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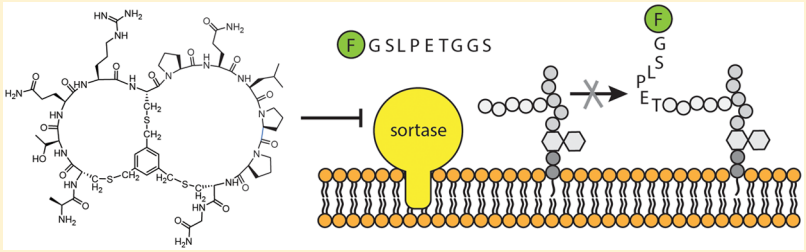
Development of Potent and Selective *S. aureus* Sortase A Inhibitors Based on Peptide Macrocycles

Inmaculada Rentero Rebollo,[†] Shawna McCallin,[‡] Davide Bertoldo,[†] José Manuel Entenza,[‡] Philippe Moreillon,[‡] and Christian Heinis*[,†]

[†]Institute of Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland

[‡]Department of Fundamental Microbiology, University of Lausanne, CH-1015 Lausanne, Switzerland

Supporting Information



ABSTRACT: Sortases are transpeptidase enzymes that anchor surface proteins, including virulence factors, to the cell wall of Gram-positive bacteria, and they are potential targets for the development of anti-infective agents. While several large compound libraries were searched by high-throughput screening, no high-affinity inhibitors of sortases could be developed to date. Here, we applied phage display to screen billions of peptide macrocycles against sortase A (SrtA) of *Staphylococcus aureus* (*S. aureus*). We were able to identify potent and selective inhibitors of SrtA that blocked SrtA-mediated anchoring of synthetic substrates to the surface of live *S. aureus* cells. A region present in all inhibitory peptides (Leu-Pro-Pro) resembled the natural substrates of SrtA (Leu-Pro-Xaa-Thr-Gly), suggesting that the macrocycles bind to the enzyme's active site and that they form similar molecular contacts as natural substrates. The evolved peptide macrocycles may be used as lead structures for the development of potent peptidomimetic SrtA inhibitors.

KEYWORDS: *Bicyclic peptide, peptide macrocycle, phage display, sortase, Staphylococcus aureus*

The development of antibiotic resistance among life-threatening human pathogens has prompted the exploration of new alternative targets beyond those exploited by conventional antibiotics. An attractive class of targets are the sortases, which are membrane-bound transpeptidases that catalyze the transfer and covalent immobilization of surface proteins to the cell wall in Gram-positive bacteria.[1−3] For example, SrtA of *S. aureus* anchors a number of important virulence factors such as protein A, clumping factors, and fibronectin-binding proteins to the cell wall.[4] The virulence factors are secreted as precursors with C-terminal sorting signals "LPXTG" that are cleaved by SrtA between threonine and glycine residues and ligated to polyglycine sequences of peptidoglycans in the bacterial cell coat.[5] These proteins enable adhesion and infection of host cells and tissues, evasion from the immune system, and biofilm formation.[6,7] SrtA of *S. aureus* has received much attention because this clinically important pathogen has developed resistance to most of the available antibiotics.[8,9] SrtA knockouts show reduced adhesion to matrix proteins and reduced pathogenicity in animal models for *S. aureus*.[10−13]

Large efforts were made to develop SrtA inhibitors in recent years.[1,2] Libraries of small organic molecules comprising 30,000,[14] 135,625,[15] and 50,240[16] chemical structures were screened for SrtA inhibition. The most active compounds identified are covalent inhibitors that react with the thiol group of the active-site Cys 184 of SrtA. While having good IC_{50}s in the submicromolar range, they typically have poor target selectivity. The most active reversible inhibitors have low micromolar IC_{50} values.[2,17,18] A recent effort combining *in silico* screening and optimization yielded a reversible SrtA inhibitor based on a triazolothiadiazole scaffold with a single-digit IC_{50} (9.3 μM).[19] The application of display techniques such as mRNA display to screen billions of peptides has so far yielded only binders of SrtA, but not inhibitors.[20] While more and more SrtA inhibitors with ever better inhibitory activities are reported, the best reversible inhibitors still have K_i values in the micromolar range. Such rather weak affinities require application of high concentrations for therapeutic intervention, therefore increasing the risk of toxicity due to off-target binding. There is a clear need of novel SrtA inhibitors with better affinity and higher target selectivity.

