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Inhibitor profiling of the *Pseudomonas aeruginosa* virulence factor LasB using *N*-alpha mercaptoamide template-based inhibitors

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ABSTRACT

We report on the synthesis and biological evaluation of a focussed library of *N*-alpha mercaptoamide containing dipeptides as inhibitors of the zinc metallopeptidase *Pseudomonas aeruginosa* elastase (LasB, EC 3.4.24.26). The aim of the study was to derive an inhibitor profile for LasB with regard to mapping the S'_1 binding site of the enzyme. Consequently, a focussed library of 160 members has been synthesised, using standard Fmoc-solid phase methods (on a Rink-amide resin), in which a subset of amino acids including examples of those with basic (Lys, Arg), aromatic (Phe, Trp), large aliphatic (Val, Leu) and acidic (Asp, Glu) side-chains populated the P'_2 position of the inhibitor sequence and all 20 natural amino acids were incorporated, in turn, at the P'_1 position. The study has revealed a preference for aromatic and/or large aliphatic amino acids at P'_1 and a distinct bias against acidic residues at P'_2. Ten inhibitor sequences were discovered that exhibited sub to low micromolar K_i values.

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Pseudomonas elastase, also known as Pseudolysin or LasB, is a metalloprotease, which has long been recognised as a key virulence factor produced by the bacterium *Pseudomonas aeruginosa*.[1] The secreted protease degrades a broad-range of host tissue proteins, and key biomolecules involved in innate immunity such as the immunoglobulins, complement factors and cytokines.[2]

In addition, LasB acts within the bacterial cell, as a key regulator in the generation of the secreted polysaccharides that constitute the bacterial biofilm. In this 'defensive' mode of growth, the bacterial cells are resistant to biocides and to the host immune response.[3] Growth as a biofilm is strongly correlated with chronic infection, and the increased resistance make such infections difficult to eradicate.[4] Resistance to anti-microbials is up to 1000-fold greater within the biofilm, and the biofilm exopolysaccharide prevents the immune system from generating a response to the bacterial cell.[5] LasB thus plays a key role in the pathogenesis of Pseudomonal infection, by mediating both the defence and virulence of this opportunistic pathogen.[6]

Inhibitors of this virulence factor have previously been demonstrated to protect host corneal tissue from the destruction that characterises Pseudomonal eye infection, and anti-LasB antibody immunization reduces the virulence of infection.[7] Inhibition of bacterial virulence factors has gained momentum recently, as an antimicrobial strategy that is non-destructive to the bacteria. It is hoped that such a 'second-generation' class of antimicrobials might not place a strong selection pressure on the bacteria for emergence of resistant strains.[8] This strategy has shown some promise recently, and so inhibition of LasB represents a rational target for attenuation of Pseudomonal virulence in a variety of pathologies of Pseudomonal infection.[9]

Against this background, we have embarked upon a comprehensive structure-function study aimed at achieving inhibitor profiling of LasB. The present work reports on the initial 'mapping' of the S'_1 binding site of the enzyme using *N*-mercaptoamide dipeptides. The generalised structure of these inhibitors is shown in Figure 1, along with the structures of previously reported compounds.

We have previously reported on the solid phase synthesis of a series of *N*-alpha mercaptoamide-containing dipeptide amides as inhibitors of matrix metalloproteases and we have employed this methodology in the present study.[10] The synthetic method is outlined in Figure 2 and includes the incorporation of *S*-trityl protected mercaptoacetic acid into the range of dipeptide target sequences, constructed using standard Fmoc-solid phase synthesis protocols, on a Rink amide resin, and employing an Advanced Chemtech 396 parallel automated peptide synthesiser. The synthesis of the target inhibitor templates proceeded without difficulty except in those sequences containing very hydrophobic amino acids. In these instances, the syntheses were repeated using a CEM Liberty™ microwave assisted peptide synthesiser.[11] The identity and purity of all peptide products were confirmed using reversed phase HPLC and electrospray mass spectrometry.

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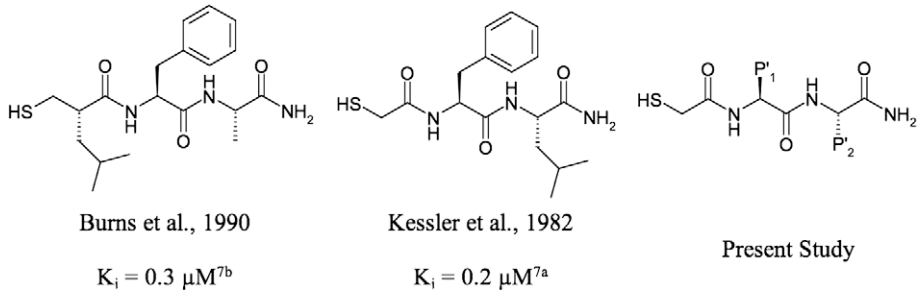

Figure 1. Comparison of chemical structures of previously reported thiol-based inhibitors of LasB, with the dipeptide templates of the present study.

