Internally quenched peptides for the study of lysostaphin: an antimicrobial protease that kills *Staphylococcus aureus*

Rachel Warfield, Philip Bardelang, Helen Saunders, Weng C. Chan, Christopher Penfold, Richard James and Neil R. Thomas*

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Lysostaphin (EC. 3.4.24.75) is a protein secreted by *Staphylococcus simulans* biovar *staphylolyticus* and has been shown to be active against methicillin resistant *S. aureus* (MRSA). The design and synthesis of three internally quenched substrates for lysostaphin based on the peptidoglycan crossbridges of *S. aureus*, and their use in fluorescence resonance energy transfer (FRET) assays is reported. These substrates enabled the gathering of information about the endopeptidase activity of lysostaphin and the effect that mutations have on its enzymatic ability. Significant problems with the inner filter effect and substrate aggregation were encountered; their minimisation and the subsequent estimation of the kinetic parameters for the interaction of lysostaphin with the substrates is described, as well as a comparison of substrates incorporating two FRET pairs: Abz–EDDnp and DABCYL–EDANS. In addition to this, the points of cleavage caused by lysostaphin in Abz-pentaglycine-EDDnp have been determined by HPLC and mass spectrometry analysis to be between glycines 2 and 3 (∼60%) and glycines 3 and 4 (∼40%).

Introduction

The emergence of virulent strains of bacteria that are resistant to antibiotics is a cause for concern in the medical profession. Since resistance to the β-lactam antibiotic penicillin G was first observed in *Staphylococcus aureus* in the 1940s, the number of cases of infections caused by resistant strains has increased markedly. As a result, the penicillin derivative methicillin was used therapeutically as it retained activity against penicillin-resistant strains of *S. aureus*. Methicillin resistant *S. aureus* (MRSA) was first reported in 1961; in 2003 over 7600 cases of MRSA were recorded in England alone and from 1993 to 2002 the number of MRSA-related deaths rose from 51 to 800 per annum. In recent years, *S. aureus* strains, such as Mu50, have been shown to have multi-drug resistance that extends beyond the β-lactams. Intermediate resistance to the glycopeptidic “antibiotic of last resort”, vancomycin was first found in Japan in 1997 and a clinical isolate of MRSA was found to also have resistance to the oxazolidinone, linezolid, shortly after the introduction of this new antibiotic in 2001. Owing to the prevalence of other effective antibiotics at the time and the difficulties encountered in producing a pure form of the protein, lysostaphin was largely ignored as a potential therapeutic agent. The emergence of multi-drug resistant bacteria and the recombinant protein technology required for ease of production has lately led to a renewed interest in lysostaphin and it is currently being developed as a new antibiotic for *S. aureus*. It is produced by another staphylococcal species, *S. simulans* biovar *staphylolyticus*, and it is reported to kill both *S. aureus* and another pathogenic bacteria, *S. epidermidis*. The mode of action appears to be the cleavage of the pentaglycine crossbridge found in the peptidoglycan of the bacterial cell wall of *Staphylococcus* species. Part of the structure of the peptidoglycan of *S. aureus* is shown in Fig. 1. It is this structure that confers rigidity to the bacterial cell wall and prevents osmotic rupture. *S. simulans* also has a glycin crossbridge, but protects itself with lysostaphin immunity factor (Lif), which enables the replacement of glycine by serine in

Fig. 1 Representation of part of the peptidoglycan of *S. aureus*, showing the sugar backbone, side chain tetrapeptide and pentaglycine crossbridge.
the pentaglycine crossbridge. The gene for this immunity factor, lif, was characterised by Thumm and Götz and was found to code for a 413 residue polypeptide. The glycine residues that are replaced in the crossbridge are numbers 3 and 5; this protects the cell wall as lysostaphin cannot hydrolyse glycyl–serine or seryl–glycine bonds.

Mature lysostaphin is a two domain structure consisting of 247 amino acid residues, the first 132 of which form the catalytic or endopeptidase domain of the protein. This is followed by a thirteen residue linker, which is attached to the N-terminal of the targeting (cell-wall binding) domain (102 residues), forming the remainder of the enzyme. Lysostaphin is related to other glycylglycyl endopeptidases, which have similar domain-type structures. These include ALE-1, a 36 kDa protein secreted by S. capitis and LytM, which is believed to be responsible for the functional autolytic activity in the Lyt– mutant of S. aureus; the crystal structures of LytM and the cell-wall binding domain of ALE-1 have recently been elucidated. In the LytM structure, an exposed triglycine sequence (Gly206–Gly 208) of one monomer is found to be inserted into a groove in the surface of an adjacent monomer in close proximity to the zinc. This helps delineate the active site of this enzyme.

Several assays for the activity of lysostaphin have been reported in the literature; the first being a turbidimetric method proposed by Schindler and Schuhardt. Spectrophotometric variations on this method have also been reported and used to examine the Michaelis–Menten kinetics of the interaction of lysostaphin with whole cells, leading to the determination of the specific activity of the enzyme. Other spectrophotometric assays described include a dye-release assay developed by Zhou et al., which gave results that were more reproducible than the turbidimetric method. Kline and co-workers developed a TNBS-based colorimetric microtitre plate assay that monitored the hydrolysis of N-acetylated hexaglycine by lysostaphin. The latter two assays are more cumbersome to perform as they are ‘stopped assays’ where the product formed is analysed by removal of samples from the assay mixture. Given that peptidoglycan has a variable composition from sample to sample due to the level of crosslinking, it was decided that a synthetic peptidoglycan has a variable composition from sample to sample was used. The substrate–enzyme interaction. A significant inner filter effect was encountered when substrate concentrations greater than 10 μM were used. The  of the bell-shaped absorbance spectrum of the substrate is 359 nm and so the substrate absorbs light, to an extent that is dictated by the [substrate], at both the excitation and emission wavelengths used for the FRET assays. This results in not all of the applied energy being available to excite the product of cleavage, and some of the fluorescence that  emits being absorbed by remaining substrate, so that it does not reach the detector. This inner filter effect means that the initial fluorescence intensity (FI) observed for the substrate, prior to enzyme addition, was not directly proportional to [substrate], and consequently, the rate of increase in FI at higher [substrate] after addition of enzyme was lower than it would have been in the absence of the inner filter effect. The effect can be compensated for by measuring the absorbance of the substrate at both the excitation (ODex) and emission (ODem) wavelengths, and applying the values obtained to the observed fluorescence (Fobs) (or rate) as dictated by eqn (1).

\[ F_{\text{corr}} = F_{\text{obs}} \times \text{anti} \log \left( \frac{OD_{\text{ex}} + OD_{\text{em}}}{2} \right) \]  

By reducing the pathlength of the cuvette used for the assays, and thus the absorbance of light by the substrate, it was possible to extend the working concentration range for the assay, although at the expense of sensitivity. A 5 mm path length cuvette enabled the use of a [substrate] up to 70 μM. After this point the correction

Results and discussion

Of the FRET pairs available, ortho-aminobenzoic acid (Abz) and \( N-(2,4\text{-dinitrophenyl})\text{ethylenediamine} \) (EDDnp) were the most readily accessible, and so we adopted these as our starting point. This pair has already been used in several studies, including the investigation of trypsin and an angiotensin converting enzyme. The substrate \( N-(\text{aminobenzoyl})\text{pentaglycyl-}[N-(2,4\text{-dinitrophenyl})\text{ethylenediamine}] \) was synthesised using solution phase methodology, as shown in Scheme 1. A convergent approach was used, consisting of the synthesis of a diglycine and a triglycine fragment labelled with EDDnp and Abz respectively, using Boc and ethyl ester protecting groups. These two compounds were coupled using an EDCl-mediated reaction to yield the desired substrate in a 20% yield over 7 steps.

