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Original article

C-terminal constrained phenylalanine as a pharmacophoric unit in peptide-based proteasome inhibitors

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Abstract

Here we report the synthesis and biological properties of peptide-based molecules bearing constrained analogues of phenylalanine at the C-terminal. Compounds were tested as proteasome subunits' inhibitors. Dehydro-peptides showed good inhibition, in particular against trypsin-like (T-L) proteasome activity while some C-terminal Tic-derivatives inhibit only caspase-like activity in enzymatic β1 subunits with a certain degree of efficacy. The best analogues of the series demonstrated good resistance to proteolysis and a capacity to permeate the cell membrane.

Keywords: Constrained phenylalanine analogues; Synthesis; Pseudopeptides; Proteasome inhibition

1. Introduction

The ubiquitin–proteasome pathway is considered to be the best means of extralysosomal cytosolic and nuclear protein degradation in cells [1–5]. The 26S proteasome expressed in eukaryotic cells is a large multicatalytic intracellular protease complex and represents the central proteolytic machinery of the system. The proteasome regulates basic cellular processes and is responsible for the degradation and proteolytic processing of proteins essential for the regulation of development, differentiation, proliferation, cell cycling, senescence, apoptosis, gene transcription, signal transduction, antigen presentation, immune activation and the inflammatory and stress responses [6]. The proteasomal pathway represents a new approach in the treatment of a range of pathologies such as cancer, inflammation, immune diseases and others [7–12].

Cell proteins must be targeted for recognition and subsequent degradation by covalent attachments of a ubiquitin polypeptide [13]. The proteolytic activities of the 26S proteasome are carried out in its barrel-shaped 20S catalytic core, which is flanked by two 19S regulatory caps. Ubiquitinated substrates are recognized and bound to the complex, then unfolded and deubiquitinated, and subsequently transferred to the catalytic chamber, where they are degraded. Proteasome 20S is composed of four axially-stacked rings; the outer ring consists of seven different non-proteolytic α subunits, which allow substrate translocation into the central cavity formed by two inner rings formed by seven β subunits [14–16]. Only the β1, β2, and β5 subunits retain proteolytic activity by means of N-terminal threonine residues which face the central cavity. Based on their specificity towards peptidyl substrates, the β1, β2, and β5 subunits have been assigned caspase-like (PGPH), trypsin-like (T-L) and chymotrypsin-like (ChT-L) peptidase activities, respectively [17,18].

Several classes of synthetic and biological compounds which inhibit the proteolytic activities of the multicatalytic-complex have been developed [19–23], and have contributed greatly to the identification of essential functions of the 26S proteasome in various processes and pathways in eukaryotic

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cells [6]. In particular, proteasome inhibitors could act as therapeutic agents in the prevention of tumoral cell proliferation and as modulators of antigen presentation [8,12,24]. The elucidation of the 3D structure of proteasomal inhibitors provides interesting informations required for improving existing inhibitors and in the design of new compounds [25].

The major family of multicatalytic-complex inhibitors has a peptide-based structure with a C-terminal functional group able to interact with proteasomal catalytic threonine. Short peptide inhibitors include synthetic and natural molecules with a pharmacophoric function, such as vinyl sulfone, boronic acid, aldehyde and epoxyketone. Other classes are comprised of peptide macrocycles and non-peptidic inhibitors with a wide structural variety [26–39].

Involvement of the different proteasomal subunits in the process of protein degradation is evaluated by the employment of selective inhibitors of the individual active sites [40]. We have previously developed new oligopeptidic proteasome inhibitors bearing different pharmacophoric units at the C-terminal [41–46]. In particular we have identified and characterized a new class of inhibitors selective for trypsin-like activity and specific for the multicatalytic 20S complex with a vinyl ester function. This class of inhibitors is able to interact with enzymatic threonine in the same way that has been suggested for the well-known vinyl sulfone peptide. The best of these derivatives inhibit the β2 subunit 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. Moreover, vinyl ester inhibitors have demonstrated good resistance to proteolysis in plasma, as well as an ability to permeate the cell membrane [42].

On the basis of our previous results we prepared and tested a new series of compounds presented in Fig. 1 with reference vinyl ester inhibitors HMB-AA_1-AA_2-Leu-VE. New oligopeptide-based molecules contain selected amino-acidic sequences derived from the most representative inhibitors of the previous series. All pseudotri- and pseudotetrapeptides are functionalized at the N-terminal position with a 2-methyl-3-hydroxybenzoyl (HMB) group, while the C-terminal pharmacophoric unit consists of constrained phenylalanine analogues. Compounds **5–8** bear an α,β-dehydro-phenylalanine (ΔPhe), meaning that the substrate for Michael addition for catalytic threonine is directly inserted into the peptidic backbone. 1,2,3,4-Tetrahydro-isoquinoline-3-ethyl acrylate (Tic-VE) in an L or D configuration is the pharmacophore in the pseudopeptides **12–19**, analogous to reference leucine-vinyl ester (Leu-VE) inhibitors.

