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Proteasome inhibitors: synthesis and activity of arecoline oxide tripeptide derivatives

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Abstract—We describe the synthesis and biological activities of a series of methyl 3,4-epoxypiperidine-3-carboxylate tripeptide derivatives that inhibit the chymotryptic and tryptic active sites of the 20*S* proteasome. Of the series, compound **2** which contains 3-hydroxy-2-methylbenzoyl group at its N-terminal position, displayed the greatest inhibitory potency (IC_{50} <1 μM). All derivatives showed favourable pharmacokinetic properties.

The proteasome is a multicatalytic protease complex and are responsible for the majority of nonlysosomal proteolysis in prokaryotes and eukaryotes.[1] The enzyme are formed of multiple subunits, with a modular structure and several activities. The core and proteolytic chamber is the 20*S* proteasome formed by four stacked rings where each of the two inner rings is composed of seven different β subunits.[2] Each β-ring contains three proteolytic active sites, the β1 subunit has a post-acidic-like (PGPH) active site, β2 is associated with a trypsin-like (T-L) activity, while the β5 subunit has a chymotrypsin-like (ChT-L) active site.[3] All the proteolytic sites utilize a N-terminal threonine residue of β subunits as nucleophile with a catalytic mechanism similar to those of serine proteases.[4] The 20*S* core structure with attached two 19*S* regulatory subunits forms 26*S* proteasome (2,4 MDa) able to recognize and to degrade ubiquitinated proteins.

Since the proteasome hydrolyze various cell cycle regulators, transcription factors and antigenic proteins, it is a promising target for the development of drug for the treatment of a range of pathologies such as cancer, inflammation, immune diseases and others.[5] The development of proteasome inhibitors into novel therapeutic agents represents a new approach and now classes of these substances are in clinical trials or used to study the role of the ubiquitin–proteasome pathway in various cellular processes. Typically inhibitors are short peptides linked to a C-terminal pharmacophore which is responsible for the interaction with catalytic threonine of the proteasome.[6]

We recently reported on the identification of a number of tripeptidic sequences derivatized at the N- and C-terminal with arecoline derivatives that were able to efficiently interact with the catalytic subsites of the proteasome 20*S*.[7] Arecoline is the major alkaloid of the betel nut (the seed of *Areca catechu*).[8] We have chosen this natural molecule as potential Michael addition substrate for the N-terminal enzymatic threonine and because it is easily usable in peptide synthesis. Biological data have suggested that the arecoline conjugate double bond in the C-terminal position does not function as substrate for the catalytic threonine, while the N-terminal 1,2,5,6-tetrahydropyridine-3-carbonyl tripeptide derivatives have shown an interesting inhibition against tryptic and chymotryptic active sites with an IC_{50} <1 μM in in vitro enzyme assays.

Here, we describe the synthesis and biological activities of a new series of arecoline tripeptide derivatives with a methyl 3,4-epoxypiperidine-3-carboxylate (guvacine oxide methyl ester) moiety representing the C-terminal

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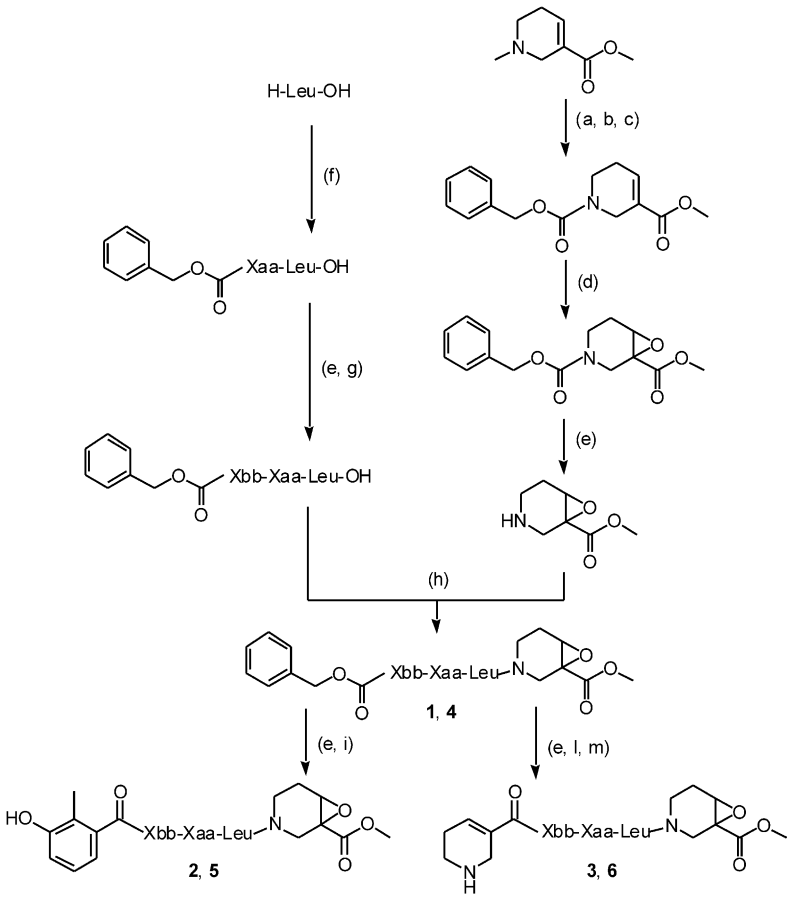

