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INHIBITORS OF THE CHYMOTRYPSIN-LIKE ACTIVITY OF PROTEASOME BASED ON DI- AND TRI-PEPTIDYL α-KETO ALDEHYDES (GLYOXALS)

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Abstract: A series of peptidyl α-keto aldehydes (glyoxals) have been synthesised as putative inhibitors of the chymotryptic-like activity of proteasome. The most potent peptides, Cbz-Leu-Leu-Tyr-COCHO and Bz-Leu-Leu-Leu-COCHO, function as slow-binding reversible inhibitors, exhibiting final K_i values of approximately 3.0 nM. These are among the lowest values so far reported for (tri)peptide-based aldehyde-releated inhibitors.

Introduction: The proteasome (multicatalytic endopeptidase complex, EC 3.4.99.46.) is a high molecular weight (~ 700 kD), multi-subunit, non-lysosomal protease of eukaryotic cells[1]. It is believed to play an important role in the catabolism of proteins within the cellular environment. As well as being perceived to be the key enzyme in the processing of antigens for MHC-class I presentation[2] and normal protein turnover via the ubiquitin-proteasome pathway[3-5].

The X-ray crystal structure of proteasome from *Thermoplasma acidophilum*[6] and a report on its inhibition by lactacystin[7], a *streptomyces* metabolite, led to the conclusion that the enzyme appears to be the first member of an entirely new proteolytic enzyme subclass, the threonine proteases.

The proposed involvement of proteasome in various pathophysiological processes, make it an interesting target for the development of synthetic inhibitors. Such inhibitors might be valuable in the delineation of the role of proteasome in normal and aberrant pathways of protein catabolism in the cell. With this in mind, and as part of our ongoing development of potent inhibitors of hydrolytic enzymes, we have discovered that peptidyl α-keto aldehydes, previously shown to be excellent inhibitors of the serine and cysteine proteases[8,9], are excellent inhibitors of the proteasome.

Chemistry: As peptidyl α-keto aldehydes share an obvious structural similarity to peptide aldehydes, which are known to be excellent inhibitors of proteasome[10,11], and since we have previously shown that the former are some 10-fold more potent than their exactly analogous aldehyde counterparts in inhibiting their serine- and cysteine protease targets[8], we reasoned that they might form attractive lead compounds for the development of potent inhibitors of the proteasome. In order to test this thesis, we have synthesized a series of di- and tri-peptidyl

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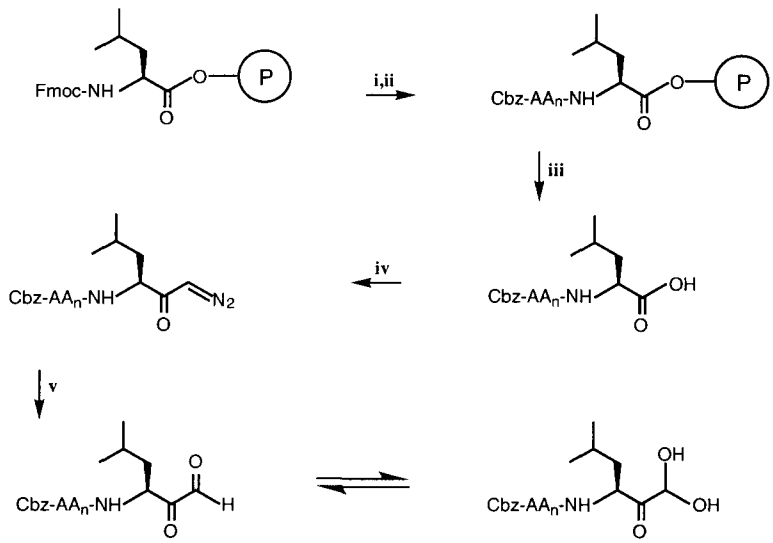
Scheme 1 Synthesis of peptidyl α-keto aldehydes



n = 1,2

Conditions

i, 20% piperidine/DMF, 45 min; **ii**, Fmoc/Cbz-amino acid (1 equiv.), HBTU (1 equiv.), HOBt (1 equiv.), DIPEA (2 equiv.), 2hr; **iii**, If Fmoc-amino acid has been used, repeat steps **i** and **ii**, if Cbz-derivative has been utilized, then treat peptide-resin with TFA/ethanedithiol/anisole (95:2.5:2.5), 1.5hr; **iv**, isobutyl chloroformate (1.1 equiv.), *N*-methylmorpholine (1.1 equiv.), 10 min @ 0°C then add ethereal diazomethane, stir to room temperature overnight; **v**, dimethyldioxirane in 0.5% H_2O/acetone, 1-3 hr at room temperature.

