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P3 and P4 position analysis of vinyl ester pseudopeptide proteasome inhibitors

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Abstract—Two small libraries of tripeptidic-based vinyl ester derivative proteasome inhibitors were synthesized and tested, starting with the Hmb-Val-Gln-Leu-VE prototype. The P3 and P4 positions were investigated with a complete set of amino acid residues, some of which showed remarkable selective inhibition of the trypsin-like (β2) subunit. In both positions, aromatic and hydrophobic residues were preferred.

The proteasome is a multicatalytic protease complex, which represents the central enzyme of intracellular protein degradation in prokaryotes and eukaryotes. It is involved in many biological processes, including the removal of abnormal, misfolded or improperly assembled proteins, and has a key role in the stress response, cell cycle control and differentiation. In addition, metabolic adaptation and generation of peptide antigens for presentation by major histocompatibility complex (MHC) class I molecules to $CD8^+$ cytotoxic T cells[1,2] are linked to an ubiquitin- and ATP-requiring protein degradation pathway involving the 26S proteasome (2.4 MDa). The 26S proteasomes are made up of a cylinder-shaped multimeric protein complex, whose core and proteolytic chamber is the 20S proteasome, capped at each end by a regulatory component termed 19S. The 20S proteasome consists of four stacked rings, where each of the two inner rings is composed of seven different β subunits. Each β-ring contains three different proteolytically active sites: the β1 subunit, which contains a post-acidic (PGPH) active site, the β2 subunit, which is associated with a trypsin-like (T-L) activity, and the β5 subunit, which has a chymotrypsin-like (ChT-L) proteolytic function. All the proteolytic sites utilize an N-ter-

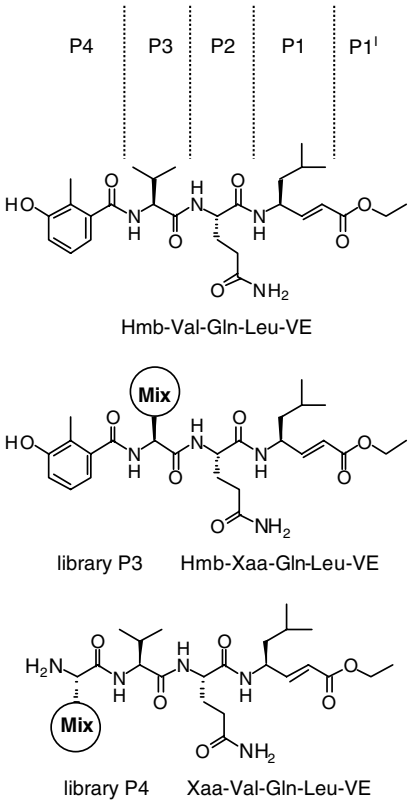

Figure 1. Structures of the reference inhibitor and libraries P3 and P4. Variable positions that contain a mixture of amino acids are indicated as Mix.

