

Recent Advances Towards New Anti-Infective Agents that Inhibit Cell Surface Protein Anchoring in *Staphylococcus aureus* and Other Gram-Positive Pathogens

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Abstract: Sortase enzymes are attractive targets for the development of new anti-infective agents against Gram-positive pathogens because they covalently anchor virulence factors to the cell wall. Here we review what is known about the mechanism of sortase mediated protein anchoring and discuss recently identified inhibitors of this new important enzyme family.

Key Words: Sortase, antibiotic, anti-infective, *Staphylococcus aureus*, Gram-positive bacteria, inhibitor, SrtA.

INTRODUCTION

The emergence of drug resistant bacteria is an increasing health problem and has driven the search for new antibiotics [1-3]. Compounds that inhibit the display of bacterial surface proteins may have great therapeutic value, since during infections surface proteins promote bacterial adhesion, evasion of the immune response, nutrient acquisition, and host cell invasion [4]. Gram-positive bacteria display surface proteins using sortase enzymes, a recently discovered family of cysteine transpeptidases that catalyze the covalent attachment of proteins to the cell wall peptidylglycan [5-11]. Sortase inhibitors could be particularly potent anti-infective agents as these enzymes have been shown to contribute to the virulence of a number of important pathogens, including among others: *Staphylococcus aureus* [12-14], *Bacillus anthracis* [15], *Listeria monocytogenes* [16, 17], and *Streptococcus pneumoniae* [18-20].

Many excellent reviews on sortase enzymes have been written [4-7, 11, 21-23]. Inhibitor development thus far has focused on the SrtA sortase from *Staphylococcus aureus*, because this microbe is the major cause of hospital-acquired infections in the United States and SrtA is essential for its virulence [12-14]. Here we discuss progress towards understanding the enzymatic mechanism of SrtA mediated transpeptidation and several recently reported small molecule inhibitors of this enzyme.

CELL SURFACE PROTEIN ANCHORING: THE *S. AUREUS* SRtA PARADIGM

The SrtA sortase from *Staphylococcus aureus* is the founding member of the sortase enzyme family and has been studied in the greatest detail [10, 24]. It is an extracellular membrane protein that consists of an N-terminal membrane anchor and an autonomously folded catalytic domain that is

conserved in other members of the enzyme family [10, 25]. Pioneering studies by Schneewind and colleagues have revealed how SrtA covalently attaches 20 distinct proteins to the cell wall. This process occurs through a transpeptidation reaction in which SrtA joins two substrates, an LPXTG motif within the C-terminal end of the surface protein precursor, and a cell wall cross-bridge peptide [10, 24]. Fig. (1) summarizes how the overall reaction is thought to occur. (1) Initially, a full-length precursor protein containing an amino terminal leader peptide is exported from the cytoplasm through the secretory (Sec) pathway [26, 27]. (2) A C-terminal cell wall sorting signal (CWS) in the precursor protein then causes it to be retained in the cell membrane [28, 29]. The CWS is ~35 residues in length and consists of three regions: the amino acid sequence LPXTG (where X is any amino acid), a hydrophobic domain, and a tail of mostly positively charged residues. The charged amino acids at the C-terminal end of the CWS are believed to prevent the protein from being released into the extracellular milieu. (3) The partially exported protein is then processed by the SrtA sortase, which recognizes the amino acid sequence LPXTG within the CWS [10, 24]. Sortase cleaves the LPXTG motif between the threonine and glycine residues, forming a thioacyl link to the threonine carbonyl carbon of the CWS [30]. (4) SrtA then recognizes its second substrate, the (Gly)₅ peptide located within lipid II [undecaprenyl-pyrophosphate-MurNac(-L-Ala-D-iGln-L-Lys(NH₂-Gly)₅-D-Ala-D-Ala)-b1-4-GlcNac], which is a membrane anchored precursor that is used in cell wall synthesis. SrtA catalyzes the formation of a peptide bond between the carbonyl carbon of the threonine and the free amine group in the peptide, resulting in the covalent attachment of the protein to lipid II [24, 31]. (5) Surface proteins tethered to lipid II by SrtA are subsequently incorporated into the peptidylglycan *via* the transglycosylation and transpeptidation reactions of bacterial cell wall synthesis. *S. aureus* also encodes a second sortase called SrtB, which attaches the IsdC protein to the pentaglycine cross-bridge within the cell wall [32]. However, SrtB recognizes a novel NPQTN motif within the CWS, and it is unclear if lipid II functions as a co-substrate because IsdC is attached

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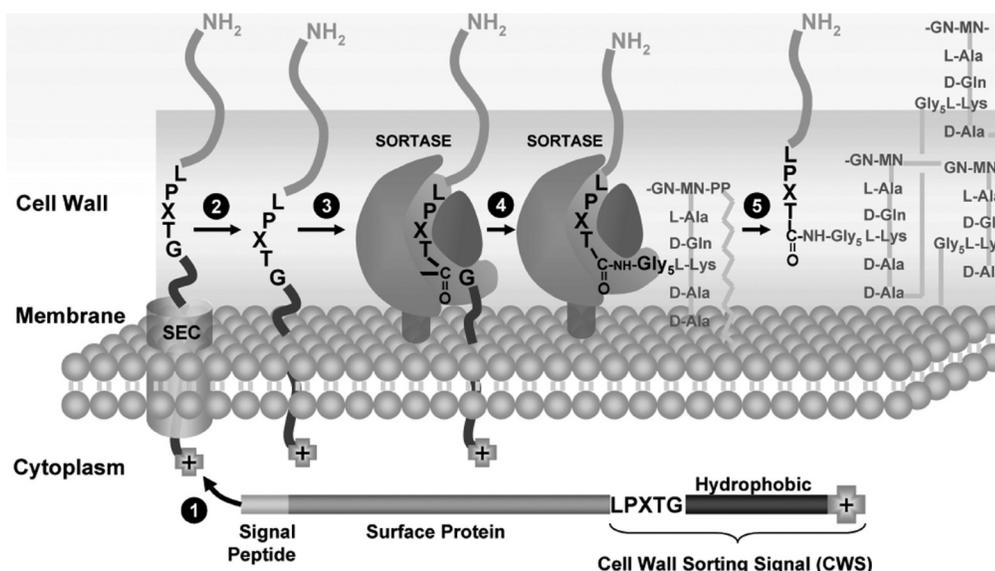


Fig. (1). Overview of sortase mediated protein anchoring to the cell wall. A protein precursor is: (1) secreted across the membrane through the Sec pathway; (2) The protein is retained in the membrane by its cell wall sorting signal (CWS); (3) The sortase enzyme cleaves the LPXTG sequence within the CWS; (4) Sortase catalyzes the formation of a new peptide bond between the carbonyl carbon of the threonine residue within the CWS and the free amine group on lipid II, forming a precursor to be used in cell wall synthesis (5). This process is universally conserved in Gram-positive bacteria. (Figure adapted from reference [22]).

to peptidylglycan that exhibits relatively little cross-linking [33, 34].

