Search and rescue: tackling antibiotic resistance with chemistry

Wade, N.

Chapter 4

Synthesis of FRET substrates for LspA activity assays

1. Introduction

Due to the emergence of multi-drug resistant pathogens, developing novel antimicrobials against gram-negative bacteria has become imperative. Gram-negative bacteria rely on a number of biological processes which are essential for optimal growth. Some of these pathways have received more attention than others, with many antibiotics working against the same targets.[1] In the search for new antibiotics, it is beneficial to tackle unexploited pathways.

The lipoprotein processing pathway is essential for the survival of gram-negative bacteria (**Figure 1**).[2] The several enzymes involved in this pathway take the lipoproteins through various processes on the inner membrane and subsequently translocate them to the outer membrane where they stay or are released.[3,4] One enzyme that contributes to this pathway is lipoprotein signal peptidase II (LspA), which cleaves a membrane-anchoring signal peptide from prolipoproteins at the so-called "lipobox", a $[LVI]^{-3}[ASTVI]^{-2}[GAS]^{-1}[C]^{+1}$ consensus sequence.[5] This is a vital step in the pathway and its inhibition leads to the accumulation of the prolipoprotein in the inner membrane.[6] The build-up of prolipoprotein and subsequent absence of mature lipoprotein caused by LspA inhibition is lethal to gram-negative bacteria, which makes this enzyme an attractive target for the development of new antibiotics.[7,8]

To aid the search for LspA-targeting compounds, it is essential to have reliable biochemical methods for quantifying the enzyme's activity as well as detecting inhibitors. Recently, two such assays were independently reported by the groups of Caffrey and Wolan, both of which utilize Förster resonance energy transfer (FRET) based substrates to observe the activity of LspA.[9,10] FRET occurs when a donor (fluorophore) and acceptor (quencher), typically present on opposing ends the same molecule, are within a suitable distance from each other (<10 nm) and have overlapping emission and excitation wavelengths respectively (known as a FRET pair).[11] A FRET substrate for a given proteolytic enzyme must contain both a donor and acceptor moiety, as well as have the ability to be cleaved by the enzyme of interest. When the FRET substrate is intact, the FRET pair are kept in close proximity, allowing for energy transfer from the donor to the acceptor (**Figure 2**). When the substrate is cleaved, the FRET pair move apart and the energy transfer can no longer occur, allowing the emission energy from the donor to be released as fluorescence which can be measured.[12]

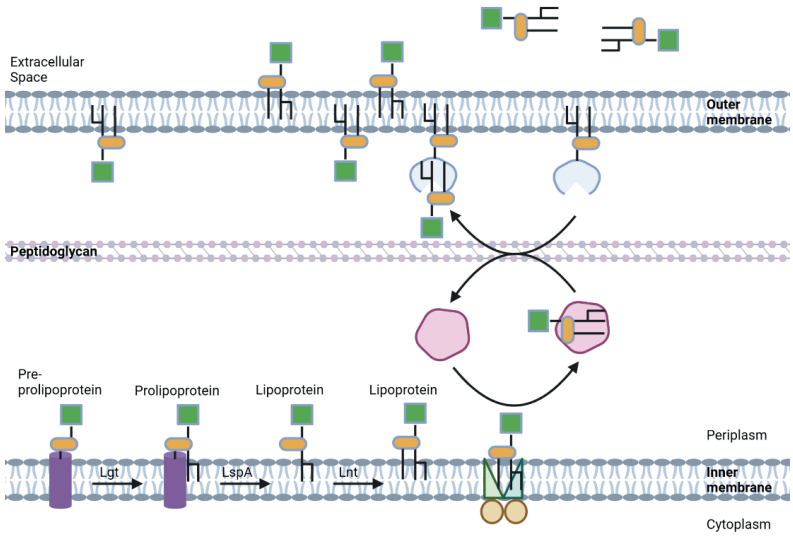


Figure 1. Lipoprotein processing pathway in gram-negative bacteria. Pre-prolipoprotein are acylated on the cysteine residue by Lgt to form the prolipoprotein, which is the substrate for LspA. LspA cleaves the signal peptide (purple) to produce the lipoprotein. The lipoprotein can then be acetylated by Lnt before being flipped to the outer membrane. Figure produced using BioRender.

While both the Caffrey and Wolan groups developed FRET-based assays for quantifying LspA activity and inhibition, the structures of the FRET substrates are different (**Figure 3**). Notably, different FRET pairs were used, with the Caffrey group using Abz/Tyr(3-NO_2) (Ex: 320 nm, Em: 420 nm) (**1**) while the Wolan group used the EDANS/DABSYL (Ex: 360 nm, Em: 490 nm) FRET pair (**2**). The source of LspA also differed between the two groups, which results in the enzymes recognizing slightly different lipobox sequences and therefore requiring FRET substrates containing distinct amino acids sequences. Caffrey and co-workers used LspA from *P. aeruginosa* which has an optimal lipobox recognition sequence of $L^{-3}A^{-2}G^{-1}C^{+1}$. By comparison, the Wolan group employed LspA from *E. coli*, and after several rounds of substrate optimization, the lipobox sequence of $V^{-3}T^{-2}G^{-1}C^{+1}$ was found to be best recognized. The sole common feature in both substrates is the presence of the cysteine with a thioether linkage to diacylglycerol (DAG), which in both cases was found to be essential for substrate recognition in LspA.[9,10]

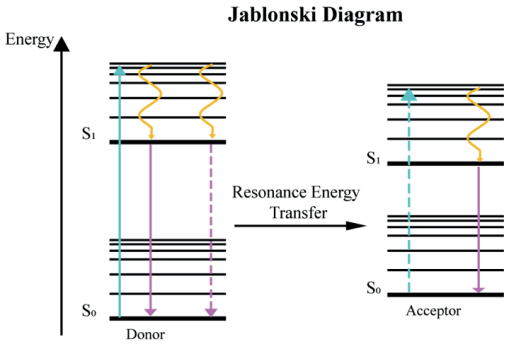


Figure 2. Jablonski diagram, showing the radiative and non-radiative transmissions between energy levels. Blue arrows show excitation from the ground state to excited state, yellow arrows signify vibrational relaxation, pink arrows represent release of fluorescence when energy moves from the excited state to the ground state. Dashed arrows signify resonance energy transfer from the donor (fluorophore) to the acceptor (quencher).

The Caffrey group used their FRET assay to characterize the half-maximal inhibitory concentration (IC_{50}) of two natural product inhibitors of LspA, globomycin and myxovirescin.[9] These macrocyclic antibiotics were discovered by searching microbial extracts for compounds which can inhibit the growth of bacteria.[13,14] Although they are incredibly potent inhibitors of LspA, with FRET assay IC_{50} values of 57 nM and 53 nM respectively against LspA from *P. aeruginosa*,[9] they are not as effective at killing bacteria with minimum inhibitory concentration (MIC) values in the µM range against wild type *E. coli*.[14] With this in mind, it is possible that other compounds which inhibit LspA may be overlooked in bacterial growth-based assay campaigns.

With the intention of searching for novel LspA inhibitors from microbial extracts, we initially elected to utilize the FRET assay protocol published by Caffrey and coworkers.[9] However, it is possible that some microbial extracts may contain compounds which interfere with the fluorescence readout of the assay. For this reason, we decided to prepare both the Caffrey group's original FRET substrate (**1**) as well as a variant bearing a different FRET pair so as to have two substrates at our disposal with different excitation and emission wavelengths. In designing the new substrate (**3**) we elected to use the same peptide sequence as for **1** but equipped with the EDANS/DABSYL FRET pair successfully used by the Wolan group (**Figure 4**). Once synthesized, both compounds were verified as substrates for the LspA by

Michaelis-Menten experiments and their performance in detecting inhibition was also assessed in test IC_{50} experiments using known LspA inhibitors.

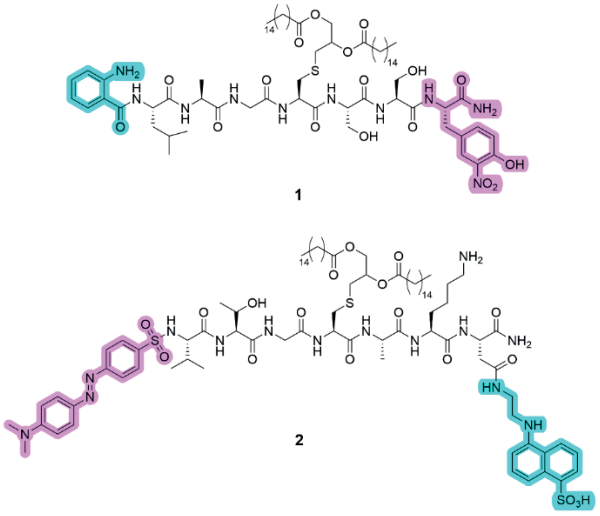


Figure 3. Structure **1** shows the substrate published by Olatunji et al. containing an Abz/Tyr(3-NO_2) FRET pair for use with LspA sourced from *P. aeruginosa*.[9] Compound **2** shows the structure of the substrate published by Kitamura et al. containing an EDANS/DABSYL FRET pair for use with LspA sourced from *E. coli*.[10] Fluorophores are highlighted in blue and the quenchers are highlighted with pink.

