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Original article

Synthesis and biological evaluation of new vinyl ester pseudotripeptide proteasome inhibitors

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Abstract

Here we report the synthesis and biological activities of new tripeptidic-based vinyl ester derivative proteasome inhibitors. Starting from Hmb-Val-Ser-Leu-VE prototype, we investigated P2 position and N-terminal substitution. The more effective inhibitors of the series showed remarkable inhibition and selectivity for the trypsin-like (β2) subunit and were revealed to be specific for the proteasome. In vitro metabolic stability studies of the new vinyl ester analogues are also reported here.

Keywords: Proteasome inhibitors; Vinyl ester pseudopeptides; Synthesis; Trypsin-like activity

1. Introduction

The 26S proteasome (2.4 MDa) is a multicatalytic protease complex which plays an essential role in cells able to recognize and degrade ubiquitinated proteins. The proteasome is made up of multiple subunits and, in mammalian cells, its modular structure consists of a 20S proteolytic chamber capped at both ends by 19S regulatory particles. The 20S portion is formed by four stacked rings, and each of the two inner rings is composed of seven different β subunits [1–3]. It has numerous physiological functions including most non-lysosomal proteolysis in prokaryotes and eukaryotes [4], and its principal activities, located respectively in the β1, β2 and β5 subunits of each β-ring, are referred to as post-acidic-like (PGPH), trypsin-like (T-L) and chymotrypsin-like (ChT-L) [5,6]. The proteolytic sites utilize the γ-hydroxyl group of the N-terminal threonine residue of β subunits as nucleophile [7–12].

The proteasome is fundamental for innumerable cellular functions such as cell cycle regulation, stress response, hydrolysis of abnormal proteins and production of antigenic peptides presented by class I-MHC [13–16], and the ubiquitin-proteasome pathway represents a promising target for the development of bioactive molecules potentially applicable in treatment of pathologies such as cancer, inflammation, immune diseases and others [17–22]. Thus far, the study of proteasome inhibitors, which are normally C-terminally functionalized oligopeptides with a pharmacophore able to interact with catalytic threonine of the proteasome, has revealed useful information about enzyme structure, catalytic mechanism and biology. Classes of these substances are being used to study the role of the proteolytic complex in various cellular processes and dipeptidyl boronic acid PS-341 has been approved by the FDA for the treatment of multiple myeloma [23–34].

We previously studied a number of tripeptidic sequences derivatized at the C-terminal with arecoline derivatives and found that these analogues showed an interesting inhibition against tryptic and chymotryptic active sites with an IC_{50} < 1 μM in in vitro enzyme assays [35,36], and we recently reported a series of peptide-based inhibitors bearing a C-terminal leucine vinyl ester (Leu-VE) as pharmacophore able to function as substrate of the N-terminal catalytic threonine [37]. The most promising derivative, Hmb-Val-Ser-Leu-VE (Fig. 1), displayed potent and selective inhibition for trypsin-like activity and good pharmacokinetic properties Fig. 2.

On the basis of these features, we describe here the synthesis and biological activities of a new series of vinyl ester pseu-

