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Synthesis and activity of isoxazoline vinyl ester pseudopeptides as proteasome inhibitors

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The ubiquitin–proteasome pathway (UPP) influences essential cellular functions including cell growth, differentiation, apoptosis, signal transduction, antigen processing and inflammatory responses. The main proteolytic component of the UPP is the 26S proteasome, which is responsible for the turnover of many cellular proteins and represents an attractive target for the treatment of pathologies such as cancer, as well as inflammatory, immune and neurodegenerative diseases. Natural and synthetic proteasome inhibitors having different chemical structures and potency have been discovered. We report herein the synthesis, proteasome inhibition and modelling studies of novel C-terminal isoxazoline vinyl ester pseudopeptides. Some new compounds that contain a C-terminal extended conjugation inhibit β1 and especially β5 proteasomal catalytic subunits with IC_{50} values ranging from 10 to 100 μM. These results will permit further optimization based on these structural moieties to develop more active and selective molecules. Copyright © 2014 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: isoxazoline vinyl ester derivatives; pseudopeptides; synthetic inhibitors; proteasome

Introduction

The ubiquitin–proteasome pathway represents the main ATP-dependent degradation system involved in the maintenance of cellular protein homeostasis. Proteasome is an enzymatic complex located in the nucleus and the cytoplasm inside all eukaryotes [1]. The main function of the proteasome is to degrade unneeded or damaged proteins, and proteasomes are part of a major mechanism by which cells regulate the concentration of essential proteins and degrade misfolded proteins. Proteins are targeted for degradation by the proteasome by covalent transfer of multiple ubiquitin moieties to a lysine residue, a process that requires the coordinated reactions of three classes of enzymes with a complex mechanism. The 26S proteasome is a cylindrical complex containing one 20S core particle structure and two 19S regulatory caps. The 20S particle is composed by four stacked rings around a central pore where the inner two rings are made of seven β subunits [2]. Three distinct catalytic activities were identified in the purified complex: chymotrypsin-like in β5 subunit, trypsin-like (β2) and peptidylglutamyl-peptide hydrolyzing located in β1 [3,4]. The mechanism of proteolysis by the β subunits of the 20S core particle is through an N-terminal threonine-dependent nucleophilic attack. This mechanism may depend on an associated water molecule for deprotonation of the catalytic threonine γ-hydroxyl. Although the three catalytic β subunits have a common mechanism, they have slightly different substrate specificities.

The proteasomal degradation pathway is essential for many cellular processes, including the cell cycle, the regulation of gene expression and responses to oxidative stress [5]. To underscore its essential role, inhibition of the proteasome has been investigated in the treatment of diverse diseases such as cancer, immune responses, inflammation and cardiovascular pathologies [6–9]. Many natural and synthetic molecules have been studied as 20S catalytic subunits inhibitors [10–21]; among those, the boronic pseudodipeptide bortezomib was the first Food and Drug Administration (FDA)-approved proteasome inhibitor for the treatment of multiple myeloma (MM) [22,23], and recently, carfilzomib became the second FDA-approved drug to treat MM in advanced state [24].

In the last decade, we developed several classes of peptide-based proteasome inhibitors containing a variety of pharma cophoric units as electrophilic groups potentially able to interact with the catalytic threonine [25–28]. Herein, we describe the synthesis, proteasome inhibition and modelling studies of novel C-terminal isoxazoline vinyl ester pseudopeptides.

The new compounds contain a C-terminal extended conjugation as electrophilic traps for the γ-hydroxy in the side chain of the catalytic threonine. The aim was to promote the interaction of this pharmacophoric conjugated system with proteasomal catalytic subunits through Michael addition in the same way as has been suggested for other well-known inhibitors [29,30].

The pseudodipeptidic or pseudotripeptidic isoxazoline vinyl ester compounds (Figure 1) are functionalized at the N-terminus

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Abbreviations: *Boc, tert-butoxycarbonyl; ChT-L, chymotrypsin-like; HOBt, N-hydroxybenzotriazole; IsxVE, isoxazoline vinyl ester; 1-Naf, α-naphthoyl; 2-Naf, α-naphthoyl; PGPH, peptidyl-glutamyl peptide hydrolyzing; TEA, triethylamine; TFA, trifluoroacetic acid; T-L, trypsin-like; WSC, water-soluble carbodiimide.*

