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Synthesis and proteasome inhibition of *N*-allyl vinyl ester-based peptides

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Inhibition of the proteasome, the multicatalytic protease complex responsible for the turnover of many cellular proteins, represents an attractive target in the development of new drug therapies, proteasome inhibitors being potentially useful tools for the treatment of pathologies such as cancer, as well as inflammatory, immune and neurodegenerative diseases. Based on our previous development of a new class of inhibitors bearing a *C*-terminal VE cluster able to interact with catalytic threonine, we report herein the synthesis and activity of new VE-based peptide analogs bearing an additional allyl pharmacophore unit at the *C*- or *N*-terminal position of the pseudotripeptide sequence. In the new series, the structural modification carried out to the prototype determine a decrease of proteasome inhibition. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: N-allyl derivatives; inhibitors; proteasome; pseudopeptides

Introduction

The proteasome, a multicatalytic protease complex [1], is an essential component of the ubiquitin–proteasome system (UPS) which is involved in prokaryotic and eukaryotic intracellular protein degradation pathways. The UPS is implicated in many biological processes, such as stress response, cell cycle control and differentiation, apoptosis and the generation of peptide antigens [2–5]. These cellular functions are linked to an ubiquitin- and ATP-dependent protein degradation pathway involving the 26S proteasome, which contains a central barrel-like core and a 20S proteolytic chamber composed of four stacked rings. These inner rings are made up of seven different β subunits, and each β-ring contains three different active sites. In particular, the $\beta 1$ subunit contains a post-acidic (PGPH) active site, the $\beta 2$ subunit expresses T-L activity, and a ChT-L proteolytic function is carried out by the $\beta 5$ subunit [6–13].

Considering the implication of the multicatalytic complex in various cellular processes, modulation of proteasomal activities is extremely interesting from a therapeutic perspective. Indeed, a variety of natural and synthetic products have been tested as inhibitors of the different proteasomal enzymatic subunits [14–26]. In particular, the boron derivative PS341 (Bortezomib) has been used in the treatment of multiple myeloma [27,28], and other such molecules have been evaluated for their effect on many disease states, including inflammation and cancer, as well as on modulation of immune responses.

Our previous studies report the design and development of a new class of peptide-based proteasome inhibitors containing a VE moiety as a potential substrate for Michael addition with the catalytic threonine through a mechanism similar to that of the well-known vinyl sulfone inhibitors. As recently reported [29–31], also the natural pseudopeptidic product Syringolin A and, even if in minor way, the analogous Syringolin B irreversibly inhibit with the same catalytic mechanism the $\beta 2$ and $\beta 5$ subunits of the proteasome. As some derivatives with the basic general structure HMB-Xaa-Xbb-Leu-VE have shown favorable pharmacokinetic properties, potent inhibition and general selectivity for the $\beta 2$ proteasome subunit [32,33], a further series of VE derivatives were synthesized substantially modifying the molecular level with respect to the basic structure. These cyclic analogs or *N*-terminal-prolonged pseudotripeptides showed differing biological profiles [34,35]. DFT calculations and docking simulations carried out on HMB-Val-Ser-Leu-VE proved that the *C*-terminal pharmacophore is surrounded by several residues; in particular, the OH group of the active threonine Thr1 interacts with the carbonyl of the VE group with a O· · ·O distance of 2.74 Å [36].

Herein we describe the synthesis and proteasome inhibition of novel *N*-allyl, VE-based peptide derivatives (Figure 1), further to an interesting study by Prof. Norm Radin which showed that anticancer and other drugs possessing potent biological activity contain polar allyl groups [37]. Our series possessed an *N*-allylic function at the *C*- or *N*-terminal position of the pseudotripeptide prototype H-Leu-Leu-Leu-VE. The aim of our work was to evaluate

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Abbreviations used: *ChT-L, chymotrypsin-like; HATU, O-(7-azabenzotriazolyl)-tetramethyl uronium hexafluorophosphate; PGPH, peptidyl-glutamyl peptide hydrolyzing; T-L, trypsin-like; WSC, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide; VE, vinyl ester; Z, benzyloxy carbonyl-N-hydroxysuccinimide..*

