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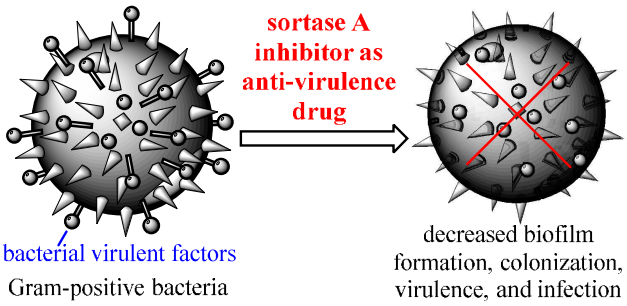

Recent progress in the development of sortase A inhibitors as novel anti-virulence drugs for antibacterial therapy has been reviewed.

Recent progress in the development of sortase A inhibitors as novel anti-bacterial virulence agents

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Abstract: Sortase A (SrtA) is a membrane-associated enzyme responsible for the covalent anchoring of many virulent factors onto the cell wall of the Gram-positive bacteria. It has been shown that SrtA plays a pivotal role in the pathogenic processes of bacterial infection. Additionally, SrtA is not indispensable for microbial growth and viability, thus its inhibition does not have a major pressure on bacteria to develop drug-resistant mechanism, and also as an extracellular membrane enzyme, SrtA can be more readily targeted by drugs, as compared to intracellular enzymes. Therefore, SrtA is an excellent target for the design and development of novel anti-virulence drugs against drug-resistant Gram-positive bacteria that have become a major and worldwide health problem. Up to date, a number of SrtA inhibitors have been identified by means of techniques including rational design of substrate mimetic inhibitors based on the structures of the enzyme and enzyme substrates, discovery of novel inhibitors among natural products, discovery and development of SrtA inhibitors via high-throughput and *in silico* screening of small molecule libraries followed by structural optimization, etc. This article has reviewed the recent progress made in the development of SrtA inhibitors as new antibacterial agents by using these techniques.

Keywords: Gram-positive bacterium, sortase, inhibitor, antibacterial, anti-virulence

1. Introduction

Gram-positive bacteria are one of the major causes of infectious diseases. The extensive use and abuse of antibiotics to treat bacterial infections in clinic have led to the rapid growth in drug resistance and thus increased morbidity and mortality of bacterial infections. How to

combat these drug-resistant bacteria, such as strains of *Staphylococcus aureus*, *Streptococcus pneumonia*, and *Enterococcus faecalis*, has become a major and worldwide health problem and a hot research area in pharmaceutical and biological sciences.[1] Owing to the increasing difficulty in the discovery and development of new antibiotics, there is an urgent need to explore novel antibacterial drug targets and strategies.[2] Recently, developing anti-virulence agents to treat bacterial infections has received increasing attention.[2] Unlike conventional antibiotics which inhibit bacterial growth or kill bacteria directly, anti-virulence agents reduce bacterial virulence and make the pathogens more susceptible to the host immune system. As a result, they impose little selective pressure on pathogens to develop drug-resistant mechanisms. Therefore, molecules associated with bacterial virulence, such as bacterial sortase (Srt), as new targets for antibacterial drug design have gained widespread interest.

Srt is a class of membrane-bound cysteine transpeptidase responsible for the covalent anchoring of surface proteins to the Gram-positive bacterial cell wall.[3-5] Phylogenetic analysis of 61 *srt* genes encoded in 22 Gram-positive bacterial genomes divided Srt into four different groups, that is, A, B, C and D.[6] SrtA is present in almost all of the low GC% Gram-positive bacterial strains. The primary SrtA-mediated protein anchoring process has been elucidated.[7-9] First, proteins destined for cell wall anchorage are secreted to associate with the extracellular membrane. These proteins contain several epitopes located at their carboxyl terminus, including a sorting signal consisting of an LPXTG motif (leucine, proline, X, threonine, and glycine, where X can be any amino acids), a membrane-binding hydrophobic region, and a tail with charged residues, which direct the secretion process. Next, the LPXTG motif is recognized by SrtA and is cleaved between Thr and Gly to form a reactive thioester between the acyl group of Thr and the Cys thiol group at the active center of SrtA. Finally, the amino terminus of the pentaglycine moiety of the cell wall precursor lipid II attacks the reactive thioester to form an amide linkage with the carboxyl terminus of the protein. By this process, surface proteins are linked to lipid II, and the products are eventually transformed into mature peptidoglycans via crosslinking reactions catalyzed by penicillin-binding proteins.

Recent studies have revealed that many bacterial virulent factors are anchored onto the cell surface by SrtA,[10-13] and these factors play an important role in the pathological process of bacterial infection, such as bacterial adhesion and invasion to the host cell, biofilm formation,

colonization, bacterial evasion of the host immune system, and so on (Table 1).[14] As a result, inhibiting the SrtA activity in bacteria caused their failure to properly display virulent factors and thereby the significant reduction of virulence and infection.[12, 15-17] For example, it has been demonstrated that an *srtA* gene knockout mutant of *S. aureus*, which was defective of various LPXTG motif-containing cell surface proteins, was unable to establish renal abscess and acute infection in mice.[10, 11] These results were also confirmed in a rat endocarditis model. Moreover, the *srtA* knockout bacterial mutant was found to be more susceptible to killing by macrophage.[18] Knockout of the *srtA* gene in other Gram-positive pathogens, such as *S. pneumoniae*,[13] *S. suis*,[19] and *Listeria monocytogenes*,[12] also led to the failure in their display of cell surface proteins, reduction of biofilm formation, and attenuation of virulence, and the mutant bacteria became more susceptible to macrophage-mediated killing.[5]

Table 1. Examples of LPXTG-containing surface proteins from *S. aureus*[a]