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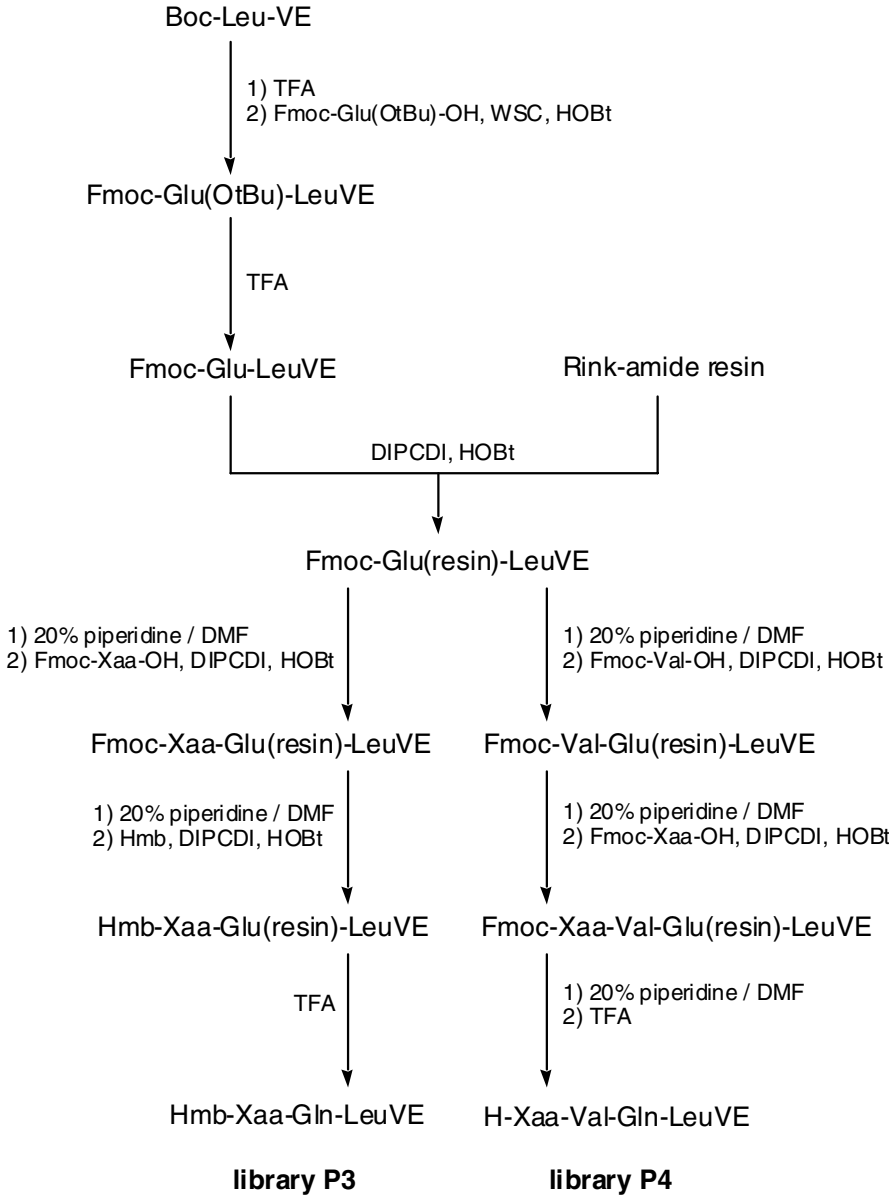
minal threonine residue of β subunits as nucleophile, employing a catalytic mechanism similar to those of the serine proteases.[3]

The development of proteasome inhibitors into novel therapeutic agents represents a stimulating approach in the treatment of many disease states including inflammation and cancer, and for the modulation of immune responses.[4] Proteasome inhibitors are usually short peptides linked to a C-terminal pharmacophore, which is responsible for the interaction with catalytic threonine.[5]

Once synthesized and tested, a series of peptide-based derivatives were found to be the peptidyl portion which derives from a screening of tripeptide sequences.[6] Vinyl ester moiety has been considered a potential substrate for the catalytic threonine.[7] The more efficacious of the series, Hmb-Val-Gln-Leu-VE, showed good inhibition, favourable pharmacokinetic properties, and remarkable selectivity for the trypsin-like activity of the 20S proteasome. These compounds increased the generation and presentation of subdominant MHC class I CTL epitopes without affecting cell viability suggesting that they may find application as immunomodulators.

The following presents a study of the P3 and P4 positions of the reference compound, employing small libraries of pseudotripeptides with the C-terminal ethyl acrylate group. These can function as substrates of catalytic threonine in Michael addition in the same way that has been suggested for the well-known peptide vinyl sulfone inhibitors.[8] As compared to the prototype, both small libraries bear a glutamine in P2 position in order to promote selectivity for the β2 subsite, in similar fashion to that previously carried out for vinyl sulfone derivatives.[9] P3 and P4 libraries contain a complete set of



Scheme 1. Synthesis of vinyl ester pseudopeptide libraries P3 and P4.

amino acid residues (minus cysteine) in the place of valine or 3-hydroxy-2-methylbenzoyl (Hmb) moiety, respectively (Fig. 1).