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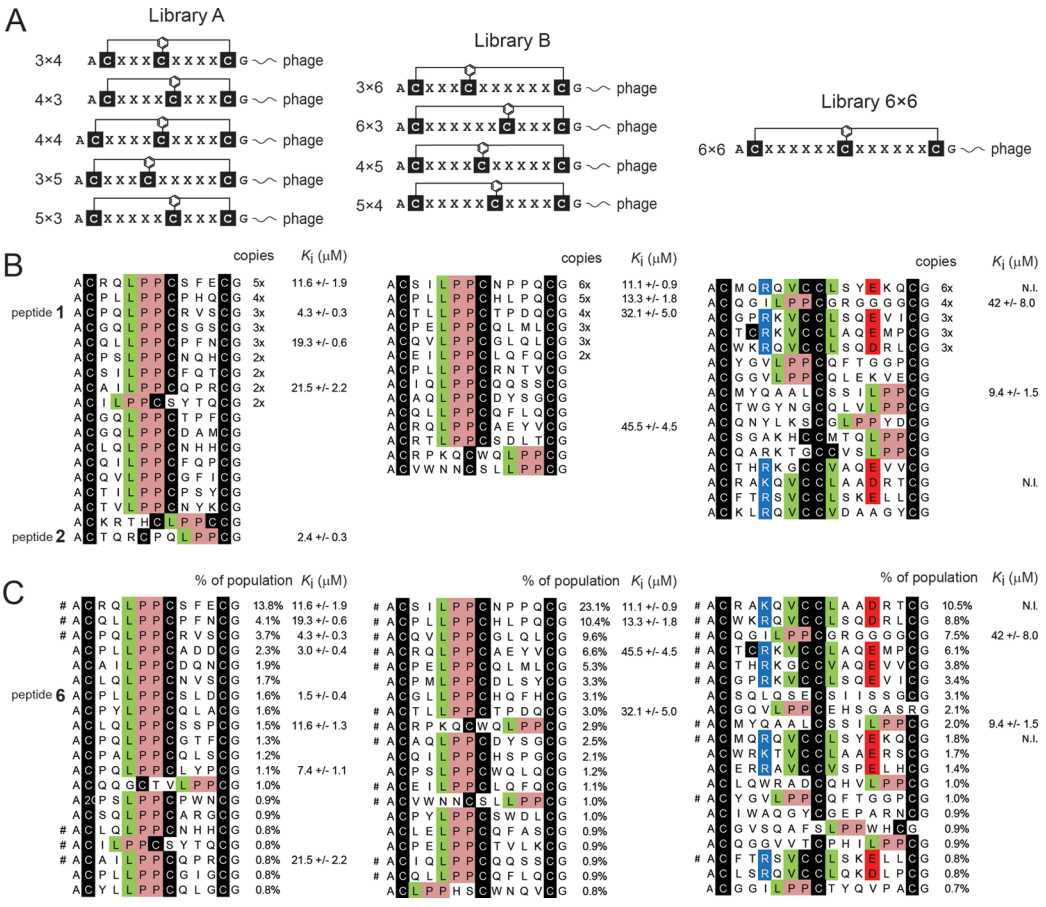

Figure 1. Phage selection of bicyclic peptides against *S. aureus* SrtA. (A) Format of libraries. (B) Peptides were sequenced after three selection rounds by Sanger sequencing. Sequence similarities are highlighted by color. Inhibitory constants K_is were determined in an activity assay using a fluorogenic substrate. Average values of at least three independent measurements and standard deviations are indicated. N.I. indicates that no inhibition was observed at the highest bicyclic peptide concentration tested (300 μM). (C) Peptides sequenced after three selection rounds by Ion Torrent high-throughput sequencing (the 20 most abundant peptides are shown; more sequences are shown in Tables S1−S3, Supporting Information). Peptides also found by Sanger sequencing are indicated with a hash symbol (#).