Name	**Abb.**	Function	Pathogenic process	Signal sequence
Collagen-binding adhesion	**Cna**	Adhesin for collagen (type Ⅰ and Ⅳ)	adhesion	**LPKTG**
Clumping factor A	**ClfA**	Platelet adhesion (fibrin-mediated); binds complement regulator factor Ⅰ	Adhesion; colonization; evasion of innate immume defense	**LPDTG**
Clumping factor B	**ClfB**	Platelet adhesion (fibrin-mediated); binds cytokeratin 10	Adhesion; colonization; evasion of innate immune defense	**LPETG**
Fibronectin-binding protein homolog	**FnbA**	Adhesin for fibrinogen, fibronectin and elastin	Adhesion; colonization; biofilm formation	**LPETG**
Fibronectin-binding protein homolog	**FnbB**	Adhesin for fibronectin and elastin	Adhesion; colonization; biofilm formation	**LPETG**
Serin-aspartate repeat protein C	**SdrC**	Adhesin	Adhesion; colonization	**LPETG**
Serin-aspartate repeat protein D	**SdrD**	Adhesin	Adhesion; colonization	**LPETG**
Serin-aspartate repeat protein E	**SdrE**	Adhesin	Adhesion; colonization	**LPETG**
Protein A	**Spa**	Binds Fc domain for immunoglobulins; binds complement protein C3	Interference with innate and adaptive immune response	**LPETG**

S. aureus surface protein C	**SasC**	Binds extracellular matrix	Adhesion; biofilm formation	**LPNTG**
S. aureus surface protein G	**SasG**	Binds extracellular matrix	Adhesion; biofilm formation	**LPDTG**
Iron-regulated surface determinant A	**IsdA**	Adhesion factor for fibronectin, fibrinogen, transferrin, hemoglobin (expressed in iron-restricted environment)	Adhesion; colonization	**LPKTG**
Plasmin sensitive protein	**Pls**	Methicillin resistant surface protein	Resistance	**LPDTG**

[a] The table is adopted from reference.[14]

Despite that SrtA is responsible for anchoring various virulent factors onto the bacterial cell surface and plays a critical role in bacterial infection and virulence, it is not indispensable for bacterial growth and viability. Therefore, SrtA inhibition does not have a big pressure on bacteria to develop drug-resistant mechanisms. Moreover, SrtA is an extracellular membrane enzyme that can be easily targeted by drugs, and currently, no eukaryotic SrtA homologs have been identified, which renders drugs targeting at SrtA specific. Therefore, SrtA is a promising target for the design and development of novel anti-virulence drugs.[14, 20]

The crystal structure of SrtA derived from different sources has been described. For example, Ilangovan et al. reported the first crystal structure of a variant of *S. aureus* SrtA, in which the 59 amino acids at the *N*-terminus of this enzyme were truncated.[21] As depicted in Figure 1, SrtA possesses an eight-stranded β-barrel fold, which includes two short helices and several loops. Strands β7 and β8 form the floor of a hydrophobic depression, with its walls constructed by amino acids located in the loops connecting strands β3–β4, β2–β3, β6–β7, and β7–β8. For all of the sortases, Cys^{184} and His^{120} are absolutely conserved and are shown to be essential for SrtA catalysis. While Cys^{184} is anchored in β7, His^{120} is located within a helical region connecting β2 and β3, with its imidazole group in the vicinity of the thiol group of Cys^{184}. Arg^{197}, which is anchored in β8, is located in close proximity and in parallel to the active-site Cys^{184} and is found to act as a hydrogen donor that interacts with the LP backbone carbonyl groups of the LPXTG substrate in the inactive form of SrtA and interacts with the TG backbone carbonyls in the active form, which may be important for holding the substrate

in position and for the catalytic process. High resolution X-ray analysis of SrtA bound to the LPETG peptide provided additional insights into the molecular interactions between SrtA and its substrate.[22] The substrate binding site resides in a concave plane molded by the β7 and β8 strands, while the scissile peptide bond between Thr and Gly is positioned between the side chains of Cys^{184} and Arg^{197}. The Leu and Pro residues of the LPETG motif are bound to the *C*-ternminal region of β7, surrounded by several highly hydrophobic amino acids. Residues perturbed after ligand binding also mapped to the *C*-terminal region of β7 strand (Thr^{180} and Ile^{182}) and to the vicinity of the loop connecting strands β3 and β4. Importantly, Thr^{180} and Ala^{118} are absolutely conserved and Ile^{182} is partially conserved among sortases. Mutation of these residues significantly impaired sortase activity *in vitro*.[23]

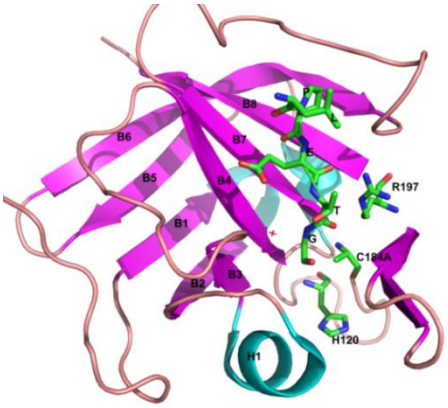

Figure 1. Crystal structure of SrtA (C184A) bound to the LPETG peptide. The structure was generated from atomic coordinates deposited in the Protein Data Bank, PDB ID: 1T2W.[22]

The elucidation of SrtA structure and its catalytic mechanism has not only facilitated the wide application of SrtA in the fields of organic synthesis and chemical biology[24-29] but also provided the molecular basis for designing and developing SrtA inhibitors as anti-virulence agents. Up to date, a number of SrtA inhibitors have been discovered through rational design and modification of analogs of the SrtA substrate. Novel inhibitors have also been discovered among natural products. In addition, modern technologies, including fluorescence resonance energy transfer-based high-throughput and *in silico* virtual screening, have been successfully utilized to design and discover SrtA inhibitors as well. This article has reviewed the recent progress made in the development of SrtA inhibitors by using various techniques.

2. Substrate mimetic SrtA inhibitors

One of the current strategies for SrtA inhibitor design is to mimic the SrtA-recognized motif of the peptide donor substrate, i.e., the sorting signal LPXTG. In this case, the inhibitor is devised to resemble the pentapeptide, so as to retain the necessary interactions between the enzyme and the resultant inhibitor, while the T-G moiety is substituted with a functionality that reacts irreversibly with the Cys^{184} thio group at the active site of SrtA. Consequently, the inhibitor can bind to SrtA and covalently modify and irreversibly deactivate the enzyme.

