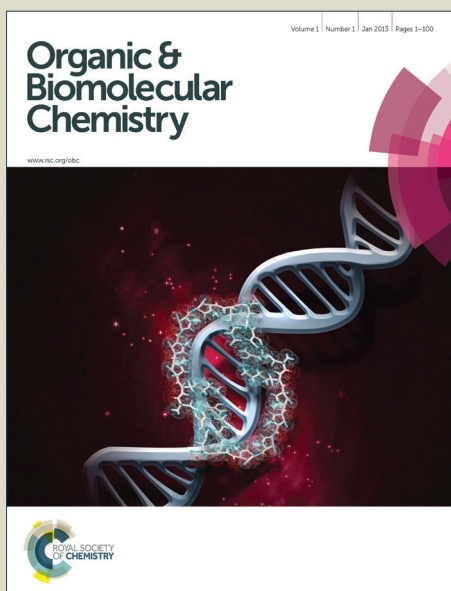


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COMMUNICATION

Chemical synthesis of Staphyloferrin A and its application for *Staphylococcus aureus* detectionRajesh K. Pandey,^{*a} Gregory G. Jarvis^a and Philip S. Low^a

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The chemical synthesis of staphyloferrin A, a siderophore used by *Staphylococcus* bacteria for ferric iron retrieval, has been achieved with 79% yield via solid phase peptide synthesis (SPPS). Biologic activity of synthetic staphyloferrin A has been confirmed by demonstrating its capture and uptake by live *S. aureus*.

Ferric iron is essential for bacterial proliferation and survival due to its requirement in the function of critical metalloenzymes.¹ To obtain sufficient iron, many bacteria have evolved high