Herein, we proposed to develop SrtA inhibitors based on bicyclic peptides because molecules of this format can bind targets with high affinity and target selectivity. Bicyclic peptides contain two peptide rings that can bind to protein targets much like antibodies bind to antigens via their complementarity determining regions.[21] Bicyclic peptides with tailored binding specificities can be developed by phage display.[22] All bicyclic peptides so far developed with this approach displayed high target selectivity.[21−24] For example, a bicyclic peptide inhibitor of the human serine protease uPA (K_i = 53 nM) was >1000-fold selective over its murine orthologue and paralogous proteases.[21] Similarly, bicyclic peptides against the targets plasma kallikrein and FXIIa showed equal or even higher selectivity.[23,24] Study of cocrystal structures of bicyclic peptides and their targets showed that the peptides adapt their shapes to be perfectly complementary to the target.[21,25] We reasoned that bicyclic peptide inhibitors, being highly selective for SrtA, could be useful as tool compounds in studies with SrtA and potentially serve as leads for the development of peptidomimetic anti-infective drugs.

Combinatorial libraries of peptides having the format ACX_mCX_nCG (C = cysteine, X = random amino acids; m, n = number of random amino acids) were displayed on phage and cyclized by reacting the cysteines with 1,3,5-tris-(bromomethyl)benzene (TBMB), as previously described.[22,26] Three libraries were in parallel subjected to affinity selections with SrtA: Library A contained bicyclic peptides with 7−8 random residues (m × n = 3 × 4, 4 × 3, 3 × 5, 5 × 3, 4 × 4), library B contained peptides with 9 random residues (4 × 5, 5 × 4, 3 × 6, 6 × 3), and library 6 × 6 contained bicyclic peptides with 12 random residues (6 × 6) (Figure 1A). After three rounds of selection, several dozens of clones were analyzed by Sanger sequencing (Figure 1B). A strong consensus sequence indicated that target-specific peptides were isolated. High-throughput sequencing (HTS) was subsequently applied to obtain a more detailed picture of enriched peptides. The 20 most abundant clones are shown in Figure 1C and more clones in Tables S1−S3, Supporting Information. Nearly all peptides isolated from libraries A and B contained the amino acid motif "LPP" (98% and 99% of the peptides, respectively). The "LPP" motif is similar to the sorting sequence "LPXTG" of the natural substrates that is recognized and cleaved by SrtA.[5] The motif was present most often in rings of five amino acids (not counting the flanking cysteines) and adjacent to the second cysteine (CXXLPPC). It was found more often in the first of the two rings. Analysis of the HTS data also showed amino acid preferences in the two positions in front of the LPP motif. In the first position, proline was found most often (30%), and in the second position, leucine, glutamine, and valine were the most frequent amino acids (21, 18, and 14%). In SrtA