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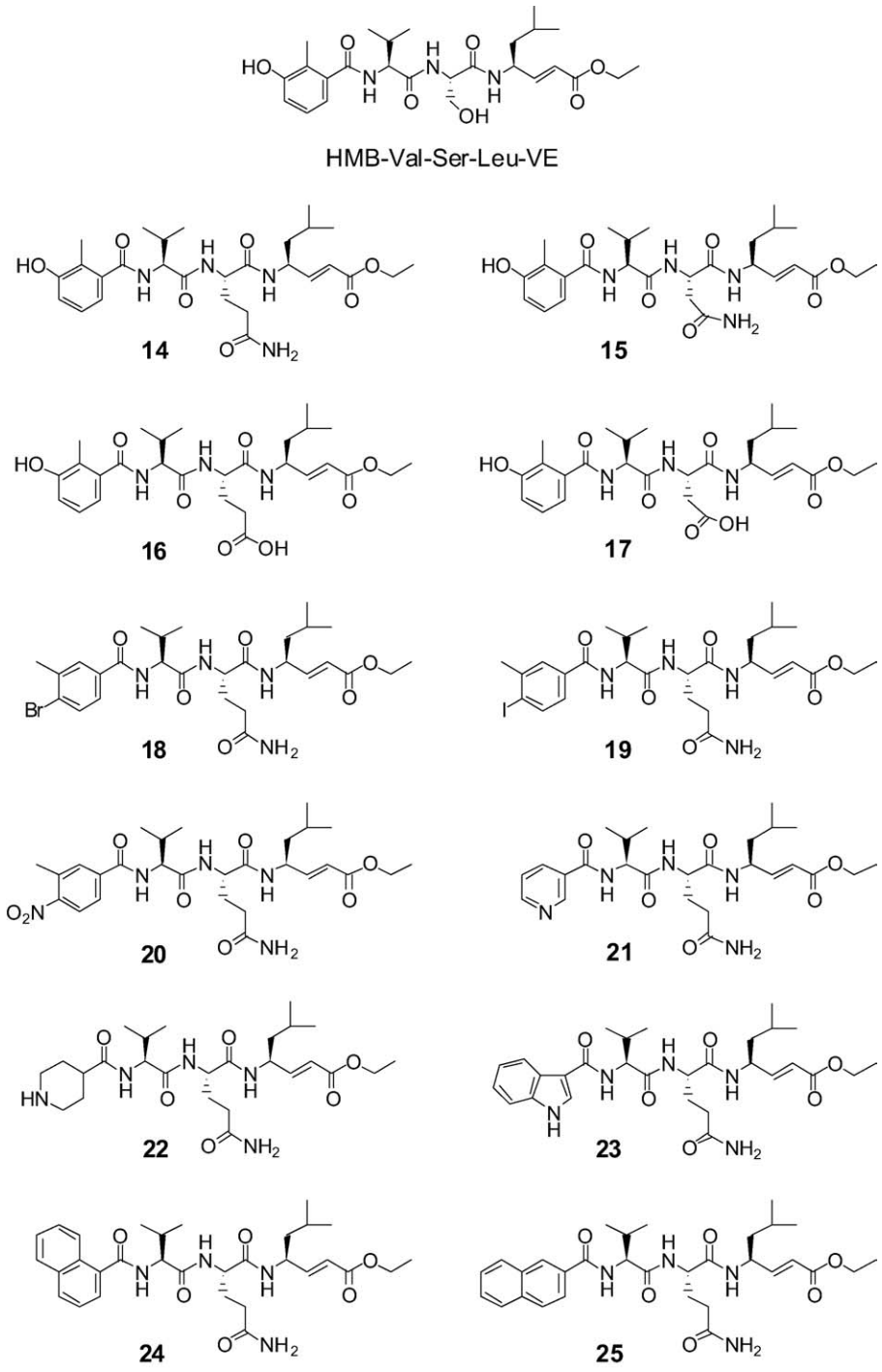
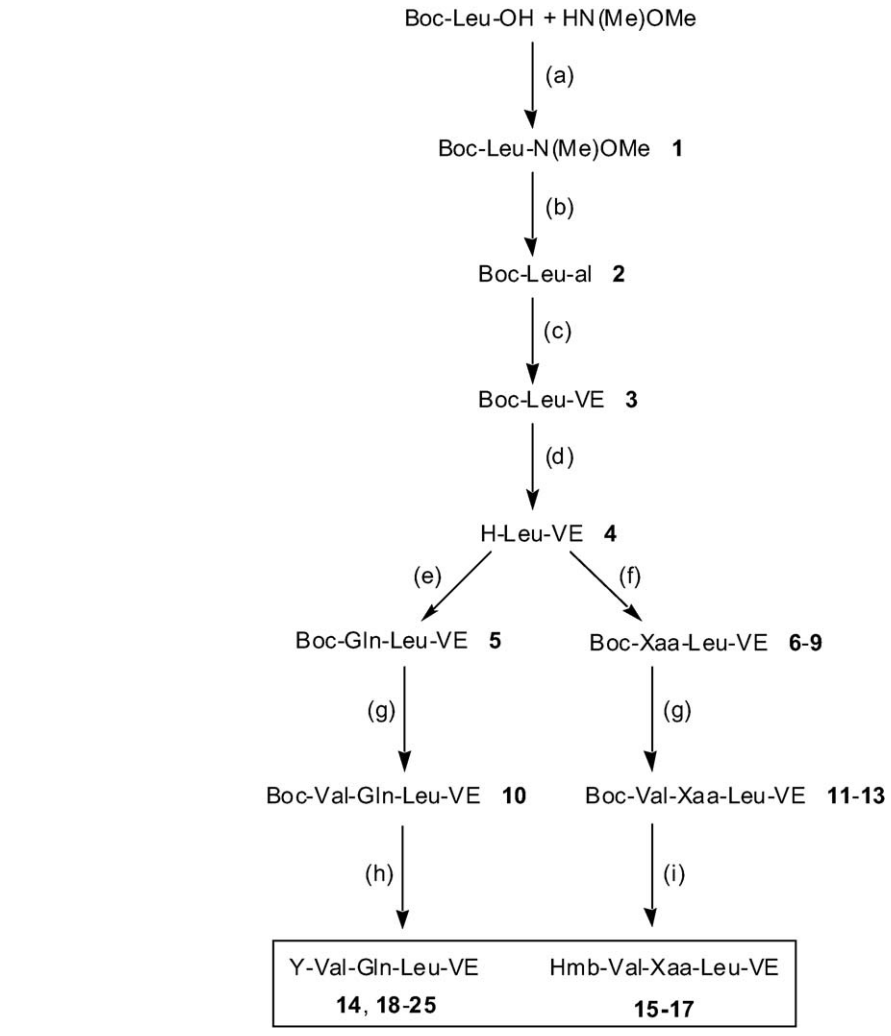


