

Irreversible inhibition of the bacterial cysteine protease-transpeptidase sortase (SrtA) by substrate-derived affinity labels

Christopher J. SCOTT, Andrew McDOWELL, S. Lorraine MARTIN, John F. LYNAS, Koen VANDENBROECK and Brian WALKER¹

Biomolecular Sciences Group, School of Pharmacy, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, U.K.

We report on the first synthesis, kinetic evaluation and application of novel substrate-derived inhibitors against the *Staphylococcus aureus* cysteine protease-transpeptidase, sortase (staphylococcal surface protein sorting A, SrtA). The peptidyl-diazomethane and peptidyl-chloromethane analogues, Cbz (benzyloxycarbonyl)-Leu-Pro-Ala-Thr-CHN₂ (I) and Cbz-Leu-Pro-Ala-Thr-CH₂Cl (II) respectively were found to act as time-dependent irreversible inhibitors of recombinant sortase (SrtA_{AN}). The peptidyl-chloromethane analogue (II) was the most powerful with an inhibitor specificity constant (k_i/K_i) of $5.3 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$, approx. 2-fold

greater than that determined for the peptidyl-diazomethane (I). Additionally, using Western-blot analysis, we have been able to demonstrate that a biotinylated version of the peptidyl-diazomethane analogue, biotin-Ahx (aminohexanoyl)-Leu-Pro-Ala-Thr-CHN₂ (III), can be used as an affinity label to detect the presence of wild-type SrtA in crude cell lysates prepared from *S. aureus*.

Key words: biotinylated probe, peptidyl-chloromethane, peptidyl-diazomethane, recombinant, Western blotting.

INTRODUCTION

In Gram-positive pathogens, the cell surface is a complex milieu of proteins that play a pivotal role in adhesion to human tissues, as well as the evasion of host-immune responses [1,2]. A number of important virulence factors, such as Protein A, fibronectin-binding proteins A and B and collagen-binding protein are covalently attached to the bacterial cell wall by a pathway that is ubiquitous amongst Gram-positive organisms. These proteins are characterized by the presence of a distinctive C-terminal 'sorting sequence' composed of three different elements: a charged tail, a preceding hydrophobic sequence and a conserved -Leu-Pro-Xaa-Thr-Gly- motif (where Xaa represents any amino acid) [3–5]. An elegant series of experiments by Schneewind et al. [6–9] have now characterized the molecular events involved in the presentation of -Leu-Pro-Xaa-Thr-Gly- proteins on the surface of Gram-positive organisms. They identified, cloned and sequenced a cytoplasmic membrane-bound cysteine protease-transpeptidase responsible for the cleavage and covalent linkage of proteins containing the conserved -Leu-Pro-Xaa-Thr-Gly- sequence motif. This constitutively expressed enzyme, known as staphylococcal surface protein sorting A (SrtA), contains a single active-site cysteine residue (Cys¹⁸⁴), which catalyses (via nucleophilic attack) a highly specific cleavage of the scissile Thr-Gly peptide bond in -Leu-Pro-Xaa-Thr-Gly-. The thiol-acyl enzyme intermediate formed between the thiol group of Cys¹⁸⁴ and the carbonyl group of Thr is subsequently linked, via an amide bond, to pentaglycine cross-bridges in the bacterial cell wall [10]. Very recently [11], Schneewind et al. identified a second sortase species (SrtB) in *Staphylococcus aureus*. This protease, which also appears to employ an active-site cysteine residue for catalysis, has a different subsite specificity from SrtA and cleaves surface proteins at an -Asn-Pro-Gln-Thr-Asn- signal sequence, before anchoring the polypeptide to the cell-wall envelope. The SrtB protease, which is transcribed from an iron-regulated operon, appears dedicated

to the acquisition of iron from the host environment, which is important for the pathogenesis of *S. aureus* infections.

Biological studies have shown that knockout mutation of the SrtA gene in *S. aureus* inhibits the organism's adherence to IgG, fibrinogen and fibronectin, and greatly reduces the capacity of the pathogen to establish an acute infection in mice [12]. In addition, inactivation of the SrtA gene in the human commensal bacterium *Streptococcus gordonii* significantly compromises the ability of the organism to colonize the oral mucosa in mice [13]. Studies with an SrtB mutant strain of *S. aureus* have revealed no significant reduction in pathogenicity during the early stages of infection in mice [11]. However, over a period of time, the level of infection in the animals injected with the mutant strain was found to decrease significantly when compared with a wild-type strain of *S. aureus*. This suggests that SrtB does not influence the initial establishment of infection, but is required for persistence of the pathogen within infected tissues. Therefore both SrtA and SrtB are important virulence factors, but they appear to play different roles in the infection process.