FRET assays to demonstrate the cleavage of 7 by lysostaphin were performed and found to be successful. The method used for the purification of lysostaphin was found to influence the activity of the enzyme: samples of the protein that had been purified using a zinc-affinity chromatography column were found to be more than three times more active than those from a nickel-affinity column (data not shown). The observed drop in activity suggested that use of the latter column led to replacement of the zinc co-factor by a nickel ion in some of the lysostaphin molecules. The results in this article are from assays using the zinc-purified enzyme.

The fragments from a full digestion of the substrate by lysostaphin were examined by mass spectrometry. The diglycyl and triglycyl derivatives of EDDnp were detected, indicating that the enzyme cleaves the substrate between both Gly2–Gly3 and Gly3–Gly4. Analysis of the digestion products by high performance liquid chromatography (HPLC) and comparison with the retention times of \( N-(2,4\text{-dinitrophenyl})\text{ethylenediamine} \)diglycine (6) and \( N-(2,4\text{-dinitrophenyl})\text{ethylenediamine} \)triglycine (8), revealed that the ratio of products 6 and 8 was 2 : 3. This indicates that cleavage is slightly favoured between glycines 2 and 3.

FRET assays at different [substrate] were performed in order to determine the Michaelis constant (\( K_{\text{m}} \)) and \( V_{\text{max}} \) for the substrate–enzyme interaction. A significant inner filter effect was encountered when substrate concentrations greater than 10 μM were used. The of the bell-shaped absorbance spectrum of the substrate is 359 nm and so the substrate absorbs light, to an extent that is dictated by the [substrate], at both the excitation and emission wavelengths used for the FRET assays. This results in not all of the applied energy being available to excite the product of cleavage, and some of the fluorescence that  emits being absorbed by remaining substrate, so that it does not reach the detector. This inner filter effect means that the initial fluorescence intensity (FI) observed for the substrate, prior to enzyme addition, was not directly proportional to [substrate], and consequently, the rate of increase in FI at higher [substrate] after addition of enzyme was lower than it would have been in the absence of the inner filter effect. The effect can be compensated for by measuring the absorbance of the substrate at both the excitation (ODex) and emission (ODem) wavelengths, and applying the values obtained to the observed fluorescence (Fobs) (or rate) as dictated by eqn (1).

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By reducing the pathlength of the cuvette used for the assays, and thus the absorbance of light by the substrate, it was possible to extend the working concentration range for the assay, although at the expense of sensitivity. A 5 mm path length cuvette enabled the use of a [substrate] up to 70 μM. After this point the correction
Scheme 1  a) SOCl₂, EtOH, 0 °C to reflux, N₂, 4 h (83%); b) Isatoic anhydride, DCM, Et₃N, DMAP, reflux, N₂, 17 h (78%); c) HCl (1 M), 40 °C, 7 h (quant.); d) Boc₂O, NaOH, dioxane, N₂, rt, 19 h (69%); e) EDDnp·HCl, PyBOP, DIPEA, DMF; N₂, rt, 20 h (48%); f) TFA–DCM (1 : 1), N₂, rt, 3 h (97%); g) EDCI, DIPEA, DMF, N₂, rt, 15 h (61%).

factor obtained by the use of eqn (1) was greater than 2, and insufficient compensation for the inner filter effect was observed. Fig. 2 demonstrates the effect that applying a correction factor has on the observed initial fluorescence: a linear relationship between [substrate] and FI is indicated.

Fig. 2  A graph of the initial substrate fluorescence intensity with and without correction for the inner filter effect. Once corrected a linear relationship between [substrate] and intensity is observed.

Fig. 3  shows the Michaelis–Menten curve obtained using Grafit® to plot the corrected rate vs. [substrate]. The kinetic constants calculated from this graph were: $K_M = (0.20 \pm 0.02) \text{ mmol dm}^{-3}$ and $V_{max} = (63 \pm 5) \text{ pmol s}^{-1} \text{ mg(Lss)}^{-1}$. A linear regression analysis of a Hanes plot gave $K_M = (0.30 \pm 0.04) \text{ mmol dm}^{-3}$ and
Grafit C-fitted Michaelis–Menten curve for the cleavage of 7 by lysostaphin.

\[ V_{\text{max}} = (76 \pm 1) \text{ pmol s}^{-1} \text{ mg(Lss)}^{-1} \ (R^2 = 0.944). \]

The rates of hydrolysis were converted from relative values to those based on the increase in [product] by use of a standard calibration curve of fluorescence intensity vs. [3].

The triglycine and tetraglycine analogues of 7 were also synthesised using similar methods to those described previously. FRET assays of these substrates with lysostaphin revealed that the triglycine substrate was not cleaved at all and the tetraglycine substrate was hydrolysed approximately five times more slowly than 7. Firczuk et al. briefly reported the testing of tetraglycine-[N-(2,4-dinitrophenyl)ethylene diamine] as a fluorescent substrate for LytM during the course of our work. In this case the product has to be separated from the substrate chromatographically. They found this compound to be a moderate substrate (\( k_{\text{cat}} = 0.003 \text{ s}^{-1}, K_M = 2.3 \text{ mmol dm}^{-3}, \text{pH 7.5} \)), which is consistent with the results we present here. It might have been expected, from the cleavage site analysis described previously, that the triglycine analogue would have been cleaved as one of the hydrolysable bonds (between Gly 2 and Gly 3) was still present; the fact that it is not suggests that decreasing the length of glycine chain reduces the ability of lysostaphin to recognise its target. Kinetic assays were performed with the tetraglycine analogue; using Grafit C the values of \( K_M \) and \( V_{\text{max}} \) were estimated to be (0.2 ± 0.05) mmol dm\(^{-3}\) and (32 ± 4) pmol s\(^{-1}\) mg(Lss)\(^{-1}\) respectively. The very slow rate of cleavage measured meant that assays with this analogue had to be quite long (at least 15 min) and were less reliable than those performed using 7 as a substrate. It was concluded that further substrates for lysostaphin should contain at least the pentaglycine motif in their structure.

Ideally, the [substrate] range used for the measurement of kinetic parameters should be in the range 20 \(\mu\)M to 1 mM given the Michaelis constant for 7 determined above. This was not possible, owing to the inner filter effect, and so attention was turned to a second substrate that incorporated a lysine residue, 1-lysine(pentaglycine-N-(2-aminobenzoyl)-(2,4-dinitrophenyl)ethylenediamine (14), the structure of which is shown in Fig. 4.

Scheme 2 illustrates the synthetic route used for its synthesis. It was hoped that the inclusion of this residue would improve the solubility of the substrate and also, as lysine is the point of attachment for one end of the pentaglycine crossbridge to the muramyl tetrapeptide side chain, improve the substrate–enzyme binding. The synthesis started from a lysine residue, the amine groups of which were protected with the orthogonal groups Boc and Fmoc. As the pentaglycine chain was to be attached to the N\(^\circ\) of the lysine group, this was protected with the Boc group. The previously prepared reagents, N-Boc-diglycine (4) and N-(2-aminobenzoyl)-triglycine hydrochloride (3) were attached consecutively following the coupling of the EDDnp group to the free C-terminal. Removal of the Fmoc group using standard basic conditions gave the substrate, 14, in 44% yield over 6 steps.

