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Inhibition of the *Staphylococcus aureus* sortase transpeptidase SrtA by phosphinic peptidomimetics

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Abstract—During pathogenesis, Gram-positive bacteria utilize surface protein virulence factors such as the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) to aid the initiation and propagation of infection through adherence to host endothelial tissue and immune system evasion. These virulence-associated proteins generally contain a C-terminal LPXTG motif that becomes covalently anchored to the peptidoglycan biosynthesis intermediate lipid II. In *Staphylococcus aureus*, deletion of the sortase isoform SrtA results in marked reduction in virulence and infection potential, making it an important antivirulence target. Here we describe the chemical synthesis and kinetic characterization of a nonhydrolyzable phosphinic peptidomimetic inhibitor of SrtA derived from the LPXTG substrate sequence.

1. Introduction

The sortase enzymes are a family of Gram-positive transpeptidases responsible for anchoring surface protein virulence factors to the peptidoglycan call wall layer (Fig. 1).[1] Two sortase isoforms have been identified in *Staphylococcus aureus*, SrtA and SrtB. While the SrtB isoform appears to be important in heme iron acquisition and iron homeostasis,[2] numerous genetic knockout experiments have shown that the SrtA isoform plays a critical role in the pathogenesis of Gram-positive bacteria by modulating the ability of the bacterium to adhere to host tissue via the covalent anchoring of adhesins and other virulence-associated proteins to the cell wall peptidoglycan.[3–9] These studies show that in the absence of SrtA activity, bacteria are unable to establish

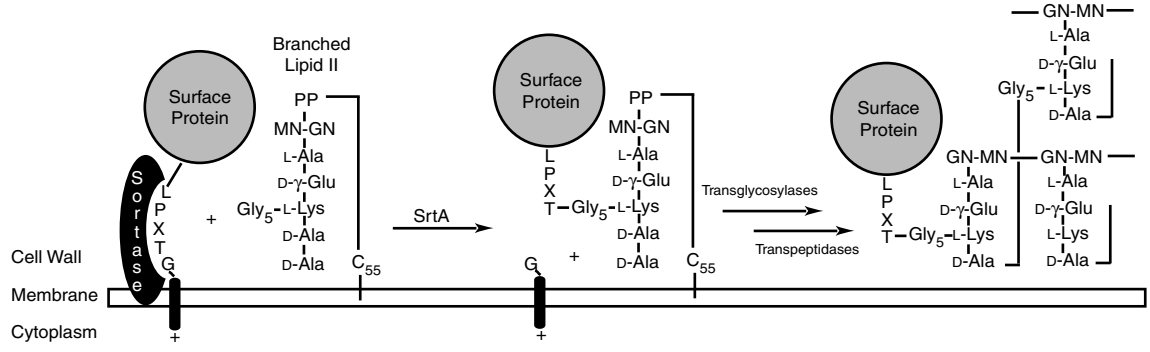

Figure 1. Sortase catalyzed surface protein attachment. Sortase cleaves surface proteins at the Thr-Gly bond of an LPXTG recognition motif, and catalyzes the transpeptidation of the resulting surface protein fragment to the Gly_5 crosslinking spacer present on branched lipid II, which then goes on to be polymerized by the transglycosylases and transpeptidases.

Keywords: Sortase; Transpeptidase; Phosphinate; Peptidomimetic; Antivirulence.
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persistent infections. Inhibitors of SrtA might consequently be promising candidates for the treatment and/or prevention of Gram-positive bacterial infections.

Several reports describing specific inhibitors of the *S. aureus* SrtA-catalyzed reaction have been published to date. Kim and co-workers evaluated 80 medicinal plant extracts for their ability to inhibit sortase activity and found that several extracts contained potent irreversible sortase inhibitors, the best being from *Cocculus trilobus*, which had an IC_{50} of 1.52 μg/mL.[10] A lipid glucose conjugate from *Fritillaria verticillata* as well as isoquinoline alkaloids from *Coptis chinensis* were identified as candidates for the inhibitory compounds in these extracts.[11,12]

As diazoketones and chloromethylketones are known to form covalent thioether adducts with cysteine nucleophiles, Scott and colleagues synthesized and tested peptides containing these functionalities as isosteres of the Thr-Gly scissile bond for SrtA inhibition (Fig. 2).