Fig. 1. Structure of the prototype and new vinyl ester pseudotripeptides inhibitors **14**–**25**.

dotripeptides (Fig. 1) possessing an ethyl acrylate group which can function as a substrate of the γ-hydroxy threonine side chain in Michael addition in a similar way to that suggested for the well-know peptide vinyl sulfone inhibitors [38]. Compared to the prototype, compound **14**–**17** bear an acidic or amidic residue in place of serine in P2 position with the aim of increasing the selectivity for the β2 subsite, analogous to previous studies on vinyl sulfone inhibitors [39]. Cyclic N-terminal substituents with different physicochemical properties replaced the 3-hydroxy-2-methylbenzoyl (Hmb) moiety at the P4 position in analogues **18**–**25**.

2. Chemistry

Vinyl ester pseudotripeptides were synthesized by the classical solution method using C-terminal stepwise elongation, as reported in Scheme 1 and the N_{α}–Boc-protected leucine vinyl ester was prepared from the corresponding aldheyde [40] by



Scheme 1. Synthesis of the vinyl ester derivatives **14**–**25**. Reagents: (a) WSC, HOBt, NMM, DMF; (b) LiAlH4, THF; (c) EtO-CO-CH=PPh3, Toluene; (d) TFA; (e) Boc-Gln-OH, HATU, NMM, DMF; (f) Boc-Xaa-OH, HATU, NMM, DMF; (g) 1. TFA; 2. Boc-Val-OH, HATU, NMM, DMF; (h) 1. TFA; 2. Y-OH, HATU, NMM, DMF; (i) 1. TFA, 2. Hmb-OH, HATU, NMM, DMF.

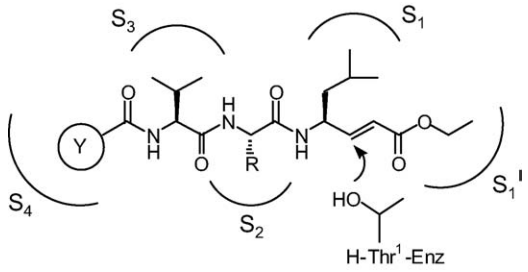


Fig. 2. Vinyl ester inhibitors: potential interactions with proteasome 20S catalytic subsites.

reaction with [(ethoxycarbonyl)methylidene]triphenylphosphorane without racemization [41]. WSC/HOBt or HATU were employed for the coupling steps, and Boc was 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^I) and other physicochemical properties of compounds **14**–**25** are summarized in Table 1.