α-keto aldehydes, by a combination of solid-phase and classical solution methodologies, for kinetic testing. A representative synthesis of these analogues is shown in **Scheme 1**. Although the particular illustrated example is for inhibitor sequences containing a *C*-terminal leucine residue, we have found the method to be of general applicability. From this scheme it can be appreciated that the peptidyl diazoketones are the key intermediates in the synthesis of α-keto aldehydes. We have previously demonstrated that the latter can be obtained, in almost quantitative yield, by the oxidative cleavage of the diazo-group of the former, using dimethyldioxirane[12]. The peptidyl diazoketones used in this study were prepared, in turn, from their corresponding *C*-terminal free acids, using a combined solid-solution phase methodology developed in our laboratory[13]. In essence, the desired target peptide sequences were prepared (typically on a 0.25 mmol scale) on a Sasrin polystyrene-based resin containing an acid-sensitive linker (2-methoxy-4-alkoxybenzyl alcohol)[14] employing standard solid phase peptide synthesis methodologies[15]. Upon completion of the synthesis, the peptides were cleaved from the support by

treatment with a dilute solution (10 ml) of trifluoroacetic acid in dichloromethane (1%, v/v), containing an appropriate carbocation scavenger cocktail, the composition of which was varied according to the amino acids contained in the peptide sequence. After standard workup, the peptide free acids were then converted into the corresponding diazoketone derivatives by reaction of their unsymmetrical anhydrides (formed by reaction with isobutylchloroformate) with ethereal diazomethane[16]. Finally, the peptidyl diazoketones were treated with a solution of dimethyldioxirane in moist acetone. The identity and purity of the final products were confirmed by a combination of TLC, ^{1}H-NMR and electrospray mass spectrometry. Invariably, the peptidyl α-keto aldehydes were obtained as their hydrates (see **Scheme 1**).

Biology: Isolation and purification of proteasome was achieved from fresh packed human red blood corpuscles (N. Ireland Blood Transfusion Service), as previously described[17]. The chymotrypsin-like activity of proteasome was assayed using the fluorogenic substrate, Suc-Leu-Leu-Val-Tyr-NHMec (Sigma Chemical Co., Poole, Dorset, England).

Kinetic studies. The peptidyl α-keto aldehydes were examined for their ability to block the proteasome-catalysed hydrolysis of Suc-Leu-Leu-Val-Tyr-NHMec. As an example, **Figure 1** shows the progress curves obtained for the proteasome-catalysed hydrolysis of the said fluorogenic substrate, carried out in the presence of varying amounts of Bz-Leu-Leu-Leu-COCHO.

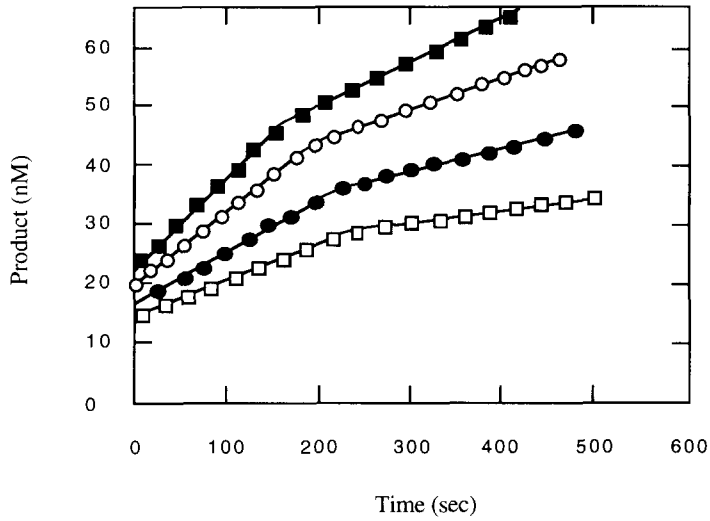
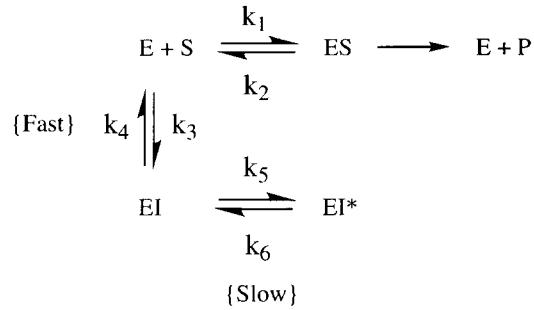


Figure 1. Progress curves for the proteasome-catalysed hydrolysis of Suc-Leu-Leu-Val-Tyr-NHMec in the presence of 0.5 μM (■), 1.5 μM (○), 2.5 μM (●) and 5.0 μM (❑) Bz-Leu-Leu-Leu-COCHO.