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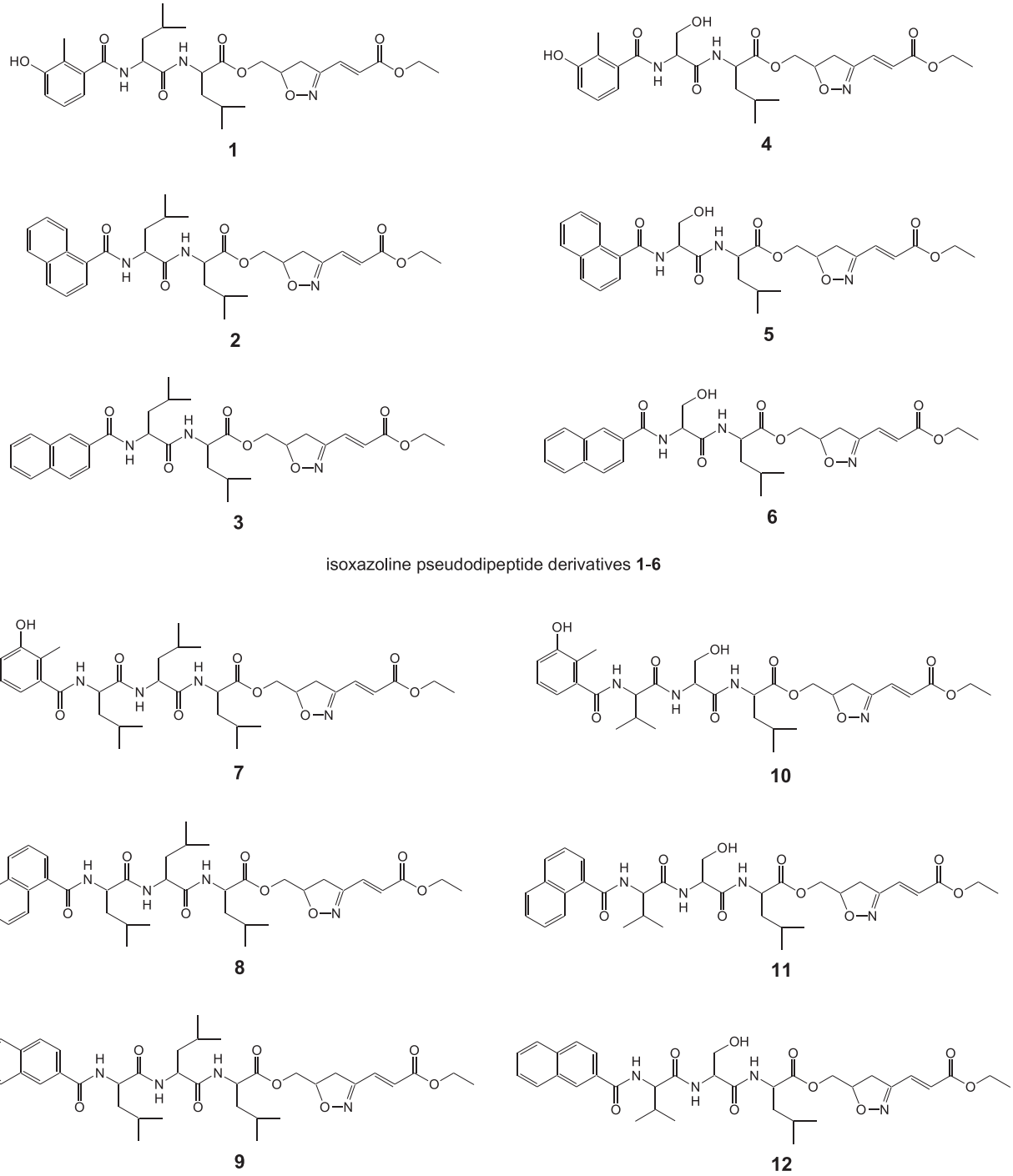


Figure 1 Structure of the isoxazoline vinyl ester pseudopeptides.