MECHANISM OF TRANSPEPTIDATION

Structural and enzyme kinetic studies have begun to reveal the mechanism of SrtA catalyzed transpeptidation. The three-dimensional structure of the catalytic domain of SrtA (SrtA_{ΔN59}, residues 60 to 206) has been determined and

adopts a novel eight-stranded β-barrel fold [25, 35]. The enzyme active site contains three proximal and conserved side chains that are essential for catalysis: His120, Cys184 and Arg197 [24, 36-38] (Fig. (2)). While Cys184 is believed to act as a nucleophile during catalysis, the function of His120 and Arg197 in catalysis is less clear (discussed in greater detail below). The structures of the SrtB enzymes from *S. aureus* (sa-SrtB) and *B. anthracis* (ba-SrtB) have also been

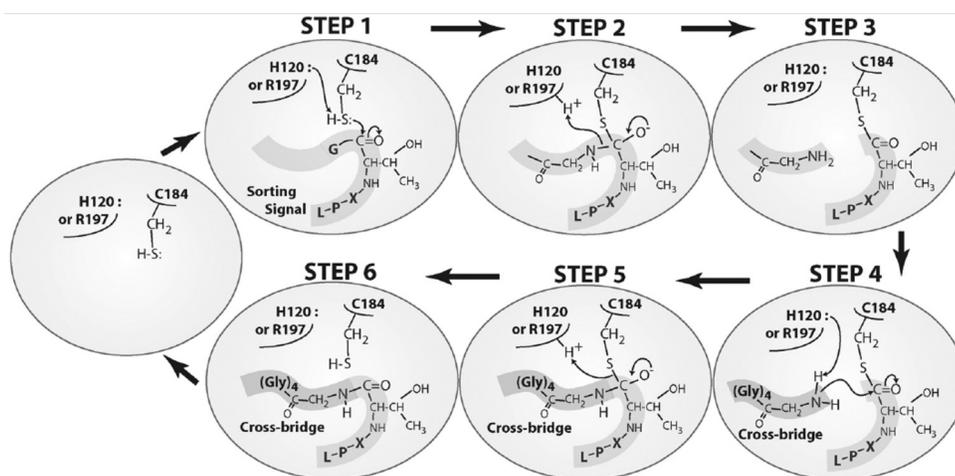


Fig. (2). Proposed chemical mechanism of SrtA catalyzed transpeptidation. Two substrates are recognized during catalysis, the LPXTG sorting signal within the protein precursor and the (Gly)₅ cross-bridge peptide in lipid II. As the LPXTG substrate enters the active site pocket, His120 or Arg197 may function as a general-base, withdrawing a proton from Cys184 (Step 1) [45]. It is also possible that in a small fraction of the protein Cys184 is in its thiolate form and stabilized by ion pairing to His120 or Arg197 [42]. The Cys184 nucleophile then attacks the carbonyl carbon of threonine in the LPXTG motif, proceeds through a tetrahedral intermediate (Step 2), and then forms a thioacyl-enzyme intermediate (Step 3). The N-terminal amine of the pentaglycine crossbridge of lipid II then enters the active site pocket (Step 4), serving as an acceptor for the acyl-enzyme intermediate. Deacylation proceeds through a second tetrahedral intermediate (Step 5), resulting in the formation of a new peptide bond between the threonine of the LPXTG substrate and the terminal glycine of lipid II (Step 6). (Adapted from references [23, 36]).

determined [39, 40]. Although their primary sequences share only 22% sequence identity with SrtA and they possess unique sorting signal motifs (NPQTN instead of LPXTG), they adopt a similar overall fold and contain the Cys, His, and Arg triad.

The SrtA mediated cell wall anchoring reaction has been reproduced *in vitro* [24]. Detailed kinetic measurements indicate that transpeptidation occurs through a ping-pong mechanism that includes a hydrolytic shunt [41, 42]. A schematic of the mechanism is shown in Fig. (2). Catalysis is initiated when the active site thiol of Cys184 nucleophilically attacks the carbonyl carbon of the threonine residue within the LPXTG sorting signal (step 1), resulting in the formation of a transient tetrahedral intermediate (step 2). This intermediate then rearranges into a more stable thioacyl enzyme-substrate linkage after breakage of the threonine-glycine peptide bond and protonation of the leaving amine group (step 3). The thioacyl intermediate is long-lived and has been directly detected by mass spectrometry [41]. In the transpeptidation reaction, the amine group of the cell wall cross-bridge (Lipid II) then attacks the carbonyl carbon (step 4), resulting in the formation of a second tetrahedral intermediate (step 5) [24, 36, 43]. Finally, protonation of the Cys184 sulfur facilitates the formation of a new peptide bond and the breakage of the enzyme-substrate bond (step 6). Interestingly, when the triglycine nucleophile is not available, SrtA catalyzes the hydrolysis of the threonine-glycine peptide bond [43]. Hydrolysis presumably occurs through a similar mechanism as transpeptidation, with a water molecule acting as the nucleophile during deacylation. The hydrolysis and transpeptidation reactions have distinct rate limiting steps [41, 44]. In transpeptidation, the formation of the acyl-intermediate is rate limiting (step 2), whereas resolution of the thioacyl-enzyme intermediate by a water molecule (step 4) is rate limiting in the hydrolysis reaction. These results would appear to be consistent with the *in vivo* function of SrtA, since the efficient hydrolysis of the sorting signal would result in the wasteful release of surface proteins, and may therefore be slowed unless lipid II, the appropriate cell wall receptor nucleophile, is available [43]. As all sortase enzymes contain the conserved catalytic domain of SrtA and appropriate active site residues, it seems likely that their mechanism of catalysis will be similar to SrtA.

The role of the His120 and Arg197 active site residues in catalysis is still unclear. Originally it was suggested that Cys184 was activated for catalysis by forming a thiolate-imidazolium ion-pair with His120, because the disposition of these side chains within the active site is reminiscent of the well characterized papain cysteine proteases [25, 36]. Subsequently however, Cys184 was shown to be fully protonated at physiological pH leading to the suggestion that His120 functions as a general-base to deprotonate Cys184 for nucleophilic attack on the threonine carbonyl carbon [45]. A general-base mechanism has been observed in the viral 3C cysteine proteases, and in the case of the SrtB type enzymes could be mediated by an Asp-His-Cys catalytic triad [40]. Recently, McCafferty and colleagues have proposed an alternative and intriguing reverse protonation activation mechanism for Cys184 [42]. Consistent with the observed ionization state of Cys184, they propose that only a small

fraction of SrtA (ca. 0.06%) contains a thiolate-imidazolium ion-pair and is enzymatically active. This mechanism is also compatible with the available kinetic data and implies that protonation of the amide leaving group in step 2 is performed by the cationic side chain of His120. Since the side chains of His120 and Cys184 do not interact with one another in the NMR and crystal structures of SrtA [25, 35], reverse protonation would seem to require the existence of transient fluctuations, and/or substrate induced reorganization in the active site that move these side chains proximal to one another for ion-pair formation. The results of recent crystallographic work suggests that the side chain of Arg197 stabilizes negatively charged tetrahedral intermediates during catalysis, since in the crystal structure of a Cys184Ala mutant of SrtA_{ΔN59} (^{C184A}SrtA_{ΔN59}) bound to a LPETG peptide the Arg197 guanidinium group is proximal to the scissile peptide bond [35].

SUBSTRATE RECOGNITION

During the anchoring reaction SrtA recognizes two substrates, the LPXTG sorting signal within the CWS of its protein substrate, and the (Gly)₅ cross-bridge peptide located within lipid II (Fig. (2)). SrtA is highly specific for the LPXTG-motif, since only the central 'X' residue can be altered without significantly impairing enzyme activity [46]. *In vitro* and *in vivo* studies have also shown that SrtA exhibits specificity for the terminal amine bearing glycine residue in the cross-bridge peptide, and to a lesser extent the penultimate residue [41, 47]. The crystal structure of a ^{C184A}SrtA_{ΔN59}-LPETG complex has localized the sorting signal binding site to a hydrophobic surface on SrtA formed by residues in strands β4, β7, and β8, and the loop that connects strands β6 to β7 (the β6/β7 loop) (Fig. (3)) [35]. The functional relevance of this surface has been independently validated by amino acid mutagenesis and NMR chemical shift perturbation studies using cyanoalkene and sulfhydryl sorting signal analogues [38]. The available data indicate that the sorting signal is bound by an induced fit mechanism involving changes in the structure and dynamics of the β6/β7 loop [35]. Interestingly, the dynamics of this loop are modulated by calcium, which activates SrtA by promoting the binding of the sorting signal substrate [48]. NMR data suggest that the loop fluctuates between a binding competent closed form, and an open state in which key substrate contacting residues are removed from the active site. This equilibrium is skewed towards the closed state by the ion, which transiently tethers the C-terminal end of the loop to the body of the protein. Additional studies of SrtA bound to its substrates are needed to elucidate the molecular basis of substrate binding, since in the crystal structure of the ^{C184A}SrtA_{ΔN59}-LPETG complex the leucine residue in the LPETG sorting signal is not contacted by the enzyme even though this part of the signal is an essential determinant for enzyme activity [35, 46]. As many sortase enzymes recognize non-LPXTG type sorting signals it will also be interesting to see how different enzymes have adjusted their active site structures and dynamics to recognize their substrates. It is not known how SrtA recognizes the terminal glycine within the cross-bridge peptide. However, recent studies of sa-SrtB suggest that in SrtA the loop connecting strands β7 to β8 interacts with this co-substrate [39].