In 2002, Scott et al. reported the synthesis, kinetic analysis, and biological evaluation of the first class of SrtA inhibitors designed on the basis of its native substrate structure.[30] In this work, peptidyl-diazomethane and peptidyl-chloromethane analogs of the LPXTG motif, i.e., benzyloxycarbonyl(Cbz)-LPAT-CHN_2 and Cbz-LPAT-CH_2Cl respectively, were found to show time-dependent irreversible inhibition on recombinant SrtA. The diazoketone or chloromethyl ketone group was chosen as a replacement of the scissile amide linkage between T and G due to their ability to alkylate the thiol group of Cys^{184} at the active site of enzyme. The inhibitory constants for the peptidyl-diazomethane and peptidyl-chloromethane analogs were 0.22 and 0.21 μM, respectively. The functional mechanisms for these inhibitors were believed to have covalent binding with SrtA to form a Michaelis complex (Figure 2) and inactivate the enzyme. Their SrtA inhibitory activities were assessed by the fluorescence resonance energy transfer (FRET) technology using a synthetic self-quenched fluorescent probe, i.e., 4-([4-(dimethyl-amino)-phenyl]azo)benzoyl(Dabcyl, the fluorescent donor)-Gln-Ala-Leu-Pro-Thr-Gly-Glu-Glu-[5-[(2-aminoethyl)amino]naphthalene-l-sulphonic acid](Edans, the fluorescent quencher), which is also a SrtA substrate. The K_m and k_{cat} values for the SrtA-catalyzed cleavage of this self-quenched substrate were calculated by fitting the data points into the Michaelis-Menten equation for substrate hydrolysis (Figure 2) using the GraFit2 software.[30] It was also found that the peptidyl-chloromethane analog with a specificity constant of 5.3×10^4 M^{-1}•min^{-1} was *ca.* 2-fold more potent than the peptidyl-diazomethane analog as SrtA inhibitors. Connolly et al. designed and synthesized a different irreversible SrtA inhibitor by replacing the scissile T-G moiety in the SrtA recognition motif LPXTG with a vinyl sulfone ($C=C-SO_2Ph$), which could covalently modify the active-site thiol group of SrtA.[31] Because the vinyl sulfone group had

lower electrophilicity than chloromethane and diazomethane keones, the inhibitor constant for the vinyl sulfone analog was significantly increased (K_i = 9 μM). Through analyzing the pH dependence of SrtA inhibition and NMR studies, they excluded the thiolate-imidazolium ion pair mechanism for the transpeptidation reaction and proposed a general base catalysis mechanism, namely that Cys^{184} as a nucleophile is neutral at the physiological pH and His^{120} functions as a general base. Subsequently, Jung et al. prepared two tetrapeptide analogs of the sorting signal motifs of SrtA and SrtB, in which (2R,3S)-3-amino-4-mercapto-2-butanol was used to replace *L*-Thr.[32] These analogs were shown to inhibit SrtA and SrtB via the reaction of their thiol group with the Cys residue at the Srt active site to generate a disulfide bond. In addition, Kruger et al. prepared a unhydrolyzable phosphinic peptidomimetic of the LPXTG motif, NH_2-YALPE-AlaΨ(PO_2H–CH_2)G-EE-NH_2, where Ψ means that the -C(=O)NH- moiety between two amino acids is replaced with the given functional group, in which a phosphinic isostere was utilized to replace the scissile T-G bond, as an analog of the transition state of the SrtA-catalyzed reaction.[33] It was shown to be a reversibly competitive inhibitor of SrtA but its inhibitory activity was relatively low (IC_{50} = 10 mM).

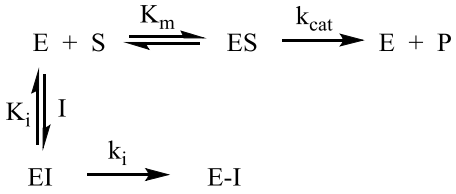

Figure 2. The mechanism for SrtA inhibition by substrate-mimetic inhibitors Cbz-LPAT-CHN_2 and Cbz-LPAT-CH_2Cl

3. Natural SrtA inhibitors

Natural products are also a rich source of SrtA inhibitors. Currently, there is no guideline for the inhibitor searching process other than activity screening. Therefore, the process can be random and time-consuming. However, it can result in novel and unexpected structures that may be used as lead compounds to carry out further optimization.

Kim et al. reported the first attempt to find SrtA inhibitors from natural sources. After screening 80 medicinal plants, they found that *Cocculus trilobus*, *Fritillaria verticillata*, *Rhus verniciflua*, and *Liriope platyphylla* had relatively strong SrtA inhibitory activities,[34] among which the ethyl acetate fraction extracted from the rhizomes of *C. trilobus* had the strongest

activity (IC_{50} = 1.52 μg/ml). Later, glucosylsterol β-sitosterol-3-*O*-glucopyranoside (Figure 3) was isolated from the bulbs of *F. verticillata* and identified as the first natural product with confirmed SrtA inhibitory activity (IC_{50} = 18.3 μg/ml or 31.72 μM).[35] However, this natural product exhibited antibacterial activities against *S. aureus*, *Bacillus subtilis*, and *Micrococcus leuteus* with MIC values of 346.71, 86.68, and 693.42 μM, respectively. In addition, after deglycosylation, the resultant aglycon, sitosterol, did not have SrtA inhibition or bacterial cell growth inhibitory activity, suggesting the importance of the glucopyranosyl residue.

