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Probing of the *cis*-5-phenyl proline scaffold as a platform for the synthesis of mechanism-based inhibitors of the *Staphylococcus aureus* sortase SrtA isoform

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ABSTRACT

cis-5-Phenyl prolinates with electrophilic substituents at the fourth position of a pyrrolidine ring were synthesized by 1,3-dipolar cycloaddition of arylimino esters with divinyl sulfone and acrylonitrile. 4-Vinylsulfonyl 5-phenyl prolinates inhibit *Staphylococcus aureus* sortase SrtA irreversibly by modification of the enzyme Cys184 and could be used as hits for the development of antibacterials and antivirulence agents.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been nominated by the Antimicrobial Availability Task Force of the Infectious Diseases Society of America as one of six high-priority problematic pathogens.[1] This nomination reflects the high incidence of MRSA infections, substantial morbidity, and peculiar virulence factors circumventing usual antimicrobial therapy. Other concerns are caused by emerging resistance of *S. aureus* strains to modern therapies and a lack of novel drug candidates, especially those with new mechanism of action. *S. aureus*, like other Gram-positive organisms, utilizes surface proteins for adhesion to host cells and invasion of tissues. The vast majority of surface proteins involved in these aspects of staphylococcal disease are substrates of sortases — cysteine transpeptidases which link surface proteins to peptidoglycan, thus incorporating them into the envelope and leading to their display on the microbial surface.[2] *S. aureus* strains in which the *srtA* gene has been deleted display no defect in survival and growth but exhibit reduced pathogenicity and virulence, due to a failure to display MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) on the bacterial surface. The same effect has been demonstrated for wild Gram-positive organisms treated with sortase inhibitors, implicating sortase enzymes as important targets for the treatment of staphylococcal disease.[2] Different types of sortase inhibitors have been recently reviewed,[3] and the most active and promising small-molecule inhibitors[4–12] are presented in Figure 1. Three types of small molecules, aryl (β-amino)ethyl ketones,[7] *trans*-3-(furan-2-yl) acrylic acid amides[8] and aaptamines,[12] have been reported since the publication of the review. This work describes our studies towards the design and synthesis of novel types of *S. aureus* SrtA inhibitors, and an understanding of their mechanisms of action.

S. aureus sortase SrtA is a 206 amino acid cysteine transpeptidase with an *N*-terminal transmembrane anchor. His120, Cys184 and Arg197 conserved side chains constitute the enzyme active site.[2,3] Of the reported small molecule inhibitors of SrtA, only a few have been subjected to detailed kinetic and mechanistic studies. Commercial vinyl sulfones[4] and aryl (β-amino)ethyl ketones[7] irreversibly modify Cys184, preventing acylation of the Thr-Gly carbonyl, and thus cleavage of the sorting signal. Diarylacrylonitriles demonstrate a competitive character of inhibition,[5] although the strong Michael acceptor moiety of these molecules does not exclude interaction with different nucleophilic species in vivo. Synthetic *trans*-3-hetaryl acrylic acid amides SrtA inhibitors have been developed from *in silico* virtual screening of commercial compound libraries and docking model interactions of inhibitors with amino acid residues in SrtA active site have been identified recently.[8] All other small-molecule inhibitors of SrtA have been isolated from natural plant extracts, and only indirect data of their mechanism of interaction with the enzyme are available.[3] Although X-ray structures of both *S. aureus* SrtA with its substrate complex[13] and *Bacillus anthracis* SrtB with and without inhibitor[7] have been solved, this information is insufficient to allow for construction of a pharmaceutically suitable inhibitor and

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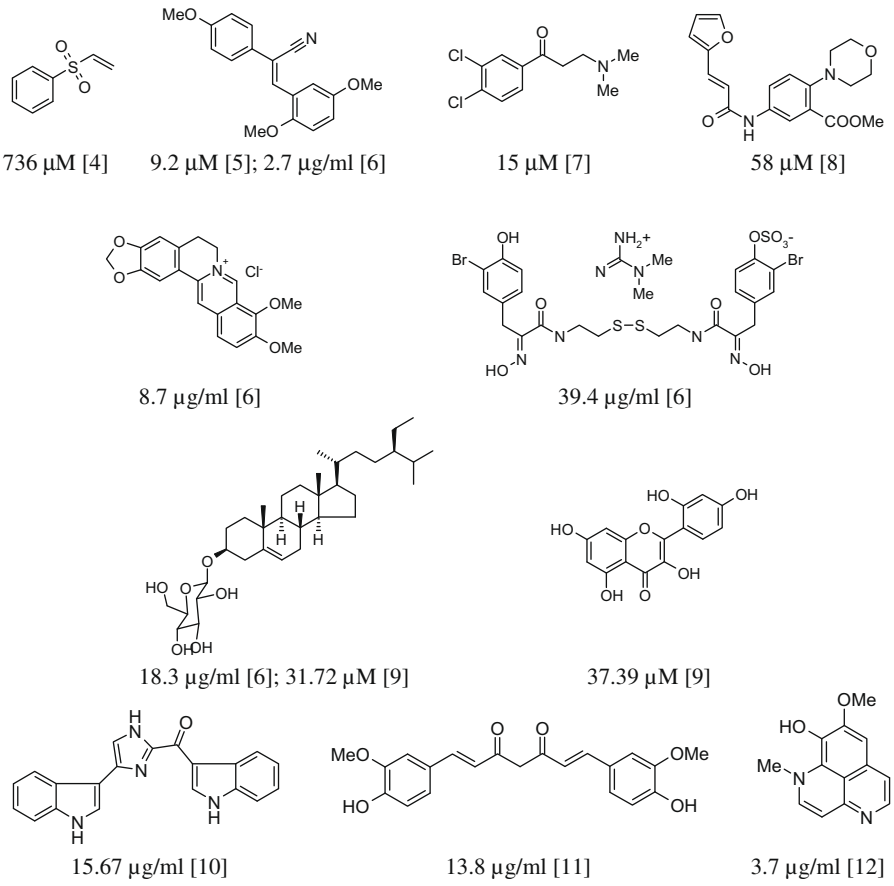


Figure 1. Small-molecule SrtA inhibitors. IC_{50} values with literature source (in square brackets) are indicated.

