Discovery of Diarylacrylonitriles as a Novel Series of Small Molecule Sortase A Inhibitors

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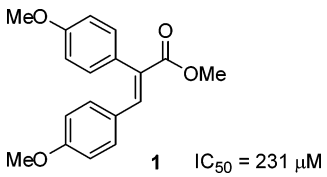
Abstract: On the basis of a hit from random screening, a novel class of small-molecule sortase A inhibitors was generated. The primary structure−activity relationship and the minimal structural requirements for potency were established through structural modifications and molecular modeling studies.

Gram-positive pathogenic bacteria display surface proteins that play pivotal roles in the adhesion to specific organ tissues, invasion of host cells, or the evasion of host-immune responses.[1] Some typical examples of these are Protein A, fibronectin-binding proteins A and B, and collagen-binding protein. A remarkably common feature of these functionally and structurally diverse surface proteins is the possession of a distinctive C-terminal 'sorting sequence', composed of a hydrophobic domain, a positively charged tail, and a conserved LPXTG (where X represents any amino acid) motif.[2−4]

The surface proteins are covalently attached to the cell wall peptidoglycan, by a mechanism ubiquitous among the entire class of the Gram-positive bacteria. Through a series of elegant experiments, Schneewind et al.[5−8] showed that sortase participates in the pathway involved in the secretion and anchoring of surface proteins. Sortase is a cytoplasmic membrane-bound cysteine protease-transpeptidase that employs an active-site cysteine residue for catalysis. Two different sortase species have been identified, sortase A (SrtA) and sortase B (SrtB), which recognize specific surface protein sorting signals.[9]

SrtA is constitutively expressed, and cleaves the amide bond between the threonine and glycine of the above-mentioned LPXTG motif, via the nucleophilic attack of the thiol group of Cys184 on the carbonyl group of the threonine.[6,7,10] The resulting acyl-enzyme intermediate is subsequently captured by the peptidoglycan amino group found at the end of the cell wall crossbridge. Conversely, SrtB, which was identified only very recently, is only transcribed under the condition of low iron and has different substrate specificity to SrtA. SrtB recognizes the surface protein substrate bearing the NPQTN motif sorting signal, and anchors to the cell wall envelope.[9]



1 IC_{50} = 231 μM

Figure 1. Structure of the screening hit **1**.

Recent extensive biological studies[9,11−14] have shown that both SrtA and SrtB are critical virulent factors that participate in the establishment and persistence of infections. *Staphylococcus aureus* mutants lacking sortase fail to display surface proteins and are defective in the establishment of infections without affecting microbial viability.[5,11] Therefore, a sortase inhibitor has the potential to be used in combination with an antibacterial agent to provide a more effective treatment for infectious diseases.[1,15]

Currently, there have only been a few reports in the literature describing inhibitors of sortase, due, in part, to the fact that importance of sortase as a new target has only recently been acknowledged. Initially, Schneewind's group tested many types of compound.[16] Of these compounds, methanethiosulfonates or organo-mercurials displayed inhibitory effects on the sorting reaction, presumably via interaction with the thiol group of the active-site cysteine. In addition, they also observed that vancomycin and moenomycin, inhibitors of cell wall polymerization into peptidoglycan strands, slowed the sorting reaction. However, these antibiotics did not interfere directly with the sorting reaction, but rather changed the physiological concentration of the peptidoglycan precursors. Recently, two groups independently reported the synthesis and evaluation of substrate-derived, irreversible peptidic inhibitors of SrtA. Walker and co-workers[17] replaced the scissile amide bond between the threonine and glycine residues of the LPXTG motif with a diazoketone or chloromethyl ketone group. Clubb et al.[18] synthesized a peptidyl-vinyl sulfone substrate mimic that irreversibly inhibited SrtA.

Herein, we report our discovery of small-molecule inhibitors of SrtA through the random screening and structural modifications based on the screening hit. The inhibitors with low micromolar inhibitory potencies, in vitro, against the recombinant SrtA have been obtained.