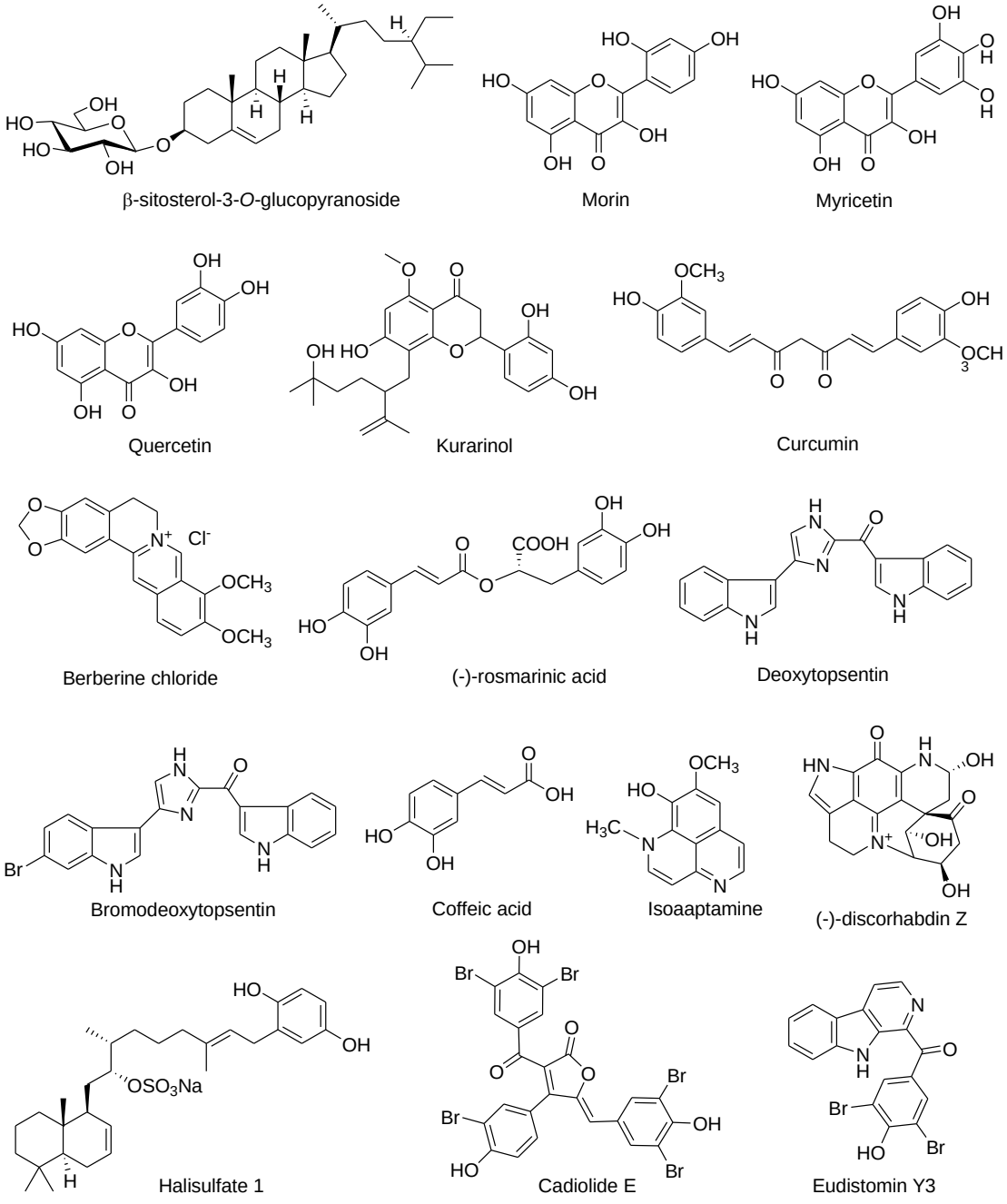

Figure 3. Structures of representative natural products SrtA inhibitors

Flavonols are another class of natural products that can inhibit SrtA. Kang et al. obtained nine flavonols from *Rhus verniciflua* with SrtA inhibitory activities, among which morin, myricetin, and quercetin (Figure 3) were the stronger ones with IC_{50} values of 11.29, 13.99, and 15.91 μg/ml (37.35, 43.96 and 52.64 μM), respectively.[36] All of these compounds did not show obvious inhibitory effect on *S. aureus* Newman growth, whereas they reduced bacterial clumping to fibrinogen in a dose dependent manner. Huang et al. demonstrated that morin had inhibitory activity to SrtA derived from *S. mutans* UA159 (IC_{50} = 8.21 μg/ml or 27.2 μM).[37] At a concentration of 9 μg/ml (30 μM), it could significantly reduce the biofilm formation of *S. mutans*, while the bacterial viability was not affected. Oh et al. isolated a series of flavonoids from the roots of *Sophora flavescens*.[38] Evaluation of their activities to inhibit SrtA and microbial growth has demonstrated that Kurarinol (Figure 3) was the most potent SrtA inhibitor, with an IC_{50} value of 48.8 μg/ml (107 μM), and had antibacterial activity against *S. aureus*, with a MIC of 99 μg/ml (219 μM).

Park et al. found that curcumin (Figure 3) extracted from *Curcuma longa* L. rhizome was a potent SrtA inhibitor, with an IC_{50} value of 13.8 ± 0.7 μg/ml or 37.5 μM, without inhibition of bacterial cell growth (MIC value greater than 200 μg/ml or 542.9 μM).[39] Moreover, similar to *srtA* gene knockout, curcumin treatment could reduce *S. aureus* cell adhesion to fibronectin in a dose dependent manner (2.5~20 μg/ml). This highlighted its potential for the treatment of *S. aureus* infections via inhibition of Srt activity. Hu et al. evaluated *in vitro* the inhibitory activity of curcumin to purified SrtA derived from *S. mutans* UA159, and its IC_{50} value was 13.8 μg/ml (10.2 μM), which was lower than the MIC value of 61.7 μg/ml (175 μM) and MBC value of 123.5 μg/ml (350 μM).[40] Subsequently, Hu et al. found that a low dose of curcumin (5.29 μg/ml or 15 μM) induced the release of Pac proteins to the supernatant and significantly reduced the biofilm formation of *S. mutant*, but it was not caused by the decrease in bacterial growth.[41]

Kim et al. isolated several isoquinoline alkaloids from the *Coptis chinenesis* rhizome and evaluated their inhibitory activities against SrtA, among which berberine chloride (Figure 3) was the most potent (IC_{50} = 8.7 μg/ml or 23.4 μM) and showed moderate antibacterial activity against Gram-positive bacteria (MIC range of 50~400 μg/ml or 134.5~1075.8 μM).[42]