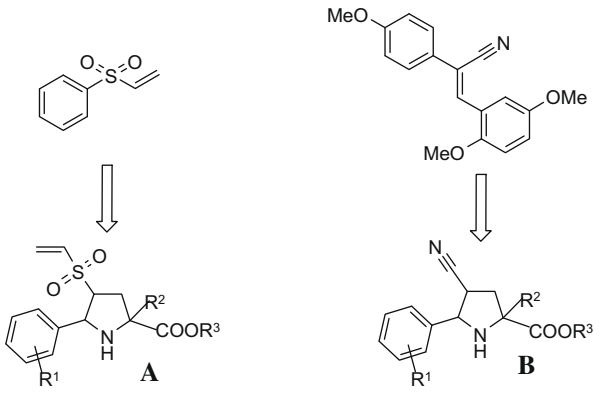


Figure 2. Structural design of novel 5-phenyl proline SrtA inhibitors.

additional studies of sortase–small molecules interactions are desired. We have employed conformationally constrained derivatives of *cis*-5-phenyl prolines with directed functional groups to evaluate the binding modes of these compounds with *S. aureus* SrtA (Fig. 2).

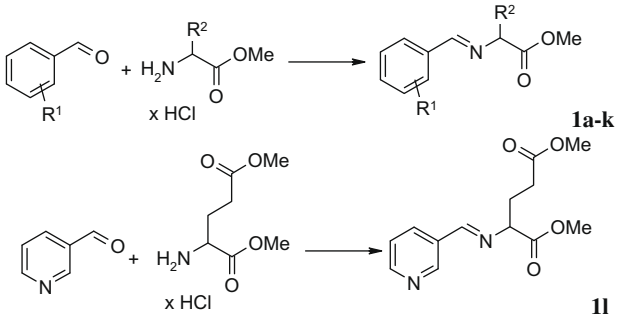
2. Chemistry

Only two types of synthetic compounds were known to inhibit SrtA at the beginning of this project — vinyl sulfones[4] and diarylacrylonitriles.[5] The utility of vinyl sulfones for different medicinal chemistry applications has been recently reviewed.[14] As effective Michael acceptors, some vinyl sulfones are in clinical studies as cysteine protease inhibitors.[15] Similarly, the nitrile group has been shown to be a key structural element for effective SrtA inhibition.[5] We planned to attach vinyl sulfonyl and nitrile groups to a functionalized pyrrolidine scaffold and to synthesize molecules **A** and **B** for subsequent testing of *S. aureus* SrtA inhibition (Fig. 2). Besides conformational constraints these molecules have the following attractive properties for drug discovery: druglikeness, the possibility of synthesis of a diverse set of analogs (combinatorial approach), an amino acid motif for possible additional interactions with the enzyme, and 5-aryl- and R^2-substituents as points for hydrophobic interactions with the enzyme. 1,3-Dipolar cycloaddition of appropriate dipolarophiles to azomethine ylides was utilized as an effective synthetic strategy[16,17] for **A** and **B** synthesis. Target compounds were synthesized from commercial benzaldehydes, α-amino acid esters, divinyl sulfone (DVS) and acrylonitrile, using established methods.[18,19] Starting imino esters **1** have been synthesized from substituted benzaldehydes and methyl esters of glycine, alanine and glutamic acid (Table 1) under dehydrating conditions (Scheme 1).

Interaction of imino esters **1** with divinyl sulfone was achieved through preliminary generation of silver(I)-azomethine ylide (Scheme 2). The subsequent cycloaddition proceeded stereospecifically as an *endo*-process, leading to racemic 5-phenyl prolinates **2** with *cis*-stereochemistry at the phenyl, methoxycarbonyl and vinylsulfonyl substituents. Structural assignments were confirmed by X-ray crystallography of **2c**[20] and **2j**.[18] In the case of glycine imino esters **1a–c**, yields of target pyrrolidinyl vinyl sulfones **2a–c** were low due to the concurrent conjugated addition of divinyl sulfone and subsequent tandem transformations.[21] Glutamate imino esters **1j–l** formed cycloadducts **2j–l** as single products with high yields and allowed for subsequent ring annulation under acidic

Table 1
Substituents in synthesized compounds

Index	a	b	c	d	e	f	g	h	j	k	l
R^1	H	2-F	3-F	3-Cl	2,3-DiF	2-Cl	4-Cl	2-Me	H	3,4-DiMeO	3-Pyridyl
R^2	H	H	H	H	H	Me	Me	Me	CH_2CH_2COOMe	CH_2CH_2COOMe	CH_2CH_2COOMe



Scheme 1. Imino esters synthesis. Reaction conditions: $MgSO_4$, Et_3N, CH_2Cl_2, rt.

conditions, and synthesis of pyrrolizidinyl vinyl sulfones **3j–l** (Scheme 2). Compounds **2k,l** and **3j–l** are novel and were exhaustively characterized by NMR and elemental analysis. Pyrrolidines **2k,l** differ from the X-ray defined **2j** only by the type of aryl substituent and all three of these analogs were characterized by well-correlated signals in ^1H NMR spectra. The stereochemistry of pyrrolizidinones **3** was confirmed by cross-peaks between H^2/H^3 and phenyl/methoxy hydrogens in NOESY experiments (Fig. 3).

Cycloadditions of acrylonitrile to azomethines **1a–h** were conducted using the published protocol of Tsuge and co-workers.[22] Lithium bromide was used as a Lewis acid for metallodipole generation, and previously subsequent stereospecific *endo*-cycloaddition with *tert*-butyl acrylate was applied for the synthesis of diverse proline-glutamate chimeras.[19] In the case of the acrylonitrile dipolarophile, the stereoselectivity of the cycloaddition is poor and two products are formed (Scheme 3). Preliminary structural assignments of cycloaddition products have been made using published spectral data for isomeric **4a** and **5a**,[22] that differs from that shown in Scheme 3 in the stereochemistry of the 4-cyano substituents.

Following transformation of major isomer **4a** into thymine-substituted pyrrolidine **9** and X-ray structural analysis of **9** revealed *cis*-stereochemistry of the cyano group with respect to the phenyl and (N^1-thyminyl)methyl substituents of the final product (Scheme 4).[23] This places the cyano group of the pyrrolidine ring of isomer **4a** and intermediates **6**–**8** with a *cis* orientation with respect to the phenyl substituent. Thus, we believe the position of the 4-cyano group in pyrrolidines **4a** and **5a** was likely assigned incorrectly,[22] and the major cycloaddition products **4** correspond to an *endo*-process as for the majority of other dipolarophiles. Minor products **5** correspond to an *exo*-interaction of *syn,syn*-azomethine ylide and dipolarophilic acrylonitrile.