Libraries P3 and P4 were obtained by a mixed solution-solid-phase synthesis approach, adhering to the strategy reported in Scheme 1. N_{α}-Boc-protected leucine vinyl ester (Boc-Leu-VE) was prepared as reported previously,[7] and after Boc removal, H-Leu-VE was acylated with N_{α}-Fmoc-glutamic acid γ-*t*-butyl ester using 1-ethyl-3-(3[I]-dimethylaminopropyl)carbodiimide (WSC) and *N*-hydroxybenzotriazole (HOBt) as coupling agents.[10] After trifluoroacetic acid (TFA) treatment, pseudodipeptide Fmoc-Glu-Leu-VE was bonded to a Rink amide resin using *N*,*N*[I]-diisopropylcarbodiimide (DIPCDI)/HOBt. Libraries were synthesized by solid phase, using an automated continuous-flow peptide synthesizer following the Fmoc/*t*-Bu-strategy. N-terminal Hmb in library P3 and Fmoc-Val-OH in library P4 were condensed to 4-fold excess with DIPCDI and HOBt as coupling agents. The isokinetic mixture of the 19 residues was created by using a ratio of equivalents of amino acids based on their reported coupling rates.[11] The total mixture was adjusted to 10-fold excess total amino acids over resin load; for both libraries, coupling of the mixture was carried out with DIPCDI/HOBt. Libraries were cleaved from the resin by addition of 88% TFA, 5% H_2O and Et_3SiH for 2 h. Cleavage solutions were treated with diethyl ether and the solid products were isolated by centrifugation followed by lyophilization. Composition of the libraries was verified by HPLC and mass spectrometry by electrospray ionisation (ESI) (MICROMASS ZMD 2000).[12]

Biological evaluation of the two small collections of vinyl ester derivatives was carried out according to the schemes reported in Figures 2 and 3. Both libraries, containing a complete set of amino acid residues at the P3 and P4 sites, were tested to assess inhibition of trypsin-like and chymotrypsin-like active sites. The inhibitory capacity of the vinyl ester libraries was tested on the 20S proteasome, previously purified from lymphoblastoid cell lines,[13] using fluorogenic substrates specific for the two main proteolytic activities of the enzymatic complex; Suc-LLVY-AMC and Boc-LRR-AMC were used to measure chymotrypsin-like and trypsin-like pro-