These progress curves are typical of an inhibitor exhibiting reversible, slow-binding kinetics where the formation of product **P** with time is described by equation (**1**)[18,19]. Each of the other active inhibitor sequences tested also yielded similar progress curves.

$$[\mathbf{P}] = v_s t - (v_s - v_o)(1 - exp(-k_{obs}t))/k_{obs} + d \qquad (1)$$

In this treatment, v_s is the final steady state velocity, v_o is the initial velocity and k_{obs} is the apparent first-order rate constant for the transition from initial to final steady state and d is a displacement term which reflects the concentration of product formed at t_0. From the resultant hyperbolic plots of k_{obs} against [I] (data not shown), we deduced that the interaction of inhibitor with protease followed a two step complexing mechanism[18] indicated below.



The inhibition constant, K^*_i for the formation of the final (tight) enzyme-inhibitor complex EI^* in the above scheme, was determined using the final steady-state rates (v_s) for substrate hydrolysis and a rearrangement of the equation (**2**) for competitive inhibition:

$$v_s = V_{max}[S]/([S] + K_m(1 + [I]/K_i)) \qquad (2)$$

The K^*_i values determined for each of the peptidyl α-keto aldehyde sequences synthesized in the present study, against the chymotrypsin-like activity of proteasome are shown in **Table 1**

Discussion: From Table 1 it is clear that peptidyl α-keto aldehydes constitute an interesting class of proteasome inhibitors. For example, while the dipeptide α-keto aldehyde sequences such as Cbz-Leu-Phe-COCHO and Cbz-Val-Phe-COCHO exhibit final K_i values in the low micromolar range, the tripeptide analogues Bz-Leu-Leu-Leu-COCHO and Cbz-Leu-Leu-Tyr-COCHO, exhibit final K_i values of 3.7 ± 0.4 nM and 3.1 ± 0.4 nM, respectively. These values compare favourably with the K_i value of 4.0 nM reported for the aldehyde Cbz-Leu-Leu-Leu-CHO (MG132)[20]. Indeed, the K_i values determined for our compounds are among the lowest so far reported for tripeptidyl aldehyde-based proteasome inhibitors. However, it should be noted that a number of groups have reported the preparation of potent inhibitors based on aldehydes with different lengths of peptide portion[11] and also based on peptidyl boronic acids with comparable or lower K_i values[21]. A number of these

inhibitors have been utilized in the study of the role of proteasome in biological systems[22]. The sequences synthesized in the present study were chosen so as to contain aromatic or large aliphatic residues at P_1 in order to fulfil the "chymotryptic-like" substrate specificity of proteasome[23]. In this regard, it was very satisfying to find that the peptidyl α-keto aldehyde sequences containing basic amino acids at P_1 were completely ineffective at blocking this "chymotryptic-like activity" thus establishing the selectivity of action of this class of inhibitor. Thus, it seems reasonable to suggest that variation of the peptide targeting sequence may lead to more potent, selective inhibitors in the future, particularly with regard to achieving inhibition of the other component activities of proteasome.

Table 1: Inhibitory constants for the inactivation of the chymotrypsin-like activity of proteasome by peptidyl α-keto aldehydes

Inhibitor	K^*_i (μM)
Cbz-Ala-Phe-COCHO	9.2
Cbz-Ala-Leu-COCHO	3.86
Cbz-Pro-Phe-COCHO	N.I.
Cbz-Val-Phe-COCHO	2.05
Ac-Arg-Arg-COCHO	N.I.
Cbz-Leu-Phe-COCHO	1.4
Ac-Val-Leu-Lys-COCHO	N.I.
Cbz-Leu-Leu-Tyr-COCHO	0.0031
Bz-Leu-Leu-Leu-COCHO	0.0037

N.I. No inhibition; values quoted are the mean of 4 determinations.

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24. Common abbreviations: Ac-: acetyl-; Bz-: benzoyl; Cbz-: benzyloxycarbonyl; DMF: *N,N*-dimethylformamide; Fmoc-: 9-fluorenylmethoxycarbonyl; HBTU: 2-(1H-Benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate; HOBt: 1-hydroxybenzotriazole; TFA: trifluoroacetic acid.