02 (dd, $J$ = 16.8, 1H); 7.15–7.24 (m, 5H); 7.85 (br s, 3H). MS: M+H$^+$ = 645.84.

**4.2.3.5. *Z*-NH-$(CH_2)_5$-CO-Leu-Leu-Leu-VE (5).** $^1$H NMR ($CDCl_3$): $\delta$ 0.99–1.04 (m, 18H); 1.27 (t, 3H); 1.55–1.85 (m, 13H); 2.15 (t, 2H); 2.94 (t, 2H); 4.07 (q, 2H); 4.25 (m, 1H); 4.50–4.59 (m, 2H); 5.38 (s, 2H); 5.98 (d, $J$ = 16.7, 1H); 6.98 (dd, $J$ = 16.5, 1H); 7.15–7.25 (m, 5H); 7.36 (br s, 3H). MS: M+H$^+$ = 659.86.

**4.2.3.6. *Z*-NH-$(CH_2)_6$-CO-Leu-Leu-Leu-VE (6).** $^1$H NMR ($CDCl_3$): $\delta$ 1.01–1.05 (m, 18H); 1.30 (t, 3H); 1.54–1.86 (m, 15H); 2.17 (t, 2H); 3.00 (t, 2H); 4.11 (q, 2H); 4.29 (m, 1H); 4.52–4.59 (m, 2H); 5.41 (s, 2H); 6.02 (d, $J$ = 16.4, 1H); 7.00 (dd, $J$ = 16.4, 1H); 7.11–7.20 (m, 5H); 7.82 (br s, 3H). MS: M+H$^+$ = 673.89.

**4.2.3.7. *Z*-NH-$(CH_2)_7$-CO-Leu-Leu-Leu-VE (7).** $^1$H NMR ($CDCl_3$): $\delta$ 1.00–1.06 (m, 18H); 1.32 (t, 3H); 1.49–1.87 (m, 17H); 2.20 (t, 2H); 3.02 (t, 2H); 4.15 (q, 2H); 4.33 (m, 1H); 4.60–4.65 (m, 2H); 5.45 (s, 2H); 6.00 (d, $J$ = 16.7, 1H); 7.01 (dd, $J$ = 16.6, 1H); 7.15–7.22 (m, 5H); 7.90 (br s, 3H). MS: M+H$^+$ = 687.92.

**4.2.3.8. *Z*-Gly-Val-Ser-Leu-VE (8).** $^1$H NMR ($CDCl_3$): $\delta$ 0.98–1.01 (m, 12H); 1.30 (t, 3H); 1.51 (m, 2H); 1.82 (m, 1H); 2.04 (br s, 1H); 2.69 (m, 1H); 3.80 (s, 2H); 3.92–4.05 (m, 2H); 4.18 (q, 2H); 4.29 (m, 1H); 4.52–4.61 (m, 2H); 5.33 (s, 2H); 5.98 (d, $J$ = 16.6, 1H); 6.99 (dd, $J$ = 16.5, 1H); 7.19–7.23 (m, 5H); 7.98 (br s, 3H). MS: M+H$^+$ = 563.66.

**4.2.3.9. *Z*-β-Ala-Val-Ser-Leu-VE (9).** $^1$H NMR ($CDCl_3$): $\delta$ 1.00–1.04 (m, 12H); 1.31 (t, 3H); 1.52 (m, 2H); 1.80 (m, 1H); 2.00 (br s, 1H); 2.40 (t, 2H); 2.67 (m, 1H); 3.23 (t, 2H); 3.95–4.02 (m, 2H); 4.20 (q, 2H); 4.26 (m, 1H); 4.50–4.57 (m, 2H); 5.35 (s, 2H); 6.02 (d, $J$ = 16.4, 1H); 7.01 (dd, $J$ = 16.5, 1H); 7.18–7.25 (m, 5H); 7.96 (br s, 3H). MS: M+H$^+$ = 577.68.

**4.2.3.10. *Z*-NH-$(CH_2)_3$-CO-Leu-Leu-Leu-VE (10).** $^1$H NMR ($CDCl_3$): $\delta$0.97–1.00 (m, 12H); 1.29 (t, 3H); 1.50 (m, 2H); 1.81 (m, 3H); 2.02 (br s, 1H); 2.21 (t, 2H); 2.65 (m, 1H); 2.98 (t, 1H); 3.93–4.01 (m, 2H); 4.17 (q, 2H); 4.25 (m, 1H); 4.48–4.58 (m, 2H); 5.32 (s, 2H); 5.99 (d, $J$ = 16.7, 1H); 7.02 (dd, $J$ = 16.6, 1H); 7.19–7.23 (m, 5H); 7.98 (br s, 3H). MS: M+H$^+$ = 591.71.

**4.2.3.11. *Z*-NH-$(CH_2)_4$-CO-Leu-Leu-Leu-VE (11).** $^1$H NMR ($CDCl_3$): $\delta$0.99–1.02 (m, 12H); 1.28 (t, 3H); 1.42–1.56 (m, 7H); 2.03 (br s, 1H); 2.19 (t, 2H); 2.68 (m, 1H); 2.95 (t, 1H); 3.90–4.02 (m, 2H); 4.15 (q, 2H); 4.21 (m, 1H); 4.50–4.59 (m, 2H); 5.33 (s, 2H); 6.03 (d, $J$ = 16.5, 1H); 7.01 (dd, $J$ = 16.6, 1H); 7.18–7.25 (m, 5H); 7.94 (br s, 3H). MS: M+H$^+$ = 605.73.

**4.2.3.12. *Z*-NH-$(CH_2)_5$-CO-Leu-Leu-Leu-VE (12).** $^1$H NMR ($CDCl_3$): $\delta$0.94–1.01 (m, 12H); 1.26–1.30 (m, 5H); 1.43–1.60 (m, 7H); 2.00 (br s, 1H); 2.20 (t, 2H); 2.67 (m, 1H); 2.97 (t, 1H); 3.85–4.03 (m, 2H); 4.13 (q, 2H); 4.20 (m, 1H); 4.48–4.60 (m, 2H); 5.32 (s, 2H); 5.97 (d, $J$ = 16.7, 1H); 6.99 (dd, $J$ = 16.7, 1H); 7.16–7.22 (m, 5H); 7.95 (br s, 3H). MS: M+H$^+$ = 619.76.

**4.2.3.13. *Z*-NH-$(CH_2)_6$-CO-Leu-Leu-Leu-VE (13).** $^1$H NMR ($CDCl_3$): $\delta$0.90–1.00 (m, 12H); 1.25–1.31 (m, 7H); 1.40–1.58 (m, 7H); 2.01 (br s, 1H); 2.16 (t, 2H); 2.66 (m, 1H); 2.94 (t, 1H); 3.80–4.05 (m, 2H); 4.15 (q, 2H); 4.22 (m, 1H); 4.50–4.59 (m, 2H); 5.29 (s, 2H); 5.90 (d, $J$ = 16.5, 1H); 6.93 (dd, $J$ = 16.6, 1H); 7.15–7.19 (m, 5H); 7.92 (br s, 3H). MS: M+H$^+$ = 633.79.

**4.2.3.14. *Z*-NH-$(CH_2)_7$-CO-Val-Ser-Leu-VE (14).** $^1$H NMR ($CDCl_3$): $\delta$ 0.87–1.02 (m, 12H); 1.28–1.33 (m, 9H); 1.43–1.62 (m, 7H); 2.03 (br s, 1H); 2.15 (t, 2H); 2.68 (m, 1H); 2.97 (t, 1H); 3.75 (m, 2H); 4.05 (q, 2H); 4.19 (m, 1H); 4.52–4.64 (m, 2H); 5.33 (s, 2H); 5.87 (d, $J$ = 16.4, 1H); 6.89 (dd, $J$ = 16.6, 1H); 7.10–7.16 (m, 5H); 7.82 (br s, 3H). MS: M+H$^+$ = 647.81.

### 4.3. Biological activity

#### 4.3.1. Purifications of proteasomes

Proteasomes were isolated from lymphoblastoid cell (LCL) and freshly isolated human peripheral blood mononuclear cell (PBMC) lines, untreated or treated for 12 h at 37 °C with the selected new N-prolonged vinyl ester derivatives, as previously described.[32]

#### 4.3.2. Proteasome inhibition assays

Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-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 pre-treated 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 is expressed as $IC_{50}$. The data were plotted as percentage control (the ratio of percentage conversion in the presence and absence of inhibitor) versus 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.

#### 4.3.3. Enzymatic stability assays

The stability to protease degradation of the selected new vinyl ester inhibitors 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 up to 360 min. Incubation was terminated by addition of ethanol (0.2 mL), the mixture poured at 21 °C, and, following centrifu-

gation (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.

### 4.3.4. Growth inhibition assays

Cells were seeded in duplicate in a 24-well plate, at a density of $50 \times 10^3$ cells/ml in a medium containing 10% FCS. A lymphoblastoid cell line (LCL) and Burkitt's lymphoma JiJoye cells were cultured either in medium alone or in a medium containing compounds **5** or **14** at incremental concentrations (from 0.1 to 10 μM); cell viability and proliferation were evaluated by trypan blue exclusion and cell counting after three days. Results are expressed as % proliferation.

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