It is clear that, in an era of growing antibiotic resistance, both SrtA and SrtB may prove exciting new targets for the development of anti-staphylococcal drugs, or even broad-spectrum agents against Gram-positive pathogens. Against this background, we now describe the first synthesis and kinetic evaluation of substrate-derived inhibitors of SrtA, including a biotinylated version that could prove valuable for the detection and disclosure of novel SrtA-like species (specific for -Leu-Pro-Xaa-Thr-Gly-motifs) in extracts from Gram-positive organisms [14].

EXPERIMENTAL

Materials

All protected amino acids, Wang Resin and coupling reagents used for peptide synthesis were purchased from Calbiochem–Novabiochem (Nottingham, U.K.). All solvents for peptide

Abbreviations used: Ahx, aminohexanoyl; BCCP, biotin carboxyl carrier protein; Cbz, benzyloxycarbonyl; Dabcyl, 4-([4-(dimethylamino)phenyl]azo)-benzoyl-; Edans, [(2-aminoethyl)-amino]naphthalene-1-sulphonyl; Fmoc, fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; LB, Luria–Bertani; Ni-NTA, Ni²⁺-nitrilotriacetate; SrtA, staphylococcal surface protein sorting A.

¹ To whom correspondence should be addressed (e-mail brian.walker@qub.ac.uk).

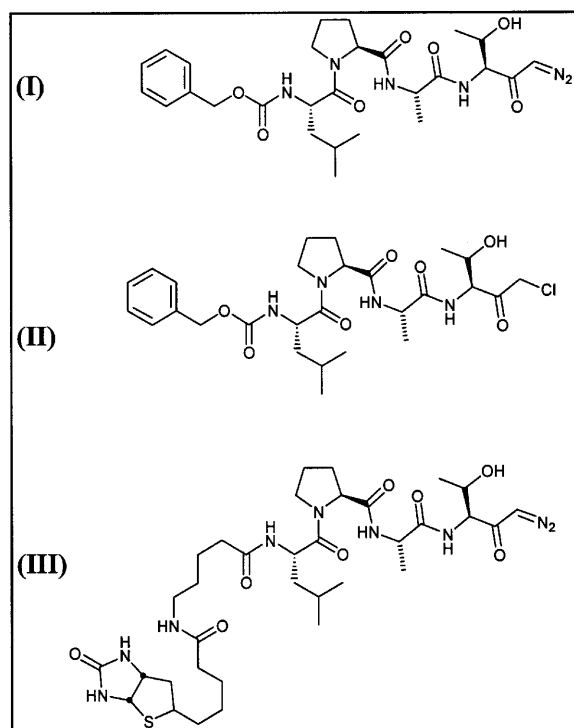


Figure 1 Molecular structures of the substrate-derived $SrtA_{\Delta N}$ inhibitors

Molecular structures correspond to the peptidyl-diazomethane inhibitor Cbz-Leu-Pro-Ala-Thr-CHN₂ (I), the peptidyl-chloromethane inhibitor Cbz-Leu-Pro-Ala-Thr-CH₂Cl (II) and the biotin-peptidyl-diazomethane inhibitor biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III).

synthesis were obtained from Applied Biosystems (Warrington, Cheshire, U.K.). *S. aureus* (Oxford strain) was from the A.T.C.C. 9144 (Manassas, VA, U.S.A.). Luria–Bertani (LB) broth was purchased from Oxoid (Basingstoke, U.K.). The pET-3d expression system, and the antibiotics ampicillin and chloramphenicol were purchased from Novagen (Madison, WI, U.S.A.). Ni²⁺-nitrilotriacetate (Ni-NTA)-agarose was purchased from Qiagen (Crawley, West Sussex, U.K.). Biotin, BSA, the substrates 5-bromo-4-chloroindol-3-yl phosphate and Nitro Blue Tetrazolium, isopropyl β -D-thiogalactoside and goat anti-rabbit IgG alkaline phosphatase were from Sigma–Aldrich (Poole, Dorset, U.K.). Novex® SDS/PAGE gels were from Invitrogen (Groningen, The Netherlands), whereas streptavidin–alkaline phosphatase was from Vector Laboratories (Peterborough, U.K.). Rabbit antiserum raised against recombinant sortase $SrtA_{\Delta N}$ was a gift from Fusion Antibodies Ltd. (Belfast, U.K.).