2. Results and discussion

2.1 Synthesis of the Fmoc-Cys[(*R*)Pam$_2$]-OH (6)

The building block used in both FRET substrates, Fmoc-Cys[(*R*)Pam$_2$]-OH (**7**), was synthesised according to previous literature with minor adjustments (**Scheme 1**).[9] The route starts with (Fmoc-Cys-O*t*Bu)$_2$ (**4**) which was reduced to the free-thiol cysteine using tributylphosphine (PBu_3). S-alkylation of the thiol, leading to compound **5**, was achieved by addition of (*R*)-(+)-glycidol by means of an epoxide ring-opening in the presence of DIPEA. According to the literature, the synthesis of diol **5** should be possible without purification between the two steps. However, significant Fmoc deprotection was observed during the S-alkylation reaction. After some experimentation, this undesired side-reaction was prevented by purifying the free thiol before the S-alkylation step, thus increasing the yield. A Steglich esterification of diol **5** with palmitic acid was achieved using EDC·HCl and catalytic amounts of DMAP to yield compound **6**. The carboxylic acid was freed by selective deprotection using

TFA which is necessary for the incorporation of compound **7** (Cys*) as a solid phase peptide synthesis (SPPS) building block.

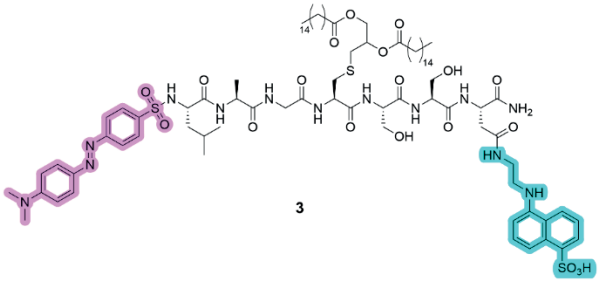
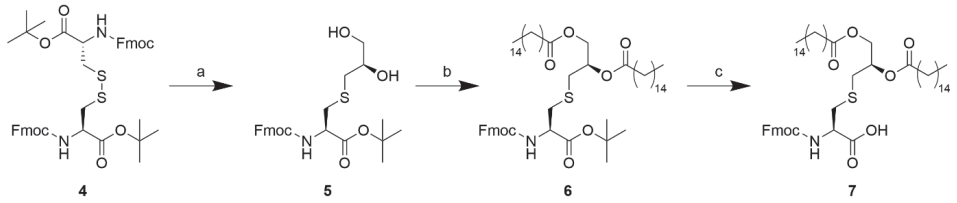


Figure 4. Proposed structure of a novel substrate **3** designed for LspA from *P. aeruginosa*. It contains the same lipobox sequence used in the original substrate structure (**1**) but utilizes the EDANS/DABSYL FRET pair present in compound **2**. The fluorophore is highlighted in blue, and the quencher is highlighted with pink.

2.2 Synthesis of FRET substrate 1

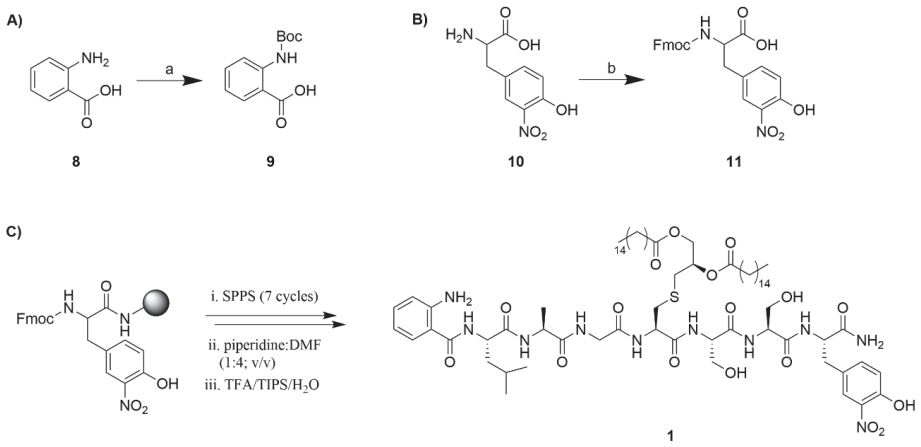
To produce peptide **1**, the FRET pair building blocks must be prepared and subsequently incorporated via SPPS. The fluorophore, 2-aminobenzoic acid (**8**), caps the *N*-terminus of the peptide. Therefore, the aniline was protected to stop polymerization during the final coupling step by using a *tert*-butyloxycarbonyl (Boc) group (**Scheme 2A**). To achieve this, 2-aminobenzoic acid was stirred with di-tert-butyl dicarbonate (Boc_2O) under basic conditions to give compound **9**. The quencher was then prepared for SPPS by Fmoc protecting the amine of commercially available Tyr(3-NO_2)-OH (**10**) (**Scheme 2B**). This was carried out under basic conditions with 9-fluorenylmethyl *N*-succinimidyl carbonate (Fmoc-OSu) to give the protected quencher, Fmoc-Tyr(3-NO_2)-OH (**11**).



Scheme 1. Reagents and Conditions: (a) i. PBu_3, H_2O, THF, rt, 1 h; ii. (*R*)-(+)-glycidol, DIPEA, DCM, rt to 40 °C, 20 h (52 %); (b) palmitic acid, EDC·HCl, DMAP, DCM, 0 °C to rt, 18 h (56 %); (c) TFA, TES, DCM, rt, 4 h (96 %).

With the necessary building blocks in hand, the manual SPPS of FRET substrate **1** was carried out. Rink amide AM resin (0.2 mmol) with a loading of 0.5 mmol g^{-1} was used as the solid support. Standard conditions used for this synthesis were piperidine/DMF (1:4; 5 mL; 2 x 10 min) for Fmoc deprotection and equimolar PyBOP/*N*-methylmorpholine (NMM) for coupling. After initial deprotection of the resin, 8 coupling/deprotections cycles were performed to build peptide **1** (**Scheme 2C**). The first amino acid coupled to the resin was Fmoc-Tyr(3-NO_2)-OH (**10**) followed by two serine residues, with LCMS monitoring confirming the successful couplings.

The addition of the Cys* residue and monitoring this reaction proved to be more challenging. When cleaved from the resin, the tetramer did not dissolve in any common solvents and could not be visualized via LCMS. Therefore, to assess this and all the subsequent couplings, the Kaiser test was employed which checks for the presence of primary amines indicated by an intense blue colour. In contrast, the lack of primary amines is revealed when the solution remains yellow.[15] For the amino acids following Cys*, it was observed that the couplings were not complete after the usual 1-hour reaction time, possibly due to steric hindrance caused by the two lipid tails of Cys*. Therefore, each amino acid was double coupled to ensure no deletions were caused in the peptide sequence.



Scheme 2. A) Synthesis of Boc-Abz-OH. Reagents and conditions: (a) NaOH, Boc_2O, THF, H_2O, rt, 16 h (82 %). B) Synthesis of Fmoc-Tyr(3-NO_2)-OH. Reagents and conditions: (b) Fmoc-OSu, Na_2CO_3, H_2O, acetone, rt, 16 h (85 %). C) Synthesis of FRET substrate **1**.

Although the N-terminus of the peptide sequence was capped with an acid labile Boc-group, a final deprotection reaction using 20% piperidine in DMF was performed. This was necessary as an unwanted side reaction involving activated amino acid and the unprotected hydroxyl group of the Tyr(3-NO_2) residue could occur during chain elongation. This was corrected by treatment with the basic deprotection conditions as this cleaved the unwanted ester by-product.

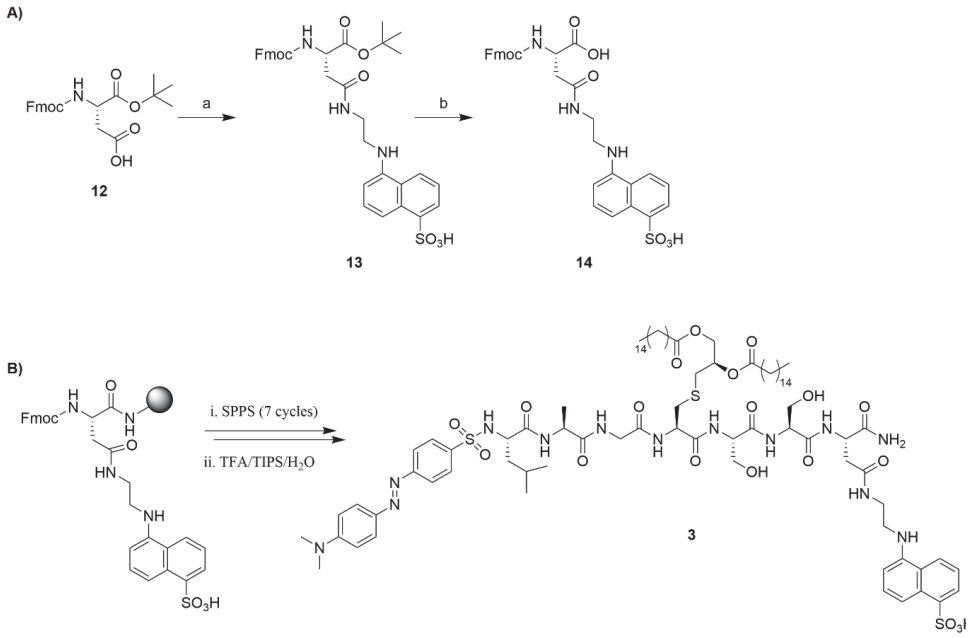
Global deprotection of the peptide and cleavage from the resin was achieved using TFA:TIPS:H_2O (95:2.5:2.5, v/v/v) before filtering to remove the resin beads. The solution was concentrated *in vacuo* and peptide **1** was precipitated by addition of cold Et_2O. The precipitate was washed with Et_2O (2 x 10 mL) and collected by centrifugation. Purification of the crude material was achieved using silica column chromatography (DCM:MeOH, 49:1 to 19:1) which afforded peptide **1** (22 mg, 8 %). Confirmation of product identity and purity assessment was carried out using NMR and MALDI. The NMR data (^1H and ^{13}C) was in agreement with data in the literature.[9]