Recently, Lee et al. isolated three new lignans along, together with eight known lignans and phenyl propanoids, from the dry roots of *Pulsatilla koreana* and found that these compounds could significantly inhibit SrtA derived from *S. mutans* OMZ65. (-)-Rosmarinic acid and coffeic acid (Figure 3) were also potent SrtA inhibitors with IC_{50} values of 7.2 and 3.6 μg/ml (20.0 and 20.0 μM), respectively.[43]

Oh et al. isolated several bis(indole) alkaloids, which have two indole moieties connected to each other via a heterocyclic unit, from the marine sponge *Spongosorites* sp. and evaluated their SrtA inhibitory activities.[44] Deoxytopsentin (Figure 3) was identified as the most potent SrtA inhibitor (IC_{50} = 15.67 μg/ml or 48.02 μM), but it had antibacterial activities (MIC = 6.25 μg/ml or 19.15 μM). Interestingly, 4,5-dihydrogenation of the imidazole ring led to total loss of the SrtA inhibitory activity (IC_{50} > 100 μg/ml), suggesting the significant influence of the imidazole ring substituents on the SrtA inhibitory activity of topsentins. Structure-activity relationship analysis suggested that the imidazole and pyrazinone skeletons were important for their activity. Bromodeoxytopsentin (Figure 3), which had potent SrtA inhibitory activity (IC_{50} = 19.4 μg/ml or 47.99 μM) and moderate antibacterial activity (MIC = 100 μg/ml or 247.4 μM), was evaluated by the fibronectin-binding assay and was shown to reduce the capacity of bacteria to adhere to fibronectin-coated surfaces dose-dependently in the range of 0-40 μg/ml (0-99.0 μM). Jang et al. isolated four aaptamines, 1*H*-benzo[*de*][1,6]-naphthyridine alkaloids, from the marine sponge *Aaptos aaptos* and evaluated their SrtA inhibitory activity.[45] Isoaaptamine (Figure 3) was a potent inhibitor of SrtA (IC_{50} = 3.7±0.2 μg/ml or 16.2 μM) and could reduce cell adhesion to fibronectin-coated surface in a dose-dependent manner (0-16 μg/ml or 0-70.0 μM). Their structure-activity relationship analysis revealed that the methyl group at the isoaaptamine N-1 position was important for their SrtA inhibitory activity. Jeon el al. isolated several pyrroloiminoquinone alkaloids of the discorhabdin class from the sponge *Sceptrella* sp.[46] Biological studies revealed that (-)-discorhabdin Z (Figure 3), which contained an unusual hemiaminal group, was a potent SrtA inhibitor (IC_{50} = 2.19 μg/ml or 6.14 μM) that did not affect microbial viability (MIC > 100 μg/ml or 280.4 μM) but was cytotoxic to the K562 cell line (IC_{50} = 0.74 μg/ml or 2.08 μM). Bae et al. isolated eight sesterterpenes and related pentaprenyl hydroquinones (halisulfates and suvanine) from the sponge *Coscinoderma* sp.,[47] and found that halisulfate 1 (Figure 3) was the most potent SrtA

inhibitor (IC_{50} = 21.34 μg/ml or 38.33 μM). In contrast to other SrtA inhibitors, halisulfates were active against both Gram-positive and Gram-negative bacteria, the mechanism of which has not been clarified yet. In addition, Won et al. isolated several furarines and beta-carboline alkaloids from *Synoicum sp*.,[48, 49] such as cadiolide E and eudistomin Y3 (Figure 3) that had moderate SrtA inhibitory activities (IC_{50} = 78.25 and 145 μM, respectively). However, these compounds had some antibacterial activity and cytotoxicity.

The natural origins, enzyme inhibitory activities, fibronectin-binding inhibitory activities, antibacterial activities, and related references of all aforementioned natural products as SrtA inhibitors are summarized in Table 2.

Table 2. The SrtA inhibition, fibronectin binding inhibition, bacterial inhibition activities of some representative natural products identified as SrtA inhibitors

Name	Source	SrtA IC_{50} (μM)	Fibronectin binding inhibition (μM)	MIC (μM)	Refs
β-sitosterol-3-O-glucopyranoside	*F. verticillata*	31.72 (*S. aureus*)	N.D.	346.7 (*S. aureus*)	35
Morin	*R. vernicifua*	37.35 (*S. aureus*)	Dose dependent	>2977 (*S. aureus*)	36, 37
		27.2 (*S. mutans*)			
Myricetin	*R. vernicifua*	43.96 (*S. aureus*)	Dose dependent	>2828 (*S. aureus*)	36
Quercetin	*R. vernicifua*	52.64 (*S. aureus*)	Dose dependent	>2977 (*S. aureus*)	36
Kurarinol	*S. flavescens*	107 (*S. aureus*)	N.D.	219 (*S. aureus*)	38
Curcumin	*C. longa* L.	37.5 (*S. aureus*)	Dose dependent 6.8-54.3	>542.9 (*S. aureus*)	39
		10.2 (*S. mutans*)	N.D.	175 (*S. mutans*)	40, 41
Berberine chloride	*C. chinensis*	23.4 (*S. aureus*)	N.D.	269 (*S. aureus*)	42
(-)-rosmarinic acid	*P. koreana*	20.0 (*S. mutans*)	N.D.	N.D.	43
coffeic acid	*P. koreana*	20.0 (*S. mutans*)	N.D.	N.D.	43

Deoxytopsentin	*Spongosorities* sp.	48.02 (*S. aureus*)	N.D.	19.15 (*S. aureus*)	44
Bromodeoxytop-sentin	*Spongosorities* sp.	47.99 (*S. aureus*)	Dose dependent 0-99.0	247.4 (*S. aureus*)	44
Isoaaptamin	*Aaptos aaptos*	16.2 (*S. aureus*)	Dose dependent 0-70.0	219.2 (*S. aureus*)	45
(-)-discorhabdin Z	*Sceptrella* sp.	6.14 (*S. aureus*)	N.D.	>280.4 (*S. aureus*)	46
Halisulfate 1	*Coscinoderma* sp.	38.33 (*S. aureus*)	N.D.	2.8-44.9 (*S. aureus*)	47
cadiolide E	*Synoicum* sp.	78.25 (*S. aureus*)	N.D.	3.9 (*S. aureus*)	48
Eudistomin Y3	*Synoicum* sp.	145 (*S. aureus*)	N.D.	14.0 (*S. aureus*)	49