Synthesis of substrate-derived inhibitors

The substrate-derived inhibitor sequences (illustrated in Figure 1) were synthesized using a combination of solid-phase and solution methodologies previously reported by our group (Figure 2; [15–17]). In essence, the -Leu-Pro-Ala-Thr- portion of each inhibitor was synthesized (0.5 mmol scale) using standard solid-phase synthesis protocols on acid-sensitive Wang resin, previously derivatized with fluoren-9-ylmethoxycarbonyl (Fmoc)-Thr-(OBu)-OH as the first amino acid [18]. Each subsequent amino acid was incorporated via single 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)-mediated couplings. Both alanine and proline residues were incorporated as their *N*- α -Fmoc derivatives. For inhibitor sequences I and II,

the common leucine residue was incorporated as an *N*-protected benzyloxycarbonyl (Cbz) derivative. For the biotinylated sequence III, the leucine residue was incorporated as an *N*- α -Fmoc derivative, as was the aminohexanoic linker residue. Biotin was incorporated into the sequence using a double-coupling step. On completion of each synthesis, the peptides were cleaved from the solid support by treatment with a 10 ml solution of 95% (v/v) trifluoroacetic acid containing 2.5% (v/v) double-distilled water and 2.5% (v/v) tri-isopropyl silane. Each cleavage reaction was concentrated to approx. 0.5 ml by evaporation under reduced pressure. The peptide product was then precipitated by dilution (1:20) with chilled diethyl ether and isolated by centrifugation (2000 *g* for 10 min). The products (yields of 80%) were dried *in vacuo*, for 12 h, before the next stage of the synthesis. The peptides (obtained as their C-terminal free acids) were then activated, via a mixed carbonic anhydride, and converted into their respective diazomethanes by reaction with ethereal diazomethane [15]. The peptidyl-chloromethane inhibitor (II) was prepared (in quantitative yield) by treating a sample (0.1 mmol) of inhibitor I with a 10 ml solution of anhydrous ethereal HCl, for 15 min at 0 °C [19]. The purity and identity of each product was confirmed by reverse-phase HPLC and matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF)-MS.

PCR cloning, expression and purification of recombinant $SrtA_{\Delta N}$

$SrtA$ is believed to be a membrane-associated protease, with an N-terminal membrane anchor. To facilitate purification and solubility, a recombinant form of sortase ($SrtA_{\Delta N}$) has previously been described and used in which the N-terminal membrane anchor segment of the enzyme (residues 2–25) was replaced with a hexahistidine (His₆) sequence [8,9,12]. In our study, we adopted a similar strategy and created a construct for $SrtA_{\Delta N}$ by replacing the N-terminal T7 tag in a pET-3d expression vector with a His₆ tag. The region of the $SrtA$ gene lacking the N-terminal membrane domain was then PCR-amplified from the genomic DNA of *S. aureus* (Oxford strain) using the primers orf6N-ds-B (5'-AAAGATCCAAACCACATATCGATAATTATC-3') and orf6C-B (5'-AAGGATCCCTATTGACTTCTGTAGCTACAA-3') [8], and the resulting amplicon was cloned into the expression vector at a unique *Bam*HI restriction site. Competent *Escherichia coli* cells (JM109) were then transformed and selected on LB agar plates containing ampicillin (100 μ g/ml). After selection and verification of positive clones by dideoxynucleotide DNA sequencing, the vector was transformed into the *E. coli* expression strain HMS174(DE3)pLysS. Transformed bacteria were propagated at 37 °C in LB broth containing ampicillin (100 μ g/ml) and chloramphenicol (35 μ g/ml), and the expression was induced with 1 mM isopropyl β -D-thiogalactoside. Cultures were harvested after a 4 h post-induction incubation at 37 °C, and the recombinant enzyme was purified on a Ni-NTA-agarose column as described previously [8]. Confirmation of $SrtA_{\Delta N}$ was achieved using a monoclonal antibody to the His₆ tag (Sigma–Aldrich) and ion-trap MS on peptides recovered from 'in-gel' tryptic digests of the recombinant protein.