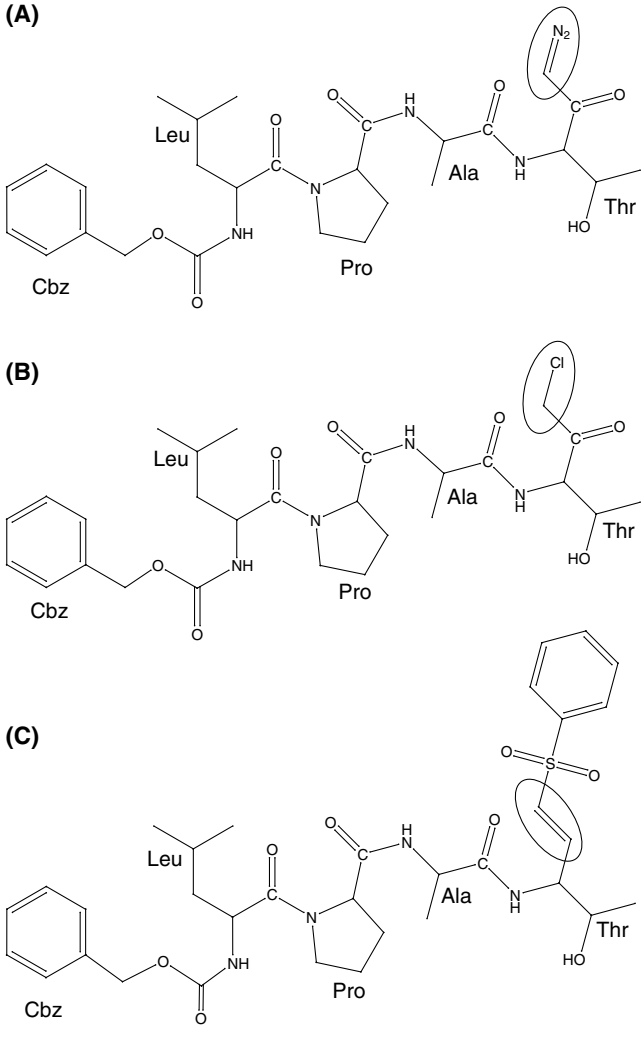

Figure 2. Structures of known irreversible SrtA inhibitors highlighting their respective functional groups. (A) Peptidyl diazoketone $K_i = 22\,nM$; $k_{inact} = 5.8 \times 10^{-3}\,min^{-1}$.[13] (B) Peptidyl chloromethylketone $K_i = 21\,nM$; $k_{inact} = 1.1 \times 10^{-2}\,min^{-1}$.[13] (C) Peptidyl vinyl sulfone $K_i = 9\,\mu M$; $k_{inact} = 4 \times 10^{-4}\,min^{-1}$.[15]

Specificity was achieved by using the sortase recognition motif LPXT terminating in the diazoketone or chloromethylketone. These inhibitors were found to have very low K_i values of 2.1×10^{-7} M for the peptidyl chloromethylketone inhibitor and 2.2×10^{-7} M for the diazoketone inhibitor, although the rates of inactivation were relatively poor, 1.1×10^{-2} and $5.8 \times 10^{-3}\,min^{-1}$, respectively.[13]

Vinyl sulfones have also been shown to irreversibly inhibit cysteine proteases, through a 1,4 conjugate addition reaction with the active site thiol.[14] Recently, a peptidyl vinyl sulfone inhibitor (Fig. 2) was tested for activity against the *S. aureus* SrtA. The K_i of the peptidyl vinyl sulfone was 9 μM while the k_{inact} was $4 \times 10^{-4}\,min^{-1}$, a rate of inactivation 10–20-fold slower than that reported for the diazoketone and chloromethylketone inhibitors.[15] Independently, our laboratory described the irreversible inhibition of SrtA-catalyzed surface protein anchoring by a series of small molecule vinyl sulfones.[16]

While progress has been made in the development of irreversible inhibitors, reversible inhibitors would be useful for kinetic and mechanistic studies of the sortase reaction. One possible design strategy for a potent reversible inhibitor would be a nonhydrolyzable peptidomimetic that would imitate the structure of the transition state (Fig. 3). Phosphorous-backbone isosteres of peptides have been shown to be effective transition state analogs for the attack of a peptide bond by water

