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New cyclic peptide proteasome inhibitors

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ABSTRACT

Here we report the study of a new series of vinyl ester cyclopeptide analogues synthesized on the basis of our previous development of a class of cyclopeptides derived from our linear prototype inhibitors. In these compounds, the exocyclic pharmacophoric unit Leu-VE was linked to the γ-carboxyl group of the glutamic acid residue at the C-terminal. The best analogues of the series have been shown to inhibit the caspase-like activity of the proteasome at nanomolar concentrations and have also demonstrated good resistance to proteolysis and a capacity to permeate the cell membrane.

The proteasome is a multicatalytic protease[1] which is involved in a number of proteolytically mediated intracellular processes, including: the constitutive turnover of many intracellular proteins, elimination of proteins with abnormal structures, control of the cell cycle and transcription, proteolytic activation of the transcription factor NF-κB, and processing of antigens for presentation by class I major histocompatibility complex proteins. The balance of protein synthesis and degradation processes is essential to preserve cellular homeostasis.

The controlled degradation of damaged or misfolded proteins and the regulation of short-lived proteins is achieved by the ubiquitin/proteasome pathway, in which the target protein is marked by a polyubiquitin chain and, after recognition, is degraded in an ATP-dependent manner by the 26 S proteasome.[2] This multicatalytic complex consists of a 20 S proteolytic core particle, which has a cylindrical shape, with the α and β subunits forming four stacked rings, and two 19 S regulatory caps which recognize ubiquitinated protein substrates and promote their entry into the central catalytic chamber. Three major proteolytic activities of proteasome can be distinguished as trypsin-like (T-L), chymotrypsin-like (CT-L), and peptidyl-glutamyl peptide hydrolase (PGPH) activities, which cleave peptide bonds on the carboxyl side of basic, hydrophobic, and acidic amino acid residues, respectively. The catalytic core of the 20 S proteasome is a Thr residue, responsible for the catalytic cleavage of substrates through nucleophilic attack.[3] Inhibition of this enzymatic activity with β-subunit-specific proteasome inhibitors may provide an anti-tumoral effect by inhibiting cell proliferation and angiogenesis, and by selectively inducing apoptosis of tumor cells.[4,5]

Several classes of synthetic and biological compounds which inhibit the proteolytic activities of the multicatalytic complex have been developed, and have contributed significantly to identification of the essential functions of the 26 S proteasome in various processes and pathways in eukaryotic cells.[6]

Most proteasome inhibitors are short peptides bearing a pharmacophoric group, such as aldehyde (e.g., MG132), boronic acid (Bortezomib), or vinyl sulphone, which forms a covalent bond with the catalytic O^{γ}-Thr 1 in three catalytic sites.[7,8] Several natural molecules (epoxomicin, lactacystin, salinosporamide) also form covalent adducts.[9] Non-covalent inhibitors, such as TMC-95A[10] (a naturally-constrained cyclic tripeptide) have been investigated in less detail, although they are thought to have weaker side effects in therapeutic applications.

We have recently been involved in the development of tri- or tetra-peptide-based derivatives with good pharmacokinetics properties and with selective activity towards the three catalytic sites. In particular, we have identified and characterized a new class of inhibitors, selective for the β2 subunit, which bear a C-terminal vinyl ester function able to interact with catalytic threonine in the same way that has been suggested for the well-known vinyl sulphone peptides. The best of these derivatives inhibit the trypsin-like activity in a nM range, are non-toxic, do not affect cell proliferation and are able to modulate the generation of antigenic peptides linked by MHC class I molecules.[11]

Elucidation of the 3D structure of proteasomal inhibitors can provide interesting information required for improving existing inhibitors and for the design of new compounds. Therefore, the most

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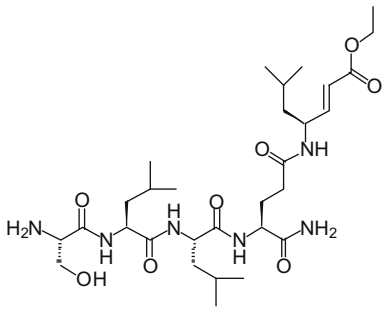
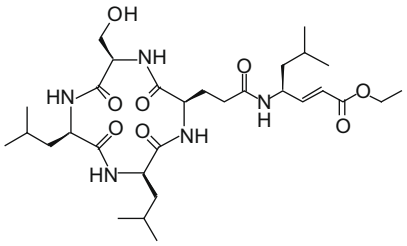