4. Synthetic small molecule SrtA inhibitors

High-throughput and *in silico* virtual screening technologies have been used to discover novel small molecule SrtA inhibitors as well. In these studies, molecular docking is typically performed on the basis of the reported crystal structures of the SrtA-pentapeptide substrate complexes (Figure 1). As discussed above, the conserved His^{120}, Cys^{184}, and Arg^{197} triad at the SrtA active site is functionally absolutely necessary. Additionally, in the vicinity, there is also a large hydrophobic binding pocket composed of the lipophilic side chains of amino acids including Val^{193}, Trp^{194}, Ala^{92}, Ala^{104}, Leu^{169}, Val^{168}, and Ile^{182}.[50] As a result, most of the SrtA inhibitors discovered so far possess not only polar functionalities located at the middle of the molecule that can form hydrogen bonds or charge-charge interactions with His^{120} and Arg^{197} and potentially Cys^{184}, but also lipophilic groups, such as aromatic rings, that can insert into and interact with the hydrophobic binding pocket. Such models of SrtA-substrate interactions are also used as a general guide during the optimization of lead compounds.

Via high-throughput screening, Oh et al. identified compound **1** (IC_{50} = 231μM) (Figure 4) as a promising lead of SrtA inhibitors.[50] They synthesized a series of its derivatives and studied their structure-activity relationships, which suggested that the positioning of the two phenyl groups and the introduction of a nitrile group to the side chain were pivotal for SrtA inhibition. Compound **2** (Figure 4), which had an IC_{50} value of 9.2 μM, was the most active

inhibitor among all of the synthetic derivatives and was almost 25 times more potent than compound **1** for the inhibition of SrtA. Kinetics studies disclosed that compound **2** was a competitive inhibitor with a K_i value of 6.81 μM. Molecular modeling was also performed to study the relationship between the compound structure and the inhibitory activity. Although compound **2** does not interact with the side chain of Cys^{184}, its nitrile group forms hydrogen bonding with the two guanidyl NH groups of Arg^{197}, and its phenyl rings have relatively strong lipophilic interactions with the large hydrophobic binding pocket at the SrtA active site. Subsequently, Oh et al. evaluated the *in vivo* biological activity of **2** in Balb/c mice.[51] After inoculation with 10^7 CFU of *S. aureus* Newman, all of the mice without treatment died within 2 weeks, but the mice receiving intraperitoneal injection of **2** at doses of 100, 20, and 4 mg/kg had survival rates of 75%, 100%, and 97%, respectively. Moreover, **2** reduced the joint and kidney infections mediated by SrtA. However, it was observed that the animal survival rate was lower for the higher dose (100 mg/kg) group than for the lower dose (20 mg/kg) group, suggesting the potential toxic and side effects of **2**. Another study further revealed that like some natural SrtA inhibitors such as β-sitosterol-3-*O*-glucopyranoside, berberine chloride and psammaplin A1, **2** could inhibit the adhesion of *S. aureus* cells to fibronectin.[52] Kudryavrsev et al. prepared some *cis*-5-phenyl prolinates with electrophilic substituents at the 4-position of the pyrrolidine ring via 1,3-dipolar cycloaddition reactions of arylimino esters with divinyl sulfone or acrylonitrile.[53] They found that 4-vinylsulfinyl 5-phenyl prolinates inhibited *S. aureus* SrtA irreversibly through modification of the enzyme Cys^{184} residue, but they were relatively weak inhibitors (IC_{50} in the order of mM). Nonetheless, they can be used as leads for the development of new antibacterial and anti-virulence agents.

After screening a library of 135,625 structures, Maresso et al. found that aryl β-aminoethyl ketones (AAEK) had SrtA inhibitory activities. AAEK1 **3** and AAEK2 **4** (Figure 4) had IC_{50} values of 47 and 15 μM for *S. aureus* SrtA and IC_{50} values of 4.8 and 5.6 μM for *Bacillus anthracis* SrtA, respectively.[54] Their proposed Srt inhibition mechanism was that under the influence of SrtA, they could transform into reactive olefins via β-elimination and the olefins could covalently modified thiol groups. Therefore, these molecules inhibited SrtA through irreversible, covalent modifications of the Cys residue at the enzyme active sites.