2. Chemistry

Pseudopeptides **5–8** contain pharmacophoric α,β-dehydrophenylalanine at the C-terminal position, as reported in Scheme 1. The ΔPhe moiety was obtained through azlactonization and dehydratation of Boc-Ser-(β-OH)Phe-OH (**1**) or Boc-Leu-(β-OH)Phe-OH (**2**), using sodium acetate in acetic anhydride [47,48]. Treatment of the azlactones with sodium ethylate resulted in the formation of N_α-protected dehydro-dipeptides **3** or **4** with ΔPhe in a *Z*-configuration [49]. Deprotection with trifluoroacetic acid and coupling steps utilizing water soluble carbodiimide and 1-hydroxybenzotriazole (WSC–HOBt) yielded the desired products.

Compounds **12–19** bearing a tetrahydro-isoquinoline vinyl ester (Tic) at the C-terminal in an L or D configuration were then synthesized stepwise by solution methods (Scheme 2). L or D N_α-protected Tic-VEs were prepared from the corresponding aldehyde, which is obtained via the Fehrentz method [50] by reaction with [(ethoxycarbonyl)methylidene]triphenylphosphorane [51]. HATU were employed for acylation of the H-Tic-VEs, the other coupling steps were accomplished using WSC/HOBt; Bocs were 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 physicochemical properties of compounds **5–8** and **12–19** are reported in Table 1.

3. Biological activity

The capacity of the ΔPhe peptides **5–8** and tetrahydro-isoquinoline vinyl ester derivatives **12–18** to inhibit the three catalytic activities of isolated 20S proteasome was tested. All compounds were assayed at different concentrations (from 0.001 to 10 μM) for their capacity to inhibit the in vitro trypsin-like (T-L), chymotrypsin-like (ChT-L) and caspase-like (PGPH) activities of proteasome purified from lymphoblastoid cell lines using a fluorogenic substrate specific for the three major proteolytic activities of the enzymatic complex [52]. IC_{50} values against proteasome subsite activities obtained after 30 min of incubation are reported in Table 2, and compared to prototype vinyl ester inhibitor HMB-Val-Ser-Leu-VE and the two known inhibitors Epoxomicin and MG132.

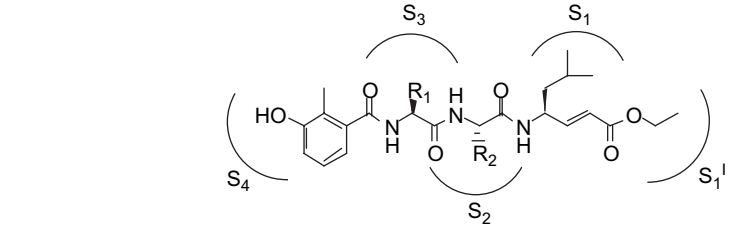
The cell membrane permeation ability of dehydro-peptides and the most active Tic-VE analogues was tested against the β1 subsite (**13**, **15**) in live cells. After cell treatment, the proteasomes were purified and assayed for proteolytic activity as described above.

The same compounds tested in vivo were evaluated for their susceptibility to enzymatic hydrolysis by incubation at 37 °C in human plasma [53]. The degradation half-lives of the pseudopeptides, reported in Table 2, were determined as described in Section 6.

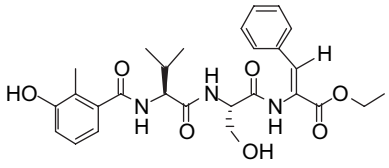
4. Results and discussion

The data obtained from enzyme inhibition tests against the catalytic subunits highlighted that the new peptide-based inhibitors generally show different potency and selectivity depending on their molecular structure.