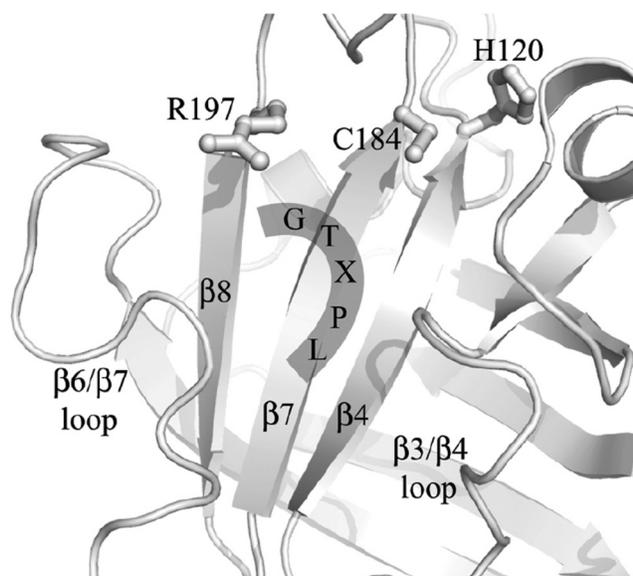


Fig. (3). The active site and LPXTG sorting signal binding site in SrtA. The positioning of the sorting signal is shown and has been localized using crystallography, NMR and amino acid mutagenesis [35, 38]. SrtA uses an induced fit mechanism to recognize the sorting signal. The $\beta 6/\beta 7$ loop is flexible in the apoenzyme, but becomes immobilized when the substrate binds [35, 48]. Residues within the $\beta 3/\beta 4$ and $\beta 6/\beta 7$ loops bind a single calcium ion, which promotes sorting signal binding by altering the structure and dynamics of the $\beta 6/\beta 7$ loop [35, 48].

SORTASE ENZYMES IN OTHER SPECIES OF GRAM-POSITIVE BACTERIA

Many species of Gram-positive bacteria encode as many as seven sortases and an even larger number of CWS-containing proteins [9]. Similar to SrtA, most homologues presumably anchor proteins to the cell wall. However, they are not restricted to this function as Ton-That has shown that sortases in *Corynebacterium diphtheriae* catalyze a protein-protein transpeptidation that assembles pilli [49]. Based on their primary sequences there are at least five types of sortases in Gram-positive bacteria (only a few sortase enzymes have been identified in Gram-negative bacteria) [50, 51]. The Gram-positive enzymes include those most closely related to the *S. aureus* SrtA and SrtB proteins, and three sortase enzyme families called SrtC-, SrtD-, and SrtE-type enzymes (also known as subfamily-3, -4 and -5 type enzymes, respectively). Interestingly, only ~20% of sortase-related proteins are most closely related to the well-characterized *S. aureus* SrtA protein, but a comparative genome analysis suggests that these sortases are prime target for inhibitor development as they are predicted to anchor the majority of surface proteins in Gram-positive bacteria [50]. In contrast, more distantly related SrtA-like proteins from other subfamilies are predicted to play a more specialized role, with each anchoring far fewer proteins that contain unusual sequence motifs.

SORTASE INHIBITORS

Here we review all reported SrtA inhibitors classified based on their type: non-specific, peptide-analogues, natural

products, and synthetic small molecules. Before this discussion we briefly survey the *in vivo* and *in vitro* assays that have been developed to measure SrtA activity. *In vitro* kinetic parameters and inhibitor IC_{50} values are typically measured using a fluorescence resonance energy transfer (FRET) assay, which monitors the SrtA enzymatic cleavage of a self-quenched fluorogenic substrate analogue [24]. In addition, HPLC-based assays have been employed to measure activity *in vitro* [52, 53]. While the HPLC assay enables the concentration of individual reaction intermediates to be tracked during catalysis, unlike the FRET assay, it is not well suited for high throughput screening. The *in vivo* potency of SrtA inhibitors can also be measured using a cell adhesion assay, which indirectly monitors enzyme activity by determining how efficiently SrtA anchors the fibronectin-binding protein to the cell wall [54-56]. IC_{50} values measured using this approach are typically ~2× fold higher than those obtained using the FRET assay [54, 55]. Several studies of SrtA inhibitors have also determined the Minimum Inhibitory Concentration (MIC), which is a semi-quantitative measurement of the concentration of the compound required to inhibit *S. aureus* growth in cell culture. As the SrtA enzyme is not required for *S. aureus* growth outside its human host, high MIC and low IC_{50} values are diagnostic for compounds that selectively inhibit SrtA.

NON-SPECIFIC SULFHYDRYL MODIFIERS

Ton-That and Schneewind have shown that SrtA activity can be inhibited by methanethiosulfonates (**1**) or *p*-hydroxymercuribenzoic acid (**2**) (Fig. (4)). These compounds modify the Cys184 side chain used in the transpeptidation reaction [57]. Although they are not specific for sortases, the identification of these compounds laid the foundation for rational inhibitor designs that targeted Cys184 and initiated the quest for inhibitors that have better potency and selectivity. The antibiotics vancomycin and moenomycin also indirectly reduce the rate of sortase mediated surface protein anchoring in whole cells [57]. These compounds inhibit the incorporation of lipid II precursors into peptidylglycan and thus act immediately downstream (Fig. (1)). In contrast, penicillin does not affect sortase activity as it inhibits cross-linking of polymerized peptidylglycan, but otherwise does not affect the physiological levels of the lipid II co-substrate [57]. Sortase enzymes are also inhibited by hydroxamate, which promotes the release of surface proteins from *S. aureus* as a result of hydroxylaminolysis of the enzyme-substrate thioester intermediate (Fig. (2), step 3) [24].

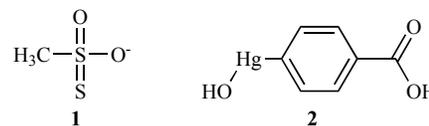


Fig. (4). Non-specific thiol modifying compounds that inhibit SrtA: Methanethiosulfonate (**1**) and *p*-hydroxymercuribenzoic acid (**2**) [57].

PEPTIDE ANALOGUES

Several peptide-based analogues of the LPXTG sorting signal have been shown to inhibit SrtA activity with modest efficacy (Fig. (5)). All of these compounds contain the