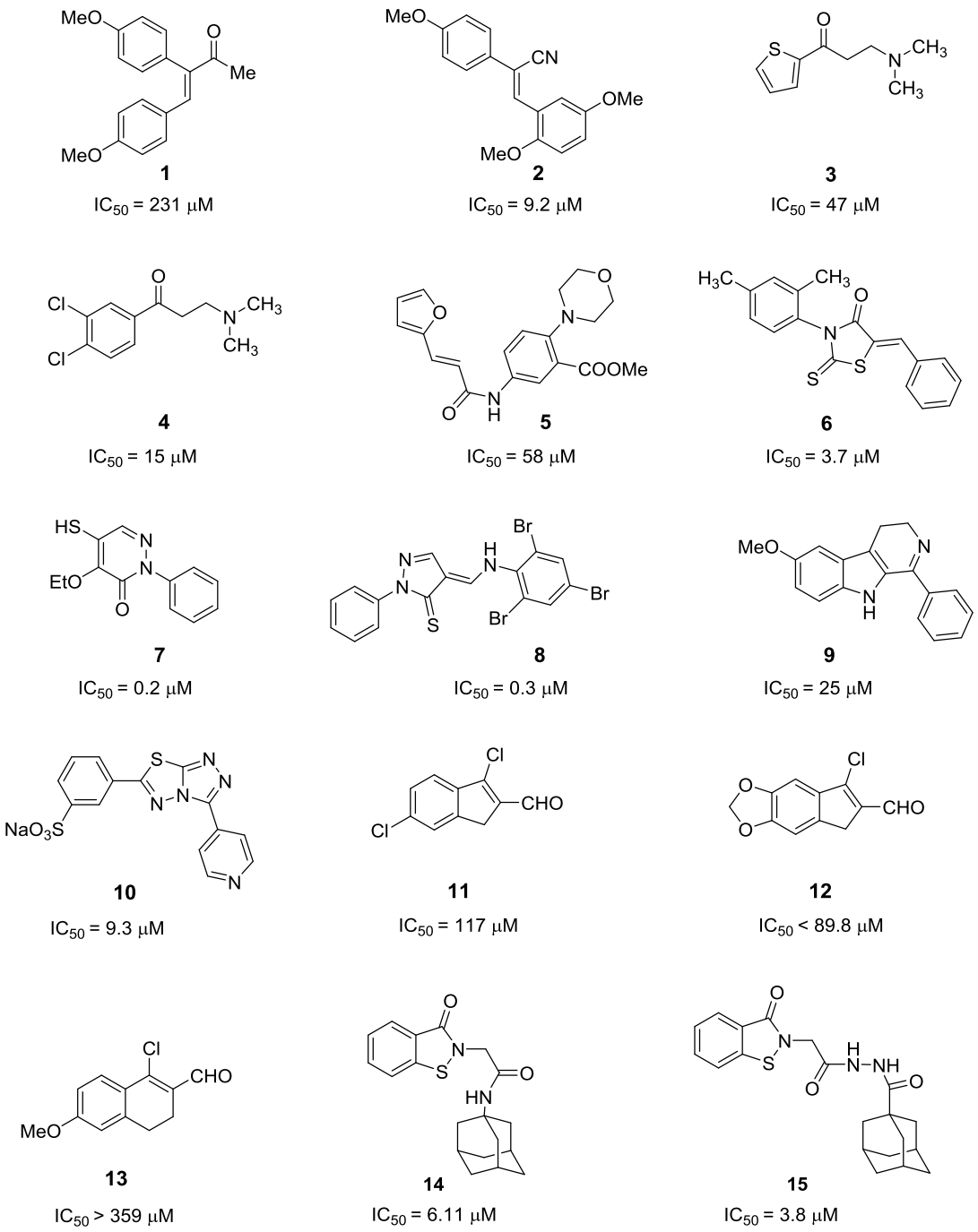

Figure 4. Structures of some synthetic small molecule SrtA inhibitors and their *S. aureus* SrtA inhibition activities

Chenna et al. identified new *S. aureus* SrtA inhibitors through *in silico* virtual screening of commercial compound libraries by means of the SYBYL software.[55] Preliminary structure-activity relationship studies on the lead compound resulted in the development of compounds with improved activity, such as **5** (Figure 4) that had an IC_{50} value of 58 μM. Subsequently, they systematically analyzed the structure-activity relationship of compound **5** and found that

the stereochemistry of the double bond was important for their bioactivity.[56] In most cases, changing the *E* double bond to the *Z* isomer or to a rigid triple bond reduced the enzyme inhibitory activity. Reducing the double bond to a C-C single bond resulted in the complete loss of activity. The amide carbonyl group, NH group, and morpholine ring oxygen were also important for the SrtA inhibitory activity.

After high-throughput screening of 30,000 compounds, Suree et al. found three classes of novel small molecule SrtA inhibitors, rhodanines, pyridazinones, and pyrazolethiones, which inhibited SrtA in a reversible manner with IC_{50} values in the sub-micromolar range.[57] The most active SrtA inhibitors were rhodanine **6**, pyridazinone **7**, and pyrazolethione **8** (Figure 4) with IC_{50} values of 3.7, 0.20, and 0.30 μM, respectively. Pyridazinone is the most potent SrtA inhibitor known to date, and it was proposed to inhibit SrtA via a thiol-disulfide exchange reaction with SrtA Cys^{184}. Its structure-activity relationship studies highlighted the significant impact of the location and nature of substituents on the pyridazinone ring on its SrtA inhibitory activity. In the meantime, cell-based assays showed that these compounds had no impact on the *S. aureus* and *B. anthracis* viability, suggesting that they are rather SrtA-specific inhibitors. This supported their potential as anti-virulence drugs for further development. On the basis of these encouraging results, as well as the results of other derivatives of **6**, **7**, and **8**,[58] Uddin et al. created a pharmacophore model and investigated the three-dimensional quantitative structure-activity relationships. The model was verified through comparative molecular field analysis and comparative molecular similarity indices analysis and could be utilized to better understand and explain the correlation between the structural features of the compounds and their biological activities.

Based on the discovery that indole-containing natural products had inhibitory activities against *S. aureus* SrtA and isocitrate lyase from *Candida albicans*,[59, 60] Lee et al. prepared a series of structural analogs of the natural products,[61] among which six compounds exhibited higher SrtA inhibitory activities than the positive control, *p*-hydroxymercuribenzoic acid. In particular, compound **9** (Figure 4) showed the highest SrtA inhibition with an IC_{50} value of 25 μM and had no influence on bacterial viability (MIC > 200 μg/ml against *S. aureus*).

Zhang et al. used virtual screening technology to identify new structures that can bind to the active site of SrtA and proved that 3,6-disubsitituted triazolothiadiazole compounds were

SrtA inhibitors through *in vitro* and *in vivo* studies.[62] Compound **10** (Figure 4) was the most active with IC_{50} values of 9.3 μM and 0.8 μM against SrtAs from *S. aureus* and *S. pyogenes*, respectively, while it had no influence on staphylococcal growth *in vitro* (MIC > 40 mM). A BALB/c mouse model was used to evaluate the *in vivo* activities of **10**, and it was found to be efficacious in preventing lethal bacteremia and infections induced by *S. aureus*.