The P1 position favours α,β-dehydro-phenylalanine (ΔPhe) to the bicyclic tetrahydro-isoquinolinic vinyl ester (Tic-VE) system. Compounds **4–8** are more active against the β2 and β5 subunits, while compounds **12–19** showed moderate inhibition of the β1 subunit of the 20S proteasome in certain cases. In terms of potency, the unsaturated residue was found to be



HMB-AA$_1$-AA$_2$-Leu-VE



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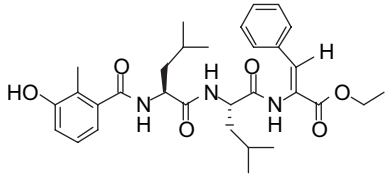

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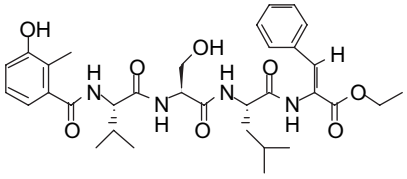

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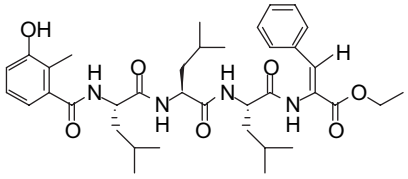

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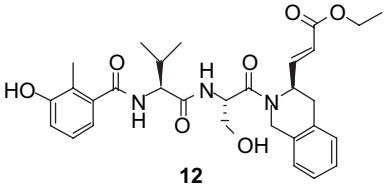

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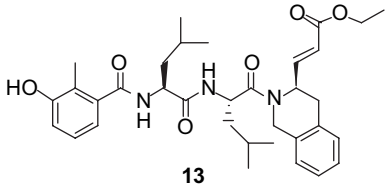

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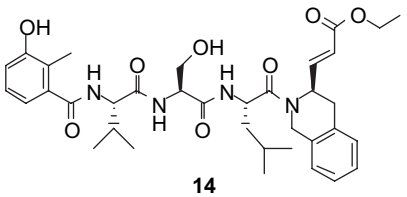

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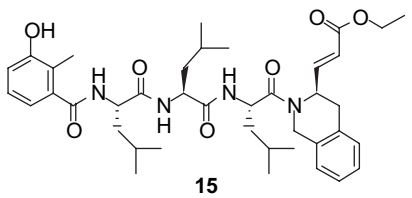

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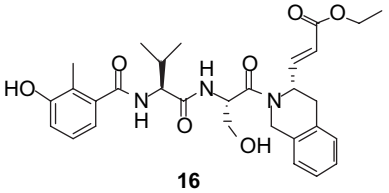

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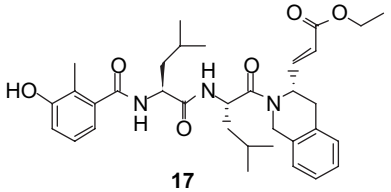

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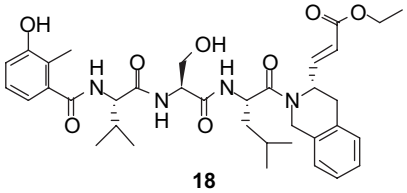

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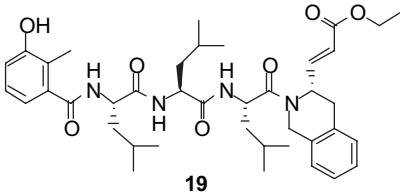