Kinetic analysis of inhibitors Cbz-Leu-Pro-Ala-Thr-CHN₂ (I) and Cbz-Leu-Pro-Ala-Thr-CH₂Cl (II)

Using methods described previously [8,9], $SrtA_{\Delta N}$ activity was measured with the internally quenched substrate 4-[(4-(dimethylamino)phenyl)azo]-benzoyl(Dabcyl)-Gln-Ala-Leu-Pro-Glu-Thr-Gly-Glu-Glu-[(2-aminoethyl)-amino]naphthalene-1-sulphonyl (Edans) (prepared 'in house' by standard Fmoc solid-phase synthesis) on a CytoFluor 4000® multi-well fluorimeter (PerSeptive Biosystems, Foster City, CA, U.S.A.). All

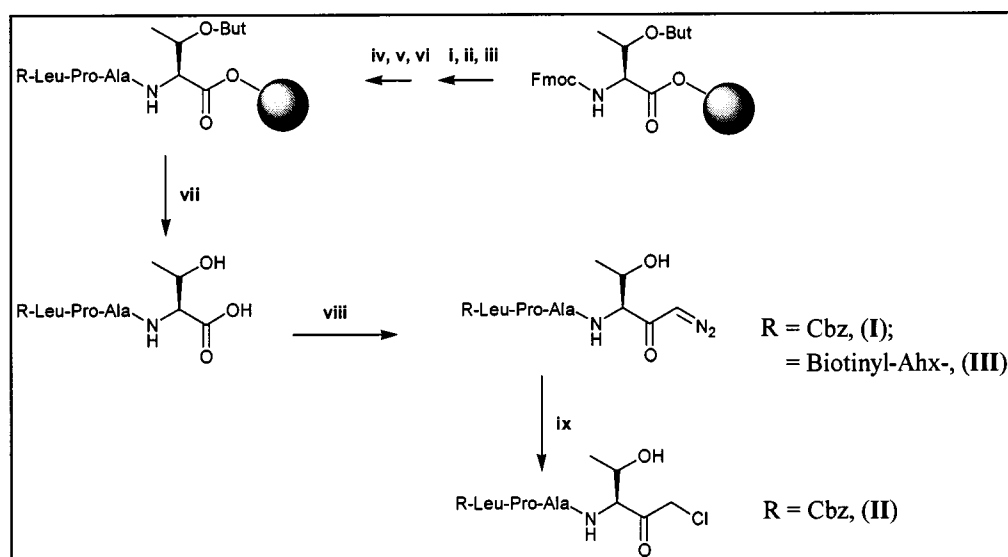


Figure 2 Strategy for solution–solid-phase synthesis of the substrate-derived $SrtA_{\Delta N}$ inhibitors

Synthetic conditions: i, 20% (v/v) piperidine-dimethylformamide for 15 min; ii, Fmoc-Ala-OH/HBTU/DIPEA (di-isopropyl ethylamine; 1:1:2, by vol.) for 30 min; iii, repeat step i; iv, Fmoc-Pro-OH/HBTU/DIPEA (1:1:2, by vol.) for 30 min; v, repeat step i; vi, for R = Cbz, Cbz-Leu-OH/HBTU/DIPEA (1:1:2, by vol.), 30 min; for R = biotinyl-Ahx-, Fmoc-Leu-OH/HBTU/DIPEA (1:1:2, by vol.), 30 min, then repeat step i, followed by biotin/HBTU/DIPEA (1:1:2, by vol.), 2×30 min; vii, trifluoroacetic acid/double-distilled water/tri-isopropyl silane (95:2.5:2.5, by vol.), 90 min at 0 °C; viii, isobutyl chloroformate (1.2 eq.), *N*-methyl morpholine (1.2 eq.), 15 min at 0 °C, then add ethereal diazomethane (6.0 eq.), stir at room temperature, overnight; ix, ethereal HCl (6.0 eq.), 3 min at 0 °C.

$SrtA_{\Delta N}$ assays were performed at 37 °C (in triplicate) in 50 mM Tris/HCl (pH 7.5) containing 150 mM NaCl, 5 mM $CaCl_2$, 5 mM NH_2-Gly_3 and 5 mM dithiothreitol ($SrtA$ buffer). Wells contained approx. 10 μM of purified $SrtA_{\Delta N}$ and 50 μM of substrate, in a final volume of 200 μl . The K_m and k_{cat} values for the $SrtA_{\Delta N}$ -catalysed cleavage of the internally quenched substrate were calculated by fitting the data points directly into the Michaelis–Menten equation for substrate hydrolysis, using GraFit® software (Erithacus Software, Horley, Surrey, U.K.).