Kahlon et al. studied tetralene and indene compounds that have shown inhibitory activity against human pathogen, *Mycobacterium tuberculosis*, as SrtA inhibitors via *in silico* analysis, which was followed by biological assays.[63] Indeed, compounds **11** and **12** (Figure 4) showed some SrtA inhibitory activities (IC_{50} values of 117 and < 89.8 μM, respectively). However, **11**-**13** also exhibited antibacterial activities against *S. aureus* with MIC values of 14.67, 14.04, and 224.5 μM, respectively, which suggested that these compounds might not be SrtA-specific and hit other targets as well. Thus, they might not be the ideal drug candidates. Nevertheless, these compounds did not exhibit significant cytotoxicity to Vero and WRL-68 cell lines.

Zhulenkovs et al. screened a library of 50,240 compounds against SrtA and identified **14** (Figure 4) as a SrtA inhibitor with an IC_{50} value of 6.11 μM, whereas its MIC value against *S. aureus* was 2.92 μM and IC_{50} value against NIH3T3 cell was 1.27 μM.[64] In order to improve the SrtA inhibitory potency and reduce cytotoxicity, structural optimization was performed, which resulted in compound **15** (Figure 4) that demonstrated higher MIC value and a ten-fold decrease in cytotoxicity. It IC_{50} value against SrtA, MIC and IC_{50} values against NIH3T3 cell were 3.8, 41.51, and 14.39 μM, respectively.

5. Conclusion

The rapid evolvement of drug-resistant bacterial strains to antibiotics currently in use has become a serious health challenge. Consequently, there is an urgent demand to develop novel antimicrobial agents and strategies. Studies have demonstrated that SrtA plays a pivotal role in the pathogenic processes of bacterial infection. Inhibition of SrtA activity in bacteria has been shown to affect the proper presentation of various virulent factors and thereby decrease bacterial biofilm formation and bacterial adhesion or invasion to the host cell, render bacteria more susceptible to the human immune system, and attenuate bacterial virulence. On the other hand, SrtA is not indispensable for bacterial growth and viability, so its inhibition does not

have a major pressure for the bacteria to develop drug-resistant mechanisms. Moreover, there has been no SrtA homolog in eukaryotes. Therefore, SrtA is a promising target for the design and development of new anti-virulence drugs. In the past decade or so, this research area has made significant process, and a variety of SrtA inhibitors have been discovered. For example, pyridazinone and pyrazolethione compounds discovered through high-throughput screening were potent SrtA inhibitors with IC_{50} values reaching the nanomolar level. In the meantime, these inhibitors did not have significant impacts on the viability of *S. aureus* and *B. anthracis*, indicating their target specificity and potential as anti-virulence drugs.

Currently, SrtA inhibitors are mainly discovered or developed by three approaches. One approach is to design SrtA inhibitors by mimicking its substrate structure. This type of SrtA inhibitors can bind specifically to the enzyme, in a reversible or irreversible manner, to block the enzymatic activity and thereby to be potent, while nontoxic. Although some progress has been made in this area, no particularly strong SrtA inhibitors have been developed yet, maybe because of the susceptibility of the synthetic peptide mimics to proteases. Nevertheless, we believe that this is a promising direction, and the key topics for future research are to further improve the binding affinity between the enzyme and the substrate analog and to improve the stability of inhibitors, such as by replacing the peptide bonds with bonds that are more stable to proteases. The other two approaches are to identify new inhibitors among natural products or by high-throughput and *in silico* screening of small molecule libraries. The key advantage for identifying SrtA inhibitors among natural products is that structurally unique inhibitors can be discovered, which can be used as lead compounds for further structural optimization and development. However, this approach is limited by the available natural resources and manpower. The small molecule library approach can take advantage of the convenience and power of high-throughput and *in silico* virtual screening technologies to probe a large number and a broad range of structures so as to improve the search efficiency, while the identified inhibitors can be further optimized through rational design. Up to date, the most potent SrtA inhibitors have been discovered through screening of small molecule libraries. With the rapid growth in molecular libraries and databases, we believe that more potent SrtA inhibitors may be identified by these technologies. Potential problems associated with small molecule drugs are that they often can interact with multiple molecular targets and thus have side effects. As

each approach has its own advantages and disadvantages, we believe that the combination of different approaches should be a promising strategy for future discovery and development of SrtA inhibitors. For example, new and better SrtA inhibitors may be designed by combining the promising specificity and targeting ability of substrate-based inhibitors with the concept of small molecule pharmacophores.

Another important research topic in the development of SrtA-based anti-virulence drugs is the methods employed to evaluate SrtA inhibitors. Currently, the SrtA inhibitory activities are assessed by means of FRET technology utilizing a synthetic self-quenche fluorescent probe, which is also a cleavable SrtA substrate. However, like other anti-virulence drugs, an ideal Srt inhibitor-based antibacterial agent may not have a significant influence on bacterial growth and viability. Consequently, to ultimately evaluate the therapeutic potential of a specific SrtA inhibitor and determine whether that inhibitor is worthy further development, it had to be assessed by other methods as well, such as evaluation of their impact on bacterial biofilm formation, evaluation of their influence on bacterial adhesion to the host cell by means of fibronectin-binding assay, evaluation of their capacities to effect macrophage-mediated killing of bacteria, and *in vivo* anti-infectious assays. These assays are time-consuming, which affects the efficiency of drug screening. In addition, the screening models for these studies are based on drug-resistant *S. aureus*, whereas whether the SrtA inhibitors are effective to combat other bacteria remains to be verified. Consequently, establishing a more convenient and effective method for rapid evaluation and high-throughput screening of the anti-virulence activities of SrtA inhibitors, or other similar antibacterial agents, is an important topic, and any progress in this direction will have a great impact on the area.

Finally, because unlike conventional antibiotics, anti-virulence agents do not directly kill bacteria but attenuate their virulence, interrupt their adhesion and invasion to the host cell, and make them more susceptible to the human immune system, for these antibacterial agents to work well, the host's immune system has to function properly to help remove the pathogens. As a result, they are not suitable for immunocompromised patients. To treat these patients and to further improve the overall therapeutic efficacies of SrtA inhibitors as anti-virulence drugs, another future research direction may be the combined usage of anti-virulence drugs with immunostimulant agents.

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