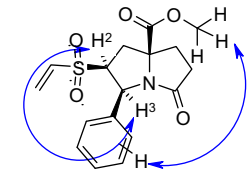
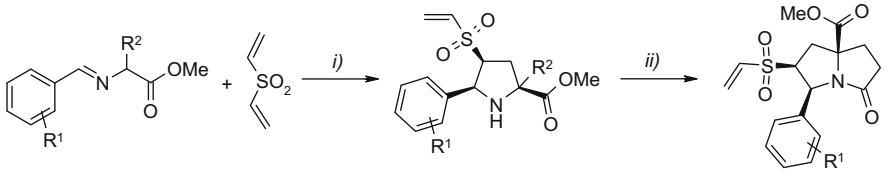


Figure 3. NOESY interactions in **3j**.

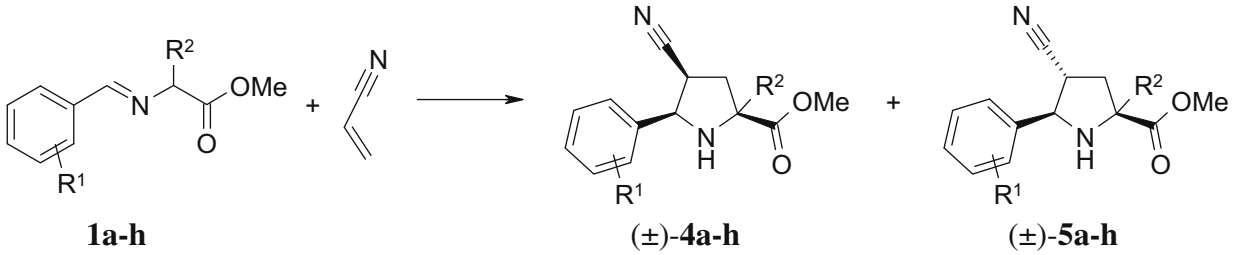
3. Inhibition studies with $SrtA_{\Delta N24}$

As a first test, the compounds were assayed for the ability to inhibit SrtA activity in an HPLC-based assay at three separate concentrations: 39.1 μM, 625 μM, and 5 mM. Compounds **2a–c** displayed complete inhibition at a concentration of 5 mM, while the remainder showed more modest inhibition. However, only the vinyl sulfone compounds were potent inhibitors at mid-micromolar concentrations. The carbonitriles **4**–**6** and **9** failed to completely inhibit SrtA even at a concentration of 5 mM and were not evaluated further.

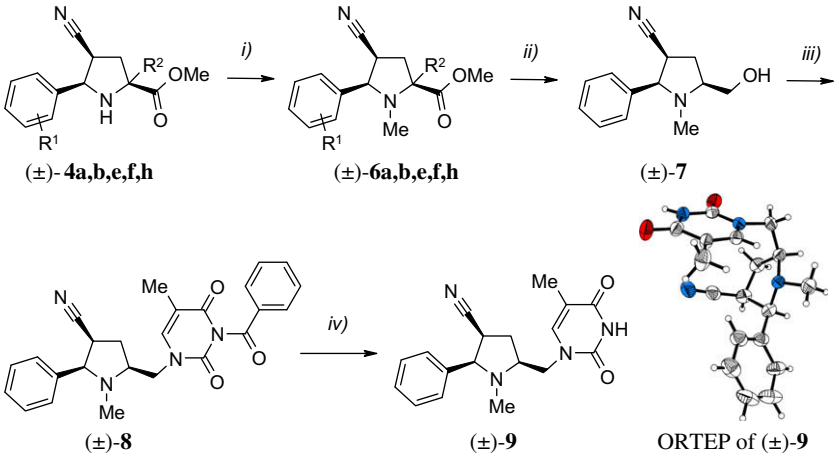
In order to more accurately rank the potencies of our inhibitory compounds, we performed dose–response studies to determine IC_{50} values for each of the compounds. Compounds **2a–c**, **k**, **l**, and **3j–l** were incubated at various concentrations with 1 μM SrtA and assayed as described above. For each of the compounds, the fractional activity remaining after treatment was measured and plotted versus compound concentration using GRAFIT v.4.03 (Erithacus Software), and fit as described in materials and methods. Compounds **2a–c** exhibited modest activity, and had IC_{50} values ranging from 850 μM to 1.32 mM (Table 2). The more highly-substituted compounds **2l** and **3j–l** were less potent and had IC_{50} values of 1.86–2.68 mM (Table 2). Compound **2k** showed significantly lower inhibition, with an IC_{50} value of greater than 5 mM. For compounds **2a**–**2c**, fitting the resultant curves to a single-exponential function allowed the calculation of the observed rate constants for inactivation (k_{obs}), which were then plotted as an inverse function of inhibitor concentration to determine $k_{inact}^{app}/K_I^{app}$ values, the *apparent* second-order rate constant for inactivation (Table 2). Because of the nature of the reverse protonation mechanism of SrtA and the markedly enhanced rate of vinyl sulfones to undergo conjugate addition with a Cys thiolates over a Cys thiol, true k_{inact}/K_I values were determined from the apparent values by correcting for the fraction of enzyme in the enzymatically active thiolate-imidazolium form (determined previously to be 0.06%).[24]



Scheme 2. Reaction conditions: (i) AgOAc, Et_3N, toluene, rt; (ii) AcOH, toluene, 110 °C.



Scheme 3. Reaction conditions: LiBr, Et_3N, THF, rt.



Scheme 4. Reaction conditions: (i) MeI, K_2CO_3, DMF, rt; (ii) $LiBH_4$, THF; (iii) DEAD, Ph_3P, THF; (iv) NH_3, MeOH.

Table 2
Inhibition of *S. aureus* SrtA in vitro by pyrrolidinyl vinyl sulfones **2** and **3**

Compound	IC_{50} (mM)	Hill coefficient	$k_{inact}^{app}/K_I^{app}$ (M^{-1} min^{-1})	k_{inact}/K_I (M^{-1} min^{-1})[a]
2a	0.85 ± 0.05	1.23 ± 0.07	12.73 ± 0.71	(2.12 ± 0.12) × 10^4
2b	1.32 ± 0.27	1.32 ± 0.27	9.18 ± 1.55	(1.53 ± 0.26) × 10^4
2c	1.04 ± 0.27	1.04 ± 0.12	10.67 ± 1.28	(1.78 ± 0.21) × 10^4
2k	> 5.0	n.d.	n.d.	n.d.
2l	1.86 ± 0.42	1.40 ± 0.33	n.d.	n.d.
3j	2.20 ± 0.17	1.90 ± 0.23	n.d.	n.d.
3k	2.68 ± 0.59	1.39 ± 0.28	n.d.	n.d.
3l	2.47 ± 0.27	1.42 ± 0.14	n.d.	n.d.