Affinity labelling of recombinant and wild-type $SrtA$ with biotin-Ahx (aminohexanoyl)-Leu-Pro-Ala-Thr-CHN₂ (III)

For the detection and disclosure of $SrtA_{\Delta N}$ with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III), crude lysates of transfected *E. coli* cells (freshly induced) were prepared, followed by purification of the recombinant enzyme on a Ni-NTA–agarose column as previously described [8]. For the disclosure of wild-type $SrtA$ in *S. aureus*, a 50 ml overnight culture of the organism in LB broth was prepared. The bacterial cells were centrifuged at 3000 *g* for 15 min and the resulting pellet was washed in 5 ml of $SrtA$ buffer. The washed bacteria were then resuspended in 1 ml of $SrtA$ buffer containing 0.1 g of glass beads. The suspension was vortex-mixed continuously for 5 min followed by centrifugation at 1500 *g* for 15 min to remove the beads and unbroken cells. To 100 μl of purified $SrtA_{\Delta N}$ and the crude bacterial preparations, the inhibitor biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III) was added (final concentration 50 μM) before incubation at 37 °C for 30 min.

To detect the affinity-labelled recombinant and wild-type $SrtA$, samples were analysed by Western-blot analysis [15,20–22]. Briefly, samples were treated with denaturing treatment buffer and the proteins separated by SDS/PAGE on 4–20% (w/v)

linear gradient gels, followed by semi-dry transfer to a nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany). After transfer, non-specific binding sites were quenched with a 3% (w/v) solution of BSA in 20 mM Tris/HCl (pH 7.4), containing 150 mM NaCl. To detect the biotin group, the membrane was incubated with a streptavidin–alkaline phosphatase conjugate (1:500). To confirm the identity of affinity-labelled recombinant and wild-type $SrtA$, the expressed protein and crude *S. aureus* extracts were respectively incubated with a rabbit antiserum (1:5000) raised against $SrtA_{\Delta N}$. Bound antibody was detected with a goat anti-rabbit IgG alkaline phosphatase conjugate (1:20000). All protein bands were revealed after incubation of the membranes with the substrates 5-bromo-4-chloroindol-3-yl phosphate and Nitro Blue Tetrazolium.

RESULTS AND DISCUSSION

In the present study, we synthesized three substrate-derived, active site-directed, irreversible inhibitors of $SrtA_{\Delta N}$. The inhibitor sequences were based on the substrate recognition motif of the protease (-Leu-Pro-Xaa-Thr-Gly-) in which the scissile amide bond between threonine and glycine residues was replaced with a diazoketone (-COCHN₂) or chloromethyl ketone (-COCH₂Cl) grouping. To facilitate synthesis, we choose to use a P_2 (X) alanine residue (nomenclature of Schechter and Berger [23]) in our inhibitor sequence since this residue is the smallest of the chiral amino acids that does not possess a nucleophilic side chain and, therefore, does not require the use of protecting groups that could complicate the synthetic strategy. Both the peptidyl-diazomethane and peptidyl-chloromethane sequences were chosen on the basis of their recognized ability to alkylate the active-site thiol group of cysteine proteases [19,24]. This is pertinent in the light of previous site-directed mutagenesis studies, which demonstrated that Cys¹⁸⁴ is essential for the catalytic

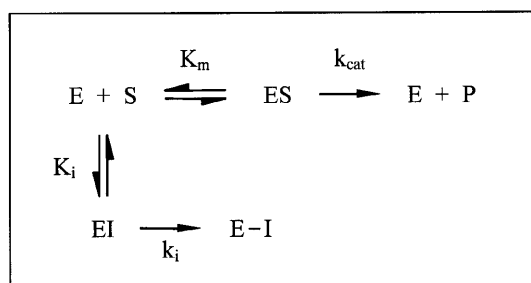


Figure 3 Mechanism of inhibition by the substrate-derived peptidyl-diazomethane (I) and peptidyl-chloromethane (II) SrtA_{AN} inhibitors

In the scheme shown, the protease E catalyses the hydrolysis of the substrate S to the fluorescent product P. The reaction is characterized by the standard kinetic constants K_m and k_{cat} . In the presence of competing inhibitor I, the protease binds the inhibitor to form the Michaelis complex EI, with a binding affinity characterized by the inhibitor constant K_i . The protease is then covalently modified to the inactivated complex E-I, with a first-order rate constant k_i .