When kinetic assays were performed using 14 as the substrate, a slight increase in cleavage rate was observed. The kinetic constants obtained by Grafit C and Hanes plot analyses of the results are shown in Table 1. The lower \( K_M \) values of 14 compared with 7 indicate that there is an increase in the binding affinity of the enzyme to this substrate. The accessible [substrate] range was the same as for 7; the overlap, between this and the ideal range for

![Fig. 4](image-url) Structures of 1-lysine(N-\(\varepsilon\)-pentaglycine-N-(2-aminobenzoyl))-([N-(2,4-dinitrophenyl)ethylenediamine] (14) and 1-lysine(N-\(\varepsilon\)-pentaglycyl-DABCYL)-EDANS (17).
Table 1  Kinetic parameters measured for the three substrates

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<th>Grafit</th>
<th>Hanes</th>
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<td>$V_{max}/\text{pmol s}^{-1} \text{mg(Lss)}^{-1}$</td>
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<td>76</td>
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<tr>
<td>Error $V_{max}/\text{pmol s}^{-1} \text{mg(Lss)}^{-1}$</td>
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<td>1</td>
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<tr>
<td>$R^2$ (Hanes)</td>
<td>0.944</td>
<td>0.967</td>
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The measurement of a Michaelis constant of $\sim 70 \mu M$, is greater, and so 14 is a better substrate for lysostaphin than 7, although the substrate–enzyme binding is still quite poor.

A second FRET pair was also investigated. 4-((4-(Dimethylamino)phenyl)azo)benzoic acid (DABCYL) and 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS) have previously been used by other groups for examining the activity of various proteases, as there is excellent overlap of the EDANS emission spectrum and the absorption spectrum of DABCYL. The extent of the overlap of these two spectra is important as a greater overlap leads to better FRET efficiency. It was hoped that the introduction of these two groups, in place of Abz and EDDnp, which have a smaller overlapping region of their emission and absorption spectra, would lead to a more sensitive assay; better FRET efficiency entails a larger fluorescence quenching effect and thus, when one substrate molecule is cleaved by the enzyme, a larger change in fluorescence intensity should be observed. The effect that use of a different FRET pair had on the binding of lysostaphin to the substrate was also explored.

Scheme 3 illustrates the solid phase-based synthetic route that was used to synthesise $N$-(1-lysyl)[N-(4-(4-(dimethylamino)phenyl)azo)benzoyl]pentaglycyl]-5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (17).

The strategy employed was similar to that used by Grahn and co-workers in their synthesis of DABCYL–EDANS-labelled substrates for trypsin: a solid phase synthesis of the DABCYL-peptide (15) that was followed by a solution coupling of EDANS to this peptide to form 16 (using the method of Pennington and Dunn). Removal of the Boc protecting groups yielded the desired substrate, 17. The solubility of 17 was very poor, even in solvents such as dimethylformamide, which made purification of the final peptide difficult. Several chromatographic methods were attempted, including silica column chromatography and preparative thin layer chromatography; the most effective method was found to be semi-preparative HPLC, using dimethylformamide as the injection solvent and an aqueous acetonitrile mobile phase. This worked well provided a small volume ($\sim 50 \mu l$) of concentrated solution was injected into a relatively large sample loop (1 ml). Larger sample volumes resulted in unacceptable quantities of peptide eluting with the dimethylformamide/solvent system front.

Experiments were conducted to compare the relative FRET efficiencies of the Abz–EDDnp and DABCYL–EDANS pairs. Fig. 5 shows the emission spectra obtained from 10 $\mu M$ solutions of 14 and 17, and 1 $\mu M$ solutions of $N$-(2-aminobenzoyl)triglycine (3) and (5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid) diglycine (18). It is clear that DABCYL quenches the emission

Scheme 3  a) Boc-K(Fmoc)-OH, DIPEA, DCM, 14 h, then MeOH, 30 min (99%); b, d, f, h, j, l) 20% piperidine in DMF, 10 min; c, e, g, i, k) TBTU, Fmoc-Gly, HOBT, DIPEA, DMF, 3 h; m) HOBT, DABCYL-OH, DIPCDI, DMF, 20 h; n) 1% TFA in DCM, 40 min, then py–MeOH (69% overall); o) EDANS·Na, EDCI, HOBT, DMF, $N_2$, 20 h; p) TFA–DCM (1 : 1), 40 min, then Et$_3$N–MeOH (6% over 2 steps).
from EDANS more efficiently than EDDnp does the emission of Abz. When the relative fluorescence intensities were compared, it was determined that there is a 3-fold increase in the efficiency of quenching for the DABCYL–EDANS pair relative to Abz–EDDnp. This was reflected in the values obtained for the relative rates of cleavage of 14 and 17 by lysostaphin; a much larger increase in emission intensity was observed over the same time period for the DABCYL–EDANS substrate than for that containing Abz and EDDnp.

Kinetic assays were performed using 17, and the $K_M$ and $V_{\text{max}}$ values for this substrate were obtained, as shown in Table 1. Unfortunately these assays were not as straightforward as had been hoped. It is suggested that as the [substrate] is increased, aggregation of substrate 17 in the buffer solution occurs. The first indication of this was the rapid alteration in the absorbance spectrum of the substrate once the stock solution (in DMSO) was added to the buffer solution. A decrease in the measured rates of cleavage for consecutive assays using the same [substrate] was also evident, and after a few hours a red precipitate was observed, suspended in the colourless buffer solution. This effect has previously been reported with a DABCYL–EDANS peptide consisting of three residues.\(^2^9\) Various methods, including alteration of temperature or pH, reduction of sodium chloride content of the buffer solution, increasing DMSO or glycerol content, or addition of chaotropic agents were tested, but these either resulted in no substrate stabilisation or inactivation of the enzyme. The zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was added to the buffer solution. It was found that if 12 mM CHAPS was used, then substrate stabilisation did occur. This is above the critical micelle concentration of the detergent ($\sim$6 mM) in the buffer solution, but lower [CHAPS] only gave partial success. Lysostaphin still appeared to be active even under these conditions, and may be activated by the presence of the detergent. When assays using the first substrate, 7, were run with and without 12 mM CHAPS, an increase in cleavage rate ($\sim$2 fold) was observed when the detergent was present, even though this substrate shows no evidence of aggregation. The results in Table 1 suggest that the use of DABCYL and EDANS does not affect the binding of the enzyme and substrate, as the Michaelis constant is almost the same as that for 7. The inner filter effect was at a similar level for this substrate as for those containing Abz and EDDnp. The maximum [substrate] possible was $\sim$63 $\mu$M, and so this means that the [substrate] range used is still not one that gives the best scope for accurate kinetic parameter determination.

Both 7 and 17 were used in FRET assays with endopeptidase domain mutants of lysostaphin: H83A and H114A (which had been shown to be inactive in turbidity and disk diffusion assays). No net increase in fluorescence was observed in these FRET assays, confirming the inactive status of the mutants and the possible use of these internally quenched substrates for the activity-screening of other lysostaphin mutants. In particular, 17 would be suitable for this, owing its greater sensitivity.