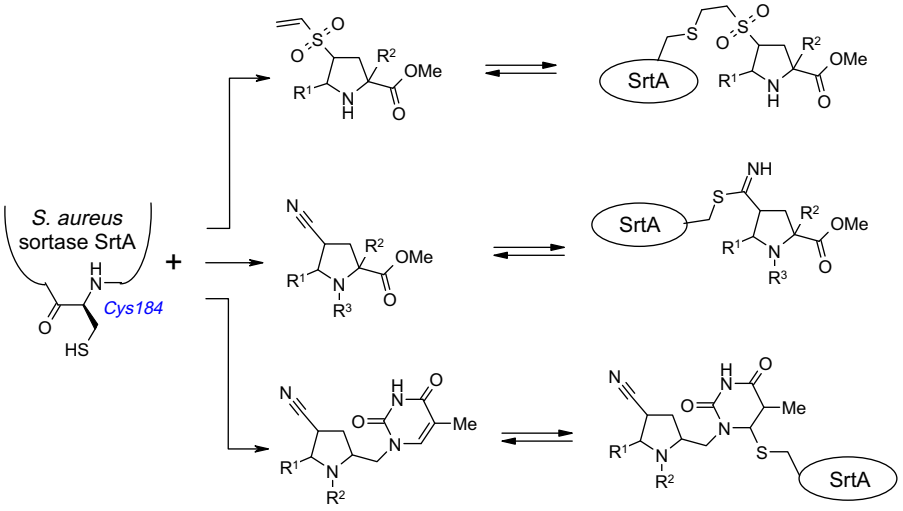
n.d. = Not determined.
[a] Corrected for the fraction of active enzyme in the reverse protonation state (0.06%).

SrtA treated with vinylsulfones depicted in Table 2 exhibited irreversible time-dependent inhibition with values for k_{inact}/K_I, ranging from 1.5 to 2.2 × 10^4 M^{-1} min^{-1} (Table 2). Of the inhibitors examined, compound **2a** proved most potent. Further inspection revealed complicated inhibition kinetics suggestive of a multistep inactivation mechanism for compounds **2a**–**2c**.

To unequivocally establish that the compounds inhibited SrtA via covalent modification of the active-site nucleophile Cys184, we used mass spectrometry to ascertain if compound **2c** formed a covalent inhibitor-SrtA adduct with SrtA. SrtA was incubated with **2c** then run on a 4–12% gradient SDS–PAGE gel. The band corresponding to SrtA was excised, trypsin digested, and analyzed by ESI-MS. A peptide was found whose mass (2256.1 before modification, 2569.4 after modification) corresponds to the fragment ^{178}QLTLITCDDYNEKTGVWEK^{196}, containing a 313.3 mass unit modification, equivalent to the mass of **2c**. Sequencing of the peptide by ESI-MS/MS confirmed the sequence, and localized the modification to Cys184 (data not shown). This result confirms that **2c** inactivates SrtA via formation of a covalent adduct with Cys184. The rest of the vinyl sulfone compounds **2** and **3** are confirmed to act in the same inhibitory manner (data not shown).

4. Discussion

Inspecting known *S. aureus* SrtA inhibitors structures (Fig. 1), it is interesting to note that many are two-dimensional molecules with limited possibility for further modification. In this regard it seems attractive to investigate the three-dimensional space of enzyme–ligand interactions within the SrtA active site and to explore a set of related structural analogs. We selected *cis*-5-phenyl proline scaffold for construction of potential inhibitors (Fig. 2) since efficient straightforward synthetic procedures and diversification methods are developed for these compounds and due to druglikeness of this compound class. An electrophilic moiety attached to this scaffold could form a reversible or an irreversible covalent bond with the enzyme cysteine residue and therefore be envisaged as a route to mechanism-based SrtA inhibitors (Scheme 5). The inhibition kinetics and mechanism of



Scheme 5. Proposed modes of action of synthesized compounds.

covalent labeling of SrtA is consistent with the proposed mechanism of thiolate mediate conjugate addition. Vinyl sulfonyl pyrrolidines **2** and pyrrolizidines **3** readily available in 2–3 synthetic steps from commercial starting materials have been equal with our expectations and inhibit *S. aureus* sortase A at concentrations similar to phenyl vinyl sulfone (PVS). The mechanism of SrtA inhibition by heterocyclic vinyl sulfones **2** and **3** includes covalent modification of the enzyme active site Cys184 analogously to PVS.[4] These compounds were more potent than simple alkyl vinyl sulfones[4] indirectly indicating an importance of hydrophobic interactions between ligands and the enzyme. It should be also concluded that substituents at second position or annulation of five-membered cycle to positions *1* and *2* of proline ring decrease affinity of vinyl sulfonyl prolinates **2** to SrtA while the character of substituents in aryl fragment at fifth position slightly influences activity (Table 2). It is remained intriguing to test both enantiopure forms of **2** and **3** and find if chiral three-dimensional environment is critical for enzyme–ligand interactions. Development of asymmetric version of dipolar cycloaddition of DVS to azomethine ylides is under active investigation in our labs to evaluate this issue. It should be also noted that vinyl sulfonyl prolinates **2a–c** are crystalline compounds stable during two years storage at +4 °C notwithstanding that the molecules contain both effective Michael acceptor (vinyl sulfonyl) and Michael donor (secondary amine) parts.

Nitrile-containing compounds are another chemotype for inhibiting enzymes with activity dependent on cysteine thiol nucleophiles.[25] A possible mechanism of inhibition could involve formation of thioimidate ester (Scheme 5). This mechanism could be also applied to discovered diarylacrylonitrile SrtA inhibitors[5] for explanation of their activity. Six of our tested pyrrolidine carbonitriles **4–6**, namely compounds **4b**, **4d**, **5a**, **5b**, **6b** and **6f**, inhibited SrtA with percent inhibition (%*I*) value 20–30% at 5 mM of inhibitor concentration. $\%I \geq 20$ was the criterion for primary selection of sortase inhibitors hits.[7] All other nitriles were less active summarizing a poor efficacy of 4-cyano-5-phenyl prolinates **4–6** towards sortase. Assuming the same or similar orientation of 5-phenyl prolinate fragment in the SrtA active site, weaker inhibitory properties of carbonitriles **4–6** compared to vinyl sulfones **2** could be accounted for by decreasing of electrophilic properties of the former compounds.