H-Ser-Leu-Leu-Glu(Leu-VE)-NH_2 **4**



Ser-Leu-Leu-Glu(Leu-VE) **8**

Figure 1.

interesting vinyl ester derivatives were subjected to conformational studies. In general, our derivatives presented a mass structure derived from a conformational level with a high degree of liberty; in the crystal state, the wrapping is similar to a β-sheet secondary structure. Through flexible alignment, our compounds showed some conformational similarities to the cyclic inhibitor TMC 95A.[12]

On the basis of these biological and structural data, we designed and prepared new vinyl ester derivatives characterized by conformational constraints.[13] These constrained analogues showed little interesting activity. However, we also synthesized a class of vinyl ester cyclopeptide analogues, and some of these latter derivatives were shown to inhibit the chymotrypsin-like activity of the proteasome at nM concentrations; their potency was found to depend on the size of the tetrapeptidic cyclic portion. Docking simulation results demonstrated that the vinyl ester moiety of these molecules is located near the Thr 1 residue, in accordance with the hypothesis that this molecular fragment can be considered the potential substrate for catalytic threonine.[14]

Here we describe the synthesis and biological activity of a new class of derivatives made up of four linear sequences (compounds **1**–**4**) and the corresponding cyclopeptides analogues (**5**–**8**) (Fig. 1). The exocyclic pharmacophoric unit Leu-VE was linked at the γ-carboxyl group of the glutamic acid residue at the C-terminal. The dipeptidic central sequence was Leu–Leu, while the glycine, alanine or a serine residues could be present at the N-terminal.

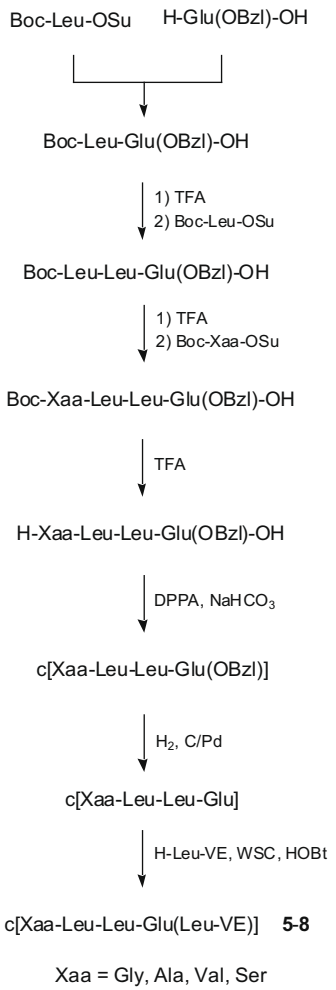
Cyclization was performed head-to-tail to obtain a 12-centre cycle related to the prototype c[Phe-Leu-Leu-Glu(Leu-VE)].

Pseudo-tetrapeptide amides with the pharmacophoric unit Leu-VE linked to the glutamic acid side chain (1–4) were prepared through solid phase synthesis starting from Rink amide resin. Fmoc-protected amino acids were condensed using WSC/HOBt and a solution of 20% piperidine/DMF was used to remove the Fmoc. The resin with the common tripeptide Glu-Leu-Leu was portioned for coupling of the N-terminal variable. After resin displacement with TFA, Fmoc-protected tetrapeptide amides were coupled to H-Leu-VE and finally treated with piperidine/DMF.

Vinyl ester cyclopeptides 5–8 were synthesized by the conventional methodology using stepwise C-terminal elongation (Scheme 1). Commencing with the glutamic acid residue monoprotected in the side chain as a benzyl ester, the other N_α-Boc-protected amino acids of the sequence were condensed as succinimidyl esters. After each coupling step, Boc was removed by TFA. Head-to-tail cyclization of the linear tetrapeptides was achieved using the activating reagent DPPA (diphenylphosphorylazide) under conditions known to incur minor racemization and minimal oligomerization.[15] After catalytic hydrogenation, the exocyclic leucine vinyl ester unit, prepared as previously described,[11a] was coupled using WSC/HOBt.