3. Biological activity

The inhibitory capacity of the new vinyl ester derivatives was tested on purified 20S proteasome using fluorogenic substrates specific for the three main proteolytic activities of the enzymatic complex [42]. The assays for a whole series of analogues was carried out at different concentrations (from 0.001 to 10 μM) to determine their capacity to inhibit the in vitro tryptic-like (T-L), chymotryptic-like (ChT-L) and post-acidic (PGPH) activities of the proteasomes purified from lymphoblastoid cell lines. IC_{50} values against proteasome subsite activities obtained after 30 min of incubation are reported in Table 2. Similar IC_{50} were obtained after 3 hours incubation.

Table 1
Analytical data, physicochemical properties and metabolic stability of vinyl ester tripeptides **14**–**25**

N°	HPLC		m.p. (°C)	$[\alpha]_D^{20}$ (c = 1, MeOH)	MS ($M + H^+$)	Half-life (min)	
	K^I (a)	K^I (b)				Medium	Plasma
14	7.42	5.97	215–220	–30.4	547.4	> 360	268
15	7.13	5.65	213–217	–25.6	533.4	> 360	294
16	7.04	5.43	135–139	–37.4	548.5	> 360	189
17	6.89	5.28	128–133	–26.3	534.6	> 360	176
18	8.22	6.41	220–224	–40.8	609.3	> 360	342
19	7.17	5.57	208–213	–43.2	657.3	> 360	287
20	7.23	5.69	222–227	–42.9	576.7	> 360	321
21	6.11	4.99	203–207	–23.5	518.7	> 360	219
22	5.78	4.78	185–188	–23.3	524.7	> 360	197
23	7.28	5.81	205–209	–35.2	555.6	> 360	> 360
24	8.35	6.62	231–234	–40.9	567.6	> 360	> 360
25	8.51	6.75	217–220	–41.3	567.6	> 360	> 360

Table 2
Subsites proteasome inhibition and other protease inhibition of vinyl ester pseudotripeptides **14**–**25**

N°	Compound	IC_{50} (μM) [a]			IC_{50} (μM) other protease [a]			
		T-L	ChT-L	PGHP	Tryp	Chym	HIV-PR	Cysteine-PR
	Hmb-Val-Ser-Leu-VE	0.033	> 10	> 10	–	–	–	–
14	Hmb-Val-Gln-Leu-VE	0.018	8.95	> 10	> 10	> 10	9.35	> 10
15	Hmb-Val-Asn-Leu-VE	0.041	> 10	> 10	> 10	8.54	6.10	> 10
16	Hmb-Val-Glu-Leu-VE	0.108	6.42	> 10	–	–	–	–
17	Hmb-Val-Asp-Leu-VE	0.121	7.79	> 10	–	–	–	–
18	Bmb-Val-Gln-Leu-VE	0.095	1.83	> 10	–	–	–	–
19	Imb-Val-Gln-Leu-VE	0.113	1.85	> 10	–	–	–	–
20	Nmb-Val-Gln-Leu-VE	0.203	1.94	> 10	–	–	–	–
21	Nic-Val-Gln-Leu-VE	0.035	0.87	> 10	> 10	> 10	> 10	> 10
22	INip-Val-Gln-Leu-VE	0.072	0.98	> 10	> 10	> 10	> 10	> 10
23	Ind-Val-Gln-Leu-VE	0.155	2.09	> 10	–	–	–	–
24	1-Naf-Val-Gln-Leu-VE	0.195	6.84	> 10	–	–	–	–
25	2-Naf-Val-Gln-Leu-VE	0.307	1.27	> 10	–	–	–	–

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

With the aim of verifying specific inhibition for the proteasome, selected compounds **14**, **15**, **21** and **22** were tested for their inhibitory capacity towards other proteases such as trypsin, chymotrypsin, HIV-PR and cysteine proteases. Inhibition of trypsin, chymotrypsin and cysteine protease activities was followed by spectrophotometric assays, the HIV-protease inhibitory capacity was analyzed in RP-HPLC. IC_{50} (Table 2) were calculated as described in experimental protocols.

The enzymatic stability of inhibitors **14**–**25** was determined by incubation at 37 °C in culture medium (RPMI) in the presence of 10% fetal calf serum or in human plasma [43]. The degradation half-lives of the derivatives reported in Table 1 were determined as described above.