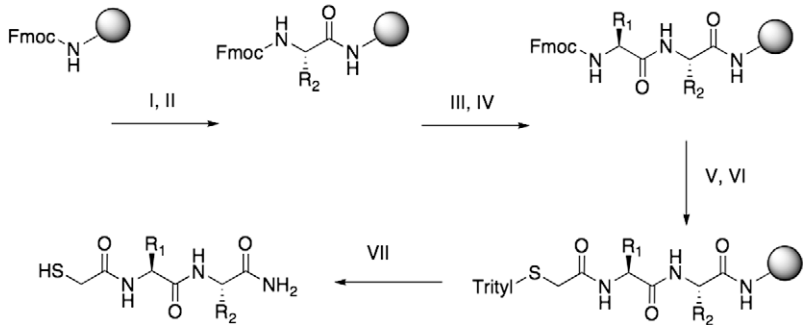

Figure 2. Reaction scheme for synthesis of focussed library of *N*-alpha mercaptoamide dipeptides. Reagents: (I) 20% piperidine/DMF (v/v); (II) Fmoc-NHCHR$_2$–CO$_2$H + HATU + DIPEA (threefold molar excess over resin loading); (III) repeat step I; (IV) Fmoc-NHCHR$_1$–CO$_2$H + HATU + DIPEA (threefold molar excess over resin loading); (V) repeat step I; (VI) *S*-trityl thioglycolic acid + HATU + DIPEA (threefold molar excess over resin loading); **VII**, TFA/TIPS/H$_2$O (95:2.5:2.5% v/v).

Each peptide was screened for inhibitory activity against LasB, obtained from Elastin Products Company, using a microtitre-based fluorimetric assay. Hydrolysis of the fluorogenic substrate Aminobenzoyl-Ala-Gly-Leu-Ala-*p*-Nitro-Benzyl-Amide by LasB was carried out in buffer containing 0.05 M Tris–HCl, 2.5 mM $CaCl_2$, 1% dimethylformamide, pH 7.2. The change in fluorescence was monitored using a BMG FLUOstar OPTIMA fluorescence microplate reader. Those peptides that exhibited inhibitory activity were then investigated further over an extended concentration range in order to determine the K_i for each. Figure 3 shows a typical dose–response relationship for SH–CH$_2$–CO–Y–V–NH$_2$, as an example. This figure clearly demonstrates the competitive nature of the inhibition, while Table 1 summarises the kinetic data that was generated in this initial inhibitor screening.

It can be appreciated from Table 1, that a number of inhibitor sequences have been identified in the present study that exhibit K_i values in the low micromolar range with SH–CH$_2$–CO–Y–V–NH$_2$ being the most potent sequence identified with a K_i value of 0.77 μM. This value is directly comparable to those reported before for LasB inhibitor templates that have been derived from studies of substrate sequences.[12] These published studies revealed a preference for aromatic or large aliphatic residues in the S'_1 and S'_2 pockets of the LasB active site.

This preference for aromatic/hydrophobic residues was also recapitulated in this present study, although an aromatic residue

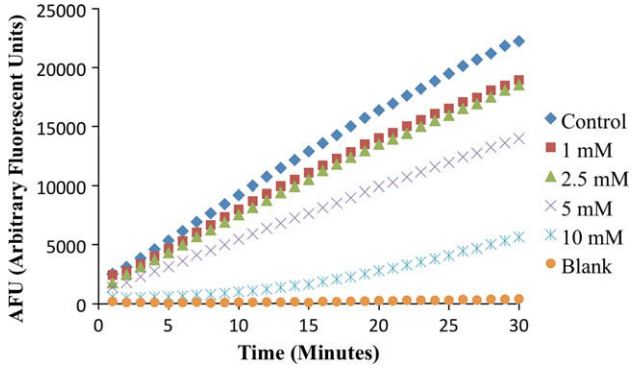

Figure 3. Typical progress curves for hydrolysis of aminobenzoyl-Ala-Gly-Leu-Ala-*p*-nitro-benzyl-amide by LasB in the presence of a range of concentrations of SH–CH$_2$–CO–Y–V–NH$_2$.