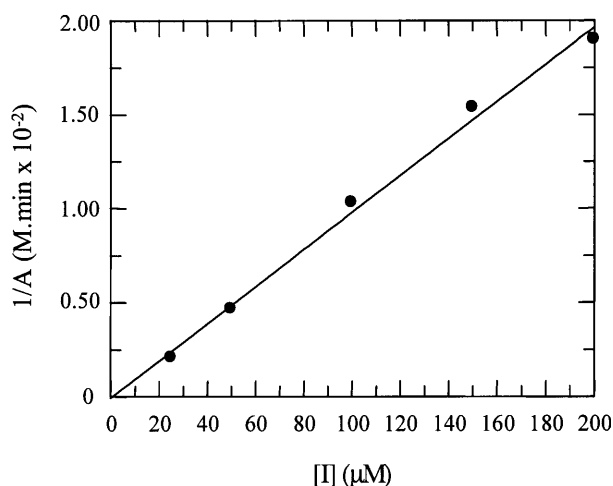


Figure 4 Inhibition of SrtA_{AN} by Cbz-Leu-Pro-Ala-Thr-CH₂Cl (II)

Plot of the reciprocal of the apparent second-order rate constant A for inactivation versus inhibitor concentration $[I]$. Hydrolysis of the internally quenched substrate Dabcyl-Gln-Ala-Leu-Pro-Glu-Thr-Gly-Glu-Glu-Edans was performed in the presence of five different concentrations (25, 50, 100, 150 and 200 μM) of the active-site directed peptidyl-chloromethane inhibitor Cbz-Leu-Pro-Ala-Thr-CH₂Cl (II). Data were fitted by non-linear regression analysis to the integrated rate equation $[P] = P_{\infty}(1 - e^{-k_{app}t})$. The P_{∞} and k_{app} values were determined and used to evaluate the apparent second-order rate constant A for inactivation of SrtA_{AN} in the presence of substrate.

activity of SrtA, and that the thiol-directed reagents methanethiosulphonates and *p*-hydroxymercuric benzoic acid (pHMB) block the catalytic function of the enzyme [7,8].

Progress curves for SrtA_{AN}-catalysed hydrolysis of the internally quenched substrate Dabcyl-Gln-Ala-Leu-Pro-Glu-Thr-Gly-Glu-Glu-Edans in the presence of different concentrations of Cbz-Leu-Pro-Ala-Thr-CHN₂ (I) and Cbz-Leu-Pro-Ala-Thr-CH₂Cl (II) typified the action of active site-directed irreversible inhibitors operating via the mechanism illustrated in Figure 3 [25,26]. Data from the curves were fitted, using non-linear regression analysis [26], to the integrated rate equation $[P] = P_{\infty}(1 - e^{-k_{app}t})$. This equation represents a first-order rate process for the formation of product P as a function of time, where k_{app} is the apparent rate constant and P_{∞} represents the concentration

Table 1 Kinetic constants for the inactivation of SrtA_{AN} by the substrate-derived peptidyl-diazomethane (I) and peptidyl-chloromethane (II) inhibitors

Data from the progress curves for SrtA_{AN}-catalysed hydrolysis of the internally quenched substrate Dabcyl-Gln-Ala-Leu-Pro-Glu-Thr-Gly-Glu-Glu-Edans in the presence of five different concentrations of inhibitors I and II (25, 50, 100, 150 and 200 μM) were fitted by non-linear regression analysis to the integrated rate equation $[P] = P_{\infty}(1 - e^{-k_{app}t})$. The P_{∞} and k_{app} values were determined and used to evaluate the apparent second-order rate constant A for inactivation of SrtA_{AN} in the presence of substrate. The individual kinetic constants k_i and K_i were then evaluated from a plot of $1/A$ versus inhibitor concentration $[I]$, and the specificity constant for each inhibitor (k_i/K_i) calculated. Values represent the means \pm S.E.M. for four determinations.

Inhibitor	k_i (min^{-1})	K_i (M)	k_i/K_i ($\text{M}^{-1} \cdot \text{min}^{-1}$)
I	$5.8 \pm 0.6 \times 10^{-3}$	$2.2 \pm 0.2 \times 10^{-7}$	$2.2 \pm 0.2 \times 10^4$
II	$1.1 \pm 0.1 \times 10^{-2}$	$2.1 \pm 0.2 \times 10^{-7}$	$5.3 \pm 0.6 \times 10^4$