4. Results and discussion

The vinyl ester pseudotripeptides were investigated for enzyme inhibition, and the data obtained (Table 2) confirm that the C-terminal ethyl acrylate pharmacophore represents a functional group which could be used for the development of new selective proteasome inhibitors. In general, all compounds were inactive against the post-acidic activity of the proteasome and showed modest inhibition of chymotryptic-like activity while efficiently inhibiting the β2 subsite. In comparison to reference compound Hmb-Val-Ser-Leu-VE, substitution of the serine at P2 position with amidic residues (**14**, **15**) had little effect on potency and selectivity for trypsin-like activity. The tripeptidic sequence of the most potent analogue, **14** (IC_{50} = 18 nM), was chosen for the construction of derivatives **18**–**25** with different cyclic moieties at P4. Pseudotripeptides with N-terminal nicotinic and isonipecotic groups (**21**, **22**) have a potency comparable to that of the prototype; bulkier cyclic substituents are less tolerated in the P4 position.

Specific proteasome inhibition was investigated for the selected compounds **14**, **15**, **21**, which were assayed in vitro against isolated trypsin, chymotrypsin and HIV protease (Table 2). Only analogue **15** with asparagines at the P2 position showed a slight inhibition for chymotrypsin and HIV-PR, thereby confirming the specificity of the vinyl ester derivatives for the multicatalytic complex.

An important pharmacokinetic parameter for the development of bioactive peptidic molecules is the stability versus degree of enzymatic hydrolysis. Susceptibility to degradation of the new inhibitors **18**–**25** was measured in in vitro experiments. The half-lives of the derivatives reported in Table 1 show great stability in cell culture medium, and good enzymatic resistance to human plasma proteases.

5. Conclusion

We designed, synthesized and tested a new series of vinyl ester pseudotripeptides able to inhibit the multicatalytic protease complex. We investigated the P2 and P4 prototype position, and our results indicated that serine is replaced by an amidic residue and that N-terminal nitrogen monocyclic moieties are well tolerated. Some derivatives display potent and selective inhibition against trypsin-like activity located in the β2 subsite of the enzyme. Specific proteasome inhibition and enzymatic stability, make these peptide-based molecules a useful tool for establishing the role of each enzymatic subunit in substrate processing, and may have potential applications as a therapeutic agents.

6. Experimental protocols

6.1. General

Amino acids, amino acid derivatives and chemicals were purchased from Bachem, Novabiochem and Fluka (Switzerland).

Crude vinyl ester tripeptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (3 0 × 4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 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 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 100% B for 25 min and b) from 30% to 90% 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 ionization (ESI) (MICROMASS ZMD 2000) and the values are expressed as MH^+. TLC was performed in precoated plates of silica gel F254 (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-Leu-VE (4)

The protected leucine vinyl ester was prepared as described in reference [34].

6.2.2. General synthetic procedures

6.2.2.1. TFA deprotection. Boc was removed by treating vinyl ester intermediates with aqueous 90% TFA (1:10, w/v) for 30–40 min. After evaporation, the residue was worked up as described triturated with Et_2O, centrifuged and the resulting solid was collected and dried.

6.2.1.2. Coupling with HATU. The deprotected α-amine intermediate (1 mmol), NMM (1 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, brine, $NaHCO_3$ 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.

6.2.3. ^{1}H NMR data of compounds *14–25*

Hmb-Val-Gln-Leu-VE (**14**). ^{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-Val-Asn-Leu-VE (**15**). ^{1}H NMR ($CDCl_3$): δ 0.92–1.19 (m, 12H); 1.30 (t, 3H); 1.49–1.57 (m, 3H); 2.00–2.15 (m, 2H); 2.42 (s, 3H); 2.81 (m, 1H); 3.99 (q, 2H); 4.24–4.39 (m, 3H); 5.11 (s, 1H); 5.63 (d, J = 16.2, 1H); 6.81 (dd, J = 16.1, 1H); 7.11–7.43 (m, 8H).