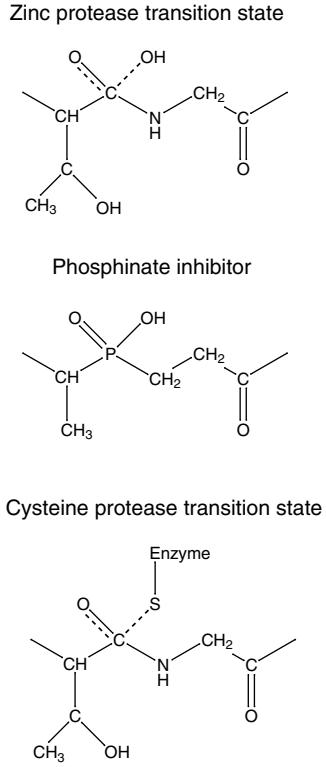

Figure 3. Structural comparison of protease transition states and phosphinate containing peptidomimetic.

and have been useful inhibitors of members of the zinc protease family.[17–19] In particular, phosphinic peptides have proven most effective, as the peptide bond is replaced by a phosphorous carbon bond [–P(O)OH–CH_2–], and are more stable than phosphonic [–P(O)OH–O–] or phosphonamidic [–P(O)OH–NH–] isosteres.[20]

We have employed the method developed by Buchardt and Meldal for the solid-phase synthesis of phosphinic peptides to synthesize a potential inhibitor for the sortase catalyzed hydrolysis reaction where the hydrolyzable Thr-Gly bond is replaced by a phosphinic isostere (**1**, Fig. 4). This method utilizes standard solid-phase techniques to synthesize the peptide backbone, followed by acryloylation of the peptide N-terminus, addition of a protected 1-aminoethylphosphinic acid, and finally elongation by standard solid-phase synthesis.[20] We would expect this phosphinic isostere to serve as an accurate transition state analog and to exhibit a high affinity for sortase only if the sortase catalyzed reaction proceeds through a sequential mechanism. If, on the other hand, the sortase reaction proceeds through an acyl-enzyme intermediate, this phosphinic isostere should not serve as a transition state analog and will likely exhibit a simple nonhydrolyzable competitive inhibitor behavior (Fig. 3).

Herein we describe the synthesis and kinetic characterization of the phosphinate containing peptidomimetic NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 (Peptide 1). Further, we show that this inhibitor can be used to aid mechanistic analysis of SrtA using global fitting of several kinetic models.

2. Results and discussion

2.1. Synthesis of NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2

Although the recognition motif of SrtA is LPXTG, prior substrate specificity studies indicated that SrtA was capable of processing alanine residues in place of threonine in the fourth position of the recognition motif.[21] Therefore, we replaced the Thr-Gly scissile bond with the AlaΨ{PO_2H–CH_2}Gly isostere, where the threonine has been replaced by alanine for ease of synthesis. To synthesize phosphinic peptides using the method of Buchardt and Meldal,[20] the amine protected 1-aminoalkylphosphinic acid that corresponds to the amino acid being replaced is added to an N-terminal acryloylated peptide. We used the Alloc protecting group due to its increased stability over the Fmoc derivative[20] for the protection of the 1-aminoalkylphosphinic acid. 1-(Allyloxycarbonylamino)ethylphosphinic acid, the alanine isostere, was synthesized on a 50 mmol scale by the method of Dingwall and co-workers.[22]

The phosphinate containing peptidomimetic NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 was assembled by solid-phase methods[20] as depicted in Figure 4. Briefly, PAL resin was loaded with two glutamic acid residues using standard automated peptide synthesis procedures. The deprotected resin was acylated with acryloyl chloride followed by the addition of **2** via the corresponding bis(trimethylsilyl) phosphonite to give resin-bound phosphinic tetrapeptide **3**. After removal of the Alloc protecting group, the efficient coupling of