A stable covalent complex is formed by *Escherichia coli* thymidilate synthase (TS) and deoxyuridine monophosphate (dUMP) by Michael addition of reactive thiolate of TS Cys198 to C-6 of dUMP that is confirmed by X-ray.[26] To test pyrimidine nucleobases as potential Michael acceptors in the sortase environment we modified 5-phenyl proline scaffold with the thymine residue (Scheme 4) with the aim to increase inhibitory properties of carbonitriles **4** and **6** (Scheme 5). Unfortunately compound **9** is practically inactive toward SrtA (%*I* = 6 at **9** concentration 1 mM).

5. Conclusions

In conclusion we have developed an effective synthetic approach to a diverse set of *cis*-5-phenyl prolinates functionalized by electrophilic groups. We have also tested these synthesized compounds on the in vitro inhibitory activity of the *S. aureus* sortase SrtA transpeptidase. Racemic vinyl sulfonyl 5-phenyl prolinates **2** inhibit *S. aureus* sortase SrtA irreversibly by modification of the enzyme Cys184. Modifications of most active compounds including asymmetric synthetic approaches are under development for increasing inhibitory properties of ligands based on *cis*-5-phenyl proline scaffold. Present work is centered on elucidating structure–activity relationships with these compound classes and on evaluating of the specificity profile of these agents against sortase activity in *S. aureus* and other Gram-positive microorganisms in vivo.

6. Experimental

6.1. Chemistry

Reagents were obtained from Alfa Aesar and used without further purification unless otherwise stated. Solvents were dried using standard procedures. Reactions were monitored by thin layer chromatography (TLC) on precoated silica gel plates (Sorbfil) with a UV indicator. Column chromatography was performed with Alfa Aesar Silica Gel 60 (0.040–0.063 mm). Melting points were determined in open capillary and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker Avance 400 MHz spectrometer. The chemical shifts (δ) are reported in parts per million upfield using residual signals of solvents as internal standards. Coupling constants (*J* values) were measured in Hertz (Hz). Combustion analyses were performed with a Carlo Erba CHN analyzer. Compounds **1**, **2a–c**, **2j**, and **4–6** have been synthesized as previously reported.[18,20] 3-Benzoylthymine was obtained from thymine according to literature protocol.[27]

6.2. General procedure for the synthesis of pyrrolidinyl vinyl sulfones (2k) and (2l)

Iminoester **1k** or **1l** (8.4 mmol) was dissolved in 20 ml of toluene under argon atmosphere and divinyl sulfone (0.89 ml, 8.4 mmol) and silver(I) acetate (1.55 g, 9.3 mmol) were added in one portions. A solution of Et_3N (1.4 ml, 10 mmol) in 40 ml of toluene was then added to the resulting suspension under stirring. The mixture was stirred for 24 h at room temperature under argon atmosphere with protection from light. The precipitate was filtered off and washed with 10 ml of toluene, the organic phase was concentrated under reduced pressure, and the residue was purified by chromatography on silica gel using hexane/AcOEt as an eluent.

6.3. Methyl (2*S**,4*S**,5*S**)-5-(3,4-dimethoxyphenyl)-4-ethenesulfonyl-2-(2-methoxycarbonylethyl)pyrrolidine-2-carboxylate (2k)

Yield 67%, colorless viscous oil. ^{1}H NMR (400 MHz; DMSO-d_6): δ 6.92 (d, *J* 2.0, 1H, Ar), 6.87 (d, *J* 8.1, 1H, Ar), 6.82 (dd, *J* 8.1, 2.0, 1H, Ar), 5.93 (dd, *J* 16.4, 9.6, 1H, C*H*=CH$_2$), 5.74–5.63 (m, 2H, CH=C*H*$_2$), 4.54 (dd, *J* 10.0, 6.0, 1H, H-5), 4.05 (m, 1H, H-4), 3.72 (s, 3H, OC*H*$_3$), 3.71 (s, 3H, OC*H*$_3$), 3.70 (s, 3H, OC*H*$_3$), 3.51 (s, 3H, OC*H*$_3$), 3.29 (d, *J* 10.0, 1H, N*H*), 2.76 (dd, *J* 15.0, 4.2, 1H, H-3), 2.38–2.26 (m, 2H, H-3, C*H*$_2$COOMe), 2.23–2.15 (m, 1H, C*H*$_2$COOMe), 2.00 (t, *J* 7.6, 2H, C*H*$_2$CH$_2$COOMe). Anal. Calcd for $C_{20}H_{27}NO_8S$: C, 54.41; H, 6.16; N, 3.17. Found: C, 54.48; H, 6.14; N, 3.25.

6.4. Methyl (2*S**,4*S**,5*S**)-4-ethenesulfonyl-2-(2-methoxycarbonylethyl)-5-pyridin-3-yl-pyrrolidine-2-carboxylate (2l)

Yield 72%, beige solid, mp 118–120 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.54 (d, *J* 2.0, 1H, Py), 8.44 (dd, *J* 4.8, 1.6, 1H, Py), 7.72 (dt, *J* 8.0, 1.6, 1H, Py), 7.33 (dd, *J* 8.0, 4.8, 1H, Py), 6.40 (dd, *J* 16.4, 9.6, 1H, C*H*=CH$_2$), 5.80 (d, *J* 9.6, 1H, CH=C*H*$_2$), 5.63 (d, *J* 16.4, 1H, CH=C*H*$_2$), 4.74 (dd, *J* 9.2, 6.8, 1H, H-5), 4.22–4.17 (m, 1H, H-4), 3.72 (s, 3H, OC*H*$_3$), 3.54 (s, 3H, OC*H*$_3$), 3.47 (d, *J* 9.2, 1H, N*H*), 2.75 (dd, *J* 14.8, 5.2, 1H, H-3), 2.40–2.32 (m, 2H, H-3, C*H*$_2$COOMe), 2.28–2.20 (m, 1H, C*H*$_2$COOMe), 2.04 (t, *J* 8.0, 2H, C*H*$_2$CH$_2$COOMe). Anal. Calcd for $C_{17}H_{22}N_2O_6S$: C, 53.39; H, 5.80; N, 7.32. Found: C, 53.33; H, 5.74; N, 7.32.