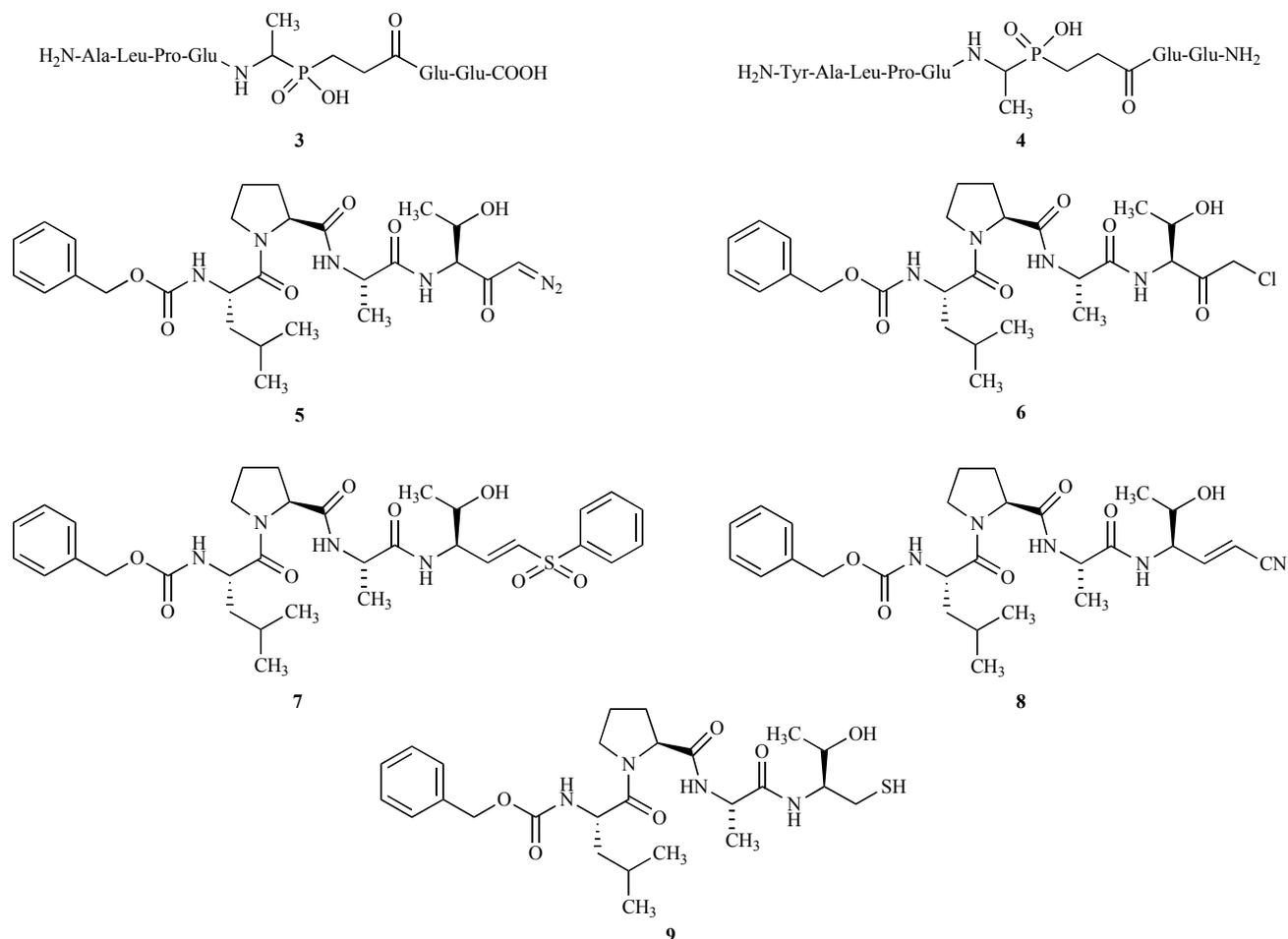


Fig. (5). Peptide-based inhibitors: a phosphinate octapeptide (3) [58] and its derivative (4) [44]; peptidyl-diazomethane (5); peptidyl-chloromethane (6) [59]; peptidyl-vinyl sulfone (7) [45]; peptidyl-cyanoalkene (8); and peptidyl-sulfhydryl (9) [38] inhibitors. All of these inhibitors contain a portion of the LPXTG motif that promotes their binding to SrtA.

LPXT recognition motif to facilitate binding to the SrtA active site, but replace the Thr-Gly scissile bond with functional groups that either mimic transition state intermediates or covalently modify Cys184. The first analogue was reported by McCafferty and colleagues and is a transition state mimic, the phosphinate octapeptide $\text{NH}_2\text{-ALPEA}\Psi$ (PO_2HCH_2)GEE-OH (3), where Ψ represents a pseudopeptide bond [58]. In the octapeptide, the SrtA substrate Thr-Gly scissile bond is replaced by a non-hydrolyzable phosphinic isostere $\text{Ala}\Psi[-\text{P}(\text{O})\text{OH}-\text{CH}_2-]\text{Gly}$, where the Thr residue has been replaced by an Ala residue for ease of synthesis. As measured using the HPLC-based assay, the phosphinate octapeptide inhibitor binds SrtA with a modest dissociation constant (K_i) of 2.5 mM. The same group also synthesized and tested a related inhibitor, $\text{NH}_2\text{-YALPEA}\Psi(\text{PO}_2\text{HCH}_2)$ GEE- NH_2 (Fig. 5. (4)), which has similar millimolar inhibitory activity ($K_i = 11.4$ mM, $\text{IC}_{50} = 10$ mM) [44]. Although the phosphinate peptides are poor inhibitors, they proved valuable in confirming that the transpeptidation reaction proceeds through a Ping-Pong hydrolytic shunt mechanism rather than a sequential (ternary complex) mechanism [44]. There appear to be at least two reasons why these transition state mimics bind with weak affinity. First, unfavorable steric interactions may occur between the phosphorous bound-

OH group on the inhibitor and the sulfur atom from Cys184 (Fig. (6)) [44]. Second, both peptides are missing a key determinant for binding as they replace the threonine side chain in the sorting signal with alanine.

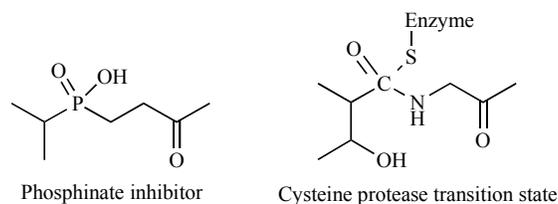


Fig. (6). Comparison of the structure of the phosphinate inhibitors (3) and (4) and the proposed transition state intermediate during catalysis. The phosphinate inhibitors non-covalently inhibit SrtA, but bind weakly. This may be caused by unfavorable steric interactions between the phosphorous -OH group of the inhibitor (left) and the side chain of Cys184 in SrtA. (Adapted from [44]).

Scott *et al.* also used sorting signal peptide analogues to inhibit SrtA, but replaced the Thr-Gly scissile bond with reactive "warheads" that covalently modified Cys184 within the active site [59]. These molecules are shown in Fig. (5) and contain diazoketone ($-\text{COCHN}_2$) (5) or chloromethylke-

tone (-COCH₂Cl) (**6**) groups appended to a peptide containing the sequence LPAT. Both the peptidyl-diazomethane and peptidyl-chloromethane peptides bind with good affinity, K_i values of 2.2×10^{-7} and 2.1×10^{-7} M, respectively. However, they inactivated SrtA at a very slow rate with first-order rate constants of inactivation (k_i) of $5.8 \times 10^{-3} \text{ min}^{-1}$ and $1.1 \times 10^{-2} \text{ min}^{-1}$, respectively. Connolly *et al.* also used a similar approach to construct irreversible peptide-based inhibitors of SrtA that had a vinyl sulfone (C=C-SO₂Ph) functional group as the reactive warhead (Fig. 5. (7)) [45]. Using the aforementioned FRET assay, the K_i and k_i of the peptidyl-vinyl sulfone were determined to be 9×10^{-6} M and $k_i = 4 \times 10^{-4} \text{ min}^{-1}$, respectively. The reduced modification rate of the peptidyl-vinyl sulfone relative to the diazomethane and peptidyl-chloromethane compounds is likely due to its lower electrophilicity, an effect which has been observed in cysteine protease inhibitors [60]. Interestingly, a study of the pH dependence of inhibition by the peptidyl-vinyl sulfone inhibitor enabled the pK_a values of the active site Cys184 residue to be determined, and revealed that this side chain is predominantly protonated at physiological pH (described above) [45]. The most recent peptide-based inhibitors were reported by Liew *et al.* [38], and used cyanoalkene (-CH=CH-CN) (**8**) and sulfhydryl (-CH₂-SH) (**9**) groups to replace the Thr-Gly scissile bond (Fig. (5)). The k_i and K_i values of both peptides are similar, 1.0×10^{-4} M and $\sim 6.3 \times 10^{-4} \text{ min}^{-1}$, respectively. Although they are poor inhibitors, NMR studies of their covalent complexes with SrtA enabled the localization of the LPXTG binding site on the enzyme and revealed that the leucine residue within the peptide por-

tion of the inhibitor is absolutely required for inhibition.