2.3 Synthesis of FRET substrate 3

Peptide **3** contains a different FRET pair than peptide **1**, incorporating 5-(2-aminoethylamino)-1-napthalenesulfonic acid (EDANS) as the fluorophore and 4-(dimethylamino)azobenzene-4′-sulfonyl chloride (DABSYL) as the quencher. Kitamura et al. incorporated the fluorophore to the peptide by using EDANS attached to the side chain of an aspartic acid.[10] There is no reported synthesis of this compound, however there are several reports of EDANS being coupled to glutamic acid, so the protocol was modified (**Scheme 3A**).[16] The synthesis started with the coupling of EDANS·Na to Fmoc-Asp-O*t*Bu (**12**) using BOP and DIPEA under light-deprived conditions and an inert atmosphere. The resulting compound **13** was deprotected using TFA and TES to free the carboxylic acid giving compound **14**, which was ready for SPPS incorporation. The DABSYL group was incorporated in the peptide using the corresponding chloride, which was used without modification.

With the synthesis of FRET substrate **1** proving successful, the same protocol was used to build peptide **2** (**Scheme 3B**). Rink amide resin MHBA (0.1 mmol) with an initial loading of 0.67 mmol g^{-1} was used as the solid support. Standard coupling conditions of PyBOP/NMM were used and piperidine/DMF mix (1:4; 5 mL; 2 x 10 min) was used for deprotection. Again, the first three amino acid couplings were monitored by LCMS and the rest were assessed using the Kaiser test. Double couplings were performed for every amino acid added after the Cys*

residue was incorporated. Following global deprotection with TFA:TIPS:H2O (95:2.5:2.5 v/v/v), the peptide was recovered as described for peptide **1**. Silica column chromatography (DCM:MeOH 49:1 to 20:3) was used to purify the peptide and yielded FRET substrate **3** (13.3 mg, 8 %). Confirmation of product identity and purity assessment was carried out using NMR and MALDI (full NMR spectra found in supporting information S1 and S2).



Scheme 3. A) Synthesis of Fmoc-Asp(EDANS)-OH (**14**). Reagents and Conditions: (a) EDANS·Na, BOP, DIPEA, DCM, rt, 16 h (64 %); (b) TFA, TES, DCM, rt, 4 h (78 %). B) Synthesis route to produce FRET substrate **3**.

2.4 LspA expression and FRET assay

LspA-pET28a plasmid and a reference sample of FRET substrate **1** were kindly provided by the groups of Martin Caffrey and Eoin Scanlan (Trinity College Dublin) respectively. LspA from *P. aeruginosa* was expressed and purified from the plasmid and a control experiment was set up to test the performance of the enzyme. If the enzyme functions correctly, it will cleave the FRET substrate, resulting in a measurable increase in fluorescence at the fluorophore emission wavelength. Conversely, if the enzyme is inhibited, the substrate will not be cleaved, resulting in energy transfer from the fluorophore to the quencher and there will be no measurable increase in fluorescence. Referring to the Caffrey group's original publication, LspA (100 nM) and reference substrate **1** (30 μM) were mixed in a black 96-well plate and

fluorescence was read immediately on a Tecan Spark plate reader (Ex: 365(35) nm, Em: 480(20) nm). The combination resulted in a measurable increase in fluorescence relative to the negative controls. Confident that the enzyme was functioning, it was then used to test the two substrates, **1** and **3**, prepared in our lab.

The activity of peptide **1** was assessed using the same conditions reported in literature resulting in K_m and V_{max} values as determined by the Michaelis-Menten model in line with expectation (K_m = 25.9 μM, V_{max} = 31.5 μM/sec, see **Figure 5A)**.

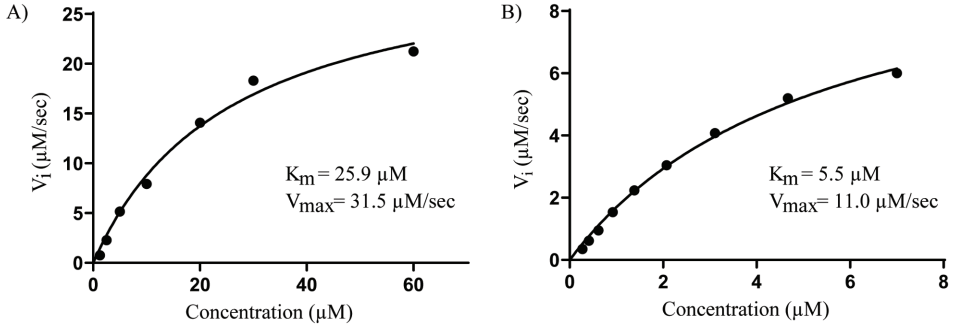


Figure 5. The Michaelis-Menten plots showing the K_m and V_{max} values calculated with GraphPad Prism 9. A) Michaelis-Menten plot for FRET substrate **1** with LspA (50 nM). B) Michaelis-Menten plot for FRET substrate **3** with LspA (50 nM).

More effort was required to validate **3** as a substrate for LspA. It was first determined whether incubation of substrate **3** with LspA would result in cleavage of the peptide. A serial dilution of compound **3** ranging from 40 μM to 1.25 μM was prepared in triplicate in a black 96-well plate. LspA (50 nM, 25 μL) was added to each well and the change in fluorescence was measured in a Tecan Spark plate reader (Ex: 365(35) nm, Em: 480(20) nm). At substrate concentrations <10 μM, the enzyme cleaves the substrate as shown by an increase in the fluorescence readout. Interestingly, at the higher concentrations tested (20 μM and 40 μM), there was an initial increase in fluorescence followed by a swift decrease and subsequent plateau of the fluorescence readout for the rest of the measurement. This suggests the substrate is cleaved at the start of the experiment, but the fluorophore is quenched as the experiment continues. A possible explanation for this phenomenon is intermolecular FRET diffusion which has been previously observed for EDANS and DABSYL.[17] Given that FRET is dependent on the distance between the donor and quencher, it does not strictly require the pair to be attached

to the same molecule (this is known as intramolecular FRET). It would appear that at concentrations >10 μM, the EDANS fragments are present in high enough concentration to interact with the DABSYL moiety (<10 nm), allowing FRET to occur continuously. A Michaelis-Menten experiment was therefore carried out at concentrations <10 μM to allow for an observable increase in fluorescence (**Figure 5B**). The K_m and V_{max} values of this substrate were determined to be 5.5 μM and 11.0 μM/sec respectively.

With the K_m values for both FRET substrates known, **1** and **3** could then be used for the determination of IC_{50} values of potential LspA inhibitors. Pepstatin A, a known inhibitor of aspartyl protease enzymes,[18–20] was first tested with FRET substrates **1** and **3**. Both were found to give results in good agreement, with IC_{50} values measured as 27.6 μM and 28.3 μM for peptide **1** and **3** respectively (**Figure 6**). Preliminary studies with globomycin, a known tight-binding inhibitor of LspA,[9] were also conducted to assess the performance of both **1** and **3**. However, the reported IC_{50} value of globomycin with LspA is lower than the concentration of enzyme used within our experiment. To determine the true IC_{50} value, more experiments would be required where the substrate concentration is increased to out compete globomycin.[21] Due to time constrictions, these extra experiments were not performed, however, a singular experiment did indeed indicate that globomycin is a tight-binder with a low nanomolar IC_{50} for both substrates (data not shown).

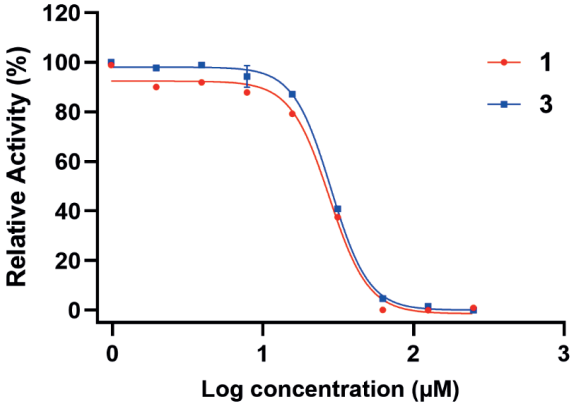


Figure 6. IC_{50} curves of Pepstatin A against LspA (50 nM) using substrates **1** and **3.**

3. Conclusion

This chapter describes the synthesis and validation of two FRET substrates for *P. aeruginosa* LspA: known substrate **1** and a novel substrate utilizing the EDANS/DABSYL FRET pair, peptide **3**. The structures of both compounds were confirmed with NMR and MALDI and validated as substrates for LspA by performing Michaelis-Menten experiments. The IC_{50} value of pepstatin A against LspA was determined using both substrates, resulting in values significantly lower than that originally published in 1984 (peptide **1**: 27.6 µM, peptide **3**: 28.3 µM, original publication: 0.32 mM).[22] Both FRET substrates are ready to be used to search for novel LspA inhibitors from bacterial extracts.