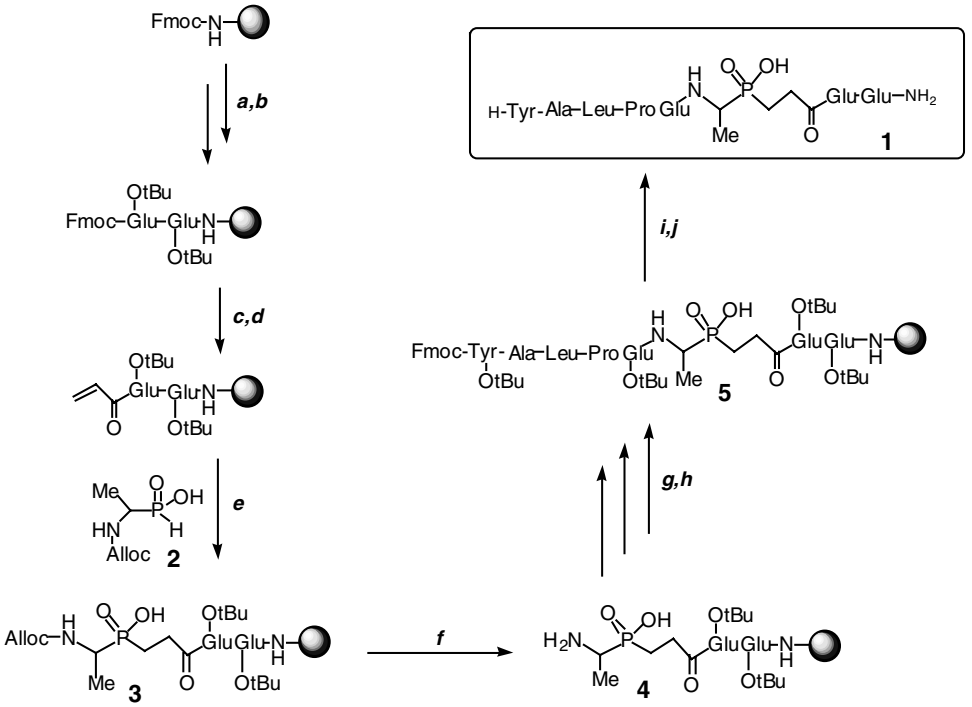

Figure 4. Synthesis of NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2. Reagents and conditions: (a) 20% piperidine/DMF; (b) Fmoc-Glu(O-*t*-Bu)-OH, HOBt, HBTU, NMM; (c) 20% piperidine/DMF; (d) acroyl chloride, Et_3N; (e) bis-(trimethylsilyl)acetamide, DCE, 100 °C; (f) $Pd(PPh_3)_4$, CH_3Cl/AcOH/NMM 92.5:5:2.5; (g) 20% piperidine/DMF; (h) Fmoc-amino acids, HBTU, NMM; (i) 20% piperidine/DMF; (j) TFA/H_2O 95:5.

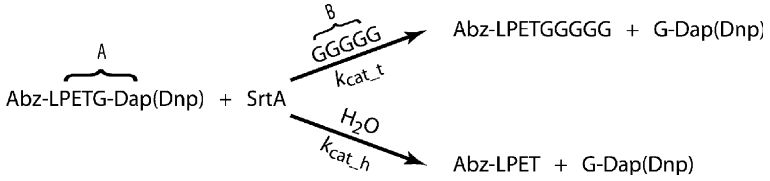

Figure 5. SrtA catalyzed reaction studied in vitro. The reaction of SrtA with substrates Abz-LPETG-Dap(Dnp) (A) and GGGG (B) was analyzed using an HPLC-based assay where the formation of G-Dap(Dnp) was monitored at 355 nm. Although transpeptidation is the major reaction (k_{cat_t}), partial hydrolysis (k_{cat_h}) is also observed.

Fmoc-Glu was accomplished using a reaction time of 16 h. The remaining four amino acids were coupled by automated solid-phase peptide synthesis. The resulting peptide was cleaved from the resin with TFA–water (95:5) and was lyophilized to afford the desired crude product **1**, NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 (75 mg, 15%). The crude product was purified by HPLC using a semipreparative C_{18} Jupiter column to $\geqslant$ 98% purity and verified by MALDI-TOF MS.