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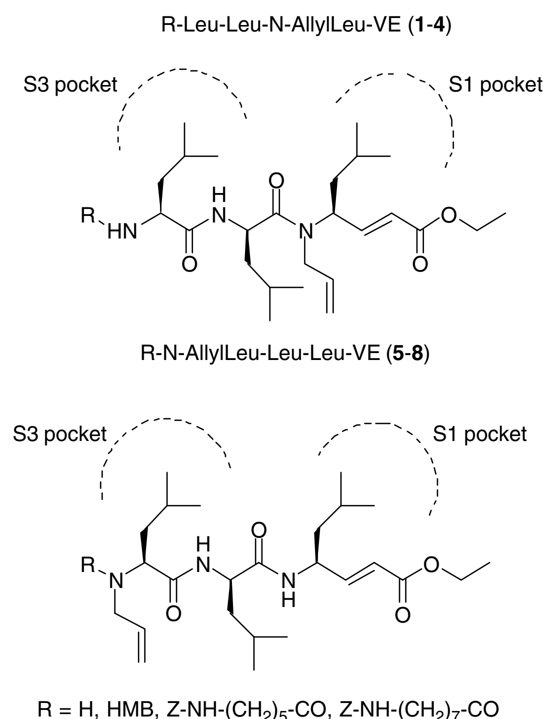

Figure 1. Structures of the N-allyl vinyl ester pseudopeptides.

whether this additional function, potentially able to interact with proteasomal catalytic threonine, can strengthen the interaction between enzyme/inhibitor and consequently increase inhibitory capacity.

The derivatives **1**–**4** bear an allyl group linked to the N of the *C*-terminal primary pharmacophore unit Leu-VE, whereas the *N*-allyl cluster was inserted at the *N*-terminal position in analogs **5**–**8**. 3-Hydroxy-2-methylbenzoyl (HMB), Z-protected 6-aminohexanoyl or 8-aminooctanoyl was the *N*-terminal functional groups, in accordance with the results obtained for the previous series.

Materials and Methods

Chemistry-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 the 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: (i) from 0 to 90% B for 25 min and (ii) 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 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: (iii) AcOEt/*n*-hexane (1 : 1, v/v), (iv) CH_2Cl_2/methanol (9.5 : 0.5, v/v), (v) CH_2CL_2/methanol (9 : 1, v/v) and (vi) 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.

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.

Coupling with HATU

The deprotected α-amine intermediate (1 mmol), NMM (2 mmol) and HATU (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 HCl 0.1 N, $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$) and evaporated to dryness. The residue was treated with Et_2O and the resulting solid separated by centrifugation.

NH-AllylLeu-VE

To a solution of 1 mmol of H-Leu-VE in DMF at rt, 2.2 mmol of K_2CO_3 was added. After 1 h of stirring, 0.8 mmol of allyl bromide was added. The reaction mixture was stirred for 1 h at 60 °C, then the solvent was evaporated and the residue was diluted with AcOEt and washed with water. The organic phase was dried ($MgSO_4$) and evaporated to dryness. The oil crude product was purified by flash chromatography (silica gel MeOH/CH_2Cl_2 1: 15).

$[\alpha]^{20}_D = +2.8$ ($c = 1$, MeOH). ^{1}H NMR ($CDCl_3$, 200 MHz): δ(ppm) 0.98–1.06 (m, 6H), 1.34 (t, 3H, $J = 7.1$), 1.58–1.62 (m, 2H), 1.74–1.83 (m, 1H), 3.40–3.48 (m, 2H), 3.60–3.68 (m, 1H), 4.25 (q, 2H, $J = 7.3$), 5.27–5.49 (m, 2H), 5.67–5.80 (m, 1H), 6.53 (d, 1H, $J = 16.6$), 6.78 (dd, 1H, $J = 16.5$). MS: $M + H^+ = 226.3$.