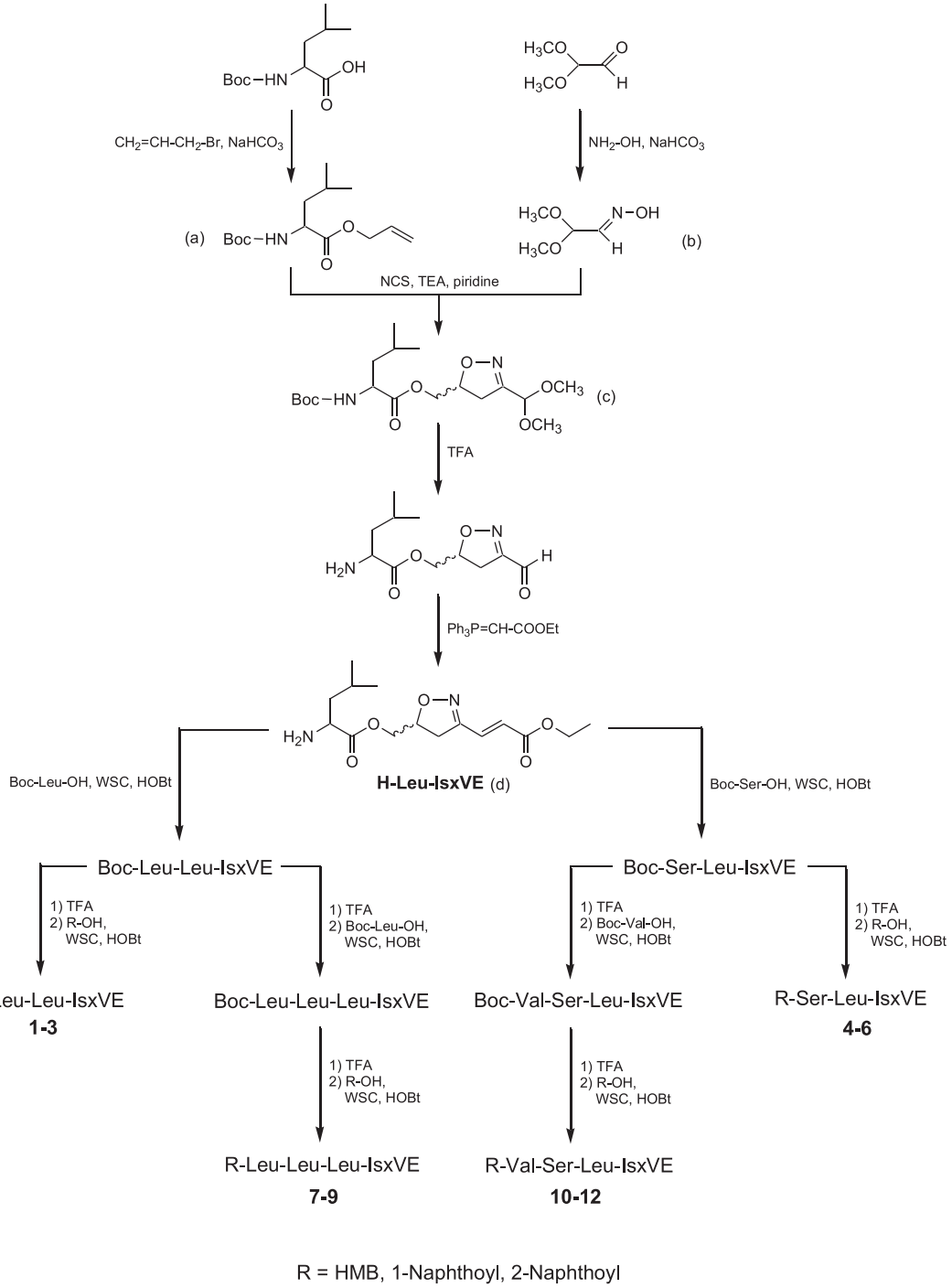
with 3-hydroxy-2-methylbenzoyl (HMB), α-naphthoyl (1-Naf) or β-naphthoyl (2-Naf) units.

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 RP-HPLC using a Waters 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) (Waters Corporation, Milford, MA, USA). 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. Diastereoisomeric mixtures of the

analogues **6** and **12** were separated by preparative HPLC at a flow rate of 20 ml/min by a linear gradient from 30% to 80% of solvent B in 50 min. HPLC analysis was performed using a Beckman System Gold (Beckman Coulter, Inc., Brea, CA, USA) with a Hypersil BDS C18 column (Thermo Electron Corporation, Waltham, MA, USA; 5 μm; 4.6 × 250 mm). Analytical determination and capacity factor (*K'*) of the peptides were assayed via HPLC conditions in the aforementioned solvent system (solvents A and B), programmed at flow rates of 1 ml/min, using the following linear gradient: from 0% to 100% B for 25 min. All pseudopeptides showed less than 1% impurity when monitored at 220 and 254 nm. The molecular weights of the compounds were determined by ESI (Micromass ZMD 2000, Waters Corporation), and the values are expressed as $[M+H]^+$. TLC was performed on pre-coated plates of silica gel F254 (Merck, Darmstadt, Germany), exploiting the following solvent systems: (a) AcOEt/*n*-hexane (1 : 1, v/v), (b) CH_2Cl_2/methanol (9.5 : 0.5, v/v), (c) CH_2CL_2/methanol (9 : 1, v/v) and (d) 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 PerkinElmer (Waltham, MA, USA) 141 polarimeter with a 10-cm water-jacketed



Scheme 1 Synthesis of the isoxazoline vinyl ester pseudopeptides.

cell. ^{1}H NMR spectroscopy was obtained using a Bruker AC 200 spectrometer (Billerica, MA, USA).