of product at a time approaching infinity. Using five different concentrations for each inhibitor (25, 50, 100, 150 and 200 μM), the values k_{app} and P_{∞} were determined and utilized to evaluate the apparent second-order rate constant A for the inactivation of SrtA_{AN} in the presence of the substrate [25]. The individual kinetic constants k_i and K_i were then evaluated for both inhibitors from a plot of $1/A$ versus inhibitor concentration $[I]$ [25], and the specificity constant k_i/K_i was calculated. Figure 4 shows the plot for inactivation of SrtA_{AN} by the peptidyl-chloromethane inhibitor Cbz-Leu-Pro-Ala-Thr-CH₂Cl (II). All the kinetic constants determined for inhibitors I and II are given in Table 1. The first point of interest was the observation that the peptidyl-chloromethane inhibitor (II) inactivates SrtA_{AN} by approx. 2-fold more rapidly than the analogous diazomethane sequence (I), as revealed by the specificity constants $k_i/K_i = 5.3 \times 10^4$ and $2.2 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ respectively. Both inhibitors I and II exhibited almost identical, sub-micromolar inhibitor constants $K_i = 2.2 \times 10^{-7}$ and 2.1×10^{-7} M respectively. This implies high affinity of interaction between the common tetrapeptide motif of each inhibitor and the SrtA_{AN} enzyme. The greater effectiveness of the chloromethane inhibitor (II) can be attributed almost exclusively to the increased rate at which it covalently modifies SrtA_{AN}. Therefore the peptidyl-diazomethane (I) covalently modifies SrtA_{AN} with a first-order rate constant $k_i = 5.8 \times 10^{-3} \text{ min}^{-1}$, whereas the peptidyl-chloromethane (II) covalently modifies the enzyme by approx. 2-fold more rapidly ($k_i = 1.1 \times 10^{-2} \text{ min}^{-1}$). This observation fits the known relative chemical reactivity of the electrophilic groupings of both inhibitors towards thiol nucleophiles [24,27]. The first-order rate constants observed are considerably smaller than those previously determined for the inactivation of cysteine proteases by active-site directed peptidyl-diazomethanes and peptidyl-chloromethanes. For example, the peptidyl-chloromethane inhibitor Cbz-Phe-Phe-CH₂Cl alkylates the active-site cysteine residue of cathepsin B with $k_i = 12.5 \text{ min}^{-1}$ [27]. This is approx. 1000-fold greater than the irreversible modification of SrtA_{AN} by the peptidyl-chloromethane sequence (II).

We have previously demonstrated that biotinylated peptidyl-diazomethanes can be used as affinity labels for the detection of cysteine proteases from diverse sources such as human breast cancer cells [20], human synovial fluid [28] and a medium conditioned by parasites [21]. On this basis, we wished to evaluate the potential of a biotinylated inhibitor of SrtA for the detection of functionally active forms of SrtA-like species in Gram-positive organisms. Towards this end, we prepared the biotinylated inhibitor sequence biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III). The strategy underlying the design of III was based on two tenets. First, we chose to prepare the peptidyl-diazomethane (I) over the

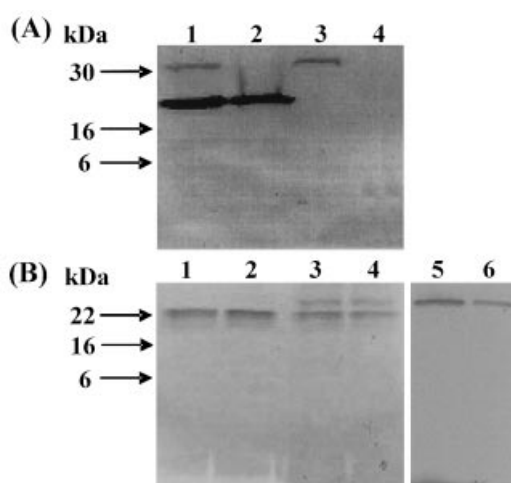


Figure 5 Western-blot analysis of recombinant and wild-type SrtA after affinity labelling with the biotin-peptidyl-diazomethane inhibitor biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III)

(A) Lane 1, crude lysate prepared from a freshly induced culture of *E. coli* cells transfected with the SrtA gene and incubated with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (I); lane 2, 'flow-through' from Ni-NTA-agarose column (during SrtA_{ΔN} purification) incubated with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (I); lane 3, bound SrtA_{ΔN} fraction eluted from Ni-NTA column with 10 mM imidazole and incubated with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (I); lane 4, sample of Ni-NTA-agarose purified SrtA_{ΔN} pre-treated with pHMB, before incubation with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (I). (B) Lanes 1 and 2, control duplicates of crude *S. aureus* cell lysate before treatment with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III); lanes 3 and 4, duplicates of crude *S. aureus* cell lysate after incubation with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III) (50 μM); lanes 5 and 6, a neat and 1:2 dilution respectively of crude *S. aureus* cell lysate treated with rabbit antiserum (1:5000) raised against SrtA_{ΔN}.

slightly more active chloromethane sequence (II), as the latter has potential to cause non-specific alkylation of biomolecules [24]. Secondly, we incorporated an aminohexanoic spacer group between the recognition motif of the inhibitor and the biotin moiety to maximize the interaction with streptavidin-alkaline phosphatase, which we routinely use for disclosure of affinity-labelled proteolytic species after SDS/PAGE and Western-blot analysis.