NH-AllylLeu-Leu-Leu-VE (5)

To a solution of 1 mmol of H-Leu-Leu-Leu-VE in DMF at rt, 3 mmol of K_2CO_3 was added. After 1 h of stirring, 2 mmol of allyl bromide was added. The reaction mixture was stirred overnight at 60 °C, then the solvent was evaporated and the residue was diluted with AcOEt and washed with water. The organic phase was dried ($MgSO_4$) and evaporated to dryness. The crude residue was directly purified by preparative RP-HPLC.

Purified yield 35%; purity estimated by HPLC >98; ^{1}H NMR ($CDCl_3$): 0.98–1.05 (m, 18H); 1.29 (t, 3H, $J = 7.1$); 1.63–1.75 (m, 6H); 1.80–1.87 (m, 3H); 3.18–3.29 (m, 2H); 4.11 (q, 2H, $J = 7.0$); 4.23–4.52 (m, 3H); 5.11 (bs, 1H); 5.18–5.24 (m, 2H); 5.72–5.80 (m, 1H); 5.99 (d, $J = 16.2$, 1H); 6.93 (dd, $J = 16.4$, 1H); 8.03 (bs, 2H).

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HMB-Leu-Leu-*N*-AllylLeu-VE (2)

Purified yield 29%; purity estimated by HPLC >98; ^{1}H NMR ($CDCl_3$): 0.96–1.08 (m, 18H); 1.35 (t, 3H, $J = 7.3$); 1.65–1.83 (m, 9H); 2.48 (s, 3H); 3.25–3.28 (m, 2H); 4.18 (q, 2H, $J = 7.2$); 4.29 (m, 1H); 4.61–4.69 (m, 2H); 5.11 (bs, 1H); 5.21–5.27 (m, 2H); 5.54–5.59 (m, 1H); 5.85 (d, $J = 16.6$, 1H); 6.58 (dd, $J = 16.5$, 1H); 7.01–7.24 (m, 3H); 7.69 (bs, 2H).

Biological investigation

Proteasome purification

Proteasomes were isolated from lymphoblastoid cell lines (LCL) as previously described [32].

Proteasome subunit inhibition assays

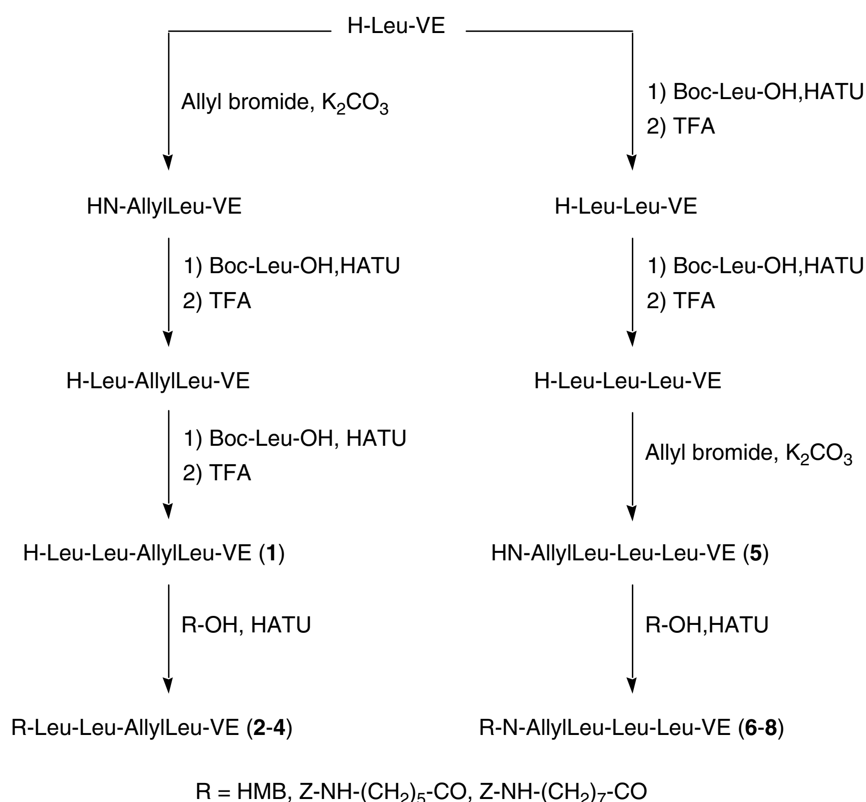
Suc-LLVY-AMC, Boc-LRR-AMC and Z-LLE-AMC (Sigma) were used to measure ChT-L, T-L and post-acidic proteasome activities, respectively. Substrates were incubated at 37 °C for 30 min with proteasomes, untreated or pre-treated with 0.001–10 μM of test compounds, in activity buffer. Fluorescence was determined 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.