NATURAL PRODUCTS INHIBITORS

Natural products are of increasing interest in drug discovery and have accounted for approximately half of the new chemical entities reported during the past two decades [61, 62]. The quest for SrtA inhibitors from natural products has been led by the Oh group, who have identified several promising compounds from fractionation studies of plants [63-67] and marine organisms [55]. The compounds are shown in Fig. (7) and include: β -Sitosterol-3-*O*-glucopyranoside from *Fritillaria verticillata* (**10**), Berberine chloride from *Coptis chinensis* (**11**), Psamaplin A1 from *Aplysinella rhax* (**12**), Bromodexytopsentin from sponge *Spongosorites sp* (**13**), Curcumin from *Curcuma long L.* (**14**), and Morin from *Rhus verniciflua* (**15**). While all of the compounds inhibit SrtA *in vitro* and in whole cells, most also inhibit SrtB, suggesting that they work by inactivating the active site thiol. Importantly, all of the compounds exhibit MIC values in excess of 200 $\mu\text{g/ml}$ (except Berberine chloride), indicating that they do not significantly affect bacterial viability and are therefore promising starting points for further development into specific anti-infective agents [14, 55]. Below we briefly discuss each molecule, and where applicable, the results of structure-activity relationship (SAR) studies.

β -Sitosterol-3-*O*-glucopyranoside (**10**) was the first potent natural product inhibitor of SrtA (Fig. (7)) [64]. It was discovered through bioassay-guided chromatographic fractionation of *Fritillaria verticillata*, a medicinal plant that has been used as an antitussive, expectorant, and antihyperten-

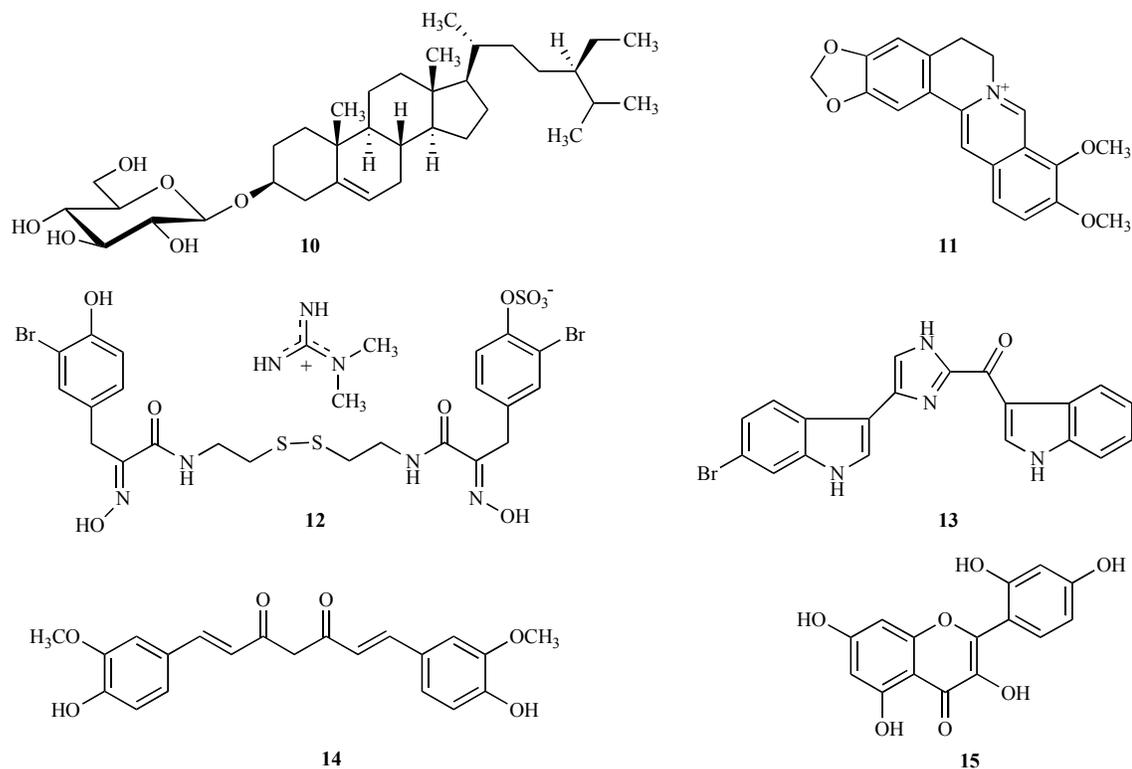


Fig. (7). Natural product inhibitors of SrtA: β -Sitosterol-3-*O*-glucopyranoside (**10**) [64]; Berberine chloride (**11**) [55]; Psamaplin A1 (**12**) [55]; Bromodexytopsentin (**13**) [55]; Curcumin (**14**) [67]; and Morin (**15**) [63].

sive drug in traditional Chinese medicine. It exhibits moderate inhibitory activity ($IC_{50} = 18.3 \mu\text{g/ml}$, or approximately $31.7 \mu\text{M}$), and its effects on microbial viability are varied (MIC values against the Gram-positive bacteria *Staphylococcus aureus*, *Bacillus subtilis*, and *Micrococcus leuteus* are 200, 50, and $400 \mu\text{g/ml}$, respectively). A limited SAR study indicates that its inhibitory potency is dependent upon the glucopyranoside side chain, since a compound that eliminates this moiety (sitosterol) is ineffective. Oh and colleagues also discovered that berberine chloride (**11**) extracted from the *Coptis chinensis* rhizome inhibits SrtA [65]. A limited SAR study of this isoquinoline alkaloid was performed by testing the potency of the structural homologs palmatine chloride and β -Hydrastine. As only β -Hydrastine exhibited reduced potency (>10-fold lower *in vitro* inhibitory activity) this result indicates that the quaternary ammonium group in berberine and/or its central ring system plays a critical role in SrtA inhibition.

Another interesting inhibitor is Psammaphin A1 (**12**), a symmetrical diamide composed of two units of an oxidized oximated bromotyrosine derivative and a cystamine unit, with *N,N*-dimethylguanidium as a counterion [55]. This compound was originally isolated from the marine sponge *Aplysinella rhax* in a search for molecules with antibacterial activity against *S. aureus*, including methicillin-resistant *S. aureus* (MRSA) [68, 69]. This molecule may reversibly modify SrtA through its acyl oxime or disulfide moieties. Nicolaou *et al.* have constructed a 3,828-membered library of related heterodimeric disulfide analogues and screened it for antimicrobial activity against *S. aureus* [70]. It would be interesting to test the effects of these compounds on SrtA activity to determine if the disulfide moiety between the subunits plays a role in Psammaphin A1's inhibitory mechanism, and structural features within each of the subunits that contribute to potency.

Bromodeoxytopsentin (**13**) inhibits SrtA and is a bis(indole) alkaloid present in the methanol extract from the marine sponge *Spongosorites* sp [55]. This compound exhibits an IC_{50} value of $19.4 \mu\text{g/ml}$ (or approximately $48 \mu\text{M}$) and has a reasonably high MIC value of $100 \mu\text{g/ml}$ against *S. aureus*. Eight natural product derivatives of Bromodeoxytopsentin have been evaluated. This work revealed that the double bond (C=C) in the imidazole ring is critical for *in vitro* activity, since 4,5-dihydrogenation of the ring leads to significant losses in potency ($IC_{50} > 100 \mu\text{g/ml}$) [55]. It is

possible that SrtA covalently modifies Bromodeoxytopsentin at this point or the presence of the double bond may simply complete the conjugated system for the overall inhibitor molecule. The imidazole ring is clearly a key determinant for activity, as hamacanthin-class derivatives that replace this group with a pyrazinone ring show diminished potency. Interestingly, the structural analogue deoxytopsentin removes the bromine atom and shows no loss in SrtA inhibitory activity, but major increases in bactericidal activity (the MIC of deoxytopsentin is $6.25 \mu\text{g/ml}$, as compared to $100 \mu\text{g/ml}$ for bromodeoxytopsentin).

Curcumin (**14**) has been shown to inhibit SrtA and is a yellow pigment found in the methanolic extract of the rhizome of turmeric, *Curcuma longa* L. (Zingiberaceae) [67]. It exhibits good *in vitro* and *in vivo* inhibitory activities ($IC_{50} = 13.8 \mu\text{g/ml}$, or approximately $37.5 \mu\text{M}$), while having no bacterial toxicity. In contrast, tetrahydrocurcumin showed no inhibitory activity against SrtA ($IC_{50} > 200 \mu\text{g/ml}$). This molecule removes the double bonds adjacent to the β -ketone, suggesting a critical role of the conjugated double bonds in the inhibition mechanism. It is possible that the enone functionalities in this compound are sites for irreversible modification by Cys184 in SrtA. Only a limited number of alterations were made to the phenol ring, and showed minimal effects on potency against SrtA. However, in a separate study the -OH groups on the phenol rings were replaced with glycinoyl moieties (-OCOCH₂NH₂) and led to significantly lower MIC values [71].