All products were purified by preparative RP-HPLC, and the homogeneity of the purified products was accessed by HPLC. Structural characterization was then achieved by electrospray ionisation (ESI) mass spectrometry (MICROMASS ZMD 2000) (Table 1) and ^{1}H NMR spectroscopy (Bruker AC 200).[16]

The activity of the vinyl ester cyclopeptides was tested to assess inhibition of the β1, β2 and β5 active sites of the 20S proteasome, previously purified from lymphoblastoid cell lines.[17,18] Suc-LLVY-AMC, Boc-LRR-AMC and Z-LLE-AMC, specific fluorogenic substrates for the three main proteolytic activities of the enzymatic complex, were used to measure chymotrypsin-like, trypsin-like and caspase-like proteasome activities, respectively. Substrates were incubated at 37 °C for 30 min with the proteasome, untreated or pre-treated with incremented concentrations (from 0.001 to 10 μM) of vinyl ester cyclopeptides, with the reference inhibitors epoxomicin and



Scheme 1. Synthesis of vinyl ester cyclopeptides **5**–**8**.

Table 1
Physicochemical data of linear and correlated vinyl ester cyclopeptides

Compound	HPLC[a]		P.f.	$[\alpha]_D^{20}$	MS
	K^l (a)	K^l (b)	(°C)	(*c* 1, MeOH)	$[M+H]^+$
1 H-Gly-Leu-Leu-Glu(Leu-VE)-NH_2	7.35	6.34	185–188	−25.4	596.4
2 H-Ala-Leu-Leu-Glu(Leu-VE)-NH_2	8.11	7.63	190–193	−28.2	610.4
3 H-Val-Leu-Leu-Glu(Leu-VE)-NH_2	8.52	8.05	195–199	−17.8	624.5
4 H-Ser-Leu-Leu-Glu(Leu-VE)-NH_2	7.21	6.04	175–178	−35.5	626.5
5 c[Gly-Leu-Leu-Glu(Leu-VE)]	8.06	7.22	113–115	−13.8	579.3
6 c[Ala-Leu-Leu-Glu(Leu-VE)]	8.94	8.38	140–143	−19.1	593.4
7 c[Val-Leu-Leu-Glu(Leu-VE)]	9.13	8.74	137–139	−17.7	607.4
8 c[Ser-Leu-Leu-Glu(Leu-VE)]	7.97	6.98	99–103	−17.4	609.4

[a] Capacity factor (K^l) of the peptides was determined by HPLC using two different solvent system gradient.

the aldehydic tripeptide derivative MG132 in activity buffer. Fluorescence was determined by a fluorimeter (Spectrafluor plus, Tecan, Salzburg, Austria) using an excitation 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.[19]

In general, the new cyclic analogues showed insignificant inhibition of chymotrypsin-like activity, with IC_{50} values above 10 μM. The inhibitory capacity of the cyclopeptides against the β2 subunit was in some cases comparable with the reference inhibitors, with IC_{50} values less than 10 μM. In particular, the cyclic compounds seemed to be more active than the corresponding linear sequences. Inhibition data of trypsin-like activity failed to reveal specific information about the structure-activity relationship, but it is evident that the more hydrophilic sequence with a serine residue at the N-terminal was preferred, analogous to previous data from our vinyl ester derivatives. It was extremely interesting to discover β1 inhibition by the new derivatives with IC_{50} values in the order of a nM range. The pseudo-peptide **8**, with an endocyclic serine residue, was found to be the most active compound of the series. Cyclization masks with β-lactam head-to-tail bonded the free aminic function of the corresponding linear sequences, and these results confirm previous data regarding compounds with a lipophilic N-terminal elongation. It is evident that the conformational constraint derived from cyclization favours interaction with the catalytic site and, in particular, the exocyclic portion with the vinyl ester pharmacophore was placed near the enzymatic Thr 1. Substitution of a phenylalanine residue with an amino acid bearing a compact lateral chain favours more complementary conformational adaptation with the structure of the catalytic site. Finally, all compounds revealed themselves to be potent and selective against the 20S proteasome β1 catalytic subsite with IC_{50} values in the nM range.