Scheme 1. Synthesis of the arecoline oxide tripeptides **1**, **6**. Reagents and conditions: (a) ClCOOCHClMe, CH_2Cl_2; (b) MeOH, Δ; (c) Z-OSu, DMF; (d) MCPBA, CH_2Cl_2; (e) H_2, C/Pd, MeOH; (f) Z-Xaa-OSu, DMF; (g) Z-Xbb-OSu, DMF; (h) WSC, HOB*t*, DMF; (i) Hmba, WSC, HOB*t*, DMF; (l) Boc-Guv, HATU, DIPEA, DMF; (m) TFA.

pharmacophore (Scheme 1). We have chosen this arecoline oxide derivative as a potential substrate for N-terminal nucleophile threonine of the catalytic β subunits, in analogy with the chemical properties of the natural epoxyketone inhibitors, such as the potent and selective epoxomycin and eponemycin.[9] Tripeptidic sequences Val-Ser-Leu (**1**–**3**) and Leu-Leu-Leu (**4**–**6**) are functionalized at N-terminal position with benzyloxycarbonyl (**1**,**4**), 3-hydroxy-2-methylbenzoyl (**2**,**5**) or 1,2,5,6-tetrahydropyridine-3-carbonyl (**3**,**6**) groups. The potential sites of interaction between the proteasome catalytic subunits and the arecoline oxide tripeptide derivative, compound **2**, are shown in Figure 1. The hydrophobic residues leucine and valine interact with S1 and S3 enzymatic pockets and the N-terminal six membered ring located in P4 position is favourable for interaction with the S4 region. Serine was inserted in the core structure to improve pharmacokinetic properties such as water solubility.

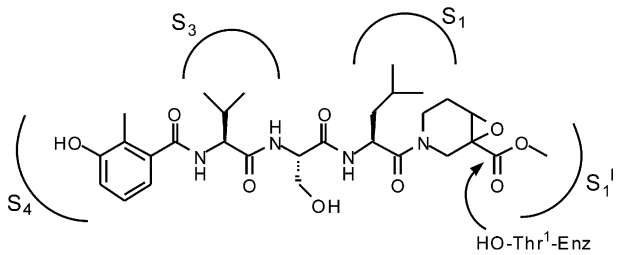

Figure 1. Arecoline oxide tripeptide derivative **2**: potential interactions with proteasome 20*S* catalytic subsites.

Arecoline oxide tripeptide derivatives **1**–**6** were synthesized by the classical solution method by means of C-terminal stepwise elongation (Scheme 1).[10] Arecoline derivatives with the C-terminal pharmacophore methyl (3*RS*,4*RS*)-3,4-epoxypiperidine-3-carboxylate and N-terminal substituent 1,2,5,6-tetrahydropyridine-3-carbonyl (Guv) were prepared according to procedures reported in literature.[11] Benzyloxycarbonyl (*Z*) protecting groups and N-protected amino acids were coupled as succinimidyl esters. WSC/HOBt were employed to introduce N-terminal 3-hydroxy-2-methylbenzoyl (Hmb) and C-terminal methyl 3,4-epoxypiperidine-3-carboxylate (AE) substituents in tripeptidic sequences. The coupling agent HATU was used to condense N-protected guvacine (Boc-Guv) in derivatives **3** and **6**. *Z* Protecting groups were removed by catalytic hydrogenation while Boc was removed by treating with aqueous TFA. After purification by preparative reversed-phase HPLC, purity of all target compounds were determined by

Table 1. Metabolic stability and inhibitory activities of compounds **1–6**

Compd	Half-life (min)		Proteasome inhibition IC_{50} (μM)		
	Culture medium	Human plasma	Chymotr.	Tryptic	Post acid.
1 Z-Val-Ser-Leu-AE	>360	148	2.14	1.13	>20
2 Hmb-Val-Ser-Leu-AE	>360	>360	0.73	0.14	>20
3 Guv-Val-Ser-Leu-AE	>360	295	0.86	0.31	>20
4 Z-Leu-Leu-Leu-AE	>360	193	0.98	13.20	>20
5 Hmb-Leu-Leu-Leu-AE	>360	>360	0.17	3.41	>20
6 Guv-Leu-Leu-Leu-AE	>360	>360	0.77	4.55	>20

HPLC and elemental analysis; structure verification was achieved by mass spectrometry and NMR spectroscopy.[12]