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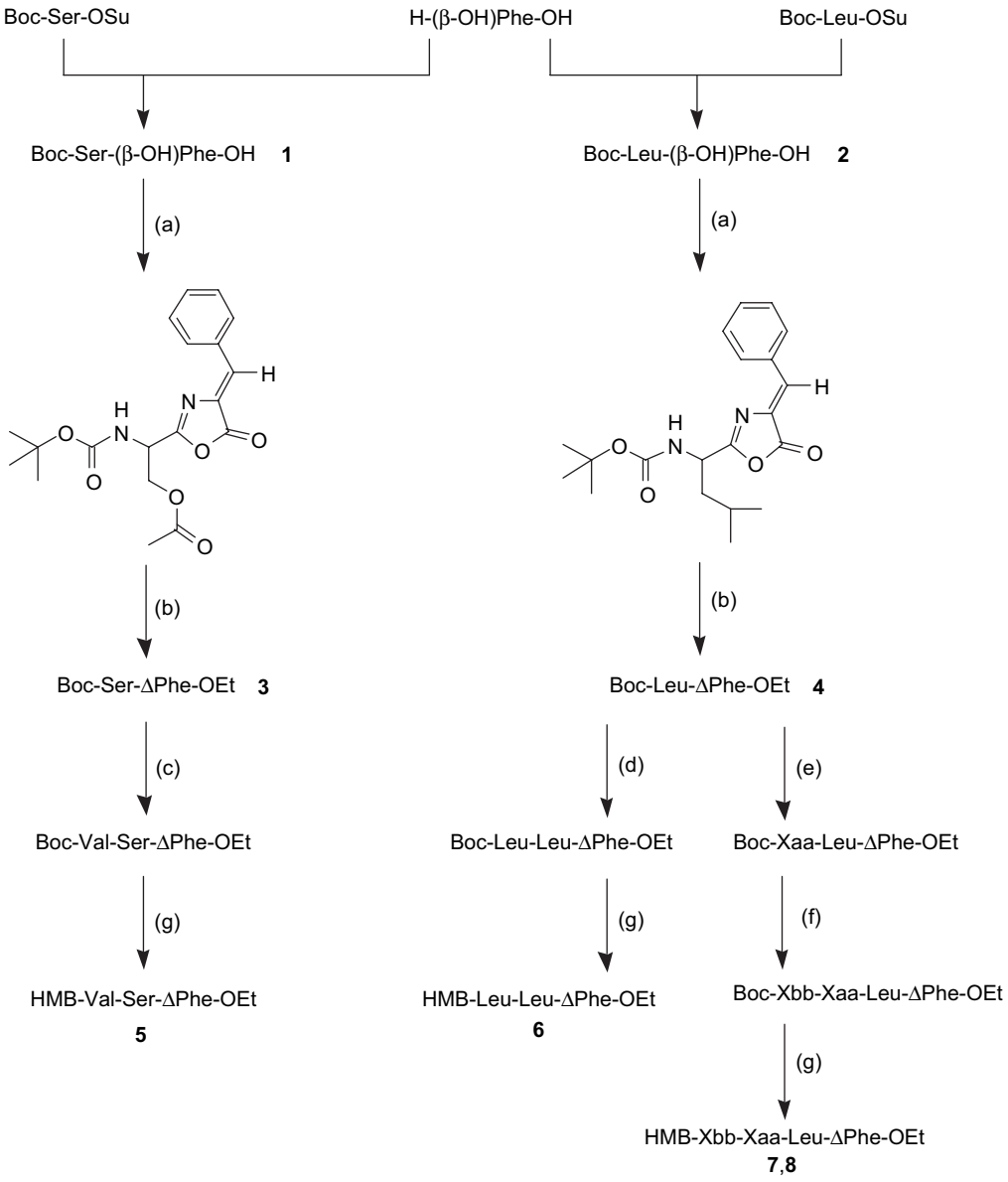
Fig. 1. Structure of the prototype and new vinyl ester pseudotripeptide inhibitors.

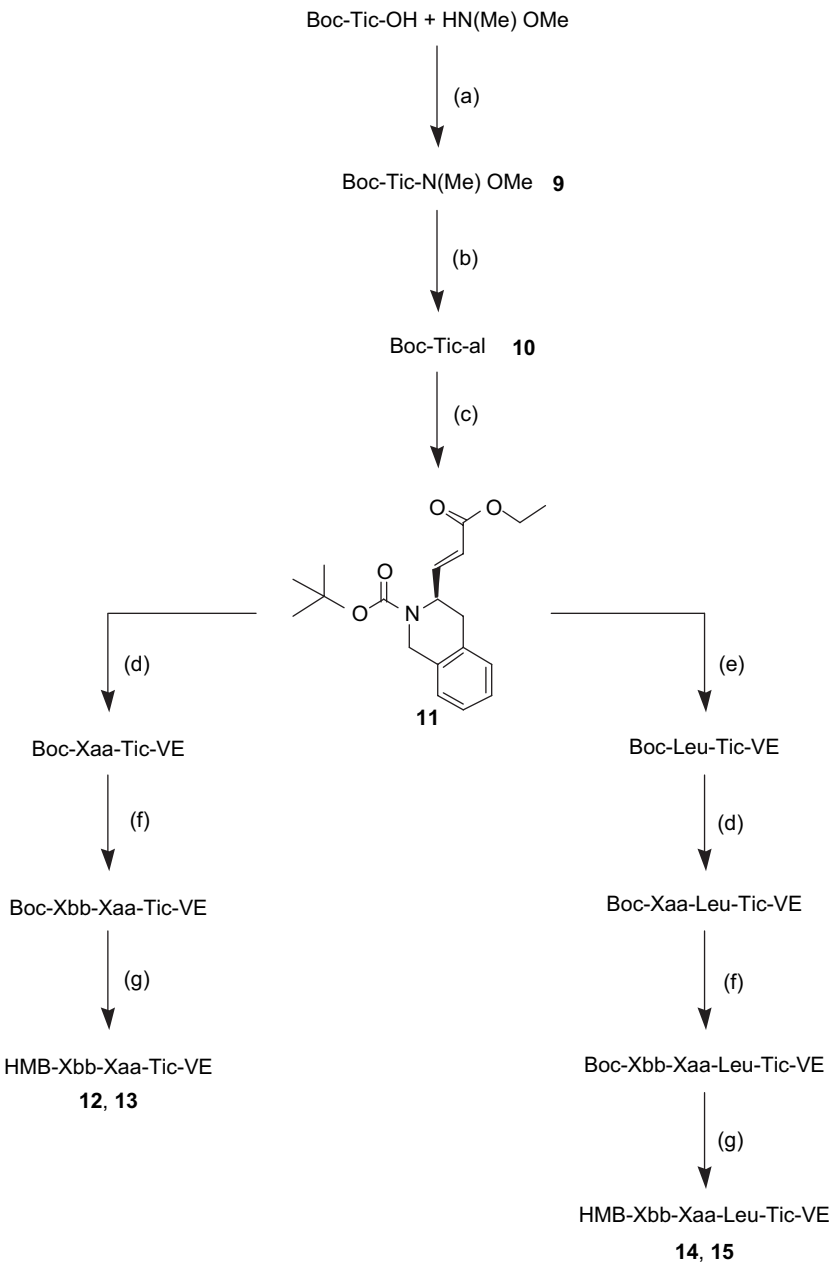
more efficacious as a C-terminal pharmacophoric unit and represents the best substrate for Michael addition. With regard to the Tic-derivatives, we noted that the configuration of the constrained analogue of phenylalanine is not so influential, the presence of the L-amino acid is slightly favoured and both of tetrahydro-isoquinolinic residue configurations of the vinyl ester chain do not reveal the correct interaction with the catalytic threonine.