### Conclusions

Three new internally quenched peptides for measuring the catalytic activity of lysostaphin have been synthesised and demonstrated to function as substrates for this enzyme. It has been shown that the pentaglycine sequence can be cleaved efficiently between both Gly2–Gly3 and Gly3–Gly4. This is in agreement with the data available on the cleavage of peptidoglycan demonstrating that the synthetic substrates are processed in a similar manner to the natural substrate. It has been found that the pentaglycine with DABCYL and EDANS groups provides a more sensitive assay that leads to shorter duration assays than those with the Abz–EDDnp FRET pair. However aggregation and precipitation of this substrate over short periods of time, when placed in aqueous solution mean that the Abz–EDDnp pentaglycine substrates give more reproducible results in kinetic assays. The change in FRET pairs does not appear to significantly affect the interaction of lysostaphin with the substrate. The relatively low affinity of lysostaphin for all of these substrates necessitates that they be used at relatively high concentrations leading to a significant inner filter effect. This can be corrected to a limited extent, but ideally internally quenched peptides with lower $K_m$ values should be identified to avoid this problem.
These internally quenched substrates provide a relatively sensitive, continuous assay for lysostaphin and despite the substrate concentrations being significantly lower than the $K_M$ values, the rates measured are directly proportional to the catalytic efficiency ($k_{cat}/K_M$) allowing the method to be used to compare the catalytic efficiency of mutants of the enzyme. The dye-release assay described by Zhou et al. is slightly more sensitive than that described in this paper and it requires a slightly lower protein concentration of lysostaphin (3 $\mu$g ml$^{-1}$) compared to the 14 $\mu$g ml$^{-1}$ for the FRET assays. However, theirs is a stopped assay and therefore much less convenient for the rapid generation of kinetic data; this is also the case for the stopped colorimetric assay of Kline et al., that requires lysostaphin in the 1.25 to 20 $\mu$g ml$^{-1}$ range and significant post-assay manipulation. Further elaboration of the substrates described in this paper to include other elements of the peptidoglycan may well improve the sensitivity of the FRET assay and be able to provide more accurate kinetic data.

In the recently reported ALE-1 (lysostaphin homologue from S. capitis) cell wall binding domain structure, the authors identified a groove in the surface of the cell wall binding domain that appears to be suitable to accommodate the pentaglycine cross-bridge as found in S. aureus and S. capitis EPK2 suggesting that both endopeptidase and cell wall binding domains bind to the same peptide motif. Further evidence for this is given by studies reported by Grundling and Schneewind who have studied the binding of lysostaphin cell wall binding domain-GFP conjugates to S. aureus peptidoglycan. Therefore an internally quenched peptide that possesses two pentaglycine sequences, one of which is modified to be non-hydrolysable may provide a higher affinity substrate provided the distance between these motifs is appropriate for the enzyme. The tetraglycine motif is reported to be cleaved by a number of other proteases including the LasA protease (staphylocysin) from Pseudomonas aeruginosa and dipeptidyl peptidases III from Rattus norvegicus that cleaves elastin. The internally quenched peptides reported here should therefore provide suitable substrates for these enzymes as well.

**Experimental**

**Instrumentation**

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV400, Bruker DRX500 or a Bruker Ultrasilhield 400 spectrometer. The $^{13}$C spectra were proton decoupled. Chemical shifts are in parts per million using a residual protonic solvent as an internal standard and coupling constants ($J$) are in Hz. Mass spectra were recorded on a VG LCT (electrospray, ES$^+$) or VG Autospec mass spectrometer (fast atom bombardment, FAB$^+$). In FAB mass spectrometry, nitrobenzyl alcohol or glycerol in water and methanol was used as the matrix. Microanalytical data were obtained on a Perkin Elmer 240B elemental analyser. IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer as solids. UV–Visible spectra were measured on a Cary 100 Bio UV–visible spectrophotometer or a HeXios $\beta$ Thermospectronic UV–visible spectrophotometer. Melting points were recorded using a Stuart Scientific SMP3 melting point apparatus and are uncorrected. Measurement of $[\alpha]_D$ values was performed using a Jasco DIP-370 polarimeter or a Bellingham-Stanley Ltd ADP 200 polarimeter.

Column chromatography was carried out using Fluorochem silica gel 60 (35–70 $\mu$m) and analytical thin layer chromatography was performed on precoated aluminium backed plates (Merck, silica gel 60 F$_{254}$). Preparative thin layer chromatography was performed using plates purchased from Sigma-Aldrich (silica, 20 × 20 cm, 2 mm). Reverse-phase high performance liquid chromatography (RP-HPLC) was performed analytically on an Agilent Eclipse XDB-C8 column (150 × 4.6 mm, 5 $\mu$m) using ChemStation for LC (Agilent Technologies) software on an Agilent 1100 series system at a flow rate of 1.0 ml min$^{-1}$. Semi-preparative RP-HPLC was performed using the same system and software and a Hichrom 100–58C column (150 × 10 mm, 5 $\mu$m), with a flow rate of 4.5 ml min$^{-1}$. The eluent was monitored by UV absorbance at 220 nm and 325 nm, 335 nm or 359 nm, depending on the fluorophore/quencher groups present. The solvents used were solvent A (0.06% v/v TFA (aq.) in Milli-Q water, de-gassed using nitrogen for 30 min) and solvent B (0.06% v/v TFA (aq.) in 9 : 1 v/v MeCN (aq.)–Milli-Q water, de-gassed by sonication for 30 min). An Edwards Modulyo freeze drier was used for lyophilisation of the products. Peptide synthesis was carried out using a NovaSyn Gem manual peptide synthesiser with post-column UV monitoring at 290 nm using a LKB Biochrom Ultrospec 4050 spectrophotometer and Fmoc deprotection profiles were recorded using a LKB Bromma 2210 chart recorder.

**Chemicals**

Chemicals were purchased from Sigma-Aldrich (Gillingham, UK), Alfa Aesar (Heysham, UK) and Fisher Scientific UK Ltd (Loughborough, UK). Fmoc amino acids and coupling agents were purchased from Novabiochem UK (Nottingham, UK). DABCYL succinimidyl ester was purchased from Invitrogen (Paisley, UK). Deuterated chloroform, DMSO, methanol and water were purchased from Sigma-Aldrich. Solvents were purchased from Fisher Scientific UK Ltd. DMF was distilled from CaH$_2$ and ethanol was distilled from magnesium turnings and iodine. Both were stored over 4 Å molecular sieves and under a nitrogen atmosphere following purification. All other reagents were used as supplied.

**Synthetic procedures**

$N$-(2,4-Dinitrophenyl)ethylene diamine hydrochloride (EDDnp). This compound was prepared according to the method of Melo et al. $R_\text{f}$ 0.09 (methanol–ethyl acetate 1 : 1); mp 268–269 °C (decomp) (Lit. 268–270 °C; $\delta_H$ (400 MHz, D$_2$O) 3.29 (2H, t, $J$ 6.1, CH$_2$), 3.82 (2H, t, $J$ 6.1, CH$_2$), 7.09 (1H, d, $J$ 9.6, CH), 8.25 (1H, dd, $J$ 2.7 and 9.6, CH), 8.99 (1H, d, $J$ 2.7, CH); $\delta_C$ (100 MHz, D$_2$O) 38.7, 40.9, 115.4, 124.5, 131.6, 131.7, 136.9, 149.3; $ml/z$ (ES$^+$): found (M+H$^+$) 227.0796. C$_{11}$H$_{11}$N$_2$O$_4$ requires M, 227.0780.