We tested the inhibitory capacity of compounds **1–6** against the isolated 20*S* proteasome using specific fluorogenic substrates. Proteasomes were isolated from lymphoblastoid cell lines and purified on affinity columns containing a derivatized matrix (specific Ab for α subunit of proteasome, Affinity).[13] The fluorogenic substrates Suc-LLVY-AMC, Boc-LRR-AMC and Ac-YVAD-AMC were used to measure chymotripsyn-like, trypsin-like and postacidic-like proteasome activities, respectively. Peptide substrates were incubated at 37 °C for 30 min with purified proteasomes, untreated or pretreated with 0.01–20 μM of test compounds and finally fluorescence was determined using an excitation of 360 nm and emission at 465 nm. Proteasome activity was evaluated in fluorescence units and the inhibitory activity of the compounds are expressed as IC_{50} (Table 1). Biological data show, if compared to natural epoxyketones, that the insertion of a potential pharmacophore at the C-terminal is not beneficial with regard to inhibitory activity. This suggests that the C-terminal arecoline oxide does not function as a substrate for the hydroxyl group in the catalytic threonine side-chain. 3-Hydroxy-2-methyl benzoyl (Hmb) N-terminal conjugates **2** and **5** are the more potent inhibitors of the series, suggesting that the substituted aromatic ring is oriented so that in may favour interactions with catalytic S4 pockets. A trileucyl sequence confers to compounds **4–6** a degree of selectivity for chymotrypsin-like subunit, derivatives **1–3** exhibit a slight preference for tryptic inhibition. Compound **2** shows an $IC_{50} < 1$ μM for both β2 and β5 subunits, while analogue **5** has a good selectivity for the chymotryptic active site. All members of the series were unable to inhibit the post-acidic-like β1 subunit. IC_{50} reported in Table 1 are relative to the effect of the inhibitors on the 1 h degradation of peptide substrates used to evaluate the different activities of the proteasome. However, we observed that the inhibitory activities of the tested compounds decreased after 2 h and was almost undetectable at 8 h, suggesting a reversible inhibition.

The cell membrane permeation and proteasome inhibitory capacities of the compounds **1–6** were tested in live cells. Lymphoblastoid cell lines were treated over 12 h with 0.01–20 μM of test compounds, followed by proteasome isolation using an affinity column and activity measured against specific fluorogenic substrates for chymotripsyn-like, trypsin-like hydrolytic proteasome capacities.[14] All compounds showed proteasome inhibition similar to that observed in the in vitro assays (data not shown). The ability of the tripeptide derivatives to permeate cell membranes is an essential pharmacokinetic property of these molecules regarding their inhibit intracellular proteolytic complexes.

In order to evaluate the susceptibility to enzymatic hydrolysis, compounds **1–6** were incubated at 37 °C in culture medium (RPMI) in the presence of 10% fetal calf serum (RPMI + 10% FCS) or in human plasma for different periods of time and kinetics were studies by HPLC.[15] 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 (Table 1). All arecoline oxide derivatives show a great stability in cell culture medium and have good enzymatic resistance against human plasma proteases; in particular Hmb N-terminal functionalized inhibitors **2** and **5** displayed a half-live of over 6 h.

In this work, we identified some tripeptidic derivatives able to efficiently interact with the catalytic subsites of the proteasome 20*S*. The C-terminal arecoline oxide pharmacophore is not particularly favourable for the primary interaction with catalytic threonine. 3-Hydroxy-2-methyl benzoyl groups situated at the N-terminal position, give remarkable inhibitory capacity against chymotryptic and tryptic activities. In summary we have developed new small peptide derivatives able to produce powerful inhibition of the proteasome having favourable pharmacokinetic properties and will prove useful tools for studying the biological role of the ubiquitin–proteasome pathway.

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12. Analytical data and physicochemical properties of the compound **2**. ^{1}H NMR ($CDCl_3$): δ 0.93–1.07 (m, 12H), 1.62–1.84 (m, 5H), 2.27 (s, 3H), 2.53 (m, 1H), 3.08 (t, 1H), 3.42 (d, 2H), 3.72–3.85 (m, 3H), 4.28–4.63 (m, 6H), 6.86–7.12 (m, 4H), 7.20–7.45 (m, 5H); HPLC: $K^I = 7.31$; MS: $M + H^+ = 591.7$. $[\alpha]_D^{20} = -39.7$ ($c = 1$, MeOH). Anal. calcd for $C_{29}H_{42}N_4O_9$: C, 58.97, H, 7.17, N, 9.49, O, 24.38. Found: C, 58.23, H, 7.04, N, 9.34. Compound **3**. ^{1}H NMR ($CDCl_3$): δ 0.89–1.11 (m, 12H), 1.62–1.84 (m, 5H), 2.07 (t, 2H), 2.53 (m, 1H), 2.84 (t, 3H), 3.08 (t, 1H), 3.29–3.41 (m, 6H), 3.68–3.80 (m, 3H), 4.18–4.42 (m, 6H), 6.71–7.05 (m, 4H); HPLC: $K^I = 8.09$; MS: $M + H^+ = 566.5$. $[\alpha]_D^{20} = -33.4$ ($c = 1$, MeOH). Anal. calcd for $C_{27}H_{43}N_5O_8$: C, 57.33, H, 7.66, N, 12.38, O, 22.63. Found: C, 56.85, H, 7.26, N, 12.03.
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