Results and Discussion

Following the strategy reported in Scheme 1, *N*-allyl VE pseudotripeptides **1**–**8** were synthesized using *C*-terminal stepwise elongation. Commencing with the leucine VE unit, prepared as previously described [32], *N*-allylation was carried out by allyl bromide in the presence of potassium carbonate. The mono-allyl intermediate was purified by flash chromatography, and the following two N_α-Boc-protected leucines were condensed using HATU to complete the pseudotripeptide sequence. After each coupling step, Boc was removed by TFA. Compounds **2**–**4** were obtained by acylation with 3-hydroxy-2-methylbenzoic, Z-protected 6-aminohexanoic or 8-aminooctanoic acids in the presence of HATU. The allyl group was introduced to the *N*-terminal of the pseudotripeptide sequence H-Leu-Leu-Leu-VE in analogs **5**–**8**, and final condensation of the *N*-elongating functions resulted in the required compounds.

All members of the series were purified and isolated by preparative RP-HPLC, and the homogeneity of the lyophilized products was assessed by HPLC. Analytical characterization was then achieved by ESI mass spectrometry (MICROMASS ZMD 2000) (Table 1) and ^{1}H-NMR spectroscopy (Bruker AC 200).

Inhibition of the $\beta 1$, $\beta 2$ and $\beta 5$ active sites of the 20S proteasome, previously purified from LCL [38,39], was determined using fluorogenic substrates specific for the three main proteolytic activities of the enzymatic complex. Suc-LLVY-AMC, Boc-LRR-AMC and Z-LLE-AMC were used to measure ChT-L, T-L and caspase-like proteasome activities, respectively. Substrates were incubated at 37 °C for 30 min, with the proteasome, pre-treated with incremented concentrations (from 0.001 to 10 μM) of the new *N*-allyl VE pseudotripeptides and the reference inhibitor HMB-Leu-Leu-Leu-VE in activity buffer. Fluorescence was determined by a



Scheme 1. Synthesis of the *N*-allylic, VE pseudotripeptides (**1**–**8**).

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Table 1. Physicochemical data of *N*-allylic, VE pseudotripeptides

No	HPLC[a] K^I (a)	K^I (b)	P.f. (°C)	$[\alpha]^{20}_D$ ($C = 1$, MeOH)	MS M+H$^+$
1	7.52	4.98	93–95	−20.5	552.6
2	9.65	7.76	84–87	−25.3	586.4
3	10.88	8.91	Oil	−44.9	565.5
4	11.31	9.75	Oil	−52.2	593.5
5	7.30	4.52	78–80	−25.8	452.6
6	9.23	8.06	67–70	−41.5	586.4
7	10.74	9.61	Oil	−70.4	565.5
8	11.48	9.56	Oil	−79.2	593.5

[a] Capacity factor (K^I) of the *N*-allylic peptides was determined by HPLC using two different solvent system gradient.