2.2. Kinetic characterization of NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2

Kinetic analysis was performed by studying the in vitro reaction of recombinant SrtA with substrates Abz-LPETG-Dap(Dnp) and GGGGG in a previously described assay (Fig. 5).[23] Preliminary characterization of the inhibitory activity of NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 indicated that it was a competitive inhibitor against the Abz-LPETG-Dap(Dnp) sortase substrate exhibiting an IC_{50} of 10 mM, which is on the same order of magnitude as the K_m for Abz-LPETG-Dap(Dnp) binding.[23] This extremely poor inhibitory activity indicates that the transition state has not been effectively mimicked and that NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 is acting as a simple reversible competitive inhibitor.

To demonstrate the utility of NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 for kinetic and mechanistic studies, bisubstrate kinetic analyses were performed in the presence of varied concentrations of the phosphinate inhibitor (Fig. 6). The resulting rates of transpeptidation product formation were globally fit to kinetic models corresponding to Ordered Sequential, Ping-Pong, and Ping-Pong with a hydrolytic shunt mechanisms in the presence of an inhibitor competitive with the first substrate (the LPETG-containing peptide) using Eqs. 1–3, respectively:

$$v_p = \frac{k_{cat}[E_0][A][B]}{K_{ia}K_{mb}\left(1+\frac{[I]}{K_I}\right)+K_{mb}[A]+K_{ma}[B]\left(1+\frac{[I]}{K_I}\right)+[A][B]} \quad (1)$$

$$v_p = \frac{k_{cat}[E_0][A][B]}{K_{mb}[A]+K_{ma}[B]\left(1+\frac{[I]}{K_I}\right)+[A][B]} \quad (2)$$

$$v_p = \frac{k_{cat\_h}K_{mb}[E_0][A]+k_{cat\_t}[E_0][A][B]}{K_{ia}K_{mb}+K_{mb}[A]+K_{ma}[B]+\frac{K_{mb}K_{ia}}{K_I}[I]+[A][B]+\frac{K_{ma}}{K_I}[B][I]} \quad (3)$$

where v_p is the velocity of the formation of G-Dap(Dnp), k_{cat} is the overall rate constant, k_{cat_h} is the overall rate constant for the hydrolytic reaction, k_{cat_t} is the overall rate constant for the transpeptidation reaction, K_{ma} is the K_m for Abz-LPETG-Dap(Dnp), K_{mb} is the K_m for GGGGG, K_{ia} is the inhibition constant for Abz-LPETG-Dap(Dnp) as a product inhibitor, K_I is the dissociation constant for NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2, [*A*] is the concentration of Abz-LPETG-Dap(Dnp), [*B*] is the concentration of GGGGG, [E_0] is the concentration of SrtA, and [*I*] is the concentration of NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2.

We chose to consider the case of a partial hydrolytic shunt since we and others have shown that in the absence of an incoming nucleophile (GGGGG for instance), SrtA catalyzes the hydrolysis of Abz-LPETG-Dap(Dnp) between the threonine and glycine residues resulting in the release of Abz-LPET and G-Dap(Dnp) (Fig. 5).[23–27] Schneewind and co-workers have proposed that SrtA proceeds through a thioester intermediate, which could be resolved through attack by water.[25] The γ-glutamyl transpeptidases, which catalyze a similar reaction, are thought to proceed through a thioester intermediate and are subject to a partial hydrolytic shunt.[28]

The inhibitory pattern observed is in strong agreement with a Ping-Pong Bi Bi hydrolytic shunt kinetic model. Furthermore, the resultant kinetic parameters (Table 1) are consistent with those obtained from analysis of