6.5. General procedure for the synthesis of pyrrolizidinones (3)

Pyrrolidine **2** (1.4 mmol) was dissolved in 20 ml of toluene, then 2 ml of glacial acetic acid was added and the reaction mixture was heated at 110 °C under stirring and argon atmosphere during 2 h. After cooling to room temperature the mixture was concentrated under reduced pressure and residue was purified by chromatography on silica gel using hexane/AcOEt as an eluent.

6.6. Methyl (2*S**,3*S**,7a*S**)-2-ethenesulfonyl-5-oxo-3-phenyltetrahydropyrrolizine-7a-carboxylate (3j)

Yield 99%, white crystals, mp 69–70 °C. ^{1}H NMR (400 MHz; DMSO-d_6): δ 7.34–7.31 (m, 2H, Ar), 7.26–7.23 (m, 3H, Ar), 6.05 (dd, *J* 16.8, 10.0, 1H, C*H*=CH$_2$), 5.73 (d, *J* 10.0, 1H, CH=C*H*$_2$), 5.62 (d, *J* 16.8, 1H, CH=C*H*$_2$), 5.27 (d, 1H, *J* 8.0, H-3), 4.52 (ddd, *J* 13.6, 8.0, 4.6, 1H, H-2), 3.74 (s, 3H, OC*H*$_3$), 3.12 (dd, *J* 13.6, 4.6, 1H, H-1), 2.75–2.66 (m, 1H, H-7), 2.54 (dd, *J* 13.6, 8.0, 1H, H-1), 2.35–2.31 (m, 2H, H-6), 2.26–2.20 (m, 1H, H-7). ^{13}C NMR (100 MHz; DMSO-d_6): δ 177.73, 173.71, 136.76, 135.93, 129.47, 129.23(2C), 128.22, 127.95(2C), 72.69, 67.70, 61.38, 52.13, 35.54, 35.07, 33.74. Anal. Calcd for $C_{17}H_{19}NO_5S$: C, 58.44; H, 5.48; N, 4.01. Found: C, 58.26; H, 5.36; N, 4.14.

6.7. Methyl (2*S**,3*S**,7a*S**)-3-(3,4-dimethoxyphenyl)-2-ethenesulfonyl-5-oxotetrahydropyrrolizine-7a-carboxylate (3k)

Yield 87%, beige solid, mp 95–97 °C. ^{1}H NMR (400 MHz; DMSO-d_6): δ 6.94 (s, 1H, Ar), 6.83 (s, 2H, Ar), 6.06 (dd, *J* 16.8, 10.0, 1H, C*H*=CH$_2$), 5.78 (d, *J* 10.0, 1H, CH=C*H*$_2$), 5.68 (d, *J* 16.8, 1H, CH=C*H*$_2$), 5.22 (d, *J* 8.0, 1H, H-3), 4.51–4.46 (m, 1H, H-2), 3.75 (s, 3H, OC*H*$_3$), 3.73 (s, 3H, OC*H*$_3$), 3.70 (s, 3H, OC*H*$_3$), 3.07 (dd, *J* 14.0, 5.6, 1H, H-1), 2.75–2.66 (m, 1H, H-7), 2.56–2.50 (m, 1H, H-1), 2.36–2.32 (m, 2H, H-6), 2.26–2.21 (m, 1H, H-7). ^{13}C NMR (100 MHz; $CDCl_3$): δ 177.62, 173.20, 149.22, 148.66, 134.69, 130.09, 127.46, 121.08, 112.31, 110.56, 72.52, 69.63, 61.15, 56.00 (2C), 53.14, 36.49, 35.92, 33.66. Anal. Calcd for $C_{19}H_{23}NO_7S$: C, 55.73; H, 5.66; N, 3.42. Found: C, 55.35; H, 5.45; N, 3.20.

6.8. Methyl (2*S**,3*S**,7a*S**)-2-ethenesulfonyl-5-oxo-3-pyridin-3-yl-tetrahydropyrrolizine-7a-carboxylate (3l)

Yield 69%, viscous oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.55 (s, 1H, Py), 8.43 (d, *J* 4.0, 1H, Py), 7.77 (d, *J* 8.0, 1H, Py), 7.30 (dd, *J* 8.0, 4.0, 1H, Py), 6.46 (dd, *J* 16.8, 10.0, 1H, C*H*=CH$_2$), 5.82 (d, *J* 10.0, 1H, CH=C*H*$_2$), 5.60 (d, *J* 16.8, 1H, CH=C*H*$_2$), 5.38 (d, *J* 8.0, 1H, H-3), 4.64–4.59 (m, 1H, H-2), 3.76 (s, 3H, OC*H*$_3$), 3.15 (dd, *J* 14.4, 3.2, 1H, H-1), 2.78–2.69 (m, 1H, H-7), 2.62 (dd, *J* 14.4, 8.8, 1H, H-1), 2.39–2.26 (m, 3H, H-6, H-7). ^{13}C NMR (100 MHz; $CDCl_3$): δ 177.31, 172.94, 149.75, 149.57, 136.73, 134.78, 131.34, 130.81, 123.36, 72.57, 68.80, 58.95, 53.25, 36.43, 36.32, 33.77. Anal. Calcd for $C_{16}H_{18}N_2O_5S$: C, 54.85; H, 5.18; N, 7.99. Found: C, 55.03; H, 5.15; N, 8.09.

6.9. (2*R**,3*S**,5*S**)-5-Hydroxymethyl-1-methyl-2-phenylpyrrolidine-3-carbonitrile (7)

$LiBH_4$ (0.200 g, 11 mmol) was added to the solution of *N*-methyl prolinate **6a** (2.690 g, 11 mmol) in 50 ml of dry THF under stirring at room temperature. After 24 h additional $LiBH_4$ (0.200 g, 11 mmol) was added and the mixture was heated at 40 °C during 18 h. After cooling to room temperature obtained suspension was filtered and concentrated at vacuum. Methanol (120 ml) was added by portions to the residue under external cooling to 0 °C and solution was kept at room temperature during 2 h. Concentration and chromatography on silica gel using hexane/AcOEt as an eluent led to 2.120 g of amino alcohol **7** as white crystals. Yield 89%, mp 98–99 °C. ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.46–7.40 (m, 4H, Ar), 7.39–7.34 (m, 1H, Ar), 3.87 (dd, *J* 11.4, 2.6, 1H, C*H*$_2$O), 3.66 (d, *J* 6.4, 1H, H-2), 3.64–3.58 (m, 1H, C*H*$_2$O), 3.24–3.19 (m, 1H, H-3), 2.75 (br, 1H, O*H*), 2.48–2.34 (m, 3H, H-4, H-5), 2.25 (s, 3H, NC*H*$_3$). ^{13}C NMR (100 MHz; $CDCl_3$): δ 128.71, 128.68 (2C), 127.98 (2C), 120.15, 72.60, 65.39, 60.94, 38.24, 35.67, 30.81. Anal. Calcd for $C_{13}H_{16}N_2O$: C, 72.19; H, 7.46; N, 12.95. Found: C, 72.20; H, 7.55; N, 12.89.