Triglycine ethyl ester hydrochloride (I). The method described was adapted from that used by Akora et al. for the preparation of methyl esters. Thionyl chloride (1.93 ml, 26.4 mmol) was added, dropwise, to ice cold ethanol (300 ml), so that the temperature remained below 5 °C. Triglycine (2.50 g, 13.2 mmol) was added, and the solution refluxed for 4 h, under nitrogen. The solvent was removed in vacuo to give a white solid that was recrystallised from boiling ethanol, giving the ester (2.78 g, 83%) as fine white needles. $R_\text{f}$ 0.06 (methanol–ethyl acetate 1 : 1); mp 214–218 °C (decomp).
N-(2-Aminobenzoyl)triglycine ethyl ester (2). Triglycine ethyl ester hydrochloride, I (0.80 g, 3.15 mmol) was dissolved in DCM (200 ml) and triethylamine (0.88 ml, 6.31 mmol). 4-Dimethylaminopyridine (39 mg, 0.32 mmol) was added and the suspension was heated to reflux temperature. Isothyride anhydride (3.57 g, 21.9 mmol) was added once the solution had cooled slightly and the resulting suspension was refluxed for 17 h, under nitrogen. The solid residue was collected by filtration. The remaining solution was washed with water (3 × 25 ml). The aqueous layer was washed with ethyl acetate (3 × 50 ml) and the organic layers were combined and dried over magnesium sulfate. The solvent was removed in vacuo and the resulting residue was combined with the solid collected by filtration. This was dissolved in methanol and DCM, evaporated onto silica (2 g) and then purified twice by column chromatography (silica, ethyl acetate, followed by ethyl acetate–methanol 9 : 1 then 1 : 1) to give the product (0.82 g, 78%) as a cream powder. Rf 0.41 (ethyl acetate–methanol 9 : 1); mp 156–157 °C; v_{\text{max}} (solid)/cm⁻¹ 3312w, 3267w, 1748s, 1673m, 1655s, 1547vs; v_{\text{max}} (CH₃OH)/nm 215 (ε/\text{dm}⁻³ mol⁻¹ cm⁻¹ 37 700), 249 (15 300) and 331 (7900); δ_H (CDCl₃/CDOD, 400 MHz) 1.30 (3H, t, J 7.2, CH₂), 3.98 (2H, s, CH), 4.00 (2H, s, CH), 4.05 (2H, s, CH), 4.21 (2H, q, J 7.2, OCH₃), 6.67 (1H, dd, J 0.9, 7.0 and 8.1, CH), 6.79 (1H, dd, J 0.9 and 8.3, CH), 7.24 (1H, d, J 1.4 and 8.3, CH), 7.59 (1H, dd, J 1.4 and 8.1, CH); δ_C (100 MHz, CDCl₃/CDOD, 400 MHz) 44.5, 80.7, 158.4, 172.8; m/z (ES⁻): found (M+Na⁺) 340.0963. C₁₅H₂₀N₄O₅ requires M, 336.1433.

N-(2-Aminobenzamido)triglycine hydrochloride (3). N-(2-Aminobenzamido)triglycine ethyl ester, 2 (0.10 g, 0.30 mmol) was stirred in HCl (1 M, 3 ml) at 40 °C for 7 h. The solvent was removed in vacuo to give the product (0.13 g, quant.) as a fine cream powder. Rf 0.40 (methanol–ethyl acetate 9 : 1); mp 128–130 °C (Lit² 128–130 °C); v_{\text{max}} (400 MHz, CDOD) 1.49 (9H, s, Bu), 3.79 (2H, s, CH₂), 3.97 (2H, s, CH); δ_C (100 MHz, CDOD) 28.7, 41.7, 44.5, 80.7, 158.4, 172.8, 172.9; m/z (FAB+) 233 (14%, M + H⁺), 177 (53), 73 (61), 69 (64) and 57 (100) (HRMS: found (M+H⁺) 233.1139; C₁₅H₂₀N₄O₅ requires M 233.1137).

N-[2-(4-Dinitrophenyl)ethyl]amine|Boc-diglycine (5). N-Boc-diglycine, 4 (0.46 g, 1.98 mmol) was dissolved in dry DMF (4 ml). PyBOP (1.03 g, 1.98 mmol) and DIPEA (0.34 ml, 1.98 mmol) were added and the yellow solution was stirred for 3 minutes. EDDnp (0.52 g, 1.98 mmol) was added and the resulting orange solution was stirred for 20 h, under nitrogen, at room temperature. The solvent was removed in vacuo and the residue was partitioned between ethyl acetate and water. The organic layer was washed with brine (2 × 5 ml) and water (2 × 5 ml) to give the impure product. This was evaporated onto silica (1 g) and purified by column chromatography (silica, ethyl acetate–methanol 9 : 1) to give the product (0.42 g, 48%) as a bright yellow powder. Rf 0.38 (ethyl acetate–methanol 9 : 1); mp 106–110 °C; v_{\text{max}} (solid)/cm⁻¹ 3360w, 3281w, 1670m, 1614s, 1583m, 1522s; δ_C (CH₂OH)/nm 210 (ε/\text{dm}⁻³ mol⁻¹ cm⁻¹ 15 800), 259 (9 600), 348 (18 100) and 405sh (6 900); δ_H (D₂O/CDOD, 400 MHz) 1.43 (9H, s, Bu), 3.59 (2H, t, J 5.8, CH₂CH₂), 3.70 (2H, t, J 5.8, CH₂CH₂), 3.78 (2H, s, CH), 3.91 (2H, s, CH), 7.26 (1H, d, J 9.6, CH), 8.35 (1H, dd, J 2.7 and 9.6), 9.06 (1H, d, J 2.7, CH); δ_C (100 MHz, D₂O/CDOD) 28.5 (CH₃), 38.7 (CH₃), 43.3 (CH₃), 43.8 (CH₃), 44.9 (CH₃), 79.7 (C), 115.8 (CH), 124.3 (CH₃), 130.7 (CH), 131.4 (C), 136.5 (C), 149.7 (C), 157.4 (C=O), 170.7 (C=O), 170.8 (C=O); m/z (FAB+) 463 (15%, M + Na⁺), 308 (22), 169 (53), 100 (25), 73 (51), 69 (100) and 65 (36) (HRMS: found (M+Na⁺) 463.1561. C₁₇H₁₈N₄O₅ requires M 463.1553).

N-[2-(4-Dinitrophenyl)ethyl]amine|diglycine trifluoroacetate (6). N-[2-(4-Dinitrophenyl)ethyl]amine|diglycine, 5 (2.36 g, 5.36 mmol) was mixed in DCM (1 : 1, 12 ml) for 3 h, under nitrogen, at room temperature. The solvent was removed in vacuo, using methanol as a co-evaporant, to give the product (2.37 g, 97%) as a bright yellow powder. Rf 0.04 (methanol–ethyl acetate 1 : 1); RP-HPLC (18% B over 8 min); Rₚ 6.76 min; mp 151–153 °C; v_{\text{max}} (solid)/cm⁻¹ 3355w, 3283w, 2988w, 2899w, 1767s, 1615s, 1582m, 1566m, 1521s; δ_C (H₂O)/nm 204sh (ε/\text{dm}⁻³ mol⁻¹ cm⁻¹ 21 000), 265 (10 200), 360 (18 700) and 413sh (8 200); δ_H (CDOD, 400 MHz) 3.60 (2H, t, J 5.8, CH₂CH₂), 3.67 (2H, t, J 5.8, CH₂CH₂), 3.67 (2H, s, CH₂), 3.87 (2H, s, CH₂), 3.98 (2H, s, CH₂), 7.25 (1H, d, J 9.7, CH), 8.32 (1H, dd, J 2.7 and 9.7, CH), 8.99 (1H, d, J 2.7, CH); δ_C (100 MHz, CDOD/CD₂O) 33.9 (CH₂), 41.4 (CH₃), 43.3 (CH₃), 43.4 (CH₃), 115.6 (CH₂), 125.2 (CH), 130.9 (C), 131.3 (CH), 136.4 (C), 149.8 (C), 168.5 (C=O), 172.5 (C=O); m/z (ES⁻): found (M+H⁺) 341.1243. C₁₂H₁₄N₁O₈ requires M 341.1210.