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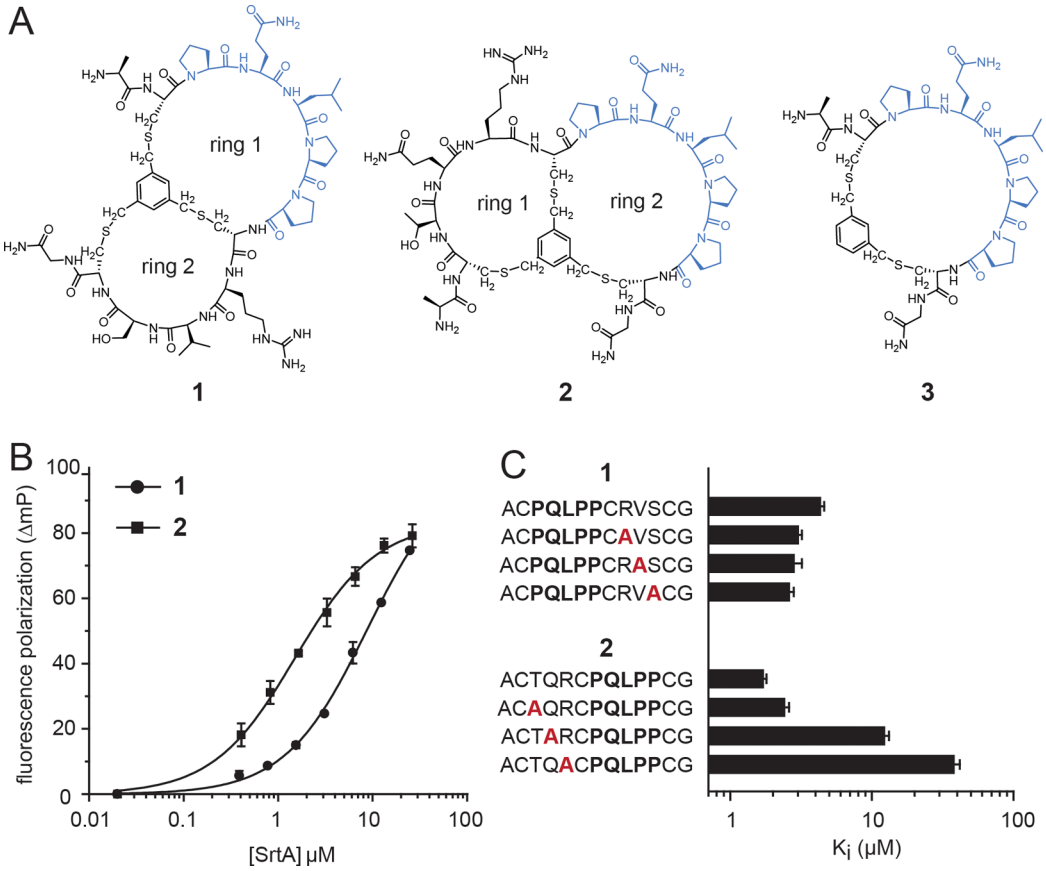

Figure 2. Structure and activity of bicyclic peptide SrtA inhibitors. (A) Chemical structures of the peptide macrocycles **1**, **2**, and **3**. (B) Binding of fluorescein-labeled bicyclic peptide **1** and **2** to SrtA measured by fluorescence polarization. Average values and standard deviations are indicated. (C) Alanine scanning of the nonconserved ring in the bicyclic peptides **1** and **2**. Average values and standard deviations are indicated.

inhibitors with the "$P^{Q}/_{L}LPP$" motif in the first ring, the second ring did not converge to a specific sequence. In contrast, in the group of peptides with the "$P^{Q}/_{L}LPP$" motif in the second ring, certain amino acids in some positions in the first ring appeared to be slightly preferred (Figure 1C and Tables S1−S3, Supporting Information). Peptides isolated from the 6 × 6 library converged to either of two consensus motifs: the first contained a fourth cysteine and the consensus sequence "$AC\underline{C}XX^{K}/_{R}XVC\underline{C}^{L}/_{V}XX^{D}/_{E}XX\underline{C}G$" and the second contained the "LPP" motif in either of the two rings. Previous studies in our laboratory showed that peptides having four cysteine residues were likely isolated in the form of bicyclic peptides with two disulfide bridges.[27] Peptides with unmodified cysteines can oxidize during phage preparation or affinity selection. Although the fraction of unmodified peptide is typically low, peptides with two disulfide bridges can be enriched in cases where they bind particularly well to the target protein.