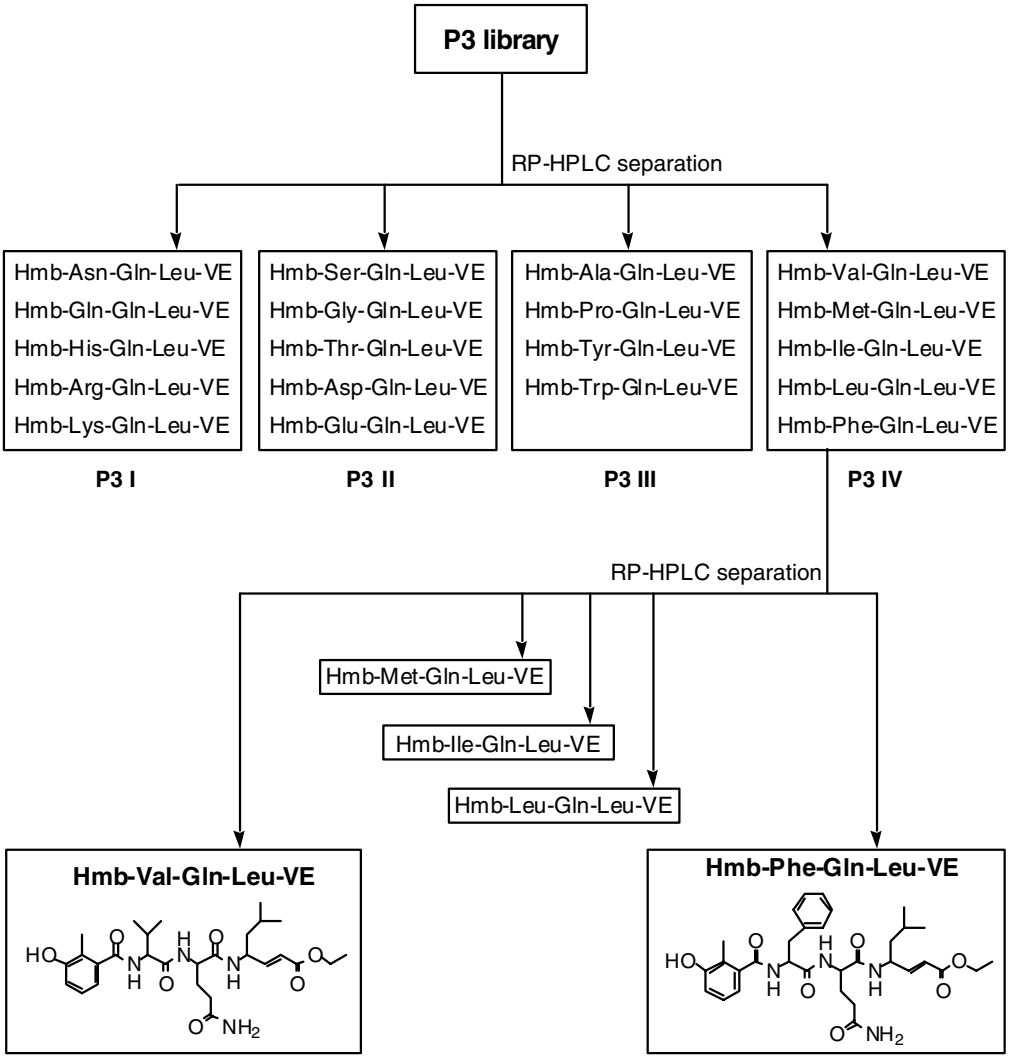


Figure 2. Separation scheme of library P3 and structure of the most active vinyl ester pseudotripeptide.