N-(2-Aminobenzoyl)pentaglycine|N-[2-(4-dinitrophenyl)ethyl]amine (7). N-(2-Aminobenzoyl)pentaglycine, 3 (0.91 g, 2.65 mmol) was added to N-(2,4-dinitrophenyl)ethylamine...
diame
diglycine trifluoroacetate, 6 (1.20 g, 2.65 mmol) in DMF (50 ml). DIPEA (1.4 ml, 8.00 mmol) and EDCI (0.51 g, 2.65 mmol) were added, and the yellow solution was stirred for 15 h, under nitrogen, at room temperature. The solvent was removed in vacuo to give a yellow solid. Ethanol and water (1: 1, 160 ml total volume) were added to this, and the precipitate that formed was collected by filtration on a sintered glass funnel, and washed with water and ethanol to give the product (1.00 g, 61%) as an orange-yellow powder. 

This powder was stirred in TFA–DCM (1 : 2, 2 ml) for 30 min, under nitrogen, at room temperature. The solvent was removed in vacuo, using methanol as a co-evaporant, to give the crude product. A sample of the crude solid (0.078 g) was purified by column chromatography (reverse-phase silica, acetonitrile–methanol 1 : 4) as an orange powder. 

Fmoc-L-lysine[Boc]–[(2,4-dinitrophenyl)ethylene diamine] trifluoroacetate (8). A solution of di-tert-butyl dicarbonate (1.00 g, 4.58 mmol) in dioxane (4 ml) was added to an ice cold stirred solution of triglycine (0.80 g, 4.23 mmol) in sodium hydroxide (1 M, 12 ml). The suspension was stirred at 5 °C for 30 min, then allowed to warm to room temperature and was stirred for 15 h, under nitrogen. The suspension was concentrated to half its original volume by evaporation in vacuo, cooled in an ice bath and the remaining aqueous solution was adjusted to pH 2 by addition of HCl (1 M). The solution was extracted with ethyl acetate (3 × 6 ml) and the acidification–extraction process was repeated with the remaining aqueous layer. The organic washings were combined and dried over magnesium sulfate. The solvent was removed in vacuo to give the product as a white powder (0.50 g, 41%). Rf 0.26 (methanol–ethyl acetate 1 : 1); mp 129–130 °C (Lit.188–90 °C); δf (400 MHz, CD,OD) 1.49 (9H, s, 'Bu), 3.78 (2H, s, CH2), 3.97 (4H, s, 2 CH2); δf (100 MHz, CD,OD) 28.7, 41.7, 43.3, 44.9, 81.0, 158.7, 172.1, 172.9, 173.1; m/z (ES+) : found (M+H)+ 290.1376. C34H40N6NaO9 requires M, 290.1359.

N-[N-(2,4-dinitrophenyl)ethylene diamine]Boc-triglycine (0.26 g, 48%) as an orange powder. 

This powder was stirred in TFA–DCM (1 : 2, 2 ml) for 30 min, under nitrogen, at room temperature. The solvent was removed in vacuo, using methanol as a co-evaporant, to give the crude product. A sample of the crude solid (0.078 g) was purified by column chromatography (reverse-phase silica, acetonitrile–methanol 1 : 4) as an orange powder. 

Fmoc-t-lysine[Boc]–[(2,4-dinitrophenyl)ethylene diamine] (9). Fmoc-L-lysine[Boc]-OH (0.50 g, 1.07 mmol) was dissolved in dry dimethylformamide (2 ml). PyBOP (0.57 g, 1.10 mmol) and DIPEA (0.20 ml, 1.09 mmol) were added, and the pale yellow solution was stirred, under nitrogen, for 3 min. EDDnp (0.32 g, 1.22 mmol) was added, and the resulting orange solution was stirred, at room temperature, under nitrogen, for 22 h. The solvent was removed in vacuo and water (50 ml) was added and the yellow solid was collected by filtration. This was washed with ethanol (20 ml) and water (20 ml) to give the product (0.55 g, 76%) as a yellow powder. 

Fmoc-t-lysine[Boc]–OH (0.50 g, 1.07 mmol) was dissolved in dry dimethylformamide (2 ml). PyBOP (0.57 g, 1.10 mmol) and DIPEA (0.20 ml, 1.09 mmol) were added, and the pale yellow solution was stirred, under nitrogen, for 3 min. EDDnp (0.32 g, 1.22 mmol) was added, and the resulting orange solution was stirred, at room temperature, under nitrogen, for 22 h. The solvent was removed in vacuo and water (50 ml) was added and the yellow solid was collected by filtration. This was washed with ethanol (20 ml) and water (20 ml) to give the product (0.55 g, 76%) as a yellow powder. 

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Fmoc-t-lysine[Boc]–OH (0.50 g, 1.07 mmol) was dissolved in dry dimethylformamide (2 ml). PyBOP (0.57 g, 1.10 mmol) and DIPEA (0.20 ml, 1.09 mmol) were added, and the pale yellow solution was stirred, under nitrogen, for 3 min. EDDnp (0.32 g, 1.22 mmol) was added, and the resulting orange solution was stirred, at room temperature, under nitrogen, for 22 h. The solvent was removed in vacuo and water (50 ml) was added and the yellow solid was collected by filtration. This was washed with ethanol (20 ml) and water (20 ml) to give the product (0.55 g, 76%) as a yellow powder. 