Scheme 1. Synthesis of ΔPhe derivatives **5**–**8**. Reagents: (a) Ac_2O, AcONa; (b) Na, EtOH; (c) (1) TFA; (2) Boc-Val-OH, WSC, HOBt, NMM, DMF; (c) (1) TFA; (2) Boc-Val-OH, WSC, HOBt, NMM, DMF; (d) (1) TFA; (2) Boc-Leu-OH, WSC, HOBt, NMM, DMF; (e) (1) TFA; (2) Boc-Xaa-OH, WSC, HOBt, NMM, DMF; (f) (1) TFA; (2) Boc-Xbb-OH, WSC, HOBt, NMM, DMF; (g) (1) TFA; (2) HMB-OH, WSC, HOBt, NMM, DMF.

The remaining part of the sequence corresponding to positions P2–P4 influences enzymatic inhibition in terms of potency and selectivity as the confirmed parameters.

A more concrete analysis of IC_{50} values of the dehydrotetrapeptides shows that the presence of a hydrophobic residue such as Val or Leu in P4 position increases inhibition; the greater length of the sequence in compounds with L or D Tic aids the interaction with the post-acidic pocket. In general, the presence of the hydrophilic Ser in P2 or P3 allows specific inhibition of the trypsin-like activity; compound **7** is the best of the series towards the β2 subunit, with an IC_{50} value a little over the reference prototype. The more hydrophobic sequences show a major selectivity for the chymotrypsin-like activity, even if the inhibition values are not so significant. The derivatives with the α,β-dehydro residue at the C-terminal are the most interesting. Their major structural rigidity could permit molecular modellistic studies with the aim to optimize the enzyme–inhibitor interaction. We postulate that the substitution of the ΔPhe with α,β-unsaturated amino acids such as ΔLeu or ΔAla with a lateral chain that is not so bulky could promote the primary interaction with the catalytic Thr. Finally, in comparison with Epoxomicin and the aldehydic inhibitor MG132, potent and selective against chymotrypsin-like (ChT-L) activity (Table 2), the dehydro-peptidic derivatives are resulted in some cases more efficacious for the β2 subunits while the compounds with the Tic-VE at the C-terminal have



Scheme 2. Synthesis of vinyl ester peptides **12**–**15**. Reagents: (a) WSC, HOBt, NMM, DMF; (b) $LiAlH_4$, THF; (c) EtO–CO–CH=PPh_3, toluene; (d) (1) TFA; (2) Boc-Xaa-OH, WSC, HOBt, NMM, DMF; (e) (1) TFA; (2) Boc-Leu-OH, WSC, HOBt, NMM, DMF; (f) (1) TFA; (2) Boc-Xbb-OH, WSC, HOBt, NMM, DMF; (g) (1) TFA; (2) HMB-OH, WSC, HOBt, NMM, DMF.

demonstrated a better inhibition of the caspase-like activity. In addition to the chymotryptic activity showed by the inhibitors used as references, it is important for the development of potent and specific molecules for the two other catalytic activities. The specificity of the new vinyl ester derivatives could be interesting in consideration of the fact that in addition to the chymotrypsin-like (β5) site, the caspase-like site has been recently identified as a secondary target of Bortezomib (VELCADE), a proteasome inhibitor being used for the treatment of multiple myeloma. Specific inhibitors of the caspase-like site are needed to address the question that whether inhibition of this site is important for anti-neoplastic activity and thus facilitate the development of new drugs of this class [54,55].