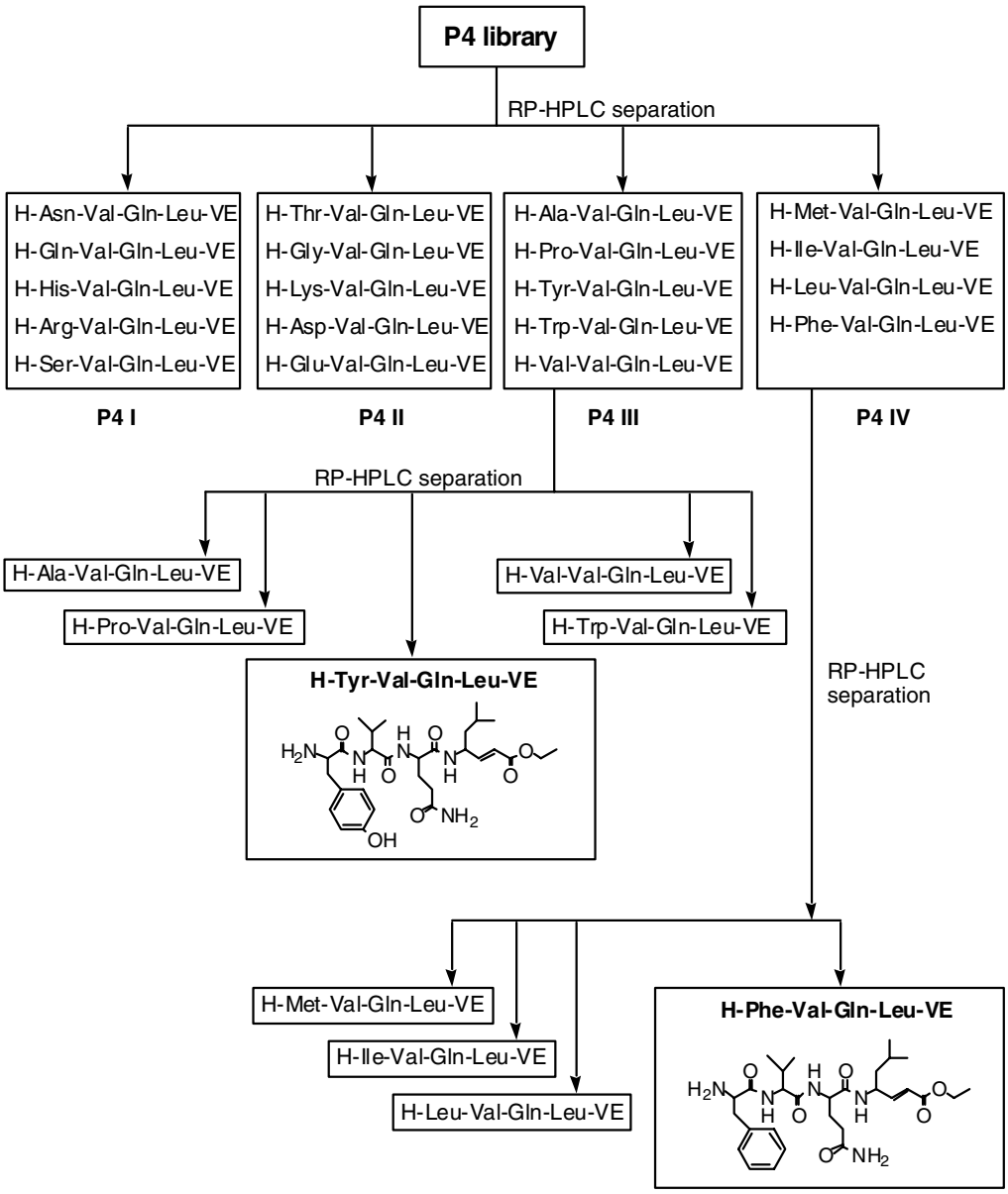


Figure 3. Sequence of separation steps of library P4 and structure of the better vinyl ester derivatives.

teasome activities, respectively. Substrates were incubated at 37 °C for 30 min with proteasomes, untreated or pretreated with 1 and 10 μM (calculated from the average of the molecular weight of the libraries' components), 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.[14] Activity was evaluated in fluorescence units, and the inhibitory activity of the libraries is expressed as percentage control (the ratio of percentage conversion in the presence and absence of inhibitor) versus inhibitor concentration (Fig. 4A).

Subsequently, the libraries were fractioned by preparative RP-HPLC. Separations were performed using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30×4 cm, 300 Å, 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 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA). Thirty-five minutes was adopted for the elution of compounds and the resulting sublibraries (P3 I–P3 IV, P4 I–P4 IV) were lyophilized and tested against proteasome catalytic subunits as described above (Fig. 4). The more hydrophobic fractions showed interesting activity for both libraries.

Finally, the more efficacious sublibraries, P3 IV, P4 III and P4 IV, were separated into their single components by preparative liquid chromatography, and each vinyl ester pseudopeptide was tested at different concentrations (from 0.001 to 10 μM). Activity was evaluated in fluorescence units as described above, and the inhibitory activity of the compounds is expressed as IC_{50}. The data were plotted as percentage control (the ratio of percent-