4. Materials and Methods

4.1 General Procedures

All reagents used were of American Chemical Society (ACS) grade or finer and were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-400 MHz or AV-500 MHz. Michaelis-Menten and IC_{50} assays were performed on a Tecan Spark plate reader. High resolution mass spectrometry (HRMS) analyses were performed on a Shimadzu Nexera X2 UHPLC system. MALDI measurements were performed on a Axima Confidence MALDI TOF (Shimadzu). For full description of analytical methods, see Supporting Information.

4.2 Synthesis

tert-Butyl-N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-((R)-2,3-dihydroxypropyl)-L-cysteinate (5)

A solution of (Fmoc-Cys-O*t*Bu)$_2$ (1.00 g, 1.25 mmol, 1.0 eq) in anhydrous THF (10 mL) under argon was combined with PBu_3 (405 µL, 1.25 mmol, 1.0 eq) and allowed to react for 5 min followed by the addition of H_2O (1.5 mL). The reaction mixture was stirred at room temperature for 1 h. TLC analysis was performed to confirm complete consumption of the disulphide. Solvents were removed under reduced pressure and purification via silica column chromatography (EtOAc:PE 1:4 – 2:1) yielded a viscous oil. This material was dissolved in DCM (20 mL) and treated with (*R*)-(+)-glycidol (830 µL, 12.5 mmol, 4.7 eq) and DIPEA (480 µL, 2.76 mmol, 1.0 eq). The reaction was stirred at 40 °C for 20 h then washed with water (3 x 10 mL) and brine (3 x 10 mL), dried over Na_2SO_4 and filtered. Solvents were removed under

reduced pressure and purification via silica column chromatography (EtOAc:PE 1:1 – 4:1) resulted in a colourless oil (576 mg, 52 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.61 (d, J = 8.1 Hz, 2H, Fmoc-Ar), 7.40 (t, J = 7.5 Hz, 2H, Fmoc-Ar), 7.32 (tt, J = 7.5, 1.20 Hz, 2H, Fmoc-Ar), 5.86 (d, J = 8.2 Hz, 1H, NH), 4.58 – 4.52 (m, 1H, Cys-αCH), 4.40 (d, J = 7.6 Hz, 2H, Fmoc-CH_2), 4.23 (t, J = 7.1 Hz, 1H, Fmoc-CH), 3.84 – 3.78 (m, 1H, *S*-glyceryl-CH), 3.68 (dd, J = 11.3, 3.7 Hz, 1H, Cys-βC$\underline{H_a}H_b$), 3.52 (dd, J = 11.3, 6.0 Hz, 1H, Cys-βC$H_a\underline{H_b}$), 3.10 – 2.92 (m, 2H, *S*-glyceryl-*O*CH_2), 2.83 (d, J = 13.9 Hz, 1H, *S*-glyceryl- C$\underline{H_a}H_b$), 2.63 (dd, J = 14.1, 8.3 Hz, 1H, *S*-glyceryl-C$H_a\underline{H_b}$), 1.49 (s, 9H, *t*-Bu-CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7 (Cys C=O), 156.1 (Fmoc-NH), 143.7 (Fmoc-qC), 141.32 (Fmoc-qC), 127.8 (Fmoc-Ar-CH), 127.1 (Fmoc-Ar-CH), 125.1 (Fmoc-Ar-CH), 120.0 (Fmoc-Ar-CH), 83.2 (*t*-Bu-qC), 70.6 (*S*-glyceryl-CH), 67.2 (Fmoc-CH_2), 65.3 (*S*-glyceryl-*O*CH_2), 54.5 (Cys-αCH), 47.1 (Fmoc-CH), 36.8 (*S*-glyceryl-CH_2), 35.7 (Cys-βCH_2), 28.0 (*t*-Bu-CH_3); *m/z* HRMS (ESI$^+$) calculated for $C_{25}H_{32}NO_6S$ $[M+H]^+$ 474.1945, found 474.1949.

*(R)-3-(((R)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(tert-butoxy)-3-oxopropyl)thio)propane- 1,2-diyl dipalmitate (**6**)*

A solution of palmitic acid (877 mg, 3.42 mmol, 2.5 eq) in dry DCM (45 mL) under argon at 0 °C was reacted with EDC·HCl (656 mg, 3.42 mmol, 2.5 eq) and DMAP (33.0 mg, 0.27 mmol, 0.2 eq) for 1 h. The reaction mixture was then combined with a solution of compound **5** (643 mg, 1.36 mmol, 1.0 eq) in dry DCM (10.5 mL). The reaction was stirred at room temperature under argon for 18 h. Solvents were removed from the reaction mixture under reduced pressure and purification via silica column chromatography (EtOAc:PE 1:12 – 1:9; 1% Et_3N) yielded a white solid (637 mg, 56 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.65 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.43 (t, J = 7.5, 2H, Fmoc-Ar), 7.35 (td, J = 7.4, 1.1 Hz, 2H, Fmoc-Ar), 5.74 (d, J = 7.7 Hz, 1H, NH), 5.22 – 5.16 (m, 1H, *S*-glyceryl-CH), 4.54 (dt, J = 7.8, 5.0 Hz, 1H, Cys-αCH), 4.48 – 4.32 (m, 3H, Fmoc-CH_2, *S*-glyceryl-*O*C$\underline{H_a}H_b$), 4.27 (t, J = 7.6 Hz, 2H, Fmoc-CH), 4.19 (dd, J = 11.4, 5.6 Hz, *S*-glyceryl-*O*C$H_a\underline{H_b}$), 3.08 (qd, J = 13.7, 5.0 Hz, 2H, Cys-βC$\underline{H_a}H_b$, Cys-βC$H_a\underline{H_b}$), 2.80 (d, J = 6.5 Hz, 2H, *S*-glyceryl-CH_2), 2.34 (p, J = 7.5 Hz, 4H, Pal-αCH_2 × 2), 1.61 (qd, J = 7.1, 4.1 Hz, 4H, Pal-CH_2 × 2), 1.52 (s, 9H, *t*-Bu-CH_3), 1.39 – 1.24 (m, 48H, Pal-CH_2), 0.95 – 0.86 (m, 6H, Pal-CH_3 × 2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.4 (Pal

C=O), 173.1 (Pal C=O), 169.5 (Cys C=O), 155.8 (Fmoc C=O), 143.8 (Fmoc-qC), 141.3 (Fmoc-qC), 127.7 (Fmoc-Ar-CH), 127.1 (Fmoc-Ar-CH), 125.2 (Fmoc-Ar-CH), 120.0 (Fmoc-Ar-CH), 83.0 (*t*-Bu-qC), 70.3 (*S*-glyceryl-CH), 67.3 (Fmoc-CH_2), 63.5 (*S*-glyceryl-*O*CH_2), 54.4 (Cys-αCH), 47.1 (Fmoc-CH), 35.4 (Cys- βCH_2), 34.3 (Pal-CH_2), 34.1 (Pal-CH_2), 33.3 (*S*-glyceryl-CH_2), 32.0 (Pal-CH_2), 29.7 (Pal-CH_2), 29.7 (Pal-CH_2), 29.7 (Pal-CH_2), 29.5 (Pal-CH_2), 29.4 (Pal-CH_2), 29.3 (Pal-CH_2), 29.2 (Pal-CH_2) 29.1 (Pal-CH_2), 28.0 (*t*-Bu-CH_3), 24.9 (Pal-CH_2), 24.9 (Pal-CH_2), 22.7 (Pal-CH_2), 14.2 (Pal-CH_3); *Various methods were utilised to detect high resolution mass, however due to solubility issues the mass was not found.*

N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-((R)-2,3-bis(palmitoyloxy)propyl)-L-cysteine (7)