At the start, to identify inhibitors of SrtA, we randomly screened our diverse small molecular library of 1000 compounds, using a recombinant SrtA,[19] expressed and purified in our laboratory. This investigation afforded several hits with high micromolar inhibitory potencies. Of these, compound **1** (Figure 1), with an IC_{50} of 231 μM, was the most attractive as a starting point for a novel class of inhibitors because of its simplistic structure and synthesis.

To understand the structure−activity relationship, we initially prepared compounds **2**−**5** (Figure 2),[20] in which the methyl ester group of compound **1** was replaced with a carboxylic acid, primary amide, and nitrile groups, and

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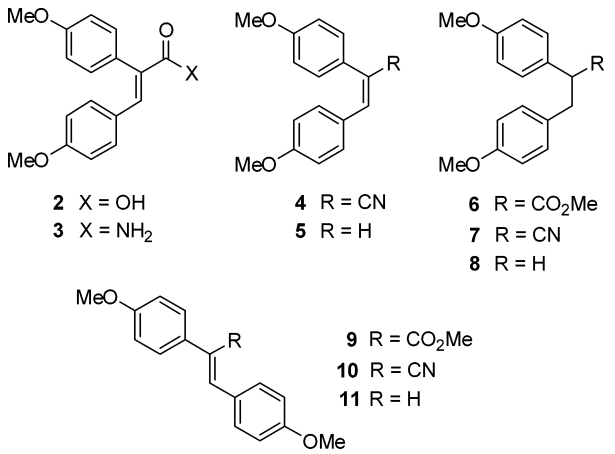


Figure 2. Structure of the prepared compounds **2**−**11**.

Table 1. Inhibition of SrtA[a]

entry	compound	IC_{50} (μM)[b]
1	**1**	231.086 ± 6.530
2	**2**	>1000
3	**3**	476.034 ± 11.083
4	**4**	187.403 ± 3.958
5	**5**	>1000
6	**6**	>1000
7	**7**	>1000
8	**8**	>1000
9	**9**	908.928 ± 26.682
10	**10**	27.892 ± 0.490
11	**11**	>1000
12	**14a**	36.222 ± 0.942
13	**14b**	17.376 ± 0.490
14	**14c**	9.244 ± 0.474
15	**14d**	22.957 ± 0.643
16	**14e**	25.463 ± 0.948

[a] Enzyme inhibitory activities were measured as described in the Supporting Information. [b] Data represents the mean ± SEM (n = 5).

a hydrogen atom, respectively, with the retention of the relative stereochemistry of the two phenyl rings. Of these, compounds **2** and **5** did not show any inhibitory activities to SrtA up to 1000 μM (Table 1). Conversely, the nitrile compound **4** showed only a slightly increased inhibitory activity (IC_{50} = 187 μM), while amide **3** exhibited a decrease in the potency of the SrtA inhibition (IC_{50} = 476 μM) than ester **1**.

To test the influence of the double bond on the activity, saturated analogues **6**−**8** were prepared from their corresponding compounds (**1**, **4**, and **5**) by hydrogenation. However, none of the three compounds exhibited measurable activity against SrtA (entry 6−8). To understand the influence of the double bond configuration further, we tested compounds **9**−**11** in which two benzene rings were located in the trans orientation. As shown in Table 1, the inhibitory potential of (*Z*)-ester **9** was diminished substantially as compared to its (*E*)-isomer **1**. The *trans*-stilbene **11** showed no activity as its cis isomer **5**. However, to our delight, we found that (*Z*)-diarylacrylonitrile **10** (IC_{50} = 28 μM) was almost 7−8 times more potent against SrtA than the screening hit **1** or the corresponding (*E*)-isomer **4**. In comparison with *trans*-stilbene **11**, which was inactive, compound **10** differed structurally by only the nitrile group at the ethylene bridge of two benzene rings. These results strongly suggested that the presence of a nitrile group