Hmb-Val-Glu-Leu-VE (**16**). ^{1}H NMR ($CDCl_3$): δ 1.02–1.18 (m, 12H); 1.40 (t, 3H); 1.52–1.60 (m, 3H); 2.02–2.22 (m, 4H); 2.45 (s, 3H); 2.80 (m, 1H); 3.97 (q, 2H); 4.25–4.44 (m, 3H); 5.13 (s, 1H); 5.67 (d, J = 16.0, 1H); 6.59 (dd, J = 16.2, 1H); 6.99–7.40 (m, 6H); 9.56 (sb, 1H).

Hmb-Val-Asp-Leu-VE (**17**). ^{1}H NMR ($CDCl_3$): δ 0.95–1.24 (m, 12H); 1.45 (t, 3H); 1.63–1.77 (m, 3H); 1.99–2.11 (m, 2H); 2.36 (s, 3H); 2.88 (m, 1H); 3.96 (q, 2H); 4.17–4.41 (m, 3H); 5.34 (s, 1H); 5.79 (d, J = 16.2, 1H); 6.85 (dd, J = 16.1, 1H); 7.03–7.50 (m, 6H); 10.23 (sb, 1H).

Bmb-Val-Gln-Leu-VE (**18**). ^{1}H NMR ($CDCl_3$): δ 1.12–1.28 (m, 12H); 1.43 (t, 3H); 1.55–1.67 (m, 3H); 1.96–2.13 (m, 4H); 2.24 (s, 3H); 2.89 (m, 1H); 4.13 (q, 2H); 4.25–4.49 (m, 3H); 5.78 (d, J = 16.3, 1H); 6.59 (dd, J = 16.2, 1H); 7.03–7.38 (m, 8H).

Imb-Val-Gln-Leu-VE (**19**). ^{1}H NMR ($CDCl_3$): δ 1.08–1.19 (m, 12H); 1.35 (t, 3H); 1.44–1.57 (m, 3H); 1.99–2.16 (m, 4H); 2.37 (s, 3H); 2.71 (m, 1H); 3.98 (q, 2H); 4.09–4.30 (m, 3H); 5.80 (d, J = 16.4, 1H); 6.63 (dd, J = 16.2, 1H); 7.08–7.41 (m, 8H).

Nmb-Val-Gln-Leu-VE (**20**). ^{1}H NMR ($CDCl_3$): δ 0.98–1.15 (m, 12H); 1.47 (t, 3H); 1.60–1.69 (m, 3H); 2.14–2.30 (m, 4H); 2.47 (s, 3H); 2.91 (m, 1H); 4.13 (q, 2H); 4.25–4.43 (m, 3H); 5.99 (d, J = 16.2, 1H); 6.85 (dd, J = 16.2, 1H); 7.02–7.38 (m, 8H).

Nic-Val-Gln-Leu-VE (**21**). ^{1}H NMR ($CDCl_3$): δ 1.02–1.14 (m, 12H); 1.52 (t, 3H); 1.70–1.77 (m, 3H); 2.21–2.38 (m, 4H); 2.95 (m, 1H); 4.19 (q, 2H); 4.35–4.51 (m, 3H); 5.92 (d, J = 16.2, 1H); 6.75 (dd, J = 16.2, 1H); 7.07–7.40 (m, 9H).

iNip-Val-Gln-Leu-VE (**22**). ^{1}H NMR ($CDCl_3$): δ 0.98–1.33 (m, 12H); 1.53–1.81 (t, 11H); 2.27–2.40 (m, 9H); 2.89 (m, 1H); 4.19 (q, 2H); 4.38–4.57 (m, 3H); 5.95 (d, J = 16.1, 1H); 6.96 (dd, J = 16.4, 1H); 7.11–7.37 (m, 5H).

Ind-Val-Gln-Leu-VE (**23**). ^{1}H NMR ($CDCl_3$): δ 1.05–1.24 (m, 12H); 1.62 (t, 3H); 1.68–1.74 (m, 3H); 2.15–2.34 (m, 4H); 3.03 (m, 1H); 4.14 (q, 2H); 4.31–4.47 (m, 3H); 5.95 (d, J = 16.2, 1H); 6.81 (dd, J = 16.2, 1H); 7.02–7.38 (m, 11H).

1-Naf-Val-Gln-Leu-VE (**24**). ^{1}H NMR ($CDCl_3$): δ 0.97–1.19 (m, 12H); 1.58 (t, 3H); 1.65–1.78 (m, 3H); 2.11–2.27 (m, 4H); 2.99 (m, 1H); 4.09 (q, 2H); 4.28–4.39 (m, 3H); 6.03 (d, J = 16.2, 1H); 6.88 (dd, J = 16.2, 1H); 7.11–7.50 (m, 12H).