Fmoc-t-lysine[Boc]–OH (0.50 g, 1.07 mmol) was dissolved in dry dimethylformamide (2 ml). PyBOP (0.57 g, 1.10 mmol) and DIPEA (0.20 ml, 1.09 mmol) were added, and the pale yellow solution was stirred, under nitrogen, for 3 min. EDDnp (0.32 g, 1.22 mmol) was added, and the resulting orange solution was stirred, at room temperature, under nitrogen, for 22 h. The solvent was removed in vacuo and water (50 ml) was added and the yellow solid was collected by filtration. This was washed with ethanol (20 ml) and water (20 ml) to give the product (0.55 g, 76%) as a yellow powder.
Fmoc-t-lysine-(N-ε-N'-Boc-diglycine)-[N-(2,4-dinitrophenyl)ethylenediame] (11). TBTU (0.74 g, 2.32 mmol), HOBt (0.16 g, 0.28 mmol) and N-Boc-glycylglycine, 4 (0.54 g, 2.32 mmol) were dissolved in DMF (6 ml) and DIPEA (0.8 ml, 4.64 mmol) was added. The mixture was allowed to stand for 3 min, until it had become a pale yellow solution, and was then added to a solution of Fmoc-t-lysine-[N-(2,4-dinitrophenyl)ethylenediame] trifluoroacetic acid, 10 (0.40 g, 0.56 mmol) in DMF (4 ml). The resultant yellow solution was stirred, under nitrogen, at room temperature for 2 h. The solvent was removed in vacuo, using methanol as a co-evaporant, to give the product as a yellow powder (0.15 g, quant.). The residue was washed with water (25 ml) and dried under vacuum to yield a yellow powder (0.01 g, 0.03 mmol) in dry DMF (0.5 ml) was added to a solution of Fmoc-t-lysine-glycylglycine-[N-(2,4-dinitrophenyl)ethylenediame] trifluoroacetic acid, 12 (0.025 g, 0.03 mmol) in dry DMF (0.5 ml). A solution of DIPEA (16 μl, 0.09 mmol) and EDCl (0.006 g, 0.03 mmol) in dry DMF (0.5 ml) was added and the yellow solution was stirred for 19 h, under a nitrogen atmosphere, at room temperature. The solvent was removed in vacuo and water (25 ml) was added to the solid residue. The resultant suspension was filtered and washed with water (25 ml). The residue was dried to give the protected peptide as a fine yellow powder (0.019 g, 64%). Rf 0.28 (1 : 1 methanol–ethyl acetate); f 0.68 (1 : 9 methanol–ethyl acetate); f 0.24 (reverse-phase silica, methanol); mp 186–188 °C (decomp); Rf (KBr) 3301 (broad, s), 3087w, 2934w, 1651v, 1620s, 1586m, 1525s; λmax (CHCl3)/nm 258 (ε/dm3mol−1cm−1 26 800), 356 (18 500), 409sh (8000); δmax (CDCl3) 1.72 (2H, broad m, CH2), 1.36 (2H, m, CH2), 1.54 (2H, broad m, CH2), 3.02 (2H, broad s, CH2), 3.38 (under water peak, CH2), 3.57 (2H, m, CH2), 3.67 (2H, d, CH2), 4.23 (3H, m, CH3), 6.43 (2H, s, NH2), 7.43 (5H, m, NH and CH2), 7.60 (2H, m, CH and 4N), 8.19 (1H, t, CH), 8.20 (5H, m, CH and CH2), 8.50 (1H, t, J 5.6, NH), 8.50 (1H, t, J 7.6, CH), 7.14 (1H, J 7.6, CH), 7.33 (3H, m, 2 CH and NH), 7.90 (2H, d, J 7.6, 2 CH), 7.43 (2H, J 7.6, 2 CH), 7.75 (2H, broad d, NH), 7.56 (2H, J 7.6, CH), 7.75 (3H, m, 2 CH and NH), 7.90 (2H, J 7.6, 2 CH), 8.09 (1H, t, J 5.6, NH), 8.20 (5H, m, 2 CH and 4N), 8.50 (1H, t, J 5.6, NH), 8.85 (1H, d, J 2.8, CH), 8.90 (1H, t, J 5.8, NH); m/z (ES−): found (M+Na)+ 981.3941. C18H16N4NaO3 requires M 981.3855.

1-Lysine-(N-ε-pentaglycine)-(N-2-amino-benzoyl) ![N-(2,4-dinitrophenyl)ethylenediame] (14). Fmoc-t-lysine-pentaglycine(N-2-amino-benzoyl)-[N-(2,4-dinitrophenyl)ethylenediame], 13 (0.20 g, 0.2 mmol) was stirred in a solution of piperidine in DMF (20%, 10 ml) for 45 min, during this time a further aliquot of piperidine (0.4 ml) was added. The solvent was removed in vacuo and the residue was triturated with ether (3 × 25 ml) and washed with water (2 × 5 ml). Following lyophilisation, the product was obtained as a yellow powder (0.15 g, quant.). Rf 0.70 (reverse-phase silica, methanol); mp 188–190 °C (decomp); [α]D20 (c = 0.57, DMSO) −7.0; λmax (KBr) 3296 (broad, s), 3087w, 2937w, 1648s, 1610s, 1590m, 1550s, 1525s; λmax (H2O)/nm 248 (ε/dm3mol−1cm−1 15 700), 359 (16 000), 415sh (6000); δmax (CDCl3) 1.25 (2H, m, CH2), 1.35 (2H, m, CH2), 1.44 (1H, m, 1H of CH2), 1.58 (1H, m, 1H of CH2), 3.01 (2H, m, CH2), 3.30 (1H, m, CH), 3.38 (under water peak, CH2), 3.59 (2H, m, CH2), 3.67 (2H, d, J 4.4, CH2), 3.74 (2H, J 4.4, CH2), 3.78 (4H, m, 2 CH), 3.88 (2H, d, J 4.4, CH2), 6.43 (2H, s, NH2), 6.52 (1H, d, J 7.6, CH), 6.70 (1H, d, J 7.6, CH), 7.16 (1H, dd, J 7.6, CH), 7.33 (1H, d, J 9.8, CH), 7.56 (1H, d, J 7.6, CH).
7.74 (1H, t, 3.54, NH), 8.11 (1H, t, 3.58, NH), 8.21 (3H, m, 3
NH), 8.28 (1H, dd, 9.8, 2.8, CH), 8.39 (1H, m, NH), 8.51 (1H, t, 3.58, NH), 8.62 (1H, d, 2.8, CH), 8.93 (1H, t, 3.58, NH); δH (100 MHz, d, DMSO-D6) 22.5 (CH2), 29.0 (CH2), 33.4 (CH3), 37.5 (CH3), 38.5 (CH3), 42.1 (CH2), 42.2 (CH2), 42.2 (CH2), 42.3 (CH2), 42.6 (CH2), 42.8 (CH2), 54.0 (CH2), 114.1 (C), 114.7 (CH), 115.4 (CH), 116.7 (CH), 123.8 (CH), 128.5 (CH), 130.0 (C), 130.1 (CH), 132.1 (CH), 135.1 (C), 145.8 (C), 150.0 (C), 168.6 (C=O), 169.2 (C=O), 169.4 (C=O), 169.5 (C=O), 169.6 (C=O), 170.0 (C=O); m/z (ES+) found: (M+H)+ 759.3204.

Boc-L-lysine(N-ε-pentaglycyl-DABCYL) (15). A solid phase protocol was used for the synthesis of this compound. The first residue was loaded onto the resin as follows: DCM (2 ml) was added to Boc-L-lysine(Fmoc)-OH (0.234 g, 0.5 mmol) and 2-chlorotritylchloride resin (0.417 g, 0.5 mmol). DIPEA (131 µl, 0.75 mmol) was added and the suspension was stirred for 15 h.

The resulting orange suspension was stirred under a nitrogen atmosphere, for 40 min, after which time it was filtered and subsequently washed with DCM and methanol (ca. 10 ml of each), before drying in vacuo. The resin was suspended in water (3 ml) and centrifuged, at 14 000 rpm, in eppendorfs (1.5 ml) four times. The solid was lyophilised to give a deep purple powder (0.041 g). Analysis by TLC indicated that both the starting material (EDANS sodium salt) and the desired product were present. The crude product was dissolved in DMF (10 ml) and loaded onto preparative silica plates. Preparative TLC (silica, 1 : 2 methanol-ethyl acetate) was performed twice and the product removed by dissolution in DMF. The solvent was removed in vacuo and the residue was lyophilised to yield N-Boc-L-lysine(N-ε-pentaglycyl-DABCYL)-EDANS, 16 (0.037 g) as a deep purple powder, contaminated with silica. Rf 0.84 (1 : 2 methanol-ethyl acetate); m/z (ES+) found: (M+H)+ 1031.4448, C43H54N12NaO10S requires M 953.3704.