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 and centrifuged, and the resulting solid was collected and dried.

Coupling with WSC/HOBt

A solution of deprotected α-amine intermediate (1 mmol) and NMM (1 mmol) in DMF (3 ml) was added to a solution of carboxylic component (1 mmol) containing coupling agents WSC (1 mmol) and HOBt (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 (yield between 70% and 80% after purification).

Preparation of the Boc-Leu-OAll (a)

To a solution of 10 mmol of Boc-Leu-OH in DMF (20 ml) was added 20 mmol of $NaHCO_3$. After 1 h of stirring, 15 mmol of allyl bromide was added. The reaction mixture was stirred overnight at rt. The DMF was evaporated, and the residue was diluted with Et_2O (90 ml) and washed with water and brine. The organic phase was dried ($MgSO_4$) and evaporated to dryness. The crude oil was used for the following cycloaddition.

Preparation of the oxime (b)

To a solution of 10 mmol of hydroxylamine hydrochloride in 60 ml of water was added dropwise a solution of $NaHCO_3$ (16 mmol) in 15 ml of water. We added 10 mmol of the 2,2-dimethoxyacetaldehyde solution (60 wt% in H_2O) and 20 ml of Et_2O. The reaction mixture was stirred overnight at rt; then, the aqueous phase was extracted with CH_2Cl_2 (2 × 20 ml). The organic phase was dried ($MgSO_4$) and evaporated to dryness to yield the desired compound (87%).

Cycloaddition (c)

To a suspension of 9.2 mmol of *N*-chlorosuccinimide in anhydrous $CHCl_3$ (11 ml) were added 0.035 ml of pyridine and a solution containing 7 mmol of the oxime (b) in anhydrous $CHCl_3$. After 30 min, TEA (1.4 ml) and Boc-Leu-OAll (9.2 mmol) were added. The reaction mixture was stirred overnight at rt; after evaporation, the residue was dissolved in AcOEt and washed with HCl 0.1 N, $NaHCO_3$ 5% (w/v) and brine. The organic phase was dried ($MgSO_4$) and evaporated to dryness. The crude product was purified by flash chromatography (silica gel MeOH/CH_2Cl_2 1 : 15) to give the desired compound in 65% yield.

Preparation of H-Leu-IsxVE (d)

The purified product of the cycloaddition was treated with aqueous 90% TFA for 30–40 min. After evaporation, the resulting aldehyde (4 mmol) was dissolved in THF (20 ml) and treated with [(ethoxycarbonyl)methylidene]triphenylphosphorane (6 mmol). The reaction mixture was stirred overnight under reflux; after evaporation, the residue was dissolved in AcOEt and washed with water and brine. The organic phase was dried ($MgSO_4$) and evaporated to dryness to give the desired product in 52% yield. $[\alpha]_D^{20} = -24.7$ (c = 1, MeOH); M + H$^+$ = 328.3; ^{1}H NMR ($CDCl_3$): δ 1.01–1.03 (m 6H); 1.27–1.30 (t 3H); 1.59–1.75 (m 3H); 2.71 (m 2H); 3.31 (m 1H); 4.09–4.33 (m 5H); 5.36 (d, J = 15.8 Hz, 1H); 6.71 (bs 2H); 7.20 (d, J = 15.8 Hz, 1H).

^{1}H NMR of the selected compounds

HMB-Leu-Leu-IsxVE (**1**): ^{1}H NMR ($CDCl_3$): δ 0.78–0.88 (m 12H); 1.20–1.27 (t 3H); 1.49–1.57 (m 4H); 1.83 (m 2H); 2.07 (s 3H); 2.94–3.17 (dd 2H); 3.9 (m 1H); 4.13–4.20 (q 2H); 4.26 (d 2H); 4.29 (m 2H); 5.01 (s 1H); 6.01 (d, J = 16.2 Hz, 1H); 6.86 (d 1H); 7.08 (t 1H); 7.39 (d 1H); 6.30 (d, J = 16.2 Hz); 8.20 (d 2H).

