Brief Articles

Arecoline Tripeptide Inhibitors of Proteasome

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The 26S proteasome is a multicatalytic protease complex that plays an essential role in intracellular protein degradation. We have synthesized and tested a series of arecoline peptide derivatives where the peptide portion derives from a screening of tripeptide sequences, and the arecoline moiety has been considered as a potential substrate for catalytic threonine. Derivatives **17**−**19** are the best compounds of the series, showing chymotryptic-like (β5) inhibition ($IC_{50} \cong 1\ \mu M$) and favorable pharmacokinetic properties.

Introduction

The ubiquitin−proteasome pathways plays a key role in the degradation of cellular proteins.[1,2] It is involved in many biological processes, including the removal of abnormal proteins, stress response, cell cycle control and differentiation, metabolic adaptation, and generation of peptide antigens presented by major histocompatibility complex (MHC) class I molecules to cytotoxic T cells.[3−6]

The proteasome is a multicatalytic protease that possess a 20S core constituted by four stacked rings, where each of the two inner rings is composed of seven different β subunits. Each β-ring contains three different proteolytically active sites: the β1 subunit contains a postacidic (PGPH) active site, the β2 subunit is associated with a trypsin-like (T−L) activity, and the β5 subunit exerts a chymotrypsin-like (ChT-L) proteolytic function. All proteolytic sites utilize an N-terminal threonine residue as nucleophile, with a catalytic mechanism similar to those of serine proteases.

The development of proteasome inhibitors into novel therapeutic agents represents a stimulating approach in the treatment of many diseases, including inflammation and cancer, and for the modulation of immune responses. Usually, inhibitors of proteasome are short peptides linked to a C-terminal pharmacophore responsible for the interaction with catalytic threonine.

Studies in this area have produced different classes of useful compounds for studying the role of the ubiquitin−proteasome pathway in various cellular processes, and some inhibitors are currently in clinical trials. C-Terminal small derivatives such as aldehyde, boronate, vinyl sulfone, or epoxyketone peptides are the most potent inhibitors with some degree of specificity.[7−14]

We report here the synthesis, enzymatic inhibition, cell membrane penetration capacity, and metabolic stability of a series of tripeptides linked to arecoline derivatives as potential pharmacophores (Table 1). We prepared and tested N-protected tripeptide benzylamides **3**−**16** which carry serine in their core structure and leucine at C-terminal position, while their N-terminal residue is a variably bulky, hydrophobic amino acid. In particular, serine was inserted to improve pharmacokinetic properties and for its potential to favor interactions at catalytic sites. We also synthesized Fmoc- and Z-protected trileucyl benzylamide peptides **1**, **2** as reference sequences. The best compounds were selected and functionalized at the C- or N-terminal with arecoline derivatives. Arecoline is the major alkaloid of the betel nut (the seed of *Areca catechu*); it is a mild central nervous system stimulant with a muscarinic agonist activity. We selected this natural molecule as a potential Michael addition substrate for catalytic threonine and for its functional groups that can be elaborated to be easily inserted in peptide sequences.

Compounds **17**−**25** were tested for their ability to inhibit all three major activities of the proteasome. We also determined the metabolic stability of all compounds in cell culture medium and in human plasma.

Results and Discussion

Synthesis. The benzylamide Z- or Fmoc-protected tripeptides **3**−**16** (Scheme 1) and reference sequences **1**, **2** were synthesized by the classical solution method using C-terminal stepwise elongation. WSC/HOBt were employed for the coupling steps, the Z-protecting group was removed by catalytic hydrogenation, and Fmoc was introduced as the succinimidyl ester.

Arecoline tripeptide derivatives were prepared following the strategies reported in Schemes 2 and 3. Compounds **20**−**25** were synthesized by initial condensation of Boc-Leu-OH and norarecoline; final coupling of the C-terminal residue with H-Xaa-Leu-norarecoline intermediate gave the required products (Scheme 2). N-Terminal 1,2,5,6-tetrahydropyridine-3-carbonyl-derivatized tripeptides **17**−**19** were obtained by the cor-