Kang *et al.* have shown that several flavonol compounds inhibit SrtA with the most potent of these molecules being Morin (**15**) ($IC_{50} = 37.4 \mu\text{M}$) (Fig. (7)) [63]. In Fig. (8) the basic structure of a flavonol compound (**16**) is displayed, along with a few of the derivatives that led to the discovery of Morin. Originally, quercetin (**16a**) was found in the ethyl acetate extract from *Rhus verniciflua* (bark), and exhibited an IC_{50} of $53 \mu\text{M}$ with no inhibition on cell growth (MIC >300 $\mu\text{g/ml}$) [63]. This prompted the researchers to test the inhibitory activity of nine other flavonols. Although all the compounds tested exhibited MIC values >300 $\mu\text{g/ml}$, their *in vitro* IC_{50} 's were dependent on the positioning of the hydroxyl groups on the B-ring (C2'-C6' phenyl ring). Flavonols containing a 4'-OH group have higher potency towards SrtA. Methylation of this 4'-OH group leads to total loss of inhibitory activity, as kaempferide (**16b**) exhibits an IC_{50} of >300 μM . In addition, the 2'-OH on the B-ring is

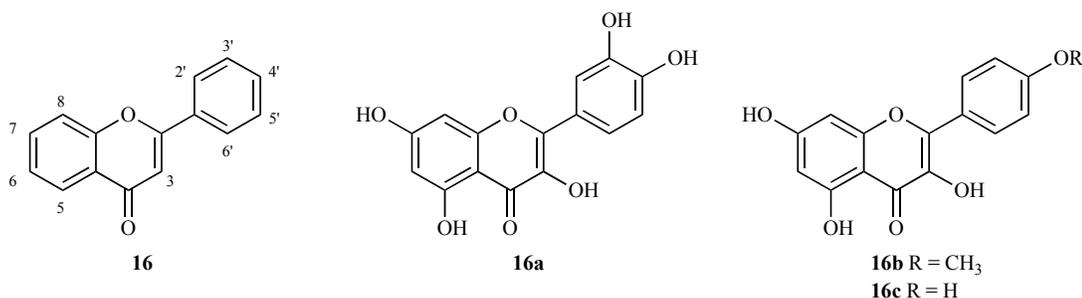


Fig. (8). Flavonol inhibitors of SrtA. Structure of flavonol (**16**) and quercetin flavonol (**16a**) SrtA inhibitor originally discovered from fractionation of *Rhus verniciflua*. The 2'-OH is critical for activity as kaempferide (**16b**) and kaempferol (**16c**) are less potent, as compared to Morin (**15**).

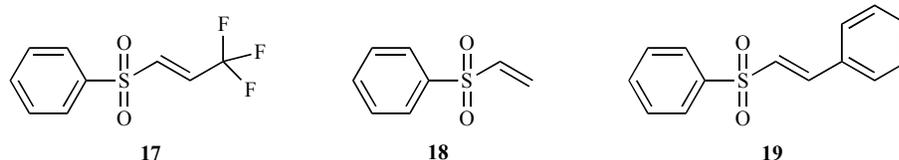


Fig. (9). Vinyl sulfone inhibitors. The compounds 3,3,3-trifluoro-1-(phenylsulfonyl)-1-propene (**17**) and phenyl vinyl sulfone (**18**) inhibit SrtA, while phenyl *trans*-styryl sulfone (**19**) is inactive.

important, as kaempferol (**16c**) ($IC_{50} = 78 \mu\text{M}$) is significantly less potent than Morin (**15**) ($IC_{50} = 37.4 \mu\text{M}$). A separate study by Xu and Lee has shown that the 2'-OH is an important determinant for MRSA toxicity [72]. By measuring the zone of inhibition they found that out of eighteen flavonols tested, only dastiscetin (3,5,7,3'-OH), kaempferol (3,5,7,4'-OH), quercetin (3,5,7,3',4'-OH), and myricetin (3,5,7,3',4',5'-OH) exhibited antibacterial activity against MRSA. These compounds share common features as they possess a 3'- or 4'-OH, but not a 2'-OH group. Although this study was performed using a different strain of *S. aureus* and did not measure inhibitory activity against SrtA, this result may be useful for further optimization efforts to create SrtA inhibitors that are not toxic to MRSA. The mechanism of SrtA inhibition is not known, but in the case of Morin may result from Cys184 modification of the α -diketone tautomeric form of the compound. The study by Kang *et al.* also revealed that the most potent flavonol inhibitors of SrtA also inhibit SrtB. Inhibition against SrtB followed the same trend as observed for SrtA, as Morin (**15**) exhibited the highest potency ($IC_{50} = 8.5 \mu\text{M}$), while kaempferide (**16b**) was least effective ($IC_{50} > 300 \mu\text{M}$). These results suggest that it might be possible to develop a dual SrtA and SrtB inhibitor, however additional studies are needed to verify that flavonols selectively inhibit sortases and not all cysteine proteases. It would also be interesting to evaluate the potency of other reported SrtA inhibitors against the activity of the SrtB enzyme, which could be useful in deriving a SAR to further understand inhibitor selectivity.

SYNTHETIC SMALL MOLECULE INHIBITORS

Two synthetic small molecule inhibitors of SrtA have been reported. Frankel *et al.* evaluated several commercially available vinyl sulfones for their ability to inactivate SrtA *in vitro* using a HPLC-based assay (Fig. (9)) [54]. They found that 3,3,3-trifluoro-1-(phenylsulfonyl)-1-propene (**17**) exhibited the lowest IC_{50} value of $190 \mu\text{M}$, 5-fold lower than the closely related phenyl vinyl sulfone (**18**) ($IC_{50} = 736 \mu\text{M}$). Presumably inhibition occurs through addition of the active site Cys184 thiol to the vinyl sulfone electrophile. Interestingly, phenyl *trans*-styryl sulfone (**19**) showed no inhibition against SrtA activity, indicating that the size of the substituents is important. Compatible with previous studies that had shown that Cys184 in SrtA acts as a nucleophile in the transpeptidation reaction [24], an analysis of the kinetic data revealed that phenyl vinyl sulfone (**18**) inhibited SrtA with an apparent single step inactivation mechanism and localized the inhibitor-SrtA covalent adduct to the active site thiol. Moreover, they found that phenyl vinyl sulfone reduced fibronectin-binding protein display in *S. aureus* at concentrations below the MIC value, indicating that it inhibited the *in*

vivo protein sorting reaction without dramatically affecting cell growth [54].

The most potent SrtA inhibitor reported to date is (*Z*)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acetonitrile (Fig. (10), (**20**), $IC_{50} = 2.7 \mu\text{M}$). This molecule was obtained by screening an 'in-house' 1,000 compound library using the FRET assay followed by optimization through synthetic chemistry approaches. The SAR study that led to this molecule revealed that the nitrile group is absolutely crucial for inhibitory activity, since replacing it with a methyl ester (-CO₂Me) reduced potency by ~100 fold. This suggests that the α,β -unsaturated nitrile serves as an electrophilic center for covalent attachment by SrtA. In addition, the *trans* conformer of the molecule was shown to be ~20 fold more active than the *cis* conformer. At present, the importance of only one of the phenyl groups has been tested by making a series of mono- and di-methoxy variants. Altering the positioning of the methoxy group resulted in modest changes in potency (2-3 fold differences). The researchers also performed a molecular modeling study in which compound (**20**) was docked into the active site of the NMR solution structure of SrtA [25]. The phenyl rings of the inhibitor were found to interact with the hydrophobic pocket in the active site, while the nitrile group formed a hydrogen bond with the side chain of Arg197. Interestingly, the active Cys184 side chain in the modeled complex does not interact with the inhibitor, a finding that is consistent with biochemical experiments that indicate that (*Z*)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acetonitrile is a reversible competitive inhibitor of SrtA [73].