A stirred solution of compound **6** (567 mg, 0.60 mmol, 1.0 eq) in DCM (2.75 mL) was reacted with TES (460 µL, 1.46 mmol, 2.5 eq) and TFA (6.50 mL) at room temperature for 4 h. Solvents were removed from the reaction mixture under reduced pressure to yield the product as a white solid (520 mg, 96 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.64 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.43 (t, J = 7.4 Hz, 2H, Fmoc-Ar), 7.34 (t, J = 7.5 Hz, 2H, Fmoc-Ar), 5.83 (d, J = 7.9 Hz, 1H, NH), 5.23-5.15 (m, 1H, *S*-glyceryl-CH), 4.69 (q, J = 5.6 Hz, 1H, Cys- αCH), 4.43 (d, J = 7.3 Hz, 2H, Fmoc-CH_2), 4.38 (dd, J = 11.9, 3.4 Hz, 1H, *S*-glyceryl-*O*C$\underline{H_a}H_b$), 4.27 (t, J = 7.0 Hz, 1H, Fmoc-CH), 4.18 (qt, J = 13.2, 6.6 Hz, 1H, *S*-glyceryl-*O*$CH_a\underline{H_b}$), 3.25 – 3.06 (m, 2H, Cys-βC$\underline{H_a}H_b$, Cys-β$CH_a\underline{H_b}$), 2.80 (dd, J = 6.6, 3.0 Hz, 1H, *S*-glyceryl-CH_2), 2.34 (q, J = 7.7 Hz, 4H, Pal-αCH_2 × 2), 1.67 – 1.57 (m, 4H, Pal-CH_2 × 2), 1.38 – 1.30 (m, 9H, *t*-Bu-CH_3), 1.27 (q, J = 3.4 Hz, 48H, Pal-CH_2), 0.94 – 0.87 (m, 6H, Pal-CH_3 × 2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.7 (Pal C=O), 173.6 (Pal C=O), 173.5 (Cys C=O), 156.0 (Fmoc-C=O), 143.7 (Fmoc-qC), 141.3 (Fmoc-qC), 127.8 (Fmoc-Ar-CH), 127.1 (Fmoc-Ar-CH), 125.2 (Fmoc-Ar-CH), 120.0 (Fmoc-Ar-CH), 70.3 (*S*-glyceryl-CH), 67.5 (Fmoc-CH_2), 63.6 (*S*-glyceryl-*O*CH_2), 53.6 (Cys-αCH), 47.1 (Fmoc-CH), 34.6 (Cys-βCH_2), 34.3 (Pal- CH_2), 33.0 (*S*-glyceryl-CH_2), 32.0 (Pal-CH_2), 29.7 (Pal-CH_2), 29.7 (Pal-CH_2), 29.7 (Pal-CH_2), 29.5 (Pal-CH_2), 29.4 (Pal-CH_2), 29.3 (Pal-CH_2), 29.2 (Pal-CH_2), 29.1 (Pal-CH_2), 24.9 (Pal-CH_2), 24.9 (Pal-CH_2), 22.7 (Pal-CH_2), 14.2 (Pal-CH_3); *Various methods were utilised to detect high resolution mass, however due to solubility issues the mass was not found.*

*2-((tert-Butoxycarbonyl)amino)benzoic acid – Boc-Abz-OH (**9**)*

A solution of 2-aminobenzoic acid **8** (2.00 g, 14.6 mmol, 1 eq) in THF:H_2O (1:1, 25 mL) was adjusted to pH 10 with aq. NaOH solution (2 M) and reacted with Boc_2O (3.50 g, 16.0 mmol, 1 eq) at room temperature for 18 h. THF was removed under reduced pressure and the remaining aqueous solution was adjusted to pH 4 with a citric acid solution (10 % w/v). The resulting white precipitate was filtered, washed with water (3 x 20 mL), and redissolved in EtOAc (40 mL) then washed with water (3 x 35 mL) and brine (2 x 15 mL), dried over Mg_2SO_4 and filtered. Organic solvents were removed under reduced pressure to yield the product as a white solid (2.82 g, 82 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.04 (s, 1H, NH), 8.47 (d, J = 7.4 Hz, 1H, Ar-H), 8.12 (dd, J = 8.0, 1.6 Hz, 1H, Ar-H), 7.57 (ddd, J = 8.8, 7.3, 1.8 Hz, 1H, Ar-H), 7.04 (ddd, J = 8.2, 7.3, 1.1 Hz, 1H, Ar-H), 1.55 (s, 9H, *t*-Bu-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.3 (COOH), 152.8 (Boc C=O), 142.9 (Ar-C), 135.6 (Ar-C), 132.0 (Ar-C), 121.4 (Ar-C), 119.0 (Ar-C), 113.4 (Ar-C), 81.0 (Boc-qC), 28.3 (Boc-CH_3); *Various methods were utilised to detect high resolution mass, however due to solubility issues the mass was not found.*

*(S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-hydroxy-3-nitrophenyl)propanoic acid – (Fmoc-Tyr(3-NO_2)-OH) (**11**)*

At 0 °C, 3-nitrotyrosine **10** (2.00 g, 8.75 mmol, 1.0 eq) was suspended in H_2O (150 mL) and a solution of Fmoc-*O*Su (2.99 g, 8.86 mmol, 1.0 eq) in acetone (100 mL) was added. The reaction was adjusted to pH 9 by addition of aq. Na_2CO_3 (10% (w/v)) and stirred at room temperature for 18 h, forming a clear red solution. Acetone was removed from the reaction mixture *in vacuo* and the remaining aqueous solution washed with Et_2O (3 x 100 mL). By addition of 5 M HCl, the aqueous layer was adjusted to pH 3 resulting in the precipitation of an orange solid. The solid was extracted with Et_2O (5 x 50 mL) and combined organic layers washed with water (3 x 100 mL) and brine (3 x 100 mL), dried over Na_2SO_4 and filtered. The remaining organic solvent was removed under reduced pressure to yield a yellow solid as the product (3.39 g, 85 %). ^{1}H NMR (400 MHz, MeOD) δ 7.97 (d, J = 2.2 Hz, 1H, Tyr-Ar), 7.71 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.55 (dd, J = 9.9, 7.5 Hz, 2H, Fmoc-Ar), 7.46 (dd, J = 8.6, 2.2 Hz, 1H, Tyr-Ar), 7.38 – 7.38 (m, 4H, Fmoc-Ar × 2), 7.03 (d, J = 8.6 Hz, 1H, Tyr-Ar), 4.44 (dd, J = 9.7, 4.7 Hz, 1H, Tyr-αCH), 4.33 (dd, J = 10.5, 7.0 Hz, 1H, Fmoc-CH_aH_b), 4.22 (dd, J = 10.6, 6.8 Hz, 1H, Fmoc-CH_aH_b), 4.11 (q, J = 7.4 Hz, 1H, Fmoc-CH), 3.21 – 2.93 (m, 2H, Tyr-βCH_2); ^{13}C NMR (101 MHz, MeOD) δ 157.0 (Fmoc C=O), 153.0 (Tyr-qC-OH), 143.8 (Fmoc-qC), 141.1 (Fmoc-qC), 137.8 (Tyr-Ar-CH), 133.8 (Tyr-qC), 127.4 (Fmoc-Ar-CH), 126.7 (Tyr-Ar-CH), 125.1

(Fmoc-Ar-CH), 124.8 (Tyr-Ar-CH), 124.7 (Fmoc-Ar-CH), 119.5 (Fmoc-Ar-CH), 66.5 (Fmoc-CH2), 55.1 (Tyr- αCH), 46.9 (Fmoc-CH), 35.9 (Tyr-βCH_2); *m/z* HRMS (ESI^+) calculated for $C_{24}H_{21}N_2O_7$ $[M+H]^+$ 449.1344, found 449.1345.

*Peptide **1***

Manual SPPS of peptide **1** was performed at room temperature. Amino acid couplings, excluding Fmoc-Cys(Dag)-OH which was gently agitated, were performed by bubbling reagents with continuous N_2 flow through the polypropylene syringe reaction vessel (25 mL). Rink amide AM resin (400 mg, 0.20 mmol, 1.0 eq) with a loading of 0.5 mmol g^{-1} was swollen in DMF (5 mL) for 1 h. Fmoc deprotection of the resin was performed using piperidine:DMF (1:4; v/v; 2 x 10 min; 5 mL) which was then washed with DMF (3 x 5 mL), DCM (3 x 5 mL) then DMF (3 x 5 mL). 3-Nitrotyrosine (179 mg, 0.40 mmol, 2.0 eq) was coupled to the resin using PyBOP (208 mg, 0.40 mmol, 2.0 eq) and NMM (40 μL, 0.40 mmol, 4.0 eq) for 2 h. The coupling solution was drained from the syringe and the resin washed with DMF (3 x 4 mL), DCM (3 x 4 mL) then DMF (3 x 4 mL). Sequential cycles of Fmoc deprotection and coupling of amino acids Fmoc-Ser(O*t*Bu)-OH (307 mg, 0.80 mmol, 4 eq) x 2, Fmoc-Cys(Dag)-OH (358 mg, 0.40 mmol, 2 eq), Fmoc-Gly-OH (238 mg, 0.80 mmol, 4 eq), Fmoc-Ala-OH (249 mg, 0.80 mmol, 4 eq), Fmoc-Leu-OH (282 mg, 0.80 mmol, 4 eq) and Boc-Abz-OH (950 mg, 0.40 mmol, 2 eq) were performed following standard Fmoc/*t*-Bu SPPS methods (Fmoc-AA : PyBOP : NMM, 1 : 1 : 2 molar eq.) in DMF (5 mL) for 1 h. Washing steps of DMF (3 x 5 mL), DCM (3 x 5 mL) then DMF (3 x 5 mL) were carried out between each deprotection and amino acid coupling. Following a final piperidine:DMF (1:4; v/v; 3 x 10 min; 5 mL) cleavage step, the resin was washed with DMF (3 x 4 mL), DCM (3 x 4 mL) and the solvent drained. Global deprotection and cleavage from resin was performed by treatment with cleavage cocktail (TFA : TIPS : H_2O (95 : 2.5 : 2.5, 5 mL)) under agitation for 1.5 h. Subsequently, the reaction mixture was filtered, collected and concentrated *in vacuo*. Precipitation from the concentrated solution occurred by addition of Et_2O (15 mL) at 0 °C. The crude peptide was washed with Et_2O (2 x 15 mL) at 0 °C before being collected by centrifugation, dried under N_2 and purified via silica column chromatography (DCM:MeOH, 49:1 – 19:1) to yield the product as a yellow solid (22 mg, 8 % over 17 steps).