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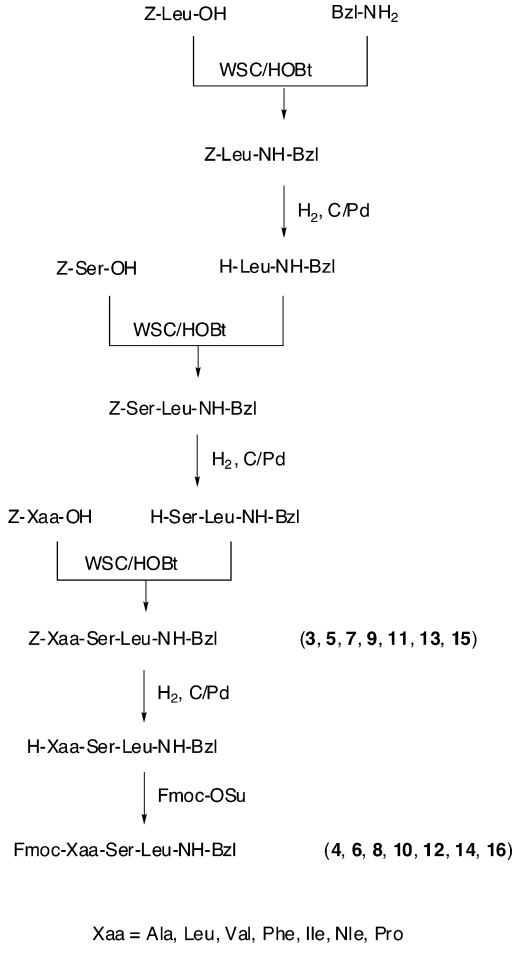
Table 1. Metabolic Stability and Inhibitory Activities of Compounds **1**−**25**

	compound	half-life (min) culture medium	half-life (min) human plasma	IC_{50} (μM) chymotrypsin	IC_{50} (μM) tryptic	IC_{50} (μM) postacid
1	Z-Leu-Leu-Leu-NH-Bzl	−	−	9.51	8.93	>20
2	Fmoc-Leu-Leu-Leu-NH-Bzl	−	−	12.24	10.72	>20
3	Z-Leu-Ser-Leu-NH-Bzl	−	−	>20	15.75	>20
4	Fmoc-Leu-Ser-Leu-NH-Bzl	−	−	>20	>20	>20
5	Z-Val-Ser-Leu-NH-Bzl	−	−	3.73	9.46	>20
6	Fmoc-Val-Ser-Leu-NH-Bzl	−	−	5.91	13.59	>20
7	Z-Phe-Ser-Leu-NH-Bzl	−	−	2.18	4.82	17.55
8	Fmoc-Phe-Ser-Leu-NH-Bzl	−	−	11.04	12.91	19.14
9	Z-Ala-Ser-Leu-NH-Bzl	−	−	>20	>20	>20
10	Fmoc-Ala-Ser-Leu-NH-Bzl	−	−	>20	>20	>20
11	Z-Pro-Ser-Leu-NH-Bzl	−	−	>20	>20	>20
12	Fmoc-Pro-Ser-Leu-NH-Bzl	−	−	>20	>20	>20
13	Z-Ile-Ser-Leu-NH-Bzl	−	−	>20	>20	>20
14	Fmoc-Ile-Ser-Leu-NH-Bzl	−	−	>20	>20	>20
15	Z-Nle-Ser-Leu-NH-Bzl	−	−	>20	18.23	>20
16	Fmoc-Nle-Ser-Leu-NH-Bzl	−	−	>20	>20	>20
17	[a]X-Leu-Leu-Leu-NH-Bzl	>360	295	1.41	2.57	>20
18	[a]X-Val-Ser-Leu-NH-Bzl	>360	>360	0.52	2.16	>20
19	[a]X-Phe-Ser-Leu-NH-Bzl	>360	>360	1.23	2.93	>20
20	Z-Leu-Leu-Leu-Y[b]	>360	164	2.33	>20	>20
21	Fmoc-Leu-Leu-Leu-Y[b]	>360	188	9.14	>20	>20
22	Z-Val-Ser-Leu-Y[b]	>360	209	13.22	>20	>20
23	Fmoc-Val-Ser-Leu-Y[b]	>360	255	>20	>20	>20
24	Z-Phe-Ser-Leu-Y[b]	>360	194	>20	>20	>20
25	Fmoc-Phe-Ser-Leu-Y[b]	>360	254	>20	>20	>20