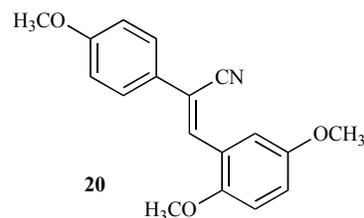


Fig. (10). The most potent SrtA inhibitor reported to date: (*Z*)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile. This compound inhibits SrtA with an $IC_{50} = 9.2 \mu\text{M}$ [73].

CONCLUSIONS AND FUTURE DIRECTIONS

The critical role of sortase enzymes in Gram-positive bacterial infections was discovered less than a decade ago [10, 24]. Since then much has been learned about the chemical mechanism of sortase-catalyzed transpeptidation and several interesting inhibitors of this reaction have been reported. Molecules derived from rational approaches have primarily been peptide-based and mimics of the LPXTG sorting signal. Although selective for SrtA, they will likely

suffer from *in vivo* instability caused by the presence of hydrolysable peptide bonds. Future work will require the synthesis of more stable cyclic analogues, which have recently been shown to be modestly effective in inhibiting SrtB [74, 75]. In addition, peptide mimetics that replace the peptide bonds with structural moieties such as thiadiazole [76], isonipecotic acid, or aminocaproic acid [74] may prove useful. Several promising natural product and synthetic inhibitors have also been identified, but only limited SAR studies have been conducted making it difficult to deduce pharmacophores required for inhibition. This will no doubt change in the future as new inhibitors are discovered and optimized, and the three-dimensional structures of sortase-inhibitor complexes are determined. Structural studies of inhibitor complexed with sortase enzymes should also facilitate the application of virtual screening approaches, yielding more insight into the inhibitory mechanism and the identification of critical pharmacophores.

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ABBREVIATIONS

| | |
|------------------|--|
| CWS | = Cell wall sorting signal |
| FRET | = Fluorescence resonance energy transfer |
| IC ₅₀ | = Inhibitory concentration at 50% |
| MIC | = Minimum Inhibitory Concentration |
| MRSA | = Methicillin-resistant <i>Staphylococcus aureus</i> |
| NMR | = Nuclear magnetic resonance |
| SAR | = Structure-activity relationship |
| SrtA | = Sortase A from <i>S. aureus</i> |
| SrtB | = Sortase B |

REFERENCES

[1] Beovic, B. *Int. J. Food. Microbiol.*, **2006**, *112*, 280-7.
 [2] Wenzel, R.P. *N. Engl. J. Med.*, **2004**, *351*, 523-6.
 [3] Finch, R.; Hunter, P.A. *J. Antimicrob. Chemother.*, **2006**, *58 Suppl 1*, i3-i22.
 [4] Navarre, W.W.; Schneewind, O. *Microbiol. Mol. Biol. Rev.*, **1999**, *63*, 174-229.
 [5] Paterson, G.K.; Mitchell, T.J. *Trends Microbiol.*, **2004**, *12*, 89-95.
 [6] Mazmanian, S.K.; Ton-That, H.; Schneewind, O. *Mol. Microbiol.*, **2001**, *40*, 1049-1057.
 [7] Cossart, P.; Jonquieres, R. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 5013-5015.
 [8] Janulczyk, R.; Rasmussen, M. *Infect. Immun.*, **2001**, *69*, 4019-4026.
 [9] Pallen, M.J.; Lam, A.C.; Antonio, M.; Dunbar, K. *Trends Microbiol.*, **2001**, *9*, 97-101.
 [10] Mazmanian, S.K.; Liu, G.; Ton-That, H.; Schneewind, O. *Science*, **1999**, *285*, 760-763.
 [11] Marraffini, L.A.; Dedent, A.C.; Schneewind, O. *Microbiol. Mol. Biol. Rev.*, **2006**, *70*, 192-221.
 [12] Weiss, W.J.; Lenoy, E.; Murphy, T.; Tardio, L.; Burgio, P.; Projan, S.J.; Schneewind, O.; Alksne, L. *J. Antimicrob. Chemother.*, **2004**, *53*, 480-6.

[13] Jonsson, I.M.; Mazmanian, S.K.; Schneewind, O.; Verdrengh, M.; Bremell, T.; Tarkowski, A. *J. Infect. Dis.*, **2002**, *185*, 1417-1424.
 [14] Mazmanian, S.K.; Liu, G.; Jensen, E.R.; Lenoy, E.; Schneewind, O. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 5510-5515.
 [15] Zink, S.D.; Burns, D.L. *Infect. Immun.*, **2005**, *73*, 5222-8.
 [16] Bierne, H.; Mazmanian, S.K.; Trost, M.; Pucciarelli, M.G.; Liu, G.; Dehoux, P.; Jansch, L.; Garcia-del Portillo, F.; Schneewind, O.; Cossart, P. *Mol. Microbiol.*, **2002**, *43*, 869-881.
 [17] Garandeau, C.; Reglier-Poupet, H.; Dubail, L.; Beretti, J.L.; Berche, P.; Charbit, A. *Infect. Immun.*, **2002**, *70*, 1382-1390.
 [18] Chen, S.; Paterson, G.K.; Tong, H.H.; Mitchell, T.J.; Demaria, T.F. *FEMS Microbiol. Lett.*, **2005**.
 [19] Paterson, G.K.; Mitchell, T.J. *Microbes. Infect.*, **2005**.
 [20] Kharat, A.S.; Tomasz, A. *Infect. Immun.*, **2003**, *71*, 2758-65.
 [21] Novick, R.P. *Trends Microbiol.*, **2000**, *8*, 148-151.
 [22] Ton-That, H.; Marraffini, L.A.; Schneewind, O. *Biochim. Biophys. Acta.*, **2004**, *1694*, 269-78.
 [23] Connolly, K.M.; Clubb, R.T. In *Structural biology of bacterial pathogenesis*, G. Waksman; M. Caparon; C. Hultgren, eds.; ASM Press: Washington DC, **2005**, pp. 101-127.
 [24] Ton-That, H.; Liu, G.; Mazmanian, S.K.; Faull, K.F.; Schneewind, O. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*, 12424-12429.
 [25] Ilangovan, U.; Ton-That, H.; Iwahara, J.; Schneewind, O.; Clubb, R.T. *Proc. Natl. Acad. Sci. USA*, **2001**, *98*, 6056-6061.
 [26] Lofdahl, S.; Guss, B.; Uhlen, M.; Philipson, L.; Lindberg, M. *Proc. Natl. Acad. Sci. USA*, **1983**, *80*, 697-701.
 [27] Uhlen, M.; Guss, B.; Nilsson, B.; Gatenbeck, S.; Philipson, L.; Lindberg, M. *J. Biol. Chem.*, **1984**, *259*, 1695-1702.
 [28] Fischetti, V.A.; Pancholi, V.; Schneewind, O. *Mol. Microbiol.*, **1990**, *4*, 1603-1605.
 [29] Schneewind, O.; Model, P.; Fischetti, V.A. *Cell*, **1992**, *70*, 267-281.
 [30] Navarre, W.W.; Schneewind, O. *Mol. Microbiol.*, **1994**, *14*, 115-121.
 [31] Ruzin, A.; Severin, A.; Ritacco, F.; Tabei, K.; Singh, G.; Bradford, P.A.; Siegel, M.M.; Projan, S.J.; Shlaes, D.M. *J. Bacteriol.*, **2002**, *184*, 2141-2147.
 [32] Mazmanian, S.K.; Ton-That, H.; Su, K.; Schneewind, O. *Proc. Natl. Acad. Sci. USA*, **2002**, *99*, 2293-2298.
 [33] Mazmanian, S.K.; Skaar, E.P.; Gaspar, A.H.; Humayun, M.; Gornicki, P.; Jelenska, J.; Joachimiak, A.; Missiakas, D.M.; Schneewind, O. *Science*, **2003**, *299*, 906-9.
 [34] Marraffini, L.A.; Schneewind, O. *J. Biol. Chem.*, **2005**, *280*, 16263-71.
 [35] Zong, Y.; Bice, T.W.; Ton-That, H.; Schneewind, O.; Narayana, S.V. *J. Biol. Chem.*, **2004**, *279*, 31383-9.
 [36] Ton-That, H.; Mazmanian, S.K.; Alksne, L.; Schneewind, O. *J. Biol. Chem.*, **2002**, *277*, 7447-7452.
 [37] Marraffini, L.A.; Ton-That, H.; Zong, Y.; Narayana, S.V.; Schneewind, O. *J. Biol. Chem.*, **2004**, *279*, 37763-70.
 [38] Liew, C.K.; Smith, B.T.; Pilpa, R.; Suree, N.; Ilangovan, U.; Connolly, K.M.; Jung, M.E.; Clubb, R.T. *FEBS Lett.*, **2004**, *571*, 221-6.
 [39] Zong, Y.; Mazmanian, S.K.; Schneewind, O.; Narayana, S.V. *Structure*, **2004**, *12*, 105-112.
 [40] Zhang, R.; Wu, R.; Joachimiak, G.; Mazmanian, S.K.; Missiakas, D.M.; Gornicki, P.; Schneewind, O.; Joachimiak, A. *Structure (Camb)*, **2004**, *12*, 1147-56.
 [41] Huang, X.; Aulabaugh, A.; Ding, W.; Kapoor, B.; Alksne, L.; Tabei, K.; Ellestad, G. *Biochemistry*, **2003**, *42*, 11307-11315.
 [42] Frankel, B.A.; Kruger, R.G.; Robinson, D.E.; Kelleher, N.L.; McCafferty, D.G. *Biochemistry*, **2005**, *44*, 11188-200.
 [43] Ton-That, H.; Mazmanian, S.K.; Faull, K.F.; Schneewind, O. *J. Biol. Chem.*, **2000**, *275*, 9876-9881.
 [44] Kruger, R.G.; Barkallah, S.; Frankel, B.A.; McCafferty, D.G. *Bioorg. Med. Chem.*, **2004**, *12*, 3723-9.
 [45] Connolly, K.M.; Smith, B.T.; Pilpa, R.; Ilangovan, U.; Jung, M.E.; Clubb, R.T. *J. Biol. Chem.*, **2003**, *278*, 34061-5.
 [46] Kruger, R.G.; Otvos, B.; Frankel, B.A.; Bentley, M.; Dostal, P.; McCafferty, D. G. *Biochemistry*, **2004**, *43*, 1541-51.
 [47] Ton-That, H.; Labischinski, H.; Berger-Bachi, B.; Schneewind, O. *J. Biol. Chem.*, **1998**, *273*, 29143-9.
 [48] Naik, M. T.; Suree, N.; Ilangovan, U.; Liew, C.K.; Thieu, W.; Campbell, D.O.; Clemens, J.J.; Jung, M.E.; Clubb, R.T. *J. Biol. Chem.*, **2006**, *281*, 1817-26.