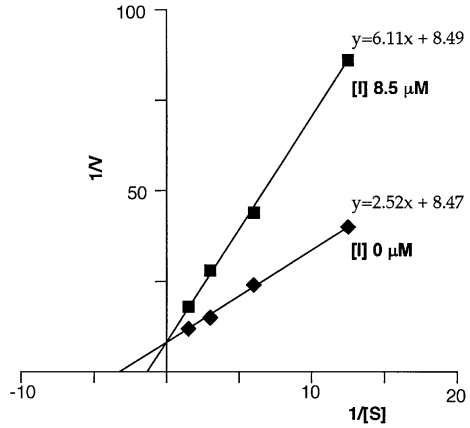
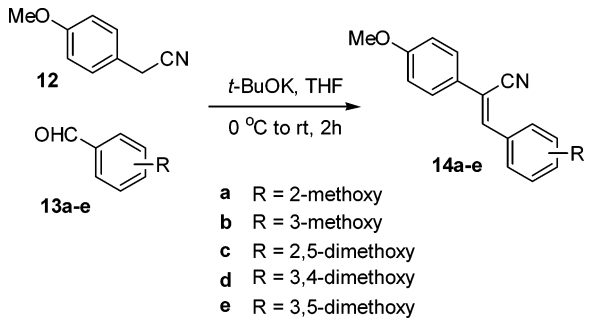


Figure 3. Lineweaver-Burk plot of SrtA inhibition by compound **14c**. [S], substrate (Dabcyl-QALPETGEE-Edans) concentration [$\times 10^{-3}$ μM]; V, reaction velocity (μM/min). Each data point represents the mean ± SD of three experiments.

Scheme 1. Synthesis of (*Z*)-Diarylacrylonitrile **14**



and the exact location of the two aromatic rings are crucial for effective inhibition of the enzyme activity.

Having established the primary structure−activity relationship, we then investigated the effects of different aromatic substituents on the new lead compound **10**. We prepared a series of compounds with the general structure **14** (Scheme 1), where one phenyl ring was fixed with a methoxy group at the 4-position, and the other phenyl ring was modified with a methoxy group at various positions. Other hydrophilic functional groups were not considered at this point, as the recent NMR structure of SrtA[8] revealed this enzyme to have a hydrophobic pocket suitable for sorting signal binding. The syntheses of this series of compounds was accomplished by the well-known condensation[21] of 4-methoxyphenylacetonitrile **12** with benzaldehyde **13** in the presence of *t*-BuOK.

All the synthesized compounds **14a**−**e** showed inhibition of SrtA, with IC_{50} values of less than 40 μM, as shown in Table 1 (entry 12−16). In particular, compound **14c** was the most active, with an IC_{50} of 9.2 μM, which could be the first small-molecule inhibitor of SrtA, with single-digit micromolar inhibitory potency, identified. The other compounds exhibited almost equipotent activities, suggesting that the potency of these compounds might not be highly dependent on the substitution pattern of the modified phenyl ring.

To determine the type of inhibition, kinetic studies were performed with compound **14c** at the concentration where approximately 50% of enzyme activity was inhibited. Inhibitory kinetics in Figure 3 show that compound **14c** only affect the slope of the Lineweaver