Thirteen bicyclic peptides that were identified by Sanger sequencing were synthesized and the inhibition of SrtA tested in an enzymatic assay using the fluorescence-quenched substrate Dabcyl-LPETG-Edans. All peptides containing the "LPP" motif, but not those containing the four cysteines, blocked SrtA (Figure 1B). The two best bicyclic peptides **1** ($AC\underline{P}QLPP\underline{C}RVS\underline{C}G$) and **2** ($AC\underline{T}QR\underline{C}PQLPP\underline{C}G$) inhibited SrtA with K_is of 4.3 ± 0.3 and 2.4 ± 0.3 μM, respectively (Figure 2A). The binding of these peptides to SrtA with single-digit micromolar affinity was confirmed in a binding assay based on fluorescence polarization. The peptides labeled at the N-terminal amino groups with fluorescein-bound SrtA with K_d values of 8.8 ± 0.7 and 1.5 ± 0.1 μM, respectively (Figure 2B). Both peptides contained the extended motif "PQLPP"; peptide **1** in the first ring and peptide **2** in the second ring. A monocyclic peptide containing the "PQLPP" motif was synthesized to test if the second ring contributes to the binding (Figure 2A; **3**). The peptide $AC\underline{P}QLPP\underline{C}G$ cyclized with 1,3-bis(bromomethyl)benzene (BBMB) inhibited SrtA with a K_i of 4.9 ± 0.5 μM and thus did so with a similar potency as bicyclic peptide **1** and 2-fold weaker than bicyclic peptide **2**. Alanine scans in the rings of bicyclic peptide **1** and bicyclic peptide **2** not containing the consensus motif were performed to assess to what extent the different amino acids contribute to binding, if at all (Figure 2C). In bicyclic peptide **1**, substitution of all three amino acids to alanine did not reduce the inhibitory activity, suggesting that the second ring was not interacting with the target. In contrast, in bicyclic peptide **2**, amino acid substitution reduced the inhibitory activity substantially. Mutation of Gln4 and Arg5 to Ala lowered the activity 7- and 22-fold, respectively (Figure 2C). The importance of Arg5 is in line with the finding that arginine was enriched in position 5 of the numerous peptides identified by high-throughput sequencing. This data suggests that peptides with the consensus motif "PQLPP" in the second ring can form contacts with SrtA via amino acids of the first ring. The high-throughput sequencing data set was subsequently searched for more peptides with the consensus motif "PQLPP" in the second ring. The most abundant peptides of this type were bicyclic peptide **4** (ACTSRCPQLPPCG; found 105 times) and bicyclic peptide

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5 (ACHSRCPQLPPCG; found 55 times). They inhibited SrtA with K_i values of 1.8 ± 0.17 and 1.1 ± 0.18 μM. It is likely that the affinity of peptides with the "PQLPP" in the second ring can be further improved by optimizing all the three amino acids in the first ring and that bicyclic peptide SrtA inhibitors with nanomolar potency can be generated. Testing the activities of more highly abundant bicyclic peptides identified by high-throughput sequencing yielded another peptide with high affinity, bicyclic peptide **6** with a K_i of 1.5 ± 0.4 μM (Figure 1C). Given the similarity of the "LPP" motif to the sortase substrate "LPXTG", the ability of SrtA to cleave the bicyclic peptides was tested. After incubation of bicyclic peptide **2** (0.5 mM) with SrtA at a high concentration (0.2 mM), no cleavage of the bicyclic peptide was observed (Figure S1, Supporting Information). The target specificity of the bicyclic peptides was assessed by testing the binding to a panel of proteins, including serum albumin, O6-alkylguanine-DNA alkyltransferase and TEV protease, all having a surface accessible cysteine residue. The bicyclic peptides did not bind to any of the proteins, indicating a high target selectivity (Figure S2, Supporting Information).

The activity of SrtA on *S. aureus* can be measured with fluorescently labeled peptides containing the LPETG sequence. The fluorophores of such substrates, when added to the culture, are incorporated into the cell wall of *S. aureus* by SrtA.[28,29] We synthesized a fluorescein-labeled LPETG substrate and analyzed its incorporation into the cell wall in the presence of bicyclic peptide. The macrocycle efficiently inhibited the incorporation of the substrate in a concentration-dependent manner with an IC_{50} of 167 μM (Figure 3). At the highest concentration used (1 mM), the transfer of fluorescent peptide to the bacterial coat was nearly as low as in the SKM12 SrtA knockout strain. Compared to the K_i value in the low micromolar range measured *in vitro*, the IC_{50} of 167 μM in the cellular assay was rather high. Potential explanations were proteolytic degradation of the inhibitor or inefficient diffusion of the peptide macrocycle into the bacterial cell wall. Bicyclic peptide incubated overnight in supernatants of *S. aureus* Newman cultures inhibited cleavage of the fluorogenic substrate by SrtA with the expected activity, showing that its functionality was not impaired by proteolytic cleavage. This experiment also showed that the inhibitory activity of the bicyclic peptides was not affected by any component in the bacterial supernatant. Inefficient diffusion of the bicyclic peptide to SrtA in the bacterial coat remained the most likely reason for the relatively high IC_{50}.