During initial Western-blotting experiments, biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III) (50 μM) was examined for its ability to disclose SrtA_{ΔN} in *E. coli* cells transfected with the SrtA gene from *S. aureus* (within the pET-3d expression vector; see Figure 5A). As shown in Figure 5, lane 1 contained a crude lysate prepared from a freshly induced culture of the transfected *E. coli* cells, whereas lane 2 contained the 'flow-through' from the Ni-NTA-agarose column used to purify the expressed His₆-tagged SrtA_{ΔN}. In lane 3, the bound fraction eluted from the column with 10 mM imidazole (purified SrtA_{ΔN}) was also analysed. In both lanes 1 and 2, the intensely stained protein species with an apparent molecular mass of 22 kDa was the endogenous biotin carbonyl carrier protein (BCCP). This species was totally absent in the fraction selectively eluted from the Ni-NTA-agarose column (Figure 5, lane 3). The band with an apparent molecular mass of approx. 30 kDa, which was present in both the crude cell lysate (Figure 5, lane 1) and eluted fraction (Figure 5, lane 3), was His₆-tagged SrtA_{ΔN}. The identity of the affinity-labelled SrtA_{ΔN} band was confirmed with a monoclonal antibody to the His₆ sequence and a rabbit antiserum raised against the recombinant enzyme (results not shown). The molecular mass determined for SrtA_{ΔN} by SDS/PAGE was in agreement with the previous reports of Ton-That et al. [8]. In lane 4, pre-treatment

of the purified SrtA_{ΔN} sample with the thiol-directed inhibitor pHMB, before 'probing' with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III), resulted in complete diminution of SrtA_{ΔN} labelling. As the enzyme contains only a single cysteine residue (Cys¹⁸⁴) essential for catalytic activity [8,9], we can confidently conclude that the binding of this biotinylated inhibitor to SrtA_{ΔN} is indeed active-site-directed.

We also performed Western-blotting experiments with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III) to detect the presence of wild-type SrtA in crude cell lysates from *S. aureus* (Oxford strain; see Figure 5B). In the duplicate control lanes 1 and 2, which contained unprobed cell lysate, the BCCP protein (22 kDa) was again observed. In the duplicate lanes 3 and 4, which contained cell lysate 'probed' with biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III), the BCCP protein was detected along with an additional protein of apparent molecular mass 24 kDa. This molecular mass was consistent with the predicted value for wild-type SrtA. That this protein band was indeed affinity-labelled SrtA was confirmed with an antiserum raised against the recombinant form of the protein (see Figure 5B).

The discrete labelling of only recombinant and wild-type forms of SrtA in crude cell lysates prepared from transfected *E. coli* cells and *S. aureus* respectively provides convincing evidence for the selectivity of action of the biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ inhibitor. As expected, biotin-Ahx-Leu-Pro-Ala-Thr-CHN₂ (III) selectively disclosed wild-type SrtA, but not SrtB in crude *S. aureus* extracts. Two aspects explain the specificity of our affinity label for SrtA only. First, SrtA is a constitutively expressed protease in *S. aureus*, whereas SrtB expression is tightly regulated by the concentration of iron, which was not added to our culture media [11]. Secondly, the previous studies of Mazmanian et al. [11] demonstrated the exquisite specificity of SrtA and SrtB towards cognate synthetic peptides modelled on their respective sorting signals. When tested against internally quenched fluorescent peptides, SrtB was shown only to cleave peptides containing the -Asn-Pro-Gln-Thr↓Asn- sorting sequence, and not the -Leu-Pro-Xaa-Thr↓Gly- sequence recognized by SrtA. In contrast, SrtA displayed exactly the contrary specificity. Consequently, we would not have expected SrtB to be inhibited by our biotinylated affinity label, even under conditions where the enzyme was expressed.

In conclusion, this paper has reported on the first substrate-derived irreversible inhibitors of SrtA that might find application in delineating the role of this cysteine protease-transpeptidase in cell surface protein sorting and adherence of Gram-positive organisms.

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