^{1}H NMR (500 MHz, DMSO) δ

Residue	-NH	Hα	HB	HY	HD
3-NT	7.94 (d, *J* = 8.3 Hz, 1H)	4.39 – 4.32 (m, 2H)	3.02 (dd, *J* = 14.0, 4.6 Hz, 1H); 2.74 (dd, *J* = 14.0, 9.5 Hz, 1H)	7.73 (d, *J* = 2.2, 1H); 7.38 (dd, *J* = 8.6, 2.3 Hz, 1H,); 7.26 (dd, *J* = 13.3, 2.1 Hz, 2H); 7.01 (d, *J* = 8.5 Hz, 1H)	
Ser		4.19 (dt, *J* = 7.3, 5.5 Hz, 1H)	3.58 – 3.51 (m, 2H); 3.48 (dt, *J* = 10.6, 5.0 Hz, 1H)	5.21 (s, 1H)	
Ser	8.23 (d, *J* = 7.6 Hz, 1H)	4.39 – 4.32 (m, 2H)	3.65 – 3.58 (m, 1H); 3.58 – 3.51 (m, 2H)	4.98 (t, *J* = 5.6 Hz, 1H)	
Cys*	8.12 – 8.06 (m, 2H)	4.54 (td, *J* = 8.5, 5.1 Hz, 1H)	2.90 (dd, *J* = 13.7, 4.9 Hz, 1H); 2.64 – 2.57 (m, 1H)	2.82 (dd, *J* = 14.1, 5.4 Hz, 1H); 2.67 (dd, *J* = 14.1, 7.5 Hz, 1H)	5.09 (dt, *J* = 10.1, 7.3 Hz, 1H); 4.31 – 4.25 (m, 2H); 4.08 (dd, *J* = 12.0, 7.1 Hz, 1H)
Lipid	2.28 – 2.20 (m, 4H); 1.55 – 1.43 (m, 7H); 1.22 (d, *J* = 2.5 Hz, 48H); 0.86 (dt, *J* = 23.8, 6.7 Hz, 12H)				
Gly	8.12 – 8.06 (m, 2H)	3.74 (t, *J* = 5.6 Hz, 2H)			
Ala	8.03 (d, *J* = 7.2 Hz, 1H)	4.31 – 4.25 (m, 2H)	1.55 – 1.43 (m, 7H)		
Leu	8.14 (d, *J* = 8.2 Hz, 1H)	4.47 (ddd, *J* = 10.7, 8.1, 4.3 Hz, 1H)	1.72 – 1.60 (td, *J* = 10.3, 4.4 Hz, 3H)	0.86 (dt, *J* = 23.8, 6.7 Hz, 12H).	
2-Abz	7.55 (dd, *J* = 8.0, 1.6 Hz, 1H); 7.13 (ddd, *J* = 8.4, 7.1, 1.5 Hz, 1H); 6.68 (dd, *J* = 8.3, 1.2 Hz, 1H), 6.51 (ddd, *J* = 8.1, 7.1, 1.2 Hz, 1H), 6.33 (s, 2H)				

m/z MALDI^{+} calculated for $C_{71}H_{116}N_{10}S$ 1412.8241, found 1412.7698.

*(S)-5-((2-(3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(tert-butoxy)-4-oxobutanamido)ethyl)amino)naphthalene-1-sulfonate (**13**)*

A suspension of EDANS·Na (500 mg, 1.73 mmol, 1.0 eq), Fmoc-Asp-OtBu (**12**) (712 mg, 1.73 mmol, 1.0 eq), BOP (770 mg, 1.73 mmol, 1.0 eq), and DIPEA (603 µL, 3.46 mmol, 2.0 eq) in dry DMF (14 mL) was stirred at room temperature for 18 h. LCMS analysis of the reaction mixture confirmed complete consumption of Fmoc-Asp-OtBu. DMF was removed from the reaction mixture under reduced pressure. The crude material was purified via silica column chromatography (DCM:MeOH 9:1) to yield the product as an off-white solid (724 mg, 64%).

^{1}H NMR (400 MHz, MeOD) δ 8.18 (dd, J = 14.6, 8.2 Hz, 3H, EDANS-Ar × 2, S(O)$_2$-OH), 7.87 (dt, J = 8.3, 1.1 Hz, 1H, EDANS-Ar), 7.81 – 7.71 (m, 3H, EDANS-Ar × 2, Asp-amide-NH), 7.66 – 7.60 (m, 2H, Fmoc-Ar), 7.55 – 7.43 (m, 1H, EDANS-amide-NH), 7.43 – 7.23 (m, 6H, Fmoc-Ar), 6.65 (d, J = 7.7 Hz, 1H, EDANS-Ar), 4.54 (dd, J = 7.6, 5.5 Hz, 1H, Asp-αCH), 4.38 – 4.14 (m, 3H, Fmoc-CH$_2$, Fmoc-CH), 3.66 – 3.50 (m, 2H, EDANS-CH$_2$), 3.37 (d, J = 1.3 Hz, 2H, EDANS-CH$_2$), 2.77 (dd, J = 15.1, 5.6 Hz, 1H, Asp-βC$\underline{\text{H}_a}$CH$_b$), 2.72 – 2.61 (m, 1H, Asp-βCH$_a$C$\underline{\text{H}_b}$), 1.43 (s, 9H, t-Bu-CH$_3$); ^{13}C NMR (101 MHz, MeOD) δ 171.6 (C=O), 170.7 (C=O), 157.0 (COOH), 144.0 (Ar-qC), 143.8 (Ar-qC), 141.1 (Ar-qC), 140.4 (Ar-qC), 130.1 (Ar-qC), 126.8 (Ar-CH), 126.6 (Ar-CH), 124.9 (Ar-CH), 124.3 (Ar-CH), 124.1 (Ar-qC), 122.2 (Ar-CH), 119.5 (Ar-CH), 117.2 (Ar-CH), 115.0 (Ar-CH), 110.2 (Ar-CH), 103.7 (Ar-CH), 81.8 (t-Bu-qC), 66.7 (Fmoc-CH$_2$), 51.7 (Asp-αCH), 43.6 (Fmoc-CH), 42.4 (EDANS-CH$_2$), 38.36 (EDANS-CH$_2$), 37.43 (Asp-βC$\underline{\text{H}_a}$CH$_b$), 26.81 (t-Bu-CH$_3$); *Various methods were utilised to detect high resolution mass, however due to solubility issues the mass was not found.*

*N^2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N^4-(2-((5-sulfonaphthalen-1-yl)amino)ethyl)-L-asparagine (**14**)*

To a solution of compound **13** (720 mg, 76.0 µmol, 1 eq) in DCM (3 mL) was added TES (850 µL, 2.70 mmol, 36 eq) and TFA (7.00 mL). The reaction was stirred at room temperature for 4 h. Acetone (3 mL) was added to the reaction mixture and the resulting off-white precipitate filtered, washed with diethyl ether (3 x 1 mL) and dried to achieve the product (520 mg, 78 %, 95 % purity, *compound was used without further purification*).

^{1}H NMR (400 MHz, DMSO) δ 8.27 (d, J = 8.6 Hz, 1H, Asp-amide-NH), 8.19 (t, J = 5.7 Hz, 1H, EDANS-amide-NH), 8.07 (d, J = 8.5 Hz, 1H, EDANS-Ar), 7.95 (dd, J = 7.2, 1.1 Hz, 1H, EDANS-Ar), 7.88 (d, J = 7.5 Hz, 1H, EDANS-Ar), 7.69 (d, J = 7.5 Hz, 2H, Fmoc-Ar), 7.63

(d, J = 8.3 Hz, 1H, EDANS-Ar), 7.46 – 7.26 (m, 7H, Fmoc-Ar x 6, EDANS-Ar), 6.76 (d, J = 7.6 Hz, 1H, EDANS-Ar), 4.41 (td, J = 8.1, 5.4 Hz, 1H, Asp-αCH), 4.31 – 4.17 (m, 3H, Fmoc-CH, Fmoc-CH_2), 3.39 (tq, J = 16.9, 5.8 Hz, 2H, EDANS-CH_2), 3.29 (t, J = 6.5 Hz, 2H, EDANS-CH_2), 2.65 (dd, J = 15.2, 5.5 Hz, 1H, Asp-βC$\underline{H_a}CH_b$), 2.53 (dd, J = 8.4, 2.4 Hz, 1H, Asp-βCH_aC$\underline{H_b}$); ^{13}C NMR (101 MHz, DMSO) δ 173.6 (C=O), 170.2 (C=O), 156.3 (COOH), 144.6 (Ar-qC), 144.3 (Ar-qC), 144.2 (Ar-qC), 141.2 (Ar-qC), 130.6 (Ar-qC), 128.1 (EDANS-CH), 127.6 (Fmoc-CH), 126.5 (Fmoc-CH), 125.7 (Fmoc-CH), 125.0 (EDANS-CH), 125.0 (EDANS-CH), 124.5 (EDANS-CH), 123.5 (Ar-qC), 123.2 (Ar-qC), 120.6 (Ar-qC), 66.1 (Fmoc-CH_2), 51.1 (Asp-αCH), 47.1 (Fmoc-CH), 44.1 (Asp-CH_2), 37.8 (Asp-CH_2), 37.6 (Asp-βC$\underline{H_a}CH_b$); *m/z* HRMS (ESI$^+$) calculated for $C_{31}H_{30}N_3O_8S$ $[M+H]^+$ 604.1748, found 604.1747.