The compounds that showed the most interesting in vitro data (**5**–**8**, **13** and **15**) were also evaluated in cellular lines. The IC_{50} values obtained on the single catalytic subunits confirmed the in vitro data, proving the capacity of these new inhibitors to penetrate the cellular membrane and the possibility of an in vivo action on the multicatalytic-complex.

Stability in the presence of peptidases is an important pharmacokinetic parameter required for molecules with

Table 1
Analytical data and physicochemical properties of pseudopeptides **5–8** and **12–19**

No	Compound	[a]HPLC		[b]$[\alpha]_D^{20}$	M.p. (°C)	$M + H^+$
		K^1 (a)	K^1 (b)			
5	HMB-Val-Ser-ΔPhe-OEt	7.44	5.97	+15.1	195–197	512.6
6	HMB-Leu-Leu-ΔPhe-OEt	8.47	6.88	+25.5	198–202	552.7
7	HMB-Val-Ser-Leu-ΔPhe-OEt	8.09	6.45	+3.7	135–138	625.5
8	HMB-Leu-Leu-Leu-ΔPhe-OEt	9.58	8.04	+14.3	211–214	665.9
12	HMB-Val-Ser-Tic-VE	6.23	4.98	+25.1	121–123	552.5
13	HMB-Leu-Leu-Tic-VE	8.87	7.34	−18.3	107–110	592.6
14	HMB-Val-Ser-Leu-Tic-VE	7.96	6.47	+15.9	136–140	665.7
15	HMB-Leu-Leu-Leu-Tic-VE	9.34	7.98	−24.8	145–148	704.7
16	HMB-Val-Ser-D-Tic-VE	6.09	4.92	+38.4	114–117	552.5
17	HMB-Leu-Leu-D-Tic-VE	8.77	7.13	−25.7	127–131	592.6
18	HMB-Val-Ser-Leu-D-Tic-VE	7.54	6.22	+21.8	156–160	665.7
19	HMB-Leu-Leu-Leu-D-Tic-VE	9.11	7.79	−34.5	120–124	704.7

[a] Capacity factors (K^1) are determined in two different solvent systems reported in Section 6.
[b] $c = 1$, MeOH.

therapeutic potential, and in particular for peptide derivatives, so the compounds of the new series with a better profile were tested by enzymatic degradation in human plasma. All the tested derivatives containing the constrained analogues of Phe in C-terminal position were found to be stable in the presence of plasmatic proteases, thereby confirming that the introduction of unusual residues results in a greater enzymatic resistance to the oligopeptidic sequence.

5. Conclusion

Considering the high therapeutic potential of molecules which are active, selective and specific for the catalytic subunits of the 20S proteasome, we designed, synthesized and tested new peptide-based compounds with new C-terminal pharmacophoric units. We inserted ΔPhe and Tic (L or D), constrained analogues of phenylalanine, as potential substrates of the catalytic γ-hydroxy threonine side-chain in Michael addition. Dehydro-peptides were demonstrated to be the most efficacious in the interaction with β2 and β5 subunits of the multicatalytic-complex with a nM range inhibition. In order to simplify the molecular structure, to improve the synthesis and pharmacokinetic properties, we inserted the α,β-dehydrophenylalanine in such a way as to insert the substrate for the enzymatic threonine directly in the peptidic backbone.

ΔPhe inhibitors can be considered to be a good target for the development of new compounds with other α,β-unsaturated residues as they possess less bulky lateral chains and are able to interact in an effective way with the catalytic subunits of the proteasome.