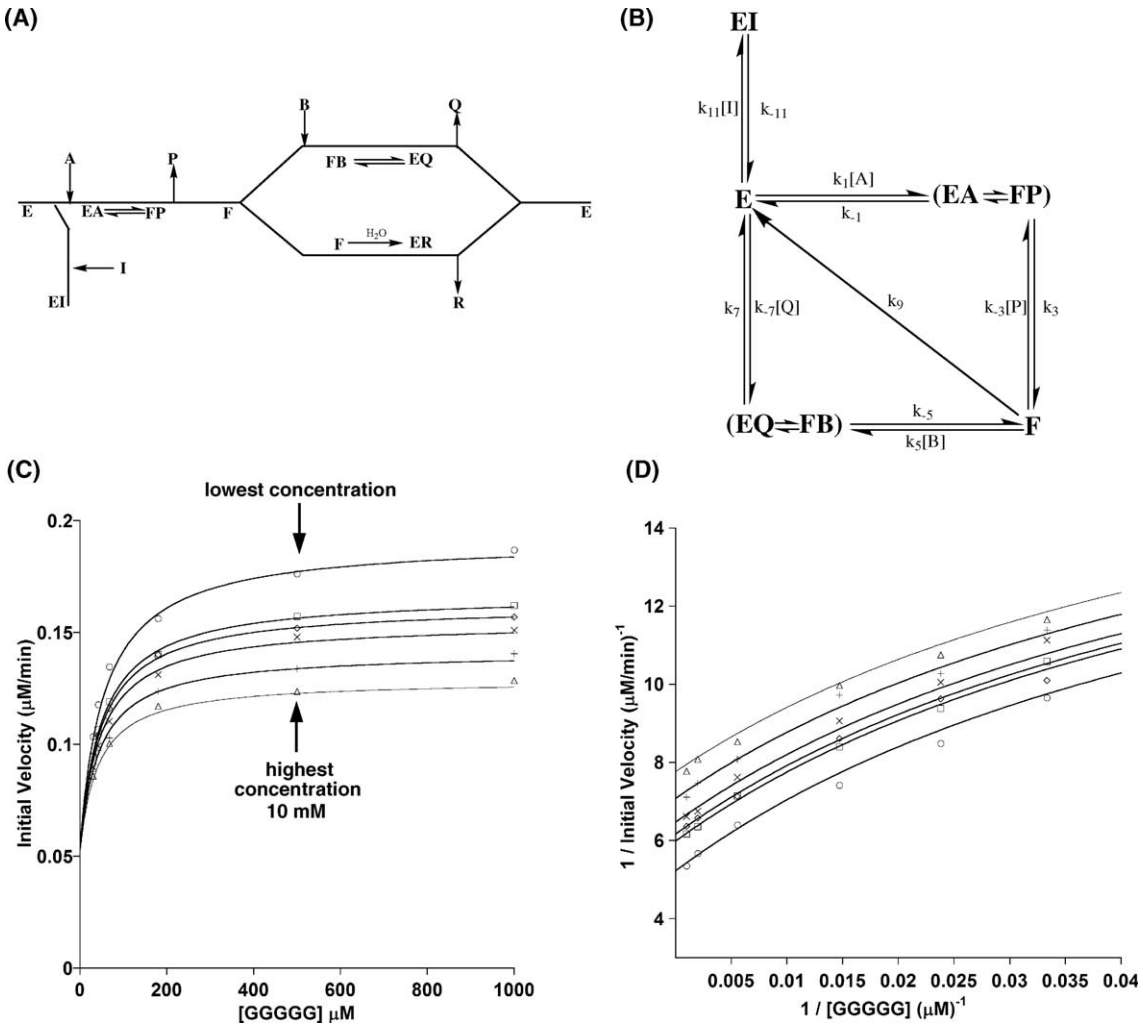

Figure 6. Kinetic analysis of transpeptidase activity in the presence of phosphinate inhibitor **1**. SrtA (100 nM) was incubated in the presence of 1 mM Abz-LPETG-Dap(Dnp) and variable concentrations of Gly_5 and phosphinate containing inhibitor **1** for 30 min, quenched, and analyzed by HPLC. Data were fit to Eq. 3. (A) Cleland diagram depicting competitive inhibitor in Ping-Pong hydrolytic shunt kinetic model. (B) King–Altman diagram showing microscopic rate constants used to derive the rate expression. (C) Initial velocity versus Gly_5 concentration, where the concentration of the phosphinate inhibitor **1** is as follows: ○ = 0 mM, □ = 3 mM, ◇ = 3.7 mM, × = 4.9 mM, + = 7.3 mM, △ = 10 mM. (D) Lineweaver-Burk reciprocal plot.

steady state bisubstrate experiments in the absence of any inhibitors.[23] *F*-test analysis, performed on the χ^2 values calculated by the Grafit program, indicates that the data statistically fit the hydrolytic shunt model significantly better than either the sequential (*F* statistic = 32.3) or Ping-Pong models (*F* statistic = 16.4). These *F*-statistics correspond to probability scores of 0.0003% and 0.0300%, respectively, demonstrating the extremely low likelihood that the hydrolytic shunt model is preferred as a result of chance.