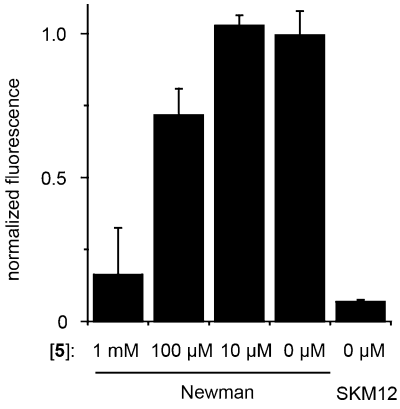

Figure 3. Inhibition of SrtA-mediated incorporation of synthetic fluorescent substrate into the cell wall. Fluorescence signal of *S. aureus* Newman cells grown for 24 h in the presence of the indicated concentrations of bicyclic peptide **5**. Cells were washed prior to fluorescence measurement. The SrtA knockout strain SKM12 was used as a control. Averages and standard deviations of at least three independent experiments are shown.

In summary, we generated SrtA inhibitors with K_i constants close to 1 μM, being substantially better than the best selective small molecule SrtA inhibitors developed so far. Key for obtaining more potent inhibitors was the choice of a different molecule format, the one of macrocycles. Peptide macrocycles have a larger size than small molecules, enabling them to form more molecular interactions with protein targets and consequently to bind more tightly. Another key in the development of better SrtA inhibitors was the screening of vast numbers of molecules. With the phage display approach, several billion different peptide macrocycles could be sampled in a short time. Compared to the small molecule high-throughput screens performed before, an around 10,000-fold larger compound library was sampled in this work. To the best of our knowledge, the peptide macrocycles developed here are the most potent noncovalent SrtA inhibitors reported so far. Given the fact that they interact through specific contacts with the active site of SrtA, it is likely that these molecules will become tool compounds for investigating the biological function of SrtA. In addition, the peptide macrocycles, and in particular the smaller monocyclic one, may serve as lead structures for the development of peptidomimetic anti-infective drugs.

EXPERIMENTAL PROCEDURES

Phage Selection. Libraries A, B, and 6 × 6 were previously described.[22,30] Phages of these libraries display around five peptides fused to the disulfide-free phage coat protein pIII.[31] Phages were produced, purified, and modified with TBMB as described before.[26] (His)6-SrtA$^{26-206}$ was expressed in XL1-blue *Escherichia coli* cells using the vector pHTT14 kindly provided by Prof. Olaf Schneewind (University of Chicago, IL, USA).[32] The protein was purified by nickel affinity chromatography and gel filtration (Figure S3, Supporting Information). Detailed protocols for the expression and purification can be found in the Supporting Information. SrtA was biotinylated by incubating the protein (32 μM) with EZ-link Sulfo-NHS-LC-biotin (128 μM; Pierce) in PBS buffer for 1 h at room temperature. Excess biotinylation reagent was removed using a PD-10 column (GE Healthcare). Three consecutive rounds of affinity selection were performed as described before.[26] Five micrograms of SrtA-biotin were immobilized on streptavidin magnetic beads (Dynabeads M-280, Life Technologies; rounds 1 and 3) or neutravidin magnetic beads (round 2). Neutravidin magnetic beads were prepared as described before.[26] After the third round of selection, the DNA of isolated phages was sequenced by Sanger sequencing (Macrogen) or by high-throughput sequencing using Ion Torrent PGM as described in the Supporting Information.

Peptide Synthesis. Peptides were synthesized in house by standard solid-phase peptide synthesis using Fmoc-protected amino acids (0.03 mmol scale). As a solid support, Rink amide AM resin (GL Biochem, Shanghai, China) was used to obtain peptides with an amidated C-terminus. Peptides were cleaved from the resin under reducing conditions (90% TFA, 2.5% H_2O, 2.5% thioanisol, 2.5% phenol, and 2.5% 1.2-ethanedithiol) and partially purified by precipitation with ether. In the case of bicyclic peptides, crude peptide at 0.5 mM was reacted with 1 mM TBMB (Sigma-Aldrich) in 80% aqueous buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0) and 20% acetonitrile for 1 h at 30 °C. The product was purified by RP-HPLC on a semiprep C18 column (ZORBAX 300SB-C18 9.4 × 250 mm 5-Micron, Agilent), H_2O/0.1% TFA, and 95% ACN/5% H_2O/0.1%

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TFA as solvents (linear gradient). The fraction containing bicyclic peptide was lyophilized and dissolved in water. The purity was assessed by analytical RP-HPLC using an analytical C18 column (Vydac C18, 218TP column, 4.6 × 250 mm) and a solvent system of 99.9% H_2O/0.1% TFA and 99.9% ACN/0.1% TFA. All peptides had a purity of >95%. The identity of the peptides was confirmed by ESI or MALDI-TOF mass spectrometry.