6. Experimental protocols

6.1. 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 Waters Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly Column C_{18} (30 × 4 cm, 300 Å,

Table 2
Proteasome inhibition and enzymatic stability of C-terminal-constrained derivatives

No	Compound	Isolated enzyme [a]IC_{50} (μM)			In vivo inhibition [a]IC_{50} (μM)			Half-life (min) plasma
		T-L	ChT-L	PGPH	T-L	ChT-L	PGPH	
	HMB-Val-Ser-Leu-VE	0.033	>10	>10	0.050	>10	>10	>360
5	HMB-Val-Ser-ΔPhe-OEt	0.183	>10	>10	0.231	>10	>10	>360
6	HMB-Leu-Leu-ΔPhe-OEt	1.761	1.187	>10	2.179	1.548	>10	>360
7	HMB-Val-Ser-Leu-ΔPhe-OEt	0.079	>10	>10	0.092	>10	>10	>360
8	HMB-Leu-Leu-Leu-ΔPhe-OEt	1.315	0.894	>10	1.815	1.045	>10	>360
12	HMB-Val-Ser-Tic-VE	3.674	>10	2.958				
13	HMB-Leu-Leu-Tic-VE	>10	3.734	1.481			2.052	>360
14	HMB-Val-Ser-Leu-Tic-VE	7.946	>10	2.354				
15	HMB-Leu-Leu-Leu-Tic-VE	>10	8.451	0.952			1.412	>360
16	HMB-Val-Ser-D-Tic-VE	2.787	>10	3.917				
17	HMB-Leu-Leu-D-Tic-VE	5.839	2.065	2.142				
18	HMB-Val-Ser-Leu-D-Tic-VE	>10	>10	2.498				
19	HMB-Leu-Leu-Leu-D-Tic-VE	>10	5.295	1.731				
	Epoxomicin	0.284	0.005	4.560				
	MG132	1.077	0.002	>10				

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

15 μm spherical particle size column). The column was perfused for 30 min at a flow rate of 30 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA); a linear gradient from 0 to 100% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) was adopted for the elution of compounds. 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 peptides 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 90% B for 25 min and (b) from 30 to 100% B for 25 min. All pseudopeptides showed less than 1% impurity when monitored at 220 and 254 nm.

The molecular weight of the compounds was determined by electrospray ionisation (ESI) (MICROMASS ZMD 2000) and the values are expressed as $[MH]^+$. TLC was performed in precoated plates of silica gel F_{254} (Merck, Darmstadt, Germany) using 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), (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 on a Bruker AC 200 spectrometer.

6.2. Chemistry

6.2.1. Boc-Xaa-DL-(β-OH)Phe-OH (**1**, **2**)

NMM (2 mmol) and Boc-Ser-OSu or Boc-Leu-OSu (1.8 mmol) were added to a solution of H-DL-(β-OH)Phe-OH (2 mmol) in DMF (10 mL). The reaction mixture was stirred for 18 h at room temperature. The residue obtained after evaporation of the solvent was dissolved with AcOEt (100 mL) and washed consecutively with HCl 0.1 N and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue was treated with Et_2O and the resulting oil (yield 85–89%) directly utilized for the following steps.

6.2.2. Boc-Xaa-ΔPhe-OEt (**3**, **4**)

Sodium acetate trihydrate was added to a solution of **1** or **2** (1.5 mmol) in 5 mL of acetic anhydride and the resulting solution was stirred at room temperature overnight. Ice-water (10 mL) was added to the reaction mixture and the precipitate azlactone was collected, washed with $NaHCO_3$ (5%) and water then dried (yield 70–75%). Azlactone (1 mmol) in absolute EtOH (5 mL) was added dropwise to a solution of sodium (1.5 mmol) in absolute EtOH (7.5 mL). After 30 min at room temperature the reaction mixture was neutralized by acetic acid, evaporated, diluted with AcOEt (50 mL) and washed consecutively with HCl 0.1 N, brine, $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue was treated with Et_2O/light petroleum (1:1) and the resulting solid separated by centrifugation (yield 80–84%).

Boc-Ser-ΔPhe-OEt (**3**): m.p. (°C) = 112–114; $[\alpha]_D^{20} = +44.5$ ($c = 1$, MeOH); ^{1}H NMR ($CDCl_3$): δ 1.52 (s, 9H), 3.8–4.2 (m, 3H), 7.14–7.32 (m, 5H), 7.60 (s, 1H); $[M + H]^+ = 378.2$.

Boc-Leu-ΔPhe-OEt (**4**): m.p. (°C) = 103–105; $[\alpha]_D^{20} = +38.4$ ($c = 1$, MeOH); ^{1}H NMR ($CDCl_3$): δ 1.05 (d, 6H), 1.38 (s, 9H), 1.59 (m, 2H), 1.95 (m, 1H), 3.64 (m, 1H), 7.11–7.33 (m, 6H), 7.63 (s, 1H); $[M + H]^+ = 403.3$.