*Peptide **3***

Manual SPPS of peptide **3** was performed at room temperature, under light-protected conditions. Amino acid couplings, excluding Fmoc-Cys(Dag)-OH which was gently agitated, were performed by bubbling reagents with continuous N_2 flow through the polypropylene syringe reaction vessel (15 mL). Rink amide MHBA resin (150 mg, 0.10 mmol, 1.0 eq) with a loading of 0.67 mmol g^{-1} was swollen in DMF (4 mL) for 1 h. Fmoc deprotection of the resin was performed using piperidine:DMF (1:4; v/v; 2 x 10 min; 4 mL) which was then washed with DMF (3 x 4 mL), DCM (3 x 4 mL) then DMF (3 x 4 mL). Fmoc-Asp(EDANS)-OH (60 mg, 0.10 mmol, 1.0 eq) was coupled to the resin using PyBOP (52 mg, 0.10 mmol, 1.0 eq) and NMM (20 µL, 0.20 mmol, 2.0 eq) for 2 h. The coupling solution was drained from the syringe and the resin washed with DMF (3 x 4 mL), DCM (3 x 4 mL) then DMF (3 x 4 mL). Sequential cycles of Fmoc deprotection and coupling of amino acids Fmoc-Ser(O*t*Bu)-OH (153 mg, 0.40 mmol, 4 eq) x2, Fmoc-Cys(Dag)-OH (179 mg, 0.20 mmol, 2 eq), Fmoc-Gly-OH (119 mg, 0.40 mmol, 4 eq), Fmoc-Ala-OH (125 mg, 0.40 mmol, 4 eq), Fmoc-Leu-OH (141 mg, 0.40 mmol, 4 eq) were performed following standard Fmoc/*t*-Bu SPPS methods (Fmoc-AA : PyBOP : NMM, 1 : 1 : 2 molar eq.) in DMF (4 mL) for 1 h. Washing steps of DMF (3 x 4 mL), DCM (3 x 4 mL) then DMF (3 x 4 mL) were carried out between each deprotection and amino acid coupling. Following the final Fmoc deprotection step, the peptide sequence was completed by the coupling of DABSYL Chloride (65 mg, 0.20 mmol, 2.0 eq) with PyBOP (104 mg, 0.20 mmol, 2.0 eq) and NMM (40 mg, 0.40 mmol, 4.0 eq) in DMF (4 mL) for 2 h. The resin was washed with DMF (3 x 4 mL), DCM (3 x 4 mL), the solvent drained and final cleavage

performed by treating resin with cleavage cocktail (TFA : TIPS : H_2O (95 : 2.5 : 2.5, 5 mL)) under agitation for 1.5 h. Subsequently, the reaction mixture was filtered, collected and concentrated *in vacuo*. Precipitation from the concentrated solution occurred by addition of Et_2O (10 mL) at 0 °C. The crude peptide was washed with Et_2O (2 x 10 mL) at 0 °C before being collected by centrifugation, dried under N_2 and purified via silica column chromatography (DCM:MeOH 49:1 – 20:3) to yield the product as an orange solid (13 mg, 8 % over 16 steps).

Residue	-NH	Hα	HB	HY	HD
EDANS	8.14 – 8.07 (m, 4H); 7.93 – 7.80 (m, 6H); 7.33 – 7.22 (m, 2H); 6.55 (d, *J* = 7.6 Hz, 1H); 5.08 (d, *J* = 7.4 Hz, 1H); 3.55 (m, 2H); 2.83 (dd, *J* = 14.0, 5.4 Hz, 1H); 2.70 – 2.57 (m, 7H)				
Asp	8.17 (t, *J* = 6.6 Hz, 2H)	4.02 – 3.93 (m, 1H)			
Ser	8.30 (d, *J* = 7.4 Hz, 1H)	4.40 – 4.34 (m, 1H)	3.85 – 3.79 (m, 1H); 3.34 – 3.21 (m, 2H)	7.18 (s, 1H)	
Ser	8.24 (t, *J* = 5.7 Hz, 1H)	4.08 (dd, *J* = 12.0, 7.0 Hz, 1H)	3.85 – 3.79 (m, 1H); 3.34 – 3.21 (m, 2H)	7.05 (s, 1H)	
Cys*	8.07 – 7.99 (m, 2H)	4.57 – 4.49 (m, 2H)	2.92 (dd, *J* = 13.8, 4.8 Hz, 1H)	2.29 – 2.20 (m, 4H)	5.22 (dt, *J* = 34.6, 5.6 Hz, 2H); 4.32 – 4.19 (m, 2H)
Lipid	6.13 (t, *J* = 5.4 Hz, 1H); 1.51 – 1.42 (m, 7H); 2.29 – 2.20 (m, 2H); 1.21 (d, *J* = 4.3 Hz, 48H); 0.87 – 0.78 (m, 9H)				
Gly	8.14 – 8.07 (m, 4H)	4.57 – 4.49 (m, 2H)			
Ala	8.07 – 7.99 (m, 2H)	3.73 – 3.63 (m, 3H)	1.03 (d, *J* = 7.0 Hz, 3H)		
Leu	8.17 (t, *J* = 6.6 Hz, 2H)	4.32 – 4.19 (m, 2H)	1.51 – 1.42 (m, 7H)	0.72 (d, *J* = 6.5 Hz, 3H)	
DABSYL	7.93 – 7.80 (m, 6H); 6.87 – 6.83 (m, 2H); 1.51 – 1.42 (m, 7H)				

m/z MALDI$^+$ calculated for $C_{85}H_{133}N_{13}O_{19}S_3$ 1735.9003, found 1735.4889.

4.3 Enzyme experiments

Expression and Purification

The plasmid LspA-pET28a was received from the Caffrey group, transformed into *E. coli* BL21(DE3) and plated on kanamycin plates. Three colonies were used to inoculate 3 x 50 mL LB supplemented with kanamycin and incubated overnight at 37 °C with shaking. The overnight cultures (20 mL/flask) were then used to inoculate 6 x 1 L of Lysogeny Broth (LB) supplemented with kanamycin (50 mg/L) and incubated at 37 °C for 2.5 h. Once the culture reached an OD600 of 0.75, LspA expression was induced by the addition of IPTG (final concentration: 1 mM) which was then incubated overnight at 30 °C. The cells were harvested by centrifugation (5000xg) at 24 °C and stored at -80 °C for two weeks. The cell pellet was resuspended in 80 mL of buffer 1 (50 mM MES pH 6.5, 150 mM NaCl, 10% (v/v) glycerol) and then lysed by sonication. The lysed cells were then centrifuged (12000xg) for 30 minutes before the supernatant was removed and centrifuged again (55000xg) for 60 minutes. The membrane pellet was resuspended using a dounce homogenizer in 12 mL of buffer 1. The enzyme was aliquoted (1 mL) and stored at -80 °C. Three aliquots were combined and diluted to 16 mL with buffer 1 then supplemented with the detergent FC-12 to a final concentration of 1% (w/v). The membranes were then centrifuged (100000xg) for 60 minutes to remove the insoluble material. The supernatant was collected and supplemented with imidazole (20 mM) before being added to nickel resin (prewashed with buffer 2 (50 mM MES pH 6.15, 150 mM NaCl, 10%(v/v) glycerol, 0.14 % (w/v) FC-12)). This suspension was mixed at 4 °C for 60 mins. The suspension was added to a gravity column and washed with buffer 3 (50 mL, 50 mM MES pH 6.15, 150 mM NaCl, 10%(v/v) glycerol, 0.14 % FC-12, 50 mM imidazole). LspA was then eluted from the resin with buffer 4 (50 mM MES pH 6.15, 150 mM NaCl, 10%(v/v) glycerol, 0.14 % FC-12, 50 mM imidazole) and collected in 1 mL fractions until it was no longer detectable by nanodrop. The eluted fractions were combined, concentrated and washed with buffer 1 until the imidazole concentration is below 1 %.