1-Lysine(N-ε-pentaglycyl-DABCYL)-EDANS (17). Boc-L-lysine(N-ε-pentaglycyl-DABCYL), 15 (0.035 g, 0.044 mmol) was dissolved in dry DMF (10 ml). A solution of EDCI (0.022 g, 0.12 mmol) and HOBt (0.018 g, 0.12 mmol) in dry DMF (8 ml) was added; this was followed by the addition of a solution of EDANS sodium salt (0.028 g, 0.096 mmol) in dry DMF (10 ml). The resulting orange suspension was stirred under a nitrogen atmosphere, at room temperature, for 20 h. The solvent was removed in vacuo and the dark red residue was suspended in water (60 ml) and centrifuged three times, at 4800 rpm. The solid was then suspended in water (3 ml) and centrifuged, at 14 000 rpm, in eppendorfs (1.5 ml) four times. The solid was lyophilised to give a deep purple powder (0.041 g). Analysis by TLC indicated that both the starting material (EDANS sodium salt) and the desired product were present. The crude product was dissolved in DMF (10 ml) and loaded onto preparative silica plates. Preparative TLC (silica, 1 : 2 methanol-ethyl acetate) was performed twice and the product removed by dissolution in DMF. The solvent was removed in vacuo and the residue was lyophilised to yield N-Boc-L-lysine(N-ε-pentaglycyl-DABCYL)-EDANS, 16 (0.037 g) as a deep purple powder, contaminated with silica. Rf 0.84 (1 : 2 methanol-ethyl acetate); m/z (ES+) found: (M+H)+ 1031.4448, C43H54N12NaO10S requires M 953.3704.
(20 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 5 mM imidazole, 1 mM PMSF) and then disrupted mechanically by French Press. Two successive centrifugations, 30 and 20 min, at 18 000 g were used to clear the cell lysate of insoluble cell debris ready for chromatography. The column was charged with zinc(ii) using a 24 ml injection of 50 mM ZnCl₂, equilibrated in buffer A (20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl, 5% (v/v) glycerol, 5 mM imidazole), then buffer B (20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl, 5% (v/v) glycerol, 0.5 M imidazole) and finally buffer A again followed by 15 ml filtered dH₂O to clear all precipitated Zn salts. Solid NaCl was added to the cleared cell lysate to a final concentration of 0.5 M and the sample applied to the column at a flow-rate of 1 ml min⁻¹. The loaded column was washed in 5% buffer B (95% buffer A) until no unbound proteins eluted from the column. Bound proteins were eluted from the column using a linear gradient of 5–85% buffer B in a total volume of 40 ml. Fractions containing the required protein, identified by SDS-PAGE, were pooled and dialysed overnight (4 °C) in the appropriate buffer for ready ion-exchange chromatography. His-tagged lysostaphin was diluted 5-fold into cold buffer cexA (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 5% (v/v) glycerol) and dialysed in buffer cexA overnight (4 °C).

After immobilised metal-affinity chromatography, His-tagged lysostaphin was subjected to cation-exchange chromatography. A 5 ml HiTRAP SP column (Pharmacia Biotech) was used. The column was equilibrated in buffer cexA (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 5% (v/v) glycerol), then buffer cexB (10 mM Tris-HCl pH 7.4, 1 M NaCl, 5% (v/v) glycerol) and finally buffer cexA again. Lysostaphin in buffer cexA was loaded on to the column at a flow-rate of 1 ml min⁻¹ and the column washed with 25 ml buffer cexA. Protein bound to the column was eluted using a linear gradient of 0–100% buffer cexB in a volume of 60 ml. Fractions containing only the required protein were identified using SDS-PAGE and stored at −80 °C.

**FRET assays**

Fluorescence data were collected with the timedrive function of a Perkin-Elmer LS55 Luminescence Spectrometer using 4 nm excitation and emission slits and a 0.8 ml quartz fluorimeter cuvette (pathlength 0.5 cm) in a thermostatted cell-holder at 37 °C. A solution of substrate was made up from stock DMSO solutions into 50 mM sodium phosphate buffer, pH 7.0, such that the DMSO content was <1% v/v. The reaction volume was 0.70 ml. The buffer–substrate solution was allowed to equilibrate for 10 minutes prior to addition of lysostaphin in a water bath at 37 °C. The buffer solution used was 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0 and was filtered using Minisart syringe filters (0.45 μm). Lysostaphin from a frozen stock was dialysed overnight into the reaction buffer, using Sigma Dialysis Tubing (cellulose membrane) and was centrifuged at 4 °C in a Sigma 3K30 centrifuge, at 12 000 rpm, before use. Determination of [lysostaphin]ₜₙᵢ₅ was performed by measuring the UV absorbance, at 280 nm, on a Cary 100 Bio UV–visible spectrophotometer. Calculation of the concentration of substrate used was performed by measurement of the absorbance of the substrate at 359 nm (Abz–EDDnp substrates) or 453 nm (DABCYL–EDANS), using a quartz cuvette (1 cm pathlength). The values obtained were divided by the extinction coefficients of the quencher groups: 17 700 mol⁻¹ dm³ cm⁻¹ and 17 800 mol⁻¹ dm³ cm⁻¹ for EDDnp and DABCYL respectively. The absorbance at the excitation and emission wavelengths were measured, at a temperature of 37 °C, maintained by a circulating water bath, so that the inner filter effect could be calibrated for at the temperature at which the assays were performed. The signal bandwidth used for these measurements was 4 nm. The excitation and emission wavelengths were as follows: 325 nm and 420 nm respectively for Abz–EDDnp assays and 335 nm and 485 nm respectively for DABCYL–EDANS assays.

**Assays with 7, 14 and 17**

A solution of substrate was made up from stock DMSO solutions into sodium phosphate buffer such that the DMSO content was <1%. (In the case of 17 the buffer contained 12 mM CHAPS and the overall DMSO content was <3%). This solution was sufficient for 4 assays to be performed and for a sample to be used to determine the concentration of substrate present and the absorbance values required for inner filter effect correction, as described in the previous section. Three assays, at least 5 min in duration, were performed using this solution. The concentration of lysostaphin was 0.5 μM and the consumption of substrate was <1% during the assay period. Once assays for the first concentration were complete, a solution of the substrate at the next concentration to be investigated was prepared and the procedure was repeated.

**Analysis of the cleavage site of 7**

**Mass spectrometry.** A sample of lyophilised lysostaphin was dissolved in ammonium bicarbonate (100 mM, 200 μl) and its concentration determined to be 52.9 μM by measuring the absorbance of the solution at 280 nm. The stock lysostaphin solution (151 μl) and an aliquot of a stock solution of 7 in DMSO (7.0 mM, 8.0 μl) was added to a solution of ammonium bicarbonate so that the final volume was 800 μl. The concentrations of 7 and lysostaphin were 70 μM and 10 μM respectively. The solution was stored at 37 °C in an incubator for 8 h and was then lyophilised for 60 h. A sample of the resultant pale yellow residue was analysed by mass spectrometry using a VG LCT (electrospray, ES⁺ and ES⁻).

**RP-HPLC**

Reverse-phase high performance liquid chromatography (RP-HPLC) was performed analytically using the instrumentation, column and solvents described in the General section. The eluent was monitored by UV absorbance at 220 nm and 359 nm. Solutions of 6 and 8 were prepared in solvent A and injected onto the column. The solvent elution program was modified until the peaks were baseline separated and could be clearly distinguished. The protocol used was: 18% B for 8 min, 18–100% B linearly over 2 min, 100% B for 2 min, 100–18% B linearly over 2 min then 18% B for 2 min. The retention times were: 6.18 min for 6 and 6.76 min for 8. A sample from the residue produced by digestion, as described in the previous section, was dissolved in solvent A, injected onto the column and eluted using the protocol outlined above. The area of the two peaks at the retention times for 6 and 8 was calculated with ChemStation and the two values compared.
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