6.2.3. Boc-Tic-VE (**11**)

A solution of aldehyde (1.18 mmol) dissolved in 5 mL of anhydrous THF was added dropwise to a solution of [(ethoxycarbonyl)methylidene]triphenylphosphorane (2.36 mmol) in 15 mL of anhydrous THF. The reaction was left to work at room temperature for 2 h, and then the solvent was removed by vacuum evaporation. The crude product was dissolved in ethyl acetate and washed twice with brine; the crude product was purified by fc (ethyl acetate/light petroleum, 1:5) with a 65% final yield. ^{1}H NMR ($CDCl_3$): δ 1.27–1.21 (t, 3H), 1.49 (s, 9H), 2.94–2.89 (m, 1H), 3.22–3.18 (dd, 1H), 4.13–4.08 (q, 2H), 4.37–4.33 (d, 1H, $J = 16.8$), 4.74–4.70 (d, 1H, $J = 16.8$), 5.00 (m, 1H), 5.81–5.76 (dd, 1H, $J = 1.6$; $J = 15.8$), 6.76–6.71 (dd, 1H, $J = 5.2$; $J = 15.6$), 7.260–7.112 (m, 4H).

6.2.4. General synthetic procedures

6.2.4.1. 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.

6.2.4.2. Coupling with WSC/HOBt. The deprotected α-amine intermediate (1 mmol), NMM (1 mmol), WSC (1 mmol) and HOBt (1 mmol) were added to a solution of the 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 room temperature, then the solution was diluted with AcOEt (80 mL) and washed consecutively with HCl 0.1 N and brine then $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue was treated with Et_2O and the resulting solid separated by centrifugation.

6.2.4.3. Coupling with HATU. The deprotected H-Tic-VE (1 mmol), NMM (1 mmol), and HATU (1 mmol) were added to a solution of Boc-Xaa-OH (1 mmol) in DMF (3 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 24 h at room temperature, then the solution was diluted with AcOEt (70 mL) and treated as described above.

6.2.5. ^{1}H NMR data of products **7** and **15**

HMB-Val-Ser-Leu-ΔPhe-OEt (**7**): ^{1}H NMR ($CDCl_3$): δ 0.99 (d, 12H), 1.30 (t, 3H), 1.73–1.78 (m, 2H), 1.85–1.89 (m, 1H), 2.01 (br s, 1H), 2.35 (s, 3H), 2.62–2.67 (m, 1H), 3.97–4.02 (m, 2H), 4.15 (q, 2H), 4.51–4.63 (m, 3H), 5.03 (br s, 1H), 6.89–7.33 (m, 9H), 8.02 (br s, 4H).

HMB-Leu-Leu-Leu-Tic-VE (**15**): ^{1}H NMR ($CDCl_3$): δ 1.01 (d, 12H), 1.06 (d, 6H), 1.28 (t, 3H), 1.72–1.81 (m, 9H), 2.30 (s, 2H), 2.80–2.84 (m, 2H), 3.42–3.50 (m, 2H), 4.09 (q, 2H), 4.47–4.53 (m, 3H), 4.98 (br s, 1H), 5.49 (d, 1H), 5.81 (d, 1H), 7.00–7.74 (m, 8H), 8.05 (br s, 3H).

6.3. Proteasome purification

Proteasome was isolated from lymphoblastoid cell lines, untreated or treated with the inhibitors for 12 h at 37 °C, as previously described [56]. A subsequent purification was carried out by affinity chromatography (mAb α-subunit, Affinity). Fractions containing proteasomes were combined and dialyzed against 25 mM Tris–HCl pH 7.5. Protein concentration was determined using BCA protocol (Pierce, Rockford, IL, U.S.A.).

6.4. Proteasome subunit inhibition assays

Suc-LLVY-AMC, Boc-LRR-AMC and Ac-YVAD-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 pretreated with 0.001–10 μM of test compounds in activity buffer. Fluorescence was determined by a fluorimeter (Spectrafluor plus, Tecan, Salzburg, Au) using an excitation of 360 nm and emission of 465 nm. Activity was evaluated in fluorescence units and 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) vs. 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.

6.5. Enzymatic stability assays

The degradation kinetics of selected C-terminal constrained inhibitors were 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 at pH 7.5. Incubation was performed at 37 °C up to 360 min. The incubation was terminated by addition of ethanol (0.2 mL), the mixture poured at 21 °C, and, following centrifugation (5000 rpm for 10 min), aliquots (20 μL) of the clear supernatant were injected into the RP-HPLC column. HPLC was performed as described in analytical determinations. The degradation half-life ($T_{1/2}$) was obtained by least-squares linear regression analysis of a plot of the logarithmic inhibitor concentration vs. time, using a minimum of five points.

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