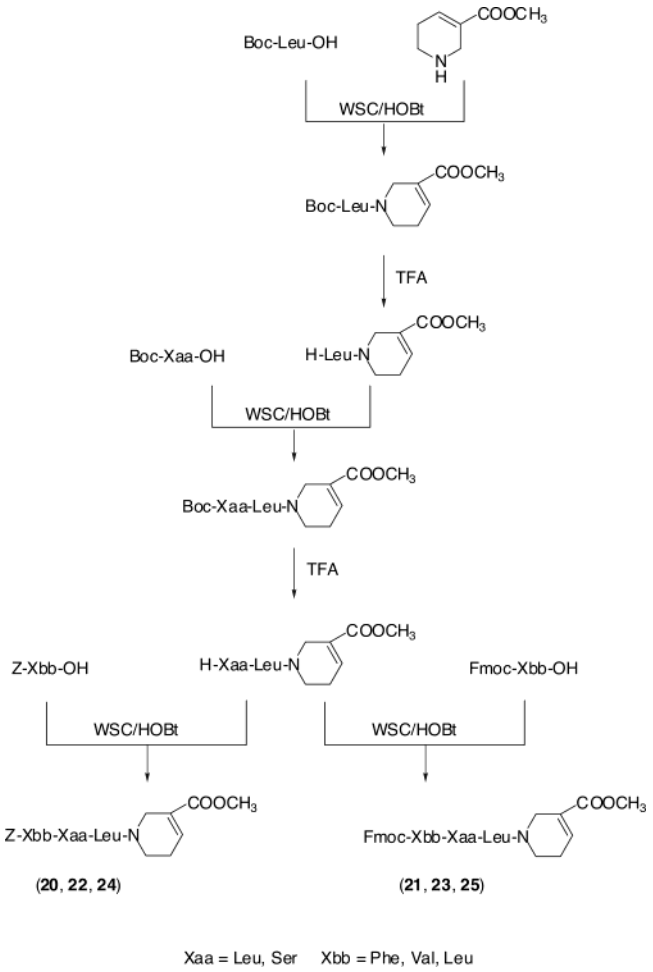
[a] X = 1,2,5,6-tetrahydropyridine-3-carbonyl. [b] Y = methyl-1,2,5,6-tetrahydropyridine-3-carboxylate.

Scheme 1. Preparation of the Tripeptides **3**−**16**



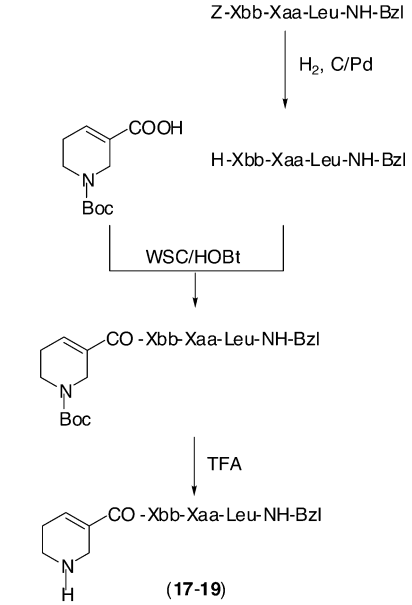
responding Z-protected benzylamide tripeptides **1**, **5**, and **7** after catalytic hydrogenation, condensation of Boc-protected norarecoline, and final displacement of Boc by TFA (Scheme 3).

Scheme 2. Synthesis of the C-Terminal Norarecoline Tripeptides **20**−**25**



Arecoline derivatives such as Boc-guvacine or norarecoline, employed in the inhibitor syntheses, were

Scheme 3. Synthesis of N-Terminal 1,2,5,6-Tetrahydropyridine-3-carbonyl Tripeptides **17**−**19**



Xaa = Ser, Leu Xbb = Phe, Val, Leu

prepared according to the procedures reported in the literature.[15,16]

Biological Results. We tested the inhibitory capacity of compounds **1**−**25** on purified 20S proteasome using specific fluorogenic substrates.[17] We first evaluated the inhibition activity of the Fmoc- or Z-tripeptide benzylamide sequences **1**−**16** (Table 1). The compounds with the best activity were the reference trileucyl (**1**, **2**), as well as tripeptides **5**, **6** and **7**, **8** which have valine and phenylalanine, respectively, at their N-terminal position. Arecoline derivatives in C- or N-terminal position were then added to these sequences.

All arecoline derivatives were tested at different concentrations (from 0.01 to 20 μM) for their capacity to inhibit the three major proteolytic activities of the proteasome. Biological data show that the insertion of potential pharmacophore at the C-terminal (**20**−**25**) does not increase the inhibitory capacity as compared to the reference sequences (Table 1). This suggests that C-terminal arecoline does not function as substrate for the catalytic threonine and, in particular, that the conjugate double bond of the tetrahydropyridine moiety is not susceptible to Michael addition of the threonine hydroxyl group. N-terminal conjugate derivatives **17**−**19** show good inhibition of the proteasome, in particular for the trypsin- and chymotrypsin-like activities (Table 1). 1,2,5,6-Tetrahydropyridine-3-carbonyl-valyl-seryl-leucyl-benzylamide (**18**) displays an interesting potency against tryptic and chymotryptic active sites, with an $IC_{50} < 1$ μM in in vitro enzyme assay. The arecoline derivative in this position represents a good moiety for binding with the catalytic sites of the 20S proteasome β subunits. In particular, we suppose that the tetrahydropyridyl ring is oriented so that it may favor interactions with catalytic S4 pockets and amino group is able to make H bond.[18,19] However, crystallographic experiments are required to exactly define the interactions between these compounds and the enzymatic subsites. The cell membrane permeation and proteasome inhibitory capacities of compounds **17**−**19** were tested in live cells. After cell treatment, proteasomes were purified and inhibition was evaluated using specific substrates. All compounds showed inhibition of chymotrypsin-like and trypsin-like activities similar to that observed in the in vitro assays (not shown). Other experiments are in progress to evaluate the antiproliferative and pro-apoptotic activities of these compounds on normal and tumor cell lines.