Fluorescein-labeled bicyclic peptides **1** and **2** were synthesized by incubating 1 mM purified peptide with 3 mM 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester (Sigma-Aldrich) in 40 μL of PBS pH 7.4 for 3 h at RT. Then, 0.96 mL H_2O containing 0.1% TFA (v/v) was added to the reaction mixture, and the peptide was purified by HPLC on an analytical C18 column as described above. The fluorescein-modified peptide was lyophilized and the mass confirmed by ESI-MS.

The substrate Fluo-GSLPETGGS was synthesized by conjugating 5(6)-carboxyfluorescein (Sigma−Aldrich) to the N-terminus of the peptide GSLPETGGS during solid-phase peptide synthesis (0.03 mmol scale, Rink amide AM resin). Two equivalents (0.06 mmol) of 5(6)-carboxyfluorescein, HOBt, and DCC, each in 0.25 mL DMF, were added to the resin and incubated at RT for 30 min at 400 rpm. The resin was washed four times with DMF and the fluorescein-labeled peptides cleaved as described above.

In Vitro SrtA Activity Assays. Inhibitory activity of bicyclic peptides was determined by incubation of 2.5 μM SrtA with various peptide concentrations and quantification of the residual activity with 20 μM of fluorogenic substrate Dabcyl-LPETG-Edans (Anaspec) and 200 μM of triglycine (Sigma-Aldrich). Residual enzymatic activities were measured in reaction buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 5 mM $CaCl_2$) containing 0.1% w/v BSA in a volume of 75 μL. Fluorescence intensity was measured with a multiwell plate reader (Infinite 200 PRO, Tecan; excitation at 350 nm, emission at 480 nm). The reactions were performed at 37 °C. The inhibitory constant K_i was calculated using equations described in the Supporting Information. The K_i was not influenced by the presence of triglycine. Average and standard deviations were calculated based on at least three measurements.

Fluorescence Polarization. SrtA was serially diluted in reaction buffer. Fluorescein-labeled peptide at a concentration of 200 nM was prepared in reaction buffer. Thirty microliters of each of the SrtA solution and fluorescein-peptide solution were transferred into a well of a black 96-well half area microplate (Greiner Bio-One international AG) and incubated at room temperature for 15 min. Fluorescence polarization of each solution was measured with a multiwell plate reader (Infinite 200 PRO) using a 485 nm excitation filter and a 535 nm emission filter. The dissociation constants (K_d) were determined by nonlinear regression analyses of fluorescence polarization versus total concentration of SrtA using the equation described in the Supporting Information.

Incorporation of Synthetic Substrates on *S. aureus*. *S. aureus* Newman or SKM12 were grown in tryptic soy broth (TSB) medium in the presence of 0.3 mM Fluo-GSLPETGGS and different concentrations of bicyclic peptides. After 24 h, cells were pelleted and washed with cold PBS. Molecules noncovalently bound to the cell wall were removed by treatment with 5% SDS at 60 °C for 5 min. Cells were pelleted and washed again twice with cold PBS. Fluorescence of the cells was measured with a multiwell plate reader (Infinite 200 PRO) using a 485 nm excitation filter and a 535 nm emission filter.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00045.

Description of procedures for SrtA expression and purification, high-throughput DNA sequencing, and calculation of K_i and K_d. Figures showing the stability and the target specificity of the bicyclic peptides. Figure documenting SrtA expression and purification. Three tables showing the 100 most abundant peptides identified by high-throughput DNA sequencing (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +41 21 692 9350. E-mail: christian.heinis@epfl.ch.

Author Contributions

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Notes

The authors declare the following competing financial interest(s): Co-founder of Bicycle Therapeutics. Please note that this role has not biased the presented work.

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