- [49] Ton-That, H.; Schneewind, O. *Trends Microbiol.*, **2004**, *12*, 228-34.
- [50] Comfort, D.; Clubb, R.T. *Infect. Immunol.*, **2004**, *72*, 2710-2722.
- [51] Dramsi, S.; Trieu-Cuot, P.; Bierne, H. *Res. Microbiol.*, **2005**, *156*, 289-97.
- [52] Aulabaugh, A.; Ding, W.; Kapoor, B.; Tabei, K.; Alksne, L.; Dushin, R.; Zatz, T.; Ellestad, G.; Huang, X. *Anal. Biochem.*, **2007**, *360*, 14-22.
- [53] Kruger, R.G.; Dostal, P.; McCafferty, D.G. *Anal. Biochem.*, **2004**, *326*, 42-48.
- [54] Frankel, B.A.; Bentley, M.; Kruger, R.G.; McCafferty, D.G. *J. Am. Chem. Soc.*, **2004**, *126*, 3404-5.
- [55] Oh, K.B.; Mar, W.; Kim, S.; Kim, J.Y.; Oh, M.N.; Kim, J.G.; Shin, D.; Sim, C.J. and Shin, J. *Bioorg. & Med. Chem. Lett.*, **2005**, *15*, 4927-4931.
- [56] Alksne, L.E.; Projan, S.J. *Curr. Opin. Biotechnol.*, **2000**, *11*, 625-36.
- [57] Ton-That, H.; Schneewind, O. *J. Biol. Chem.*, **1999**, *274*, 24316-24320.
- [58] Kruger, R., Pesiridis, S., and McCafferty, D.G. Characterization of the Staphylococcus aureus Sortase Transpeptidase: A Novel Target for the Development of Chemotherapeutics Against Gram Positive Bacteria; R. Houghten ed.; American Peptide Society: San Diego, **2001**, pp. 565-566.
- [59] Scott, C.J.; McDowell, A.; Martin, S.L.; Lynas, J.F.; Vandembroek, K.; Walker, B. *Biochem. J.*, **2002**, *366*, 953-8.
- [60] Otto, H.H.; Schirmeister, T. *Chem. Rev.*, **1997**, *97*, 133-171.
- [61] Balunas, M.J.; Kinghorn, A.D. *Life Sci.*, **2005**, *78*, 431-41.
- [62] Newman, D.J.; Cragg, G.M.; Snader, K.M. *J. Nat. Prod.*, **2003**, *66*, 1022-37.
- [63] Kang, S.S.; Kim, J.G.; Lee, T.H.; Oh, K.B. *Biol. Pharm. Bull.*, **2006**, *29*, 1751-5.
- [64] Kim, S.H.; Shin, D.S.; Oh, M.N.; Chung, S.C.; Lee, J.S.; Chang, I.M.; Oh, K.B. *Biosci. Biotechnol. Biochem.*, **2003**, *67*, 2477-9.
- [65] Kim, S.H.; Shin, D.S.; Oh, M.N.; Chung, S.C.; Lee, J.S.; Oh, K.B. *Biosci. Biotechnol. Biochem.*, **2004**, *68*, 421-4.
- [66] Kim, S.W.; Chang, I.M.; Oh, K.B. *Biosci. Biotechnol. Biochem.*, **2002**, *66*, 2751-4.
- [67] Park, B.S.; Kim, J.G.; Kim, M.R.; Lee, S.E.; Takeoka, G.R.; Oh, K.B.; Kim, J.H. *J. Agric. Food Chem.*, **2005**, *53*, 9005-9.
- [68] Kim, D.; Lee, I.S.; Jung, J.H.; Yang, S.I. *Arch. Pharm. Res.*, **1999**, *22*, 25-9.
- [69] Shin, J.; Leea, H.-S.; Seo, Y.; Rho, J.-R.; Choa, K.W.; Paul, V.J. *Tetrahedron*, **2000**, *56*, 9071-9077.
- [70] Nicolaou, K.C.; Hughes, R.; Pfeifferkorn, J.A.; Barluenga, S.; Ro-ecker, A.J. *Chemistry*, **2001**, *7*, 4280-95.
- [71] Kumar, S.; Narain, U.; Tripathi, S.; Misra, K. *Bioconjug. Chem.*, **2001**, *12*, 464-9.
- [72] Xu, H.X.; Lee, S.F. *Phytother. Res.*, **2001**, *15*, 39-43.
- [73] Oh, K.B.; Kim, S.H.; Lee, J.; Cho, W.J.; Lee, T.; Kim, S. *J. Med. Chem.*, **2004**, *47*, 2418-21.
- [74] Matsoukas, J.; Apostolopoulos, V.; Mavromoustakos, T. *Mini. Rev. Med. Chem.*, **2001**, *1*, 273-82.
- [75] Lee, H.S.; Shin, H.J.; Jang, K.H.; Kim, T.S.; Oh, K.B.; Shin, J. *J. Nat. Prod.*, **2005**, *68*, 623-5.
- [76] Leung-Toung, R.; Zhao, Y.; Li, W.; Tam, T.F.; Karimian, K.; Spino, M. *Curr. Med. Chem.*, **2006**, *13*, 547-81.