2-Naf-Val-Ser-Leu-IsxVE (**12**): ^{1}H NMR ($CDCl_3$): δ 0.81–0.98 (m 12H); 1.19–1.26 (t 3H); 1.59–1.74 (m 3H); 1.83 (m 1H); 2.94–3.17

Table 1. Physicochemical data of isoxazoline vinyl ester pseudopeptides

No.	HPLC K^l	P. f. °C	$[\alpha]_D^{20}$ (C = 1, MeOH)	MS M + H$^+$
1		Oil	−16.7	560.3
2		Oil	−9.6	580.3
3		Oil	−11.1	580.3
4		Oil	−12.5	534.2
5		Oil	−8.2	554.3
6		Oil	−8.9	554.3
7		Oil	−20.3	673.4
8		60–65	−12.1	693.3
9		72–78	−13.8	693.3
10		Oil	−24.5	633.3
11		84–89	−21.3	653.3
12		89–93	−18.4	653.3
6 (1° peak)	7.85	Oil	−9.7	653.3
6 (2° peak)	8.12	Oil	−7.4	554.3
12 (1° peak)	10.04	97–99	−16.9	653.3
12 (2° peak)	10.65	105–108	−18.9	653.3

Table 2. Subsites proteasome inhibition of isoxazoline vinyl ester derivatives

No.	Compound	IC_{50} (μM)[a] ChT-L	T-L	PGPH
1	HMB-Leu-Leu-IsxVE	>100	>100	>100
2	1-Naf-Leu-Leu-IsxVE	>100	>100	>100
3	2-Naf-Leu-Leu-IsxVE	>100	>100	>100
4	HMB-Ser-Leu-IsxVE	>100	>100	>100
5	1-Naf-Ser-Leu-IsxVE	58.1(±4.1)	>100	>100
6	2-Naf-Ser-Leu-IsxVE	27.4(±1.9)	>100	>100
7	HMB-Leu-Leu-Leu-IsxVE	>100	>100	>100
8	1-Naf-Leu-Leu-Leu-IsxVE	>100	>100	>100
9	2-Naf-Leu-Leu-Leu-IsxVE	>100	>100	>100
10	HMB-Val-Ser-Leu-IsxVE	>100	>100	95.5(±9.3)
11	1-Naf-Val-Ser-Leu-IsxVE	84.5(±6.8)	>100	83.5(±7.7)
12	2-Naf-Val-Ser-Leu-IsxVE	44.2(±3.6)	>100	65.5(±6.8)
6	1° peak	10.6(±1.1)	>100	>100
6	2° peak	>100	>100	>100
12	1° peak	13.7(±1.2)	>100	58.7(±5.2)
12	2° peak	>100	>100	>100

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

(dd 2H); 3.04–3.08 (m 2H); 3.43 (s 2H); 3.8 (m 1H); 4.19–4.23 (q 2H); 4.26 (d 2H); 4.83 (m 3H); 5.22 (bs 1H); 6.01 (d, $J = 16$ Hz, 1H); .13 (d, $J = 16$ Hz, 1H); 7.58 (m 3H); 7.87 (m 4H); 8.91 (m 1H).

Biological Investigation

Proteasome purification

Proteasomes were isolated from lymphoblastoid cell lines (LCL), which are known to contain both proteasomes and immunoproteasomes [31].

Proteasome subunit 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 [32]. Fluorescence was determined after 60 min by a fluorimeter (Spectrafluor Plus, Tecan, Salzburg, Austria), using an excitation of 360 nm and emission of 465 nm. Competitive inhibition for each catalytic subsite was measured 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.