6.10. (2*R**,3*S**,5*S**)-5-(3-Benzoyl-5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-ylmethyl)-1-methyl-2-phenylpyrrolidine-3-carbonitrile (8)

Amino alcohol **7** (0.865 g, 4.0 mmol), PPh_3 (2.100 g, 8.0 mmol) and 3-benzoylthymine (1.842 g, 8.0 mmol) were suspended in 50 ml of anhydrous THF under argon atmosphere. A solution of diethyl azodicarboxylate (1.400 g, 8.0 mmol) in 60 ml of anhydrous THF was added within 5 h. After stirring 48 h at room temperature all volatiles were removed under vacuum. The residue was purified by column chromatography. Yield 0.240 g (14%), white crystals, mp 224–226 °C. ^{1}H NMR (400 MHz; DMSO-d_6): δ 8.00 (d, *J* 7.6, 2H, Ar), 7.79–7.75 (m, 2H, Ar, C*H*=), 7.56 (t, 4H, *J* 7.6, 2H, Ar), 7.38–7.30 (m, 5H, Ar), 4.12 (dd, *J* 14.4, 2.8, 1H, C*H*$_2$N), 3.84 (dd, *J* 14.4, 4.4, 1H, C*H*$_2$N), 3.73 (d, *J* 6.0, 1H, H-2), 3.54–3.50 (m, 1H, H-3), 3.00–2.93

(m, 1H, H-5), 2.50–2.41 (m, 1H, H-4), 2.23 (s, 3H, NCH_3), 1.99–1.93 (m, 1H, H-4), 1.93 (s, 3H, CH_3). Anal. Calcd for $C_{25}H_{24}N_4O_3$: C, 70.08; H, 5.65; N, 13.08. Found: C, 70.04; H, 5.68; N, 13.12.

6.11. (2*R**,3*S**,5*S**)-1-Methyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-ylmethyl)-2-phenylpyrrolidine-3-carbonitrile (9)

Methanol (25 ml) was saturated with gaseous ammonia. Compound **8** (0.214 g, 0.5 mmol) was added to this solution and stirred 48 h at room temperature. All volatiles were removed under reduced pressure. The residue was purified by column chromatography. Yield 0.128 g (79%), white crystals, mp 209–211 °C. ^{1}H NMR (400 MHz; DMSO-d_6): δ 11.40 (br, 1H, N*H*), 7.49 (s, 1H, C*H*=), 7.38–7.36 (m, 4H, Ar), 7.35–7.28 (m, 1H, Ar), 4.06 (dd, *J* 14.2, 3.4, 1H, CH_2N), 3.72–3.68 (m, 2H, CH_2N, H-2), 3.50–3.45 (m, 1H, H-3), 2.93–2.89 (m, 1H, H-5), 2.39 (dt, *J* 13.6, 9.2, 1H, H-4), 2.19 (s, 3H, NCH_3), 2.02–1.97 (m, 1H, H-4), 1.83 (s, 3H, CH_3). ^{13}C NMR (100 MHz; DMSO-d_6): δ 164.81, 152.18, 143.05, 138.52, 128.72(2C), 128.44, 128.23(2C), 120.93, 108.57, 71.45, 63.53, 48.70, 38.75, 35.20, 31.49, 12.40. Anal. Calcd for $C_{18}H_{20}N_4O_2$: C, 66.65; H, 6.21; N, 17.27. Found: C, 66.79; H, 6.06; N, 17.15.

6.12. Crystal Structure Determination of Compound (9)

The single crystal of **9** of approximate dimensions 0.30 × 0.20 × 0.20 mm was mounted in inert oil on the top of glass fiber and transferred on the Bruker SMART APEX diffractometer. Crystal data: $C_{18}H_{20}N_4O_2$, M = 324.38, monoclinic, a = 12.097(3), b = 7.6630(18), c = 18.508(4) Å, β = 105.559(3)°, V = 1652.7(7) Å^3, space group $P2_1/c$, Z = 4, D_c = 1.304 g/cm^3, $F(000)$ = 688, μ(Mo Kα) = 0.088 mm^{-1}. Total of 12,508 reflections (3244 unique, R_{int} = 0.0617) were measured using graphite monochromatized Mo Kα radiation (λ = 0.71073 Å) at 293(2) K. Data were collected in the range $2.89 < \theta < 26.00$ ($-14 \le h \le 14$, $-9 \le k \le 9$, $-22 \le l \le 22$). Omega scan mode with the step of 0.3° (30 s per step) was used. The structure was solved by direct methods[28] and refined by full matrix least-squares on F^2.[29] All H atoms (except ^{1}H) were placed in calculated positions and refined using a riding model. The final residuals were: R_1 = 0.0577, wR_2 = 0.1403 for 1638 reflections with $I > 2\sigma(I)$ and 0.1228, 0.1686 for all data and 223 parameters. Goof = 1.047, maximum $\Delta\rho$ = 0.215 e/Å^3.

7. Expression and purification of 6-His tagged SrtA$_{\Delta N24}$

N-Terminally His$_6$-tagged SrtA lacking the amino-terminal 24 amino acids was expressed in BL21(DE3) cells containing the plasmid pET15bSrtA$_{\Delta N24}$. Cells were grown in Luria Broth containing 100 μg/ml of ampicillin at 37 °C until the OD$_{600}$ reached 0.5–0.6. Protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and the cells were grown for an additional 3 h at 37 °C then harvested by centrifugation at 3000 *g* for 10 min. Cells were resuspended in 150 mM NaCl, 50 mM Tris-Cl, 5 mM imidazole, 10% glycerol, pH 7.5, and lysed with an EmulsiFlex-C5 high-pressure homogenizer (Avestin, Inc.). Lysate was clarified by centrifugation and applied to a chelating sepharose fast flow column. The column was washed with 50 mM imidazole, SrtA$_{\Delta N24}$ was eluted using a linear gradient of 50–500 mM imidazole and fractions were collected. SrtA$_{\Delta N24}$-containing fractions were pooled, concentrated, and dialyzed overnight into 150 mM NaCl, 50 mM Tris-Cl, 5 mM $CaCl_2$, 0.1% β-mercaptoethanol, 10% glycerol, pH 7.5. Purified SrtA$_{\Delta N24}$ was concentrated using 10,000-MWCO Centriplus centrifugal filters (Amicon, Inc.). SrtA$_{\Delta N24}$ concentration was determined using a calculated extinction coefficient (ε_{280} = 17,420 M^{-1} cm^{-1}).