Michaelis-Menten Kinetics

Michaelis-Menten analyses were carried out for each enzyme/substrate combination. The substrates were serially diluted in 25 µL (peptide **1**: 60 µM, 40 µM, 30 µM, 20 µM, 10 µM, 5 µM; peptide **3**: 8 µM, 6 µM, 4 µM, 3 µM, 2 µM, 1 µM, 0.5 µM, 0.25 µM, 0.125 µM). LspA (50 nM, 25 µL) was added to each well and the fluorescence was monitored for 1 hour, automixing 3 seconds every 30 seconds (peptide **1**: λ_{ex} 320 nm, λ_{em} 420 nm; peptide **3**: λ_{ex} 360 nm, λ_{em} 490 nm) on a Tecan Spark plate reader. The initial velocity data was used to determine

the K_m and V_{max} values of the enzyme/substrate combination. The buffer used in the experiments was 100 mM MES/NaOH pH 5.4, 150 mM NaCl containing 0.05%(w/v) lauryl maltose neopentyl glycol (LMNG). The microplates used were µClear®, black half-area 96-well plate (Greiner Bio-one).

Half maximal inhibition concentration tests (IC_{50})

The compounds were serially diluted (25 µL) and incubated at 37 °C with LspA (50 nM, 12.5 µL) for 10 min with shaking every 30 seconds. Substrate was added (25 µM for peptide **1**, 5.5 µM for peptide **3**, final concentrations, 12.5 µL in each case), fluorescence was monitored for 1 hour, automixing 3 seconds every 30 seconds (peptide **1**: λ_{ex} 320 nm, λ_{em} 420 nm; peptide **3**: λ_{ex} 360 nm, λ_{em} 490 nm) on a Tecan Spark plate reader. The initial velocity data was used to produce the IC_{50} curves using GraphPad prism 7 software. The buffer used in the experiments was 100 mM MES/NaOH pH 5.4, 150 mM NaCl containing 0.05%(w/v) LMNG. The microplates used were µClear®, black half-area 96-well plate (Greiner Bio-one).

5. Supporting Information

General Notes

HRMS analysis was performed on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 μm) at 30 °C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1 % formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 6 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 3 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionisation) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine) diluted to achieve a mass count of 10000. MALDI-TOF analysis was performed on a Bruker ultrafleXtreme spectrometer. Samples were prepared by spotting 1 μL of analyte (0.1 M) followed by 1 μL of tetraammonium ethylenediaminetetraacetate solution (20 mM). Once dry, 1 μL of the matrix solution (20 mg/mL 5-chloro-2-mercaptobenzothiazole (CMBT) in $CHCl_3$/MeOH/H_2O, 2/3/1, v/v/v) was spotted on the analyte.

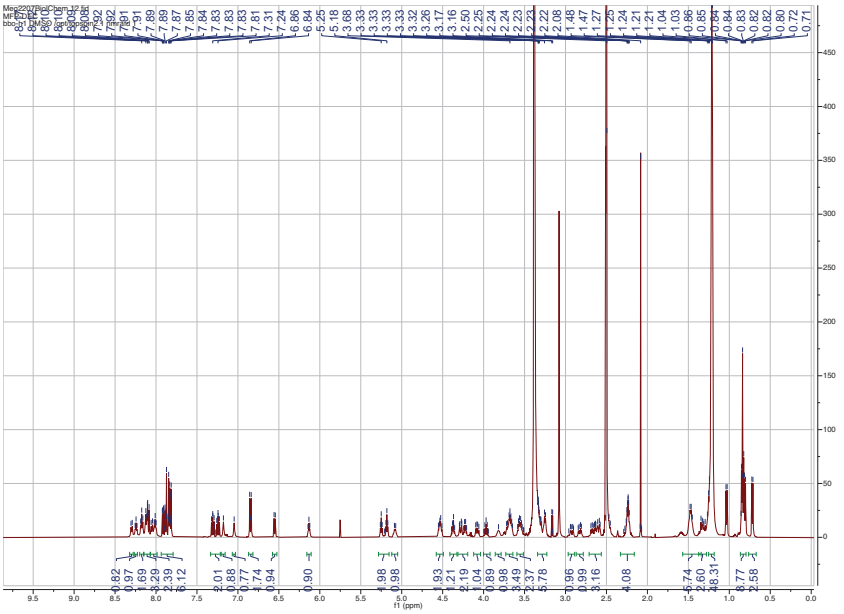


Figure S1. ^{1}H NMR (500 MHz) spectrum of peptide **3** (DMSO-d_6).

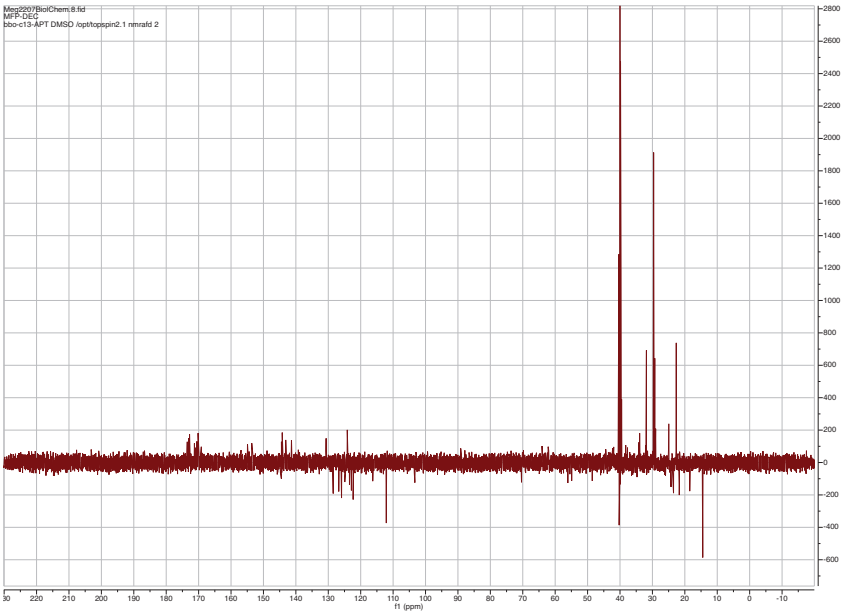


Figure S2. ^{13}C NMR (500 MHz) spectrum of peptide **3** (DMSO-d_6).

6. References

1 M. I. Hutchings, A. W. Truman and B. Wilkinson, *Curr. Opin. Microbiol.*, 2019, **51**, 72–80.

2 N. Buddelmeijer, *FEMS Microbiol. Lett.*, 2015, **39**, 246–261.

3 M. M. Wilson and H. D. Bernstein, *Trends Microbiol.*, 2016, **24**, 198–208.

4 W. R. Zückert, *Biochim. Biophys. Acta - Mol. Cell Res.*, 2014, **1843**, 1509–1516.

5 M. M. Babu, M. L. Priya, A. T. Selvan, M. Madera, J. Gough, L. Aravind and K. Sankaran, *J. Bacteriol.*, 2006, **188**, 2761–2773.

6 M. Hussain, S. Ichihara and S. Mizushima, *J. Biol. Chem.*, 1980, **255**, 3707–3712.

7 L. Vogeley, T. El Arnaout, J. Bailey, P. J. Stansfeld, C. Boland and M. Caffrey, *Science.*, 2016, **351**, 876–880.

8 T. El Arnaout and T. Soulimane, *Trends Biochem. Sci.*, 2019, **44**, 701–715.

9 S. Olatunji, X. Yu, J. Bailey, C. Y. Huang, M. Zapotoczna, K. Bowen, M. Remškar, R. Müller, E. M. Scanlan, J. A. Geoghegan, V. Olieric and M. Caffrey, *Nat. Commun.*, 2020, **11**, 1–11.

10 S. Kitamura, A. Owensby, D. Wall and D. W. Wolan, *Cell Chem. Biol.*, 2018, **25**, 301–308.

11 A. Kaur, P. Kaur and S. Ahuja, *Anal. Methods*, 2020, **12**, 5532–5550.

12 H. Hu, S. Gehrig, G. Reither, D. Subramanian, M. A. Mall, O. Plettenburg and C. Schultz, *Biotechnol. J.*, 2014, **9**, 266–281.

13 M. Inukai, R. Enokita, A. Torikata, M. Nakahara, S. Iwado and M. Arai, *J. Antibiot.*, 1978, **31**, 410–420.

14 Y. Xiao, K. Gerth, R. Müller and D. Wall, *Antimicrob. Agents Chemother.*, 2012, **56**, 2014–2021.

15 E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.*, 1970, **34**, 595–598.

16 L. L. Maggiora, C. W. Smith and Z. Zhang, *J. Med. Chem.*, 1992, **35**, 3727–3730.

17 C. D. F. Martins, M. M. M. Raposo and S. P. G. Costa, *Proceedings*, 2020, **41**, 1–4.

18 H. Umezawa, T. Aoyagi, H. Morishima, M. Matsuzaki, M. Hamada and T. Takeuchi, *J. Antibiot.*, 1970, **23**, 259–262.

19 S. Seelmeier, H. Schmidt, V. Turkt and K. von der Helm, *Proc. Natl. Acad. Sci.*, 1988, **85**, 6612–6616.

20 C. G. Knight and A. J. Barrett, *Biochem. J.*, 1976, **155**, 117–125.

21 J. Strelow, W. Dewe, P. W. Iversen, H. B. Brooks, J. A. Radding, J. McGee and J. Weidner, *Mechanism of Action Assays for Enzymes*, 2004.

22 I. K. Dev and P. H. Ray, *J. Biol. Chem.*, 1984, **259**, 11114–11120.