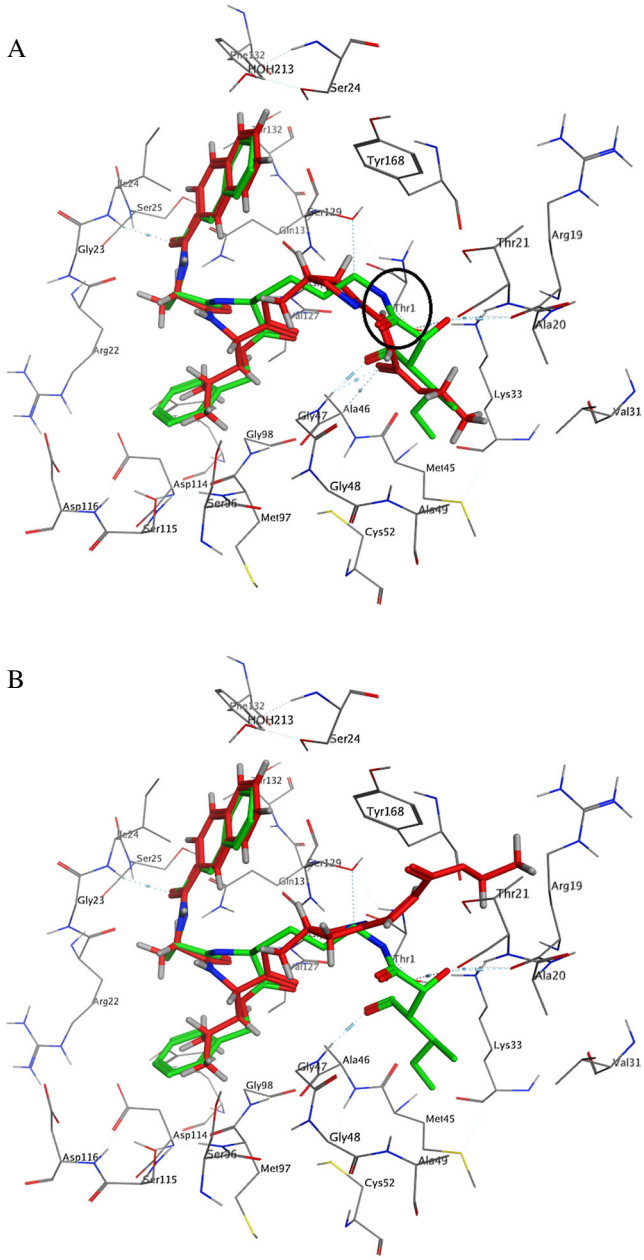


Figure 2 Molecule 6 (red) in S (a) and R (b) configuration and homobelactosin (green) superimposed in the active site of the beta 5 subunit. The black circle encloses the active Thr residue.

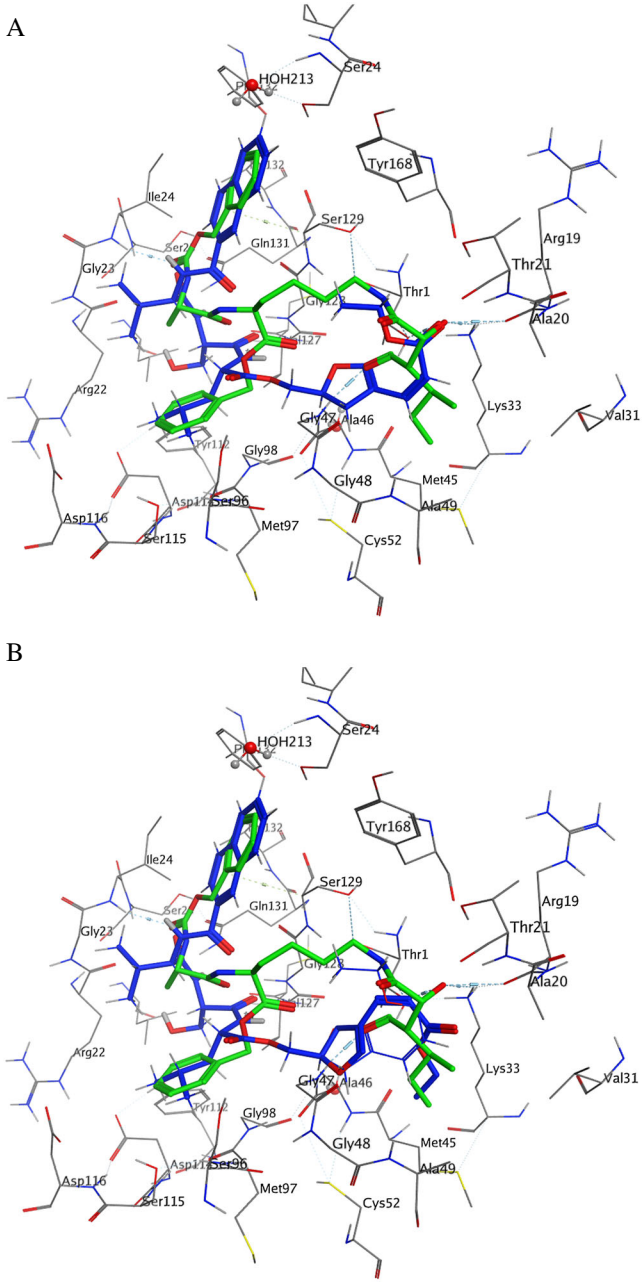


Figure 3 Molecule 12 (blue) in S (a) and R (b) configuration and homobelactosin (green) superimposed in the active sute of beta 5 subunit.