3. Conclusions

Peptides containing nonhydrolyzable phosphinic isosteres have previously been shown to be effective transition state inhibitors of proteases that do not proceed through an acyl-enzyme intermediate (Fig. 3).[17–19] We have employed the synthetic scheme of Meldal in the synthesis of a potential transition state mimetic, where we replaced the Thr-Gly scissile bond from the LPXTG substrate with the nonhydrolyzable AlaΨ{PO_2H–CH_2}Gly phosphinic isostere. The resulting peptidomimetic NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 was found to have a very poor inhibitory activity (IC_{50} = 10 mM) indicating that the transition state has not been accurately mimicked in this design.

Although NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 is not a high affinity transition state analog, preliminary kinetic evidence is consistent with it being a simple reversible competitive inhibitor with millimolar affinity to SrtA. To demonstrate the utility of such an inhibitor for the kinetic characterization of sortase, NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 was employed as a tool to verify that the reaction mechanism was consistent with the Ping-Pong hydrolytic shunt kinetic model. The inhibitory pattern observed is consistent with the presence of a hydrolytic shunt since the data in Figure 6 verify the formation of product in the

Table 1. Summary of kinetic parameters from bisubstrate kinetic analysis

	k_{cat_t}[a] (min^{-1})	k_{cat_h}[b]	K_{ma}[c] (mM)	K_{mb}[d] (μM)	K_I (mM)	χ^2
Sequential	6.33	NA[e]	11.8	97	15.6	0.0004
Ping-Pong	4.59	NA[e]	7.2	74	13.5	0.0004
Ping-Pong hydrolytic shunt	4.05	0.40 min^{-1}	5.5	107	11.4	0.0002

[a] Rate constant for transpeptidation.
[b] Rate constant for hydrolysis.
[c] K_m for Abz-LPETG-Dap(Dnp).
[d] K_m for GGGGG.
[e] Not applicable.

absence of Gly_5, the second substrate. In addition, the Lineweaver–Burk reciprocal plot shown in Figure 6D is nonlinear, as predicted by Eq. 3.

As Figure 3 illustrates, the Ala$\Psi\{PO_2H–CH_2\}$ isostere preserves the tetrahedral geometry at the phosphate atom, but a phosphorous bound OH is present where the cysteine sulfur would reside in the transition state of a cysteine protease. Steric interactions between the phosphorous bound OH from the inhibitor and the sulfur atom in the active site likely prevent optimal binding geometry. We have shown that phosphinate containing peptidomimetics are not good transition state analogs of the sortase catalyzed reaction, supporting the notion that sortase does not proceed through a sequential (ternary complex) mechanism.

4. Experimental methods

4.1. Materials and reagents

Buffer salts were purchased from Sigma. Recombinant *S. aureus* SrtA enzyme was prepared as previously described. Sortase activity was monitored by HPLC according to the method of McCafferty and co-workers.[23] Standard Fmoc amino acids were purchased from Novabiochem or Bachem and used without further purification. 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid-MBHA (PAL) resin (Advanced Chemtech) was used for solid-phase peptide synthesis. Solid-phase peptide synthesis was performed on an automated 433A synthesizer (Applied Biosystems). All mass spectrometry data were obtained on an Voyager™ MALDI-TOF Spectrometer (Applied Biosystems). HPLC was performed using a Thermo Separation Products SpectraSYSTEM™ HPLC equipped with an autosampler and either a semipreparative Jupiter™ octadecyl silica column (Phenomenex), or an analytical (4.6 × 250 mm, 5 μm) or fast analytical (4.6 × 50 mm, 3 μm) octadecyl silica column (Vydac).