Table 2. Subsites proteasome inhibition of *N*-allylic, VE derivatives **1**–**8** and reference inhibitor HMB-Leu-Leu-Leu-VE

No	Compound	IC_{50} (μM)[a] ChT-L	T-L	PGPH PPG
1	H-Leu-Leu-*N*-AllylLeu-VE	>10	2.65 (±0.33)	>10
2	HMB-Leu-Leu-*N*-AllylLeu-VE	>10	0.29 (±0.045)	>10
3	Z-NH-$(CH_2)_5$-CO-Leu-Leu-*N*-AllylLeu-VE	>10	0.44 (±0.062)	>10
4	Z-NH-$(CH_2)_7$-CO-Leu-Leu-*N*-AllylLeu-VE	>10	0.59 (±0.081)	>10
5	HN-AllylLeu-Leu-Leu-VE	>10	4.34 (±0.51)	>10
6	HMB-*N*-AllylLeu-Leu-Leu-VE	>10	5.87 (±0.70)	>10
7	Z-NH-$(CH_2)_5$-CO-*N*-AllylLeu-Leu-Leu-VE	8.98 (±0.90)	7.62 (±0.82)	>10
8	Z-NH-$(CH_2)_7$-CO-*N*-AllylLeu-Leu-Leu-VE	6.73 (±0.69)	7.13 (±0.77)	>10
	HMB-Leu-Leu-Leu-VE	4.47 (±0.58)	0.059 (±0.003)	>10

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

fluorimeter (Spectrafluor Plus, Tecan, Salzburg, Austria) using an excitation setting of 360 nm and an emission of 465 nm. Activity was evaluated in fluorescence units, and the inhibitory activity of the compounds is expressed here as IC_{50}. The data were then plotted as percentage control (the ratio of percentage 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 values reported in Table 2 are the means of two independent evaluations.

General analysis of the activity profile shows that the new VE derivatives have a minimal capacity to inhibit proteasome subunits. Disappointingly, the additional polar allyl structure, adjacent to or on the opposite side to the primary pharmacophore unit, hinders interaction with the catalytic cavities. In fact, all compounds of the series exhibited lesser inhibition of the multicatalytic complex with respect to the reference inhibitor HMB-Leu-Leu-Leu-VE. The structural modification enforced by the presence of the allylic group in the base structure results harmful at the *C*-terminal position where a complementarity of the peptidic backbones inhibitor–proteasome is essential. The same group also is not useful for the formation of additional bonds with the catalytic tasks.

In summary, ChT-L activity was slightly inhibited by *N*-terminal-elongated derivatives **7** and **8**. Similar to VE prototypes, the *N*-allyl pseudotripeptides showed a certain selectivity for the $\beta 2$ subunit, although at a lower potency than the parent compound. Analog **2** was the more active compound in the series, with an IC_{50} value against the proteasome T-L activity five times higher than HMB-Leu-Leu-Leu-VE. Generally, T-L inhibition was improved by the presence of *C*-terminal *N*-allylated VE peptides; the correspondent *N*-terminal modification was shown to be less well tolerated. The different functionalization of the *N*-terminal part of the amino acidic sequence had a poor influence on the biological profile.

All compounds were unable to inhibit PGPH activity in the $\beta 1$ subunit of the enzyme complex.

Conclusions

The ubiquitin–proteasome pathway plays an important role in numerous basic cellular processes, and modulation of proteasome activity by specific inhibitors may be crucial for new therapeutic approaches to many diseases. However, clinical trials indicate that proteasome inhibitors exhibit remarkable toxicity during prolonged drug treatment. Hence, the availability of new molecules able to interact with the multicatalytic enzyme subsite in a potent and selective manner without side effects is even today a primary necessity. In this context, we prepared a new series of VE derivatives on the basis of the encouraging previously obtained results. We inserted an additional *N*-allyl function on the base structure with the aim of providing another substrate able to undergo nucleophilic attack by the catalytic *N*-terminal Thr1. However, contrary to the expected results, the molecular modification of the structural prototype determined a marked reduction of proteasome inhibition. We can suppose that, as previously described by Kaiser *et al.* [40] regarding the cyclic inhibitor TMC-95A and analogs, the presence of the *N*-allyl function in our VE derivatives does not allow the formation of an antiparallel β-sheet structure with the active site of the multicatalytic complex. In conclusion, the results indicate that it is more convenient to increase the capacity of the primary *C*-terminal pharmacophore unit to react with catalytic threonine than to add a further cluster, which can destabilize enzyme interaction.

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