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Calculation Methods

The equilibrium structure of molecules **6** and **12** has been obtained using PM3 semi-empirical energy function [33]. Because the molecules chosen for the modelling study present a stereocenter of undetermined chirality at the C atom of the isoxazoline ring, both R and S configurations have been considered obtaining two different geometries for each molecule. The molecules were then superimposed to homobelactosin C in its active conformation, as found in the structure of the proteasome–homobelactosin complex retrieved from the Protein Data Bank database [34]. The alignment has been performed using the Flex-Align application [35] of the Molecular Operating Environment software [36]. This procedure combines a force field and a 3D similarity function based upon Gaussian descriptions of shape and pharmacophore features such as H-bond donor and acceptors, aromaticity and partial charges to produce an ensemble of possible (scored) alignments. The alignments with the highest score were considered. The bound homobelactosin ligand was used as a template by keeping fixed its coordinates, while the conformation of the candidate ligands was allowed to change.

Results and Discussion

Following the strategy reported in Scheme 1, isoxazoline vinyl ester pseudopeptides **1–12** were synthesized by C-terminal stepwise elongation of the common intermediate H-Leu-IsxVE (d). The C-terminal pharmacophoric unit was prepared in a diastereoisomeric mixture through cycloaddition of the N-protected L-leucine allyl ester (a) with oxime (b) promoted by *N*-chlorosuccinimide. After Boc deprotection, the pharmacophoric unit was obtained by Wittig reaction using [(ethoxycarbonyl)methylidene]triphenyl phosphorane. Completed pseudopeptidic sequences **1–12** were obtained by condensation of the selected *N*-Boc residues and N-terminal functions using WSC/HOBt as coupling agents. After each acylation step, Boc was removed by TFA.

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

Biological evaluation of the new pseudopeptides was carried out to assess their capacity to inhibit the trypsin-like, chymotrypsin-like and post-acidic activities of the proteasome. Their inhibitory capacity was assayed on proteasomes purified from LCL, using fluorogenic substrates specific for the three main proteolytic activities of the enzyme complex [31]. All compounds were assayed at different concentrations (from 0.001 to 10 mM), and the IC_{50} values reported in Table 2 were obtained after 30 min of incubation (the values are reported as the average ± SD of three independent determinations). The results obtained show that none of these new isoxazoline vinyl ester peptide-based derivatives inhibited the trypsin-like activities of proteasomes isolated from LCL. Inhibition of the β1 subunit was also absent except for compounds **10–12**, which exhibited a mild capacity to inhibit the post-acidic activity with an IC_{50} values under 100 μM. Pseudodipeptides **5** and **6** and tripeptidic analogues **11** and **12** in diastereoisomeric form showed a mild inhibitory capacity of the chymotryptic activity attributed to the β5 subunit. The different activities of the new compounds seem to be due to the aminoacidic sequence and N-terminal substituent. Indeed, more hydrophilic peptide sequences due to the presence of a L-serine as well as the presence of the aromatic bulky naphthyl group at the N-terminal seem to be associated with a higher inhibitory capacity. The diastereoisomeric mixtures of the isoxazoline vinyl ester derivatives **6** and **12**, the most potent inhibitors of the series, were successfully separated in preparative HPLC. Using experimental HPLC conditions reported in the section on Chemistry – General, we obtained two peaks named 1 and 2 completely baseline resolved. The activity of the separated isomers of the selected analogues **6** and **12** was tested to assess inhibition of the β1, β2 and β5 active sites of the 20S proteasome. Peak 1 for both compounds displayed inhibition of the chymotrypsin-like activity better than the corresponding