To determine their susceptibility to enzymatic hydrolysis, compounds **17**−**25** were incubated at 37 °C in culture medium (RPMI) in the presence of 10% fetal calf serum or in human plasma. The half-lives of arecoline derivatives reported in Table 1 show great stability in cell culture medium and good enzymatic resistance against human plasma proteases; in particular, N-terminal functionalized inhibitors **18** and **19** display half-lives exceeding 6 h.

Conclusion

In this work, we first identified some tripeptidic sequences able to efficiently interact with the catalytic subsites of proteasome 20S, then we derivatized these molecules at N- and C-terminal with arecoline derivatives. The N-terminal conjugates displayed significant inhibitory activity, remarkable metabolic resistance, and good capacity to permeate cell membrane. This new class of small peptide derivatives open a new way for further studies on the biological role of the ubiquitin−proteasome pathway.

Experimental Section

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.[20] A subsequent purification was done by affinity chromatography (mAb α-subunit, Affinity). Fractions containing proteasomes were combined and dialized against 25 mM Tris-HCl pH 7.5. Protein concentration was determined using BCA protocol (Pierce, Rockford, IL).

Enzyme Assays. Suc-LLVY-AMC, Boc-LRR-AMC and Ac−YVAD-AMC (Sigma) were used to measure chymotrypsin-like, trypsin-like, and postacidic proteasome activities, respectively. Substrates were incubated at 37 °C for 30 min with proteasomes, untreated or pretreated with 0.01−20 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}.

Metabolic Stability Assays. The degradation kinetics of new inhibitors 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 time: 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 was 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.

Inhibitors Purification and Analytical Determinations. Crude compounds were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 × 4 cm, 300 A, 15 μm spherical particle size column). HPLC analysis was performed by a Beckman System Gold with a Beckman ultrasphere ODS column (5 μm; 4.6 × 250 mm). Molecular weight of compounds was determined by a MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight) analysis using a Hewlett-Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as a matrix. The values are expressed as MH^+. Elemental analyses were carried out on a TermoQuest CE instruments EA 1110-CHNS−O elemental analyzer. Optical rotations were determined by a Perkin-Elmer 241 polarimeter with a 10 cm cell using methanol as solvent and at an analyte concentration of 1%. ^{1}H NMR spectroscopy was obtained on a Bruker spectrometer (Bruker WM 500 MHz).

Chemistry. General Procedures. Coupling with WSC/HOBt. To a solution of the carboxy component (1 mmol) in DMF (10 mL) were added the amino component (1 mmol), NMM (*N*-methylmorpholine, 1 mmol), HOBt (1.1 mmol), and WSC (1-ethyl-3-(3^I-dimethylaminopropyl)carbodiimide, 1.1 mmol) in this order 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 EtOAc (100 mL) and washed consecutively with 0.1 N HCl, 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 resulting solid separated by centrifugation.

Hydrogenation Deprotection. The Z (benzyloxycarbonyl) protecting group was removed by treating the protected intermediates in methanol (5 mL) with hydrogen (120 min) in the presence of 10% Pd/C. The reaction mixture was filtered, the solvent was evaporated, the resulting crude product was treated with Et_2O, and resulting solid was separated by centrifugation.

Introduction of Fmoc. To a solution of tripeptide benzylamide (0.5 mmol) in DMF (5 mL) was added Fmoc-OSu (fluorenylmethyloxycarbonyl-*N*-hydroxysuccinimide, 0.5 mmol) at 0 °C. The mixture was stirred for 1 h at 0 °C and 15 h at room temperature and then evaporated. The resulting target compounds were purified by preparative HPLC.

TFA Deprotection. *tert*-Butyloxycarbonyl (Boc) protection was removed from *N*-arecoline tripeptide derivatives by treating intermediates with aqueous 90% TFA (trifluoroacetic acid 1:10, w/v) for 30−40 min. After evaporation, the residue was treated with Et_2O and resulting solid separated by centrifugation.

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Supporting Information Available: Analytical data of arecoline inhibitors are available free of charge via the Internet at http://pubs.acs.org.

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(21) Abbreviations: Fmoc, fluorenylmethoxycarbonyl; Boc, *tert*-butoxycarbonyl; Fmoc-OSu, 9-fluorenylmethoxycarbonyl-*N*-hydroxy succinimide; HOBt, N-hydroxybenzotriazole; Suc, succinyl; TFA, trifluoroacetic acid; WSC (water soluble carbodiimide), 1-ethyl-3-(3^I-dimethylaminopropyl)carbodiimide; Z, benzyloxycarbonyl.

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