Table 1
K_i Values (μM) for inhibitor library. 'NI' (no inhibition) has been stated for values over 1000 μM. Values in grey identify a general trend for low K_i values in inhibitors containing P'_1 Trp and Tyr residues

P'_2	K_i (μM)							
	Basic		Aromatic		Large aliphatic		Acidic	
	Lys	Arg	Phe	Trp	Val	Leu	Asp	Glu
P'_1								
His	332	(NI)	21	18	47	306	(NI)	(NI)
Arg	135	(NI)	224	125	(NI)	(NI)	650	(NI)
Lys	433	(NI)	126	(NI)	555	123	971	(NI)
Ile	190	(NI)	(NI)	366	1.8	1.3	142	(NI)
Phe	76	(NI)	146	206	11	645	(NI)	(NI)
Leu	14	623	113	300	(NI)	53	587	(NI)
Trp	10	25	1.1	49	4.1	3.7	38	91
Ala	153	115	(NI)	395	51	21	316	(NI)
Met	3.9	6.6	867	204	98	(NI)	7.0	(NI)
Pro	766	56	(NI)	562	157	246	(NI)	(NI)
Cys	274	646	131	108	161	(NI)	(NI)	(NI)
Asn	289	280	37	70	180	508	(NI)	(NI)
Val	22	69	72	(NI)	10	69	(NI)	(NI)
Gly	451	641	51	122	457	138	(NI)	(NI)
Ser	(NI)	444	75	(NI)	229	510	(NI)	(NI)
Gln	380	217	(NI)	91	937	540	(NI)	(NI)
Tyr	8.5	3.0	6.5	14	0.77	33	5.5	27
Asp	491	551	(NI)	207	(NI)	118	(NI)	(NI)
Glu	553	(NI)	146	704	730	(NI)	(NI)	(NI)
Thr	(NI)	616	225	65	377	(NI)	(NI)	(NI)

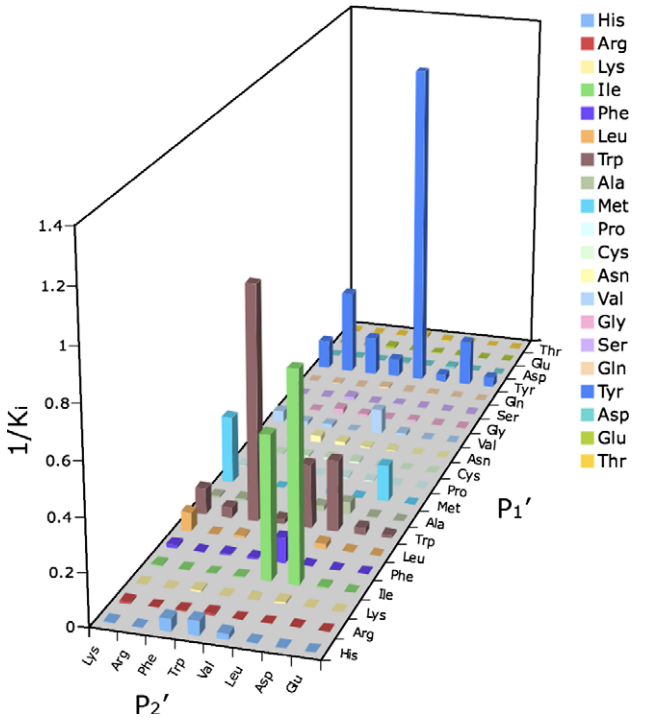

Figure 4. Relative potency of individual compounds displayed as the reciprocal of the micromolar K_i value ($1/K_i$) to distinguish the most potent inhibitors. The library has been arranged according to the dipeptide amino acid sequences, and colour coded according to P'_1 residue.

at P'_1 did not guarantee a successful inhibitor in all cases. P'_1 Phenylalanine for example, although routinely employed in previous studies, produced only moderate inhibition (K_i = 11–645 μM across this group) as part of the C-terminal amide containing mercaptoacetyl dipeptides of this study. We have displayed the relative potency of each inhibitor as the reciprocal of its K_i value in Figure 4, with values arranged according to the amino acid residues occupying the P'_1 and P'_2 positions in each case. It is immediately apparent from this figure that for the most active compounds from this library, the P'_1 position is almost exclusively occupied by aromatic or bulky aliphatic residues, with Tyr, Trp and Ile appearing in nine of the top ten inhibitors. Methionine is also represented at this position. It can thus be concluded that the S'_1 pocket is highly specific for bulky hydrophobic residues, but this is again dependent on the presence of a suitable residue at P'_2 within the overall inhibitor structure.

The same general preference also exists for the P'_2 residue, with P'_2 Phe-, Leu-, and Val-based compounds occupying seven out of the top 10 inhibitors. This confirms the known preference for aromatic or large aliphatic residues in the S'_2 pocket, indicating that this region of the active site is also predominantly hydrophobic. The acidic residues Asp and Glu performed poorly in the P'_2 position, while the basic residues produced moderate inhibitors. However, no single amino acid residue occupying the P'_2 position of the inhibitors produced active compounds in every instance, suggesting that the P'_1 position is the driving determinant for recognition.

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