8. Solid-phase synthesis of Abz-LPETG-Dap(DNP)-NH$_2$

The peptide Abz-LPETG-Dap(DNP)-NH$_2$ was synthesized by the Fmoc/piperidine strategy on PAL resin on a 0.25 mmol scale. Cleavage from the resin was achieved via incubation with a 95:2.5:2.5 TFA/water/triisopropylsilane (TIPS) mixture for 2.5 h. The peptide was precipitated using cold diethyl ether following the removal of excess TFA via rotary evaporation. After filtration, the precipitate was dissolved in a 50:50 water/acetonitrile mixture and lyophilized to yield crude peptide. Crude peptide was purified by HPLC using a semi-preparative C18 Jupiter™ column (21.2 × 250 mm, 10 μm, Phenomenex, Inc.) to ⩾98% purity. MALDI-TOF mass spectrometry was used to verify the identity of the purified product (Abz-LPETG-Dap(DNP)-NH$_2$: m/z = 885.3). The purified, lyophilized peptide were stored at −20 °C.

9. Inhibition assays

9.1. Steady-state activity assays

Purified recombinant SrtA$_{\Delta N24}$ at 1 μM was incubated with 2 mM Abz-LPETG-Dap(DNP)-NH$_2$, 2 mM NH$_2$-Gly$_5$-OH, and increasing amounts of inhibitor at 37 °C in 2% DMSO, 0.1% CHAPS, 150 mM NaCl, 5 mM mM $CaCl_2$ and 300 mM Tris (pH 7.5), in a total volume of 100 μL. To control for the possibility of time-dependent inactivation, the enzyme and inhibitor were pre-incubated in buffer at 37 °C for 30 min. Reactions were initiated by addition of substrate mix (Abz-LPETG-Dap(DNP)-NH$_2$ and NH$_2$-Gly$_5$-OH), and allowed to incubate at 37 °C. After 30 min, the reactions were quenched by removal of 80 μL of reaction mix into 40 μL (1/2 volume) of 1.2 M HCl. Next, 70 μL of the quenched reaction mix was injected onto a reverse phase octadecylsilica analytical fast-flow HPLC column (4.6 × 50 mm, 3 μm, Vydac, Inc.). Products were separated using a linear gradient from 0% to 45% CH_3CN/0.1%TFA over 5 min. The elution of the dinitrophenol-containing substrate (Abz-LPETG-Dap(DNP)-NH$_2$) and product (NH$_2$-G-Dap(DNP)-NH$_2$) were monitored at 355 nm, and integration of the areas under the substrate and product peaks was used to determine the percentage of substrate converted to product.

9.2. IC$_{50}$ determination

For IC$_{50}$ determination, 1 μM SrtA$_{\Delta N24}$ was pre-incubated with the inhibitor for 30 min at 37 °C, to allow for any time-dependent inactivation to occur. Assays were then initiated by addition of a mixture of Abz-LPETG-Dap(DNP)-NH$_2$ and NH$_2$-Gly$_5$-OH. Assays were performed in 100 μL final volume, were run for 30 min, and then were quenched by addition of 50 μL (1/2 volume) of 1.2 M HCl. All measurements were performed in triplicate. 70 μL of each quenched reaction mixture was analyzed by HPLC using the method described above. Fractional activity remaining relative to uninhibited controls (v_i/v_o) was calculated by comparing the difference in percent product formation (as measured at 355 nm). GRAFIT v.4.0 (Erithacus Software) was used to generate plots of fractional activity remaining versus inhibitor concentration, which were fit using the equation:

$$\frac{v_i}{v_o} = \frac{1}{1 + \left(\frac{[I]}{IC_{50}}\right)^h}$$

where v_i is the initial velocity in the presence of the inhibitor at concentration [I], v_o is the initial velocity in the absence of inhibitor, IC$_{50}$ is the concentration of inhibitor at which one-half of the original activity remains, and h is the Hill coefficient.

9.3. Measurement of second-order rate constants for SrtA inactivation

Assays were performed in 100 μL volume, containing 1 μM $SrtA_{\Delta N24}$, inhibitor, 2 mM Abz-LPETG-Dap(DNP)-NH_2, 2 mM NH_2-Gly_5-OH, 2% DMSO, 0.1% CHAPS, 150 mM NaCl, 5 mM $CaCl_2$, and 300 mM Tris (pH 7.5). No pre-incubation of enzyme with inhibitor was performed. Assays were initiated by addition of enzyme and allowed to run at 37 °C. At 10, 20, 30, 40, 50 and 60 min, assays were quenched by removing 90 μL of reaction mix into 45 μL (1/2 volume) of 1.2 M HCl. Quenched samples (70 μL) were injected onto an octadecylsilica column and run using the standard HPLC method described above. Progress curves were generated, and fit using SIGMAPLOT v.8.0 to the following equation:

$$P = A\left(1 - e^{-k_{obs}t}\right)$$

where P is product concentration, A is the amplitude (v_i/k_{obs}), k_{obs} is the observed rate constant for inactivation, and t is time. For several cases outlying values of k_{obs} were observed due to a lack of curvature in the progress lines, and these were removed prior to subsequent analysis. Plots of k_{obs} versus inhibitor concentration were generated, and SIGMAPLOT v.8.0 was used to fit these plots to either a linear fit or a saturation fit, using the equations below:

$$k_{obs} = \left(\frac{k_{inact}}{K_I}\right)[I] \qquad \text{(Linear)}$$

$$k_{obs} = \frac{k_{inact} \cdot [I]}{K_I + [I]} \qquad \text{(Saturation)}$$

Subsequent analysis determined the best fit, and provided the apparent second-order rate constant for inactivation, $k_{inact}^{app}/K_I^{app}$. This value was subsequently corrected for the fraction of active enzyme concentration of 0.06% previously determined for SrtA[24] to yield the second-order rate constant of inactivation k_{inact}/K_I.

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