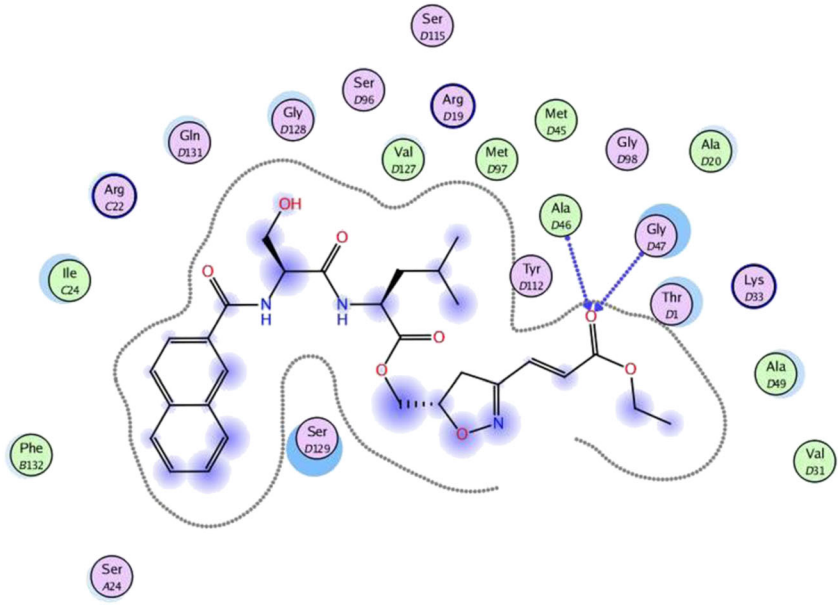

Figure 4 Arrangement of the residues of the binding pocket around molecule 6 (S configuration).

diastereoisomeric mixture. Only peak 1 of derivative **12** showed a detectable activity against β1 catalytic subunit. Peak 2 of the selected isoxazoline vinyl ester analogues inhibited all proteasome catalytic subunits with a $IC_{50} > 100$ (μM), demonstrating that only isomers present in peak 1 are active as proteasome inhibitors.

Molecular modelling studies applied to isomers derived from R or S configuration of the C5 of the isoxazoline ring confirm the biological data. The results of the superposition of molecules **6** and **12** to the bound homobelactosin ligand through flexible alignment procedure are shown in Figures 2 and 3, respectively. The template ligand (in green) specifically binds to the chymotryptic-like active site, represented in the figures by the interacting residues; in particular, the position of the catalytic Thr residue of subunit β5 is indicated by a black circle in Figure 2A. The superimposed molecule **6** in its S configuration appears to fit homobelactosin into the binding pocket quite nicely (Figure 2A) being able to model the shape of the active site and matching the most important functional groups; actually, it is worth considering that the acrylate group occupies the region near Thr1, as clearly shown by the illustrative scheme of Figure 4. Moreover, many of the residues interacting directly with homobelactosin, such as Thr1, Ile24 (hydrogen bonded to a carboxylic group) and Gly47, are positioned in such a way to approach complementary groups of molecule **6**, as shown by the illustrative scheme of Figure 4. In spite of its size, even molecule **12** (S configuration), thanks to its high flexibility, can adapt itself to the shape of homobelactosin bringing the terminal vinyl-ester group near the active threonine (Figure 3A). On the contrary, for both molecules, the change of configuration at isoxazoline C5 atom from S to R results in a definitely worse superposition, in particular as far as the molecular fragment interacting with the active threonine residue is concerned, as shown in Figures 2B and 3B, respectively. It is hence reasonable to suppose that the configuration of the carbon atom in position 5 at the isoxazoline is not irrelevant.

Thus, taking into consideration the biological profiles and the results of molecular modelling of these compounds, it is reasonable to speculate that the active optical isomer (peak 1) for both analogues **6** and **12** present S configuration at C5 in the isoxazoline ring.

Conclusions

Proteasome inhibition is an attractive area of research and has a significant potential to become a new therapeutic protocol for many diseases and disorders. Indeed, proteasome inhibitors, in addition of being of great interest in the cancer field by preventing also angiogenesis, metastasis and resistance to apoptosis, may also find applications in the treatment of cardiac disease, organ transplant rejection and neurodegenerative disorders.

The development in proteasome inhibitor discovery is in continuous progress using different paths such as screening of large libraries, repurposing of existing therapeutics and development of new selective compounds. Hence, the availability of new molecules able to interact with the multicatalytic enzyme subsites in a potent and selective manner without side effects is a primary requirement to reduce toxicity during drug treatment. With this purpose, we have developed a small series of peptide-based proteasome inhibitors generally bearing a C-terminal pharmacophoric unit. In this work, we have studied a new series of oligopetidic molecules with a C-terminal isoxazoline vinyl ester as pharmacophore with the aim that the extended conjugation could result in a favourable substrate for the nucleophilic attack by the catalytic N-terminal Thr1. Some new compounds that contain at the C-terminal extended conjugation inhibit especially β5 proteasomal catalytic subunits with an IC_{50} around the micromolar concentration in diastereoisomeric form due to asymmetric C5 of the isoxazoline ring. The inhibitory activity determined for the separated isomers of the selected compounds **6** and **12** and molecular modelling studies showed that probably the S configuration at carbon 5 of the isoxazoline moiety produce active analogues.

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