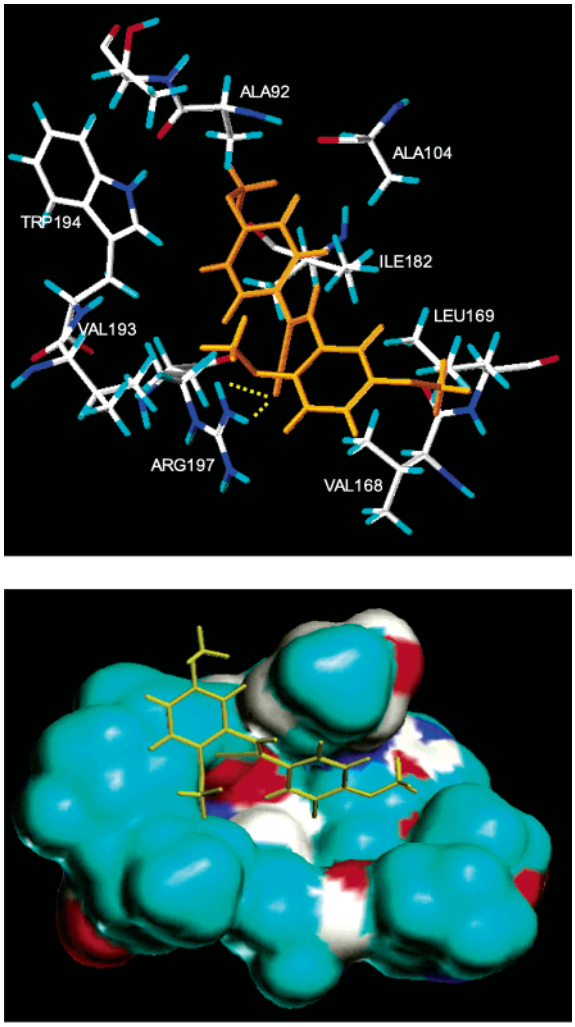


Figure 4. Docking model of compound **14c** (yellow) within the SrtA is shown. The structure of SrtA was obtained from the Protein Data Bank (1IJA). (up) The H-bond between the CN of **14c** and the hydrogen of the NH of Arg 197 backbone is shown as a yellow dotted line. Two substituted aromatic rings can establish hydrophobic interaction with the alkyl side chains of Val 168, Leu 169, Ile 182, Ala 104, Ala 92, Trp 194, and Val 193. (down) **14c** is positioned to a MOLCAD surface representation of binding site of SrtA. The colors represents as follows: red for oxygen atoms, blue for nitrogen atoms, and cyan for hydrogen atoms.

and Burk plot,[22] unambiguously demonstrating that it behaved as a competitive inhibitor ($K_i = 6.81 \pm 0.41$ μM). Moreover, the binding of compound **14c** to enzyme was reversible because the enzyme activity was indeed recovered by dialysis within 2 h, excluding the possible existence of a covalent bond between inhibitor and enzyme.

To study and understand the relationship between the inhibitory activity and compounds with the general structure **14**, molecular modeling studies were performed. Figure 4 shows the binding mode of compound **14c** in the active site of SrtA. The structure of SrtA was obtained from the Protein Data Bank (1IJA), and the energy-minimized conformation of **14c** was obtained using the semiempirical PM3 method in MOPAC package.

As previously revealed,[8] the large hydrophobic binding pocket in the active site was composed of lipophilic side chains of the amino acids, such as Val 193, Trp 194, Ala 92, Ala 104, Leu 169, Val 168, and Ile 182. These hydrophobic side chains have relatively strong lipophilic interactions with the phenyl rings of **14c**. However, it seems that the side chain of Cys 184, which is crucial for the transpeptidation activity, does not interact with the ligand, even though the methoxy group of **14c** is positioned near the Cys 184 (2.463 Å). Of note, in this model, the nitrile group of compound **14c** has a hydrogen bonding interaction with two guanidyl NH of Arg 197, with a distance of 1.662 and 2.579 Å. Our model predicts the effective inhibition of the enzyme activity by the compounds containing a nitrile group.

In conclusion, based on a hit from random screening, we have discovered, for the first time, a novel class of small-molecule SrtA inhibitors. We have established the primary structure−activity relationship, and defined the structural requirements for potency, through structural modifications and molecular modeling studies. These results open the avenue toward novel antivirulence chemotherapeutic agents against Gram-positive pathogenic infection. Further structure−activity relationship and mechanistic studies on this newly disclosed class of inhibitors are ongoing and will be reported in due course.

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Supporting Information Available: Experimental details corresponding to the synthesis of the compounds described in this paper, spectral data for all relevant compounds, details of the biological assay protocol, and molecular modeling methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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