4.2. Synthesis of 1-aminoethylphosphinic acid

Synthesis of 1-aminoethylphosphinic acid was prepared according to the method of Baylis et al. with slight modifications.[22] Acetaldehyde (4.4 g, 100 mmol) in water (25 mL) was added dropwise to a refluxing solution of diphenylmethylamine hydrochloride (**7**, 22.0 g, 100 mmol) and 50% aqueous hypophosphorous acid (13.2 g, 100 mmol) in water (50 mL). Heating was continued for 2 h, the solution was cooled, and the solid was collected by filtration and washed with acetone giving 1-(benzhydrylamino)ethylphosphinic acid (20 g, 70%). 1-(Benzhydrylamino)ethylphosphinic acid (20 g, 70 mmol) was mixed with 48% HBr (66.8 mL, 8.2 equiv) and brought to 126 °C for 15 min. The reaction mixture was cooled to room temperature and concentrated in vacuo. The resulting oil was partitioned between water (100 mL) and DCM (100 mL). The aqueous layer was washed three times with DCM, adjusted to pH 7 with NaOH, stirred for 30 min at 0 °C, and filtered. Solid Na_2CO_3 (15.4 g, 2 equiv) was added to the filtrate giving a solution of 1-aminoethylphosphinic acid. Allyl chloroformate (9.2 mL, 2 equiv) was added dropwise at room temperature with vigorous stirring. After 30 min, the pH was lowered to 0 with HCl, and 100 g NaCl was added to create a supersaturated solution. Extraction with ethyl acetate (12 × 100 mL) was performed. The combined extracts were dried with $MgSO_4$ and concentrated to dryness. The resulting product was dissolved in ethyl acetate and recrystallized from petroleum ether to give **2** (9.1 g, 64%).

4.3. Synthesis of NH_2-YALPE-Ala$\Psi\{PO_2H–CH_2\}$Gly-EE-NH_2

The phoshinate containing peptide mimetic **1**, NH_2-YALPE-Ala$\Psi\{PO_2H–CH_2\}$Gly-EE-NH_2, was synthesized from a procedure adapted from Buchardt and Meldal[20] using the alanine analog precursor, 1-aminoethylphosphinic acid, which is readily available.[22] Briefly, PAL resin (0.5 mmol) was loaded with two glutamic acid residues using standard automated peptide synthesis procedures. The deprotected resin was washed successively with DMF and DCM and acylated with acryloyl chloride (412.5 μL, 0.5 mmol) in DCM. The resin was again washed with DMF and DCM and was placed in a 250 mL reaction vessel with a solution of **2** (1.16 g, 6 mmol) and bis(trimethylsilyl)acetamide (4.5 mL, 18 mmol) in degassed DCE (50 mL). The vessel was purged with Ar and refluxed at 100 °C for 4 h, and cooled to room temperature. The Alloc protecting group was removed by adding a solution of $Pd(PPh_3)_4$ (160 mg, 1.5 mmol) in $CHCl_3$–AcOH–NEM 92.5:5:2.5 (10 mL) and stirring for 10 min. The resin was washed successively with $CHCl_3$, DMF, (0.5% Et_2NCS_2Na in DMF), and DMF. The next Fmoc protected amino acid, Fmoc-Glu, was coupled using standard procedures, but was left to react overnight. The remaining four amino acids

were coupled by automated procedures. The peptide was cleaved from the resin using TFA–water (95:5) for 3 h. Excess TFA was removed by rotary evaporation, the peptide was precipitated using cold diethyl ether, filtered with a fine porosity fritted glass filter, dissolved in water, and lyophilized to afford the desired crude product **1**, NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 (75 mg, 15%). The crude product was purified by HPLC using a semipreparative C_{18} Jupiter column to $\geqslant$98% purity and verified by MALDI-TOF MS.

4.4. Kinetic characterization of the NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 inhibitor

NH_2-YALPE-AlaΨ{PO_2H–CH_2}Gly-EE-NH_2 inhibitory patterns were determined with 1 mM Abz-LPETG-Dap(Dnp) and Gly_5 concentrations from 50 to 1000 μM using 100 nM $SrtA_{\Delta N24}$. The enzyme was assayed using the HPLC based method[23] monitoring the formation of the G-Dap(Dnp) product at 355 nm. The data were fit to the Ping-Pong Bi Bi hydrolytic shunt kinetic mechanism (Eq. 3) using the Grafit global fitting program (Erithacus Software).

Note added in proof

During the final stages of publication of this manuscript, Oh and coworkers [*J. Med. Chem.* **2004**, *47*, 2418–2421] recently reported the discovery of reversible inhibition of SrtA by small molecule diarylacrylonitriles from random library screening efforts. In this report, the primary structure–activity relationship and minimal structural requirements for potency were established through structural modifications and modeling studies.

Acknowledgements

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