Subsequently, the cell membrane permeation of the most representative compounds, **5**–**8**, was tested in live cells. After cell treatment, proteasomes were purified and assayed for proteolytic activity using specific substrates for T-L, ChT-L and caspase-like activities as previously described.[19] The results obtained (Table 2) were comparable to those observed in the in vitro assay, thereby demonstrating that vinyl ester cyclopeptides are cell-permeable and able to inhibit the proteasome in vivo.

Resistance to proteolysis of the selected cyclic derivatives was then studied in human plasma. The degradation kinetics were determined by incubation in human plasma at 37 °C for time increments up to 360 min, and the incubation was halted by addition of ethanol. After centrifugation, an aliquot of the clear supernatant was analysed in HPLC.[19] The degradation half-lives were obtained by a least-squares linear regression analysis of a plot of logarithmic compound concentrations versus time, using a minimum of five points. The data, reported in Table 2, as expected on the basis of the molecular features determined by cyclization, show that all derivatives analyzed possess great stability against human plasma protease, with a half-life of over 6 h.

In conclusion, we have presented herein a new series of cyclopeptide vinyl ester proteasome inhibitors. The new oligomeric derivatives have shown to selectively and efficiently inhibit the β1 enzymatic subsite. The specificity and potency of this inhibition appears to be directly related to the conformational constraint imposed by cyclization. The distinctive characteristics of these new molecules could represent an interesting springboard for future investigation into β1-mediated biological activity, and for analysis of the structural features essential for a precise interaction with the post-acidic site.

Considering the high therapeutic potential of molecules which are active, selective and specific for the three catalytic subunits of the proteasome, we have obtained new inhibitors selective for

Table 2
Subsites proteasome inhibition and enzymatic stability of peptides **1**–**8** and reference inhibitors

Compound	Isolated enzyme IC_{50}[a] (μM)			In vivo inhibition IC_{50}[a] (μM)			Half-life (min) plasma
	ChT-L	T-L	PGPH	ChT-L	T-L	PGPH	
Epoxomicin	0.009	0.311	5.640				
MG132	0.005	1.398	>10				
1 H-Gly-Leu-Leu-Glu(Leu-VE)-NH_2	>10	6.470	>10				48.5
2 H-Ala-Leu-Leu-Glu(Leu-VE)-NH_2	>10	4.530	>10				73.2
3 H-Val-Leu-Leu-Glu(Leu-VE)-NH_2	>10	>10	>10				117.4
4 H-Ser-Leu-Leu-Glu(Leu-VE)-NH_2	>10	1.390	>10				86.1
5 c[Gly-Leu-Leu-Glu(Leu-VE)]	>10	3.580	0.172	>10	5.830	0.221	>360
6 c[Ala-Leu-Leu-Glu(Leu-VE)]	>10	8.830	0.121	>10	>10	0.123	>360
7 c[Val-Leu-Leu-Glu(Leu-VE)]	>10	>10	0.205	>10	>10	0.273	>360
8 c[Ser-Leu-Leu-Glu(Leu-VE)]	>10	2.980	0.065	>10	6.940	0.091	>360

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

caspase-like activity, stable to enzymatic degradation, and able to permeate cell membranes. Selective inhibitors for the β1 catalytic subsite could be useful due to their biological profiles related to the boronic derivative PS-341 (Velcade^{R}), which are used as an anti-tumoral therapy in the treatment of multiple myeloma.[6a]

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16. 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'*) of the vinyl ester cyclopeptides 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.c[Ser-Leu-Leu-Glu(Leu-VE)] (8). Purified yield 29%; purity estimated by HPLC > 98%; ^{1}H NMR ($CDCl_3$, 200 MHz): δ (ppm) 0.99 (d, 6H), 1.11 (m, 12H), 1.31 (t, 3H, J = 7.2), 1.39 (m, 2H), 1.63–1.75 (m, 7H), 1.99 (m, 2H), 2.18 (d, 2H), 2.84 (m, 2H), 3.87 (m, 2H), 4.08 (q, 2H, J = 7.3), 4.31–4.54 (m, 5H), 6.01 (d, 1H, J = 16.2), 7.06 (dd, 1H, J = 16.1), 8.02 (bs, 5H).
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19. Purification of the proteasomes and enzyme assays in vitro and in live cells are described in 'Supporting information' of Ref. 11a.