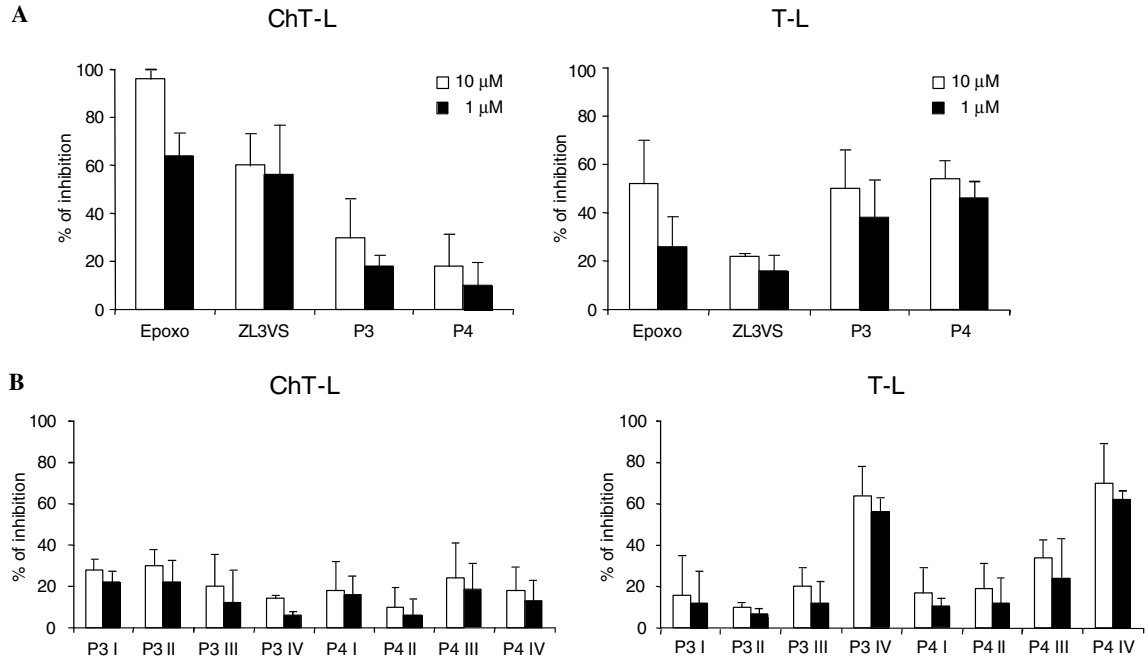


Figure 4. % of inhibition of the P3 and P4 libraries compared to epoxomicin and ZL3VS (A) and corresponding sublibraries after RP-HPLC separation (B) at 10 and 1 μM concentrations against chymotrypsin-like activity (ChT-L) and trypsin-like activity (T-L) of the proteasome.

age conversion in the presence and absence of the inhibitor) versus the 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.

The most active derivatives, Hmb-Phe(Val)-Gln-Leu-VE for P3 and H-Tyr(Phe)-Val-Gln-Leu-VE for P4 libraries, were identified by mass spectrometry and confirmed by NMR.[15] Analytical data and inhibitory capacity against β2 and β5 proteasome subunits of the four better analogues are reported in Table 1.

Biological data confirm that the C-terminal ethyl acrilate pharmacophore is a moiety which could be used for the development of new selective proteasome inhibitors. In general, libraries, fractioned sublibraries and separated vinyl ester pseudopeptides show modest inhibition against chymotryptic-like activity while efficiently inhibit the β2 subsite. In comparison to reference compound (Hmb-Val-Ser-Leu-VE), the presence of the phenylalanine at the P3 position confers similar potency and selectivity for trypsin-like activity. Pseudotetrapeptides, in which the N-terminal Hmb is substituted by aromatic residues (Tyr, Phe) in the P4 position, show a nearly identical biological profile to the prototype. In summary, the N-terminal pseudodipeptidic portion determines the selectivity for the β2 subunit, confirming the data previously obtained. In the P3 position, the presence of hydrophobic residues promotes the interaction with the catalytic site. Finally, we observed that amino acids with aromatic side chains in position P4 are able to replace cyclic substituents without greatly altering the biological profile.

In conclusion, small libraries of vinyl ester pseudopeptides able to selectively inhibit trypsin-like activity of the proteasome ($IC_{50} < 0.1$ μM) were designed, synthesized and tested. This proved to be a rapid method for the analysis of the P3 and P4 positions of a reference

Table 1. Analytical data and inhibition of trypsin-like and chymotrypsin-like activities of the proteasome by better vinyl ester derivatives selected from libraries P3 and P4 and reference inhibitors epoxomicin and ZL3VS