2-Naf-Val-Gln-Leu-VE (**25**). ^{1}H NMR ($CDCl_3$): δ 1.00–1.20 (m, 12H); 1.55 (t, 3H); 1.66–1.81 (m, 3H); 2.07–2.29 (m, 4H); 3.11 (m, 1H); 4.09 (q, 2H); 4.35–4.50 (m, 3H); 5.84 (d, J = 16.2, 1H); 6.70 (dd, J = 16.2, 1H); 7.01–7.40 (m, 12H).

6.3. Purification of proteasomes

Partially purified proteasomes were obtained from lymphoblastoid cell lines, untreated or treated for 12 h at 37 °C with the inhibitors, as previously described [44]. 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, USA).

6.4. Proteasome 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, 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) 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. Trypsin and chymotrypsin inhibition

Selected compounds **14**, **15**, **21** and **22** were tested for their ability to inhibit trypsin and chymotrypsin in the concentration range 0.1–100 μM. BAPNA and α-ZTNPE substrates were used to measure trypsin and chymotrypsin enzyme activity respectively. The kinetics of degradation was followed by spectrophotometry at 405 nm for BAPNA and 360 nm for the α-ZTNPE substrates. The data were expressed as IC_{50} calculated as described above.

6.6. Test for the inhibition of cysteine proteases

Selected compounds **14**, **15**, **21** and **22** were tested for their ability to inhibit cysteine proteases from total cell lysate in the concentration range 0.1–100 μM using the specific Suc-LY-AMC substrate (Bachem). The substrate was incubated at 37 °C with 50 μg of cell lysate, untreated or pretreated with the indicated compounds, in activity buffer. Fluorescence was determined by a fluorimeter using an excitation of 360 nm and emission of 465 nm. The data were expressed as IC_{50} calculated as described above.

6.7. Test for the inhibition of HIV-1 protease

To determine IC_{50} values, affinity-purified HIV-1 protease (Bachem Bioscience) at a final concentration of 1.1 nM was added to a solution (100 μl final volume) containing inhibitor **14**, **15**, **21** or **22**, 4 mM peptide substrate (His-Lys-Ala-Arg-Val-Leu-*p*-nitro-Phe-Glu-Ala-Nle-Ser, Bachem Bioscience), and 1.0% dimethyl sulfoxide in assay buffer: 1.0 mM dithiothreitol, 0.1% glycerol, 80 mM sodium acetate, 160 mM sodium chloride 1.0 mM EDTA, all at pH 4.7. The solution was mixed and incubated for 25 min at 37 °C and the reaction quenched by addiction of trifluoroacetic acid, 2% final concentration. The Leu-Phe(*p*-NO_2) bond of the substrate was cleaved by the enzyme and the cleavage products and substrate were separated by RP-HPLC. Absorbance was measured at 220 nm, peak areas were determined, and percentage conversion to product was calculated using relative peak areas. The data were expressed as IC_{50} calculated as described above.

6.8. Enzymatic stability assays

The degradation kinetics of new vinyl ester tripeptides were studied in culture medium (RPMI) and human plasma. 0.1 ml of a solution of each compound (10 mg/ml in acetonitrile/H_2O 1:1) was added to 1 ml of RPMI containing 20% FCS. Alternatively, test compounds were incubated with plasma (0.6 ml) in a total volume of 1.5 ml of 10 mM Tris–HCl buffer, pH 7.5. Incubation was performed at 37 °C for different durations: up to 360 min in the case of human plasma, and up to 2 days in the case of RPMI containing 20% FCS. The incubation was terminated by addition of ethanol (0.2 ml), the mixture poured at 21 °C, and, after centrifugation (5000 rpm for 10 min) aliquots (20 μl) of the clear supernatant were injected into RP-HPLC column. HPLC was performed as described in analytical determinations. The degradation half-life ($T_{1/2}$) was obtained by a least-squares linear regression analysis of a plot of the logarithmic inhibitor concentration versus time, using a minimum of five points.

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