Compound	HPLC[b]		MS (ESI) $[M+H]^+$	IC_{50}[a] (nM)	
	K^I (a)	K^I (b)		T-L	ChT-L
Hmb-Val-Gln-Leu-VE	7.64	5.97	547.5	20 ± 4	6670 ± 387
Hmb-Phe-Gln-Leu-VE	9.45	8.03	595.5	32 ± 4	2230 ± 202
H-Tyr-Val-Gln-Leu-VE	7.23	5.88	576.6	83 ± 6	4320 ± 322
H-Phe-Val-Gln-Leu-VE	10.38	8.76	560.6	42 ± 6	4150 ± 299
Epoxomicin				980 ± 53	47 ± 3
ZL3VS				2430 ± 124	61 ± 5

[a] The values reported are the average ± SD of two independent determinations.
[b] Capacity factor (K^I) of the compounds was determined by HPLC using two different solvent system gradients.[13]

compound which could be very useful for further studies to optimize the structural requirement of this class of the 20S proteasome inhibitors.

Acknowledgments

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10. Spectroscopic data for Fmoc-Glu(OtBu)-Leu-VE: MS: 593 (M+1); ^{1}H NMR ($CDCl_3$, 200 MHz): δ (ppm) 1.02 (d, 6H), 1.26 (t, 3H, J = 7.2), 1.44 (s, 9H), 1.56 (m, 2H), 1.81 (m, 1H), 2.15 (m, 2H), 2.32 (d, 2H), 4.09 (q, 2H, J = 7.3), 4.31–4.58 (m, 5H), 5.96 (d, 1H, J = 16.0), 6.92 (dd, 1H, J = 16.1), 7.19–7.80 (m, 10H).
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15. Hmb-Phe-Gln-Leu-VE. Purified yield 46%; purity estimated by HPLC > 98%; ^{1}H NMR ($CDCl_3$): δ 0.94–1.17 (m, 12H); 1.37 (t, 3H); 1.48–1.59 (m, 3H); 1.98–2.18 (m, 4H); 2.39 (s, 3H); 2.73 (m, 1H); 4.05 (q, 2H); 4.19–4.40 (m, 3H); 5.20 (s, 1H); 5.82 (d, J = 16.3, 1H); 6.71 (dd, J = 16.4, 1H); 7.08–7.41 (m, 8H). Hmb-Phe-Gln-Leu-VE. Purified yield 42%; purity estimated by HPLC > 98%; ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm) 1.10 (d, 6H); 1.37 (t, J = 7.2, 3H); 1.45–1.59 (m, 3H); 1.92–2.09 (m, 4H); 2.34 (s, 3H); 2.63 (m, 2H); 4.02 (q, J = 7.1, 2H); 4.20–4.38 (m, 3H); 5.13 (s, 1H); 5.80 (d, J = 16.1, 1H); 6.62 (dd, J = 16.3, 1H); 7.05–7.43 (m, 13H). H-Tyr-Val-Gln-Leu-VE. Purified yield 32%; purity estimated by HPLC > 98%; ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm 0.93–1.15 (m, 12H); 1.32 (t, J = 7.0, 3H); 1.45–1.58 (m, 3H); 1.98–2.18 (m, 6H); 2.31 (br s, 2H); 2.78 (m, 1H); 4.02 (q, J = 7.1, 2H); 4.22–4.38 (m, 3H); 5.17 (s, 1H); 5.77 (d, J = 16.3, 1H); 6.67 (dd, J = 16.4, 1H); 7.08–7.41 (m, 9H). H-Phe-Val-Gln-Leu-VE. Purified yield 37%; purity estimated by HPLC > 98%; ^{1}H NMR ($CDCl_3$, 200 MHz): δ ppm 0.97–1.16 (m, 12H); 1.29 (t, J = 7.2, 3H); 1.39–1.53 (m, 3H); 1.92–2.09 (m, 6H); 2.29 (br s, 2H); 2.77 (m, 1H); 4.04 (q, J = 7.2, 2H); 4.25–4.40 (m, 3H); 5.15 (s, 1H); 5.80 (d, J = 16.1, 1H); 6.71 (dd, J = 16.2, 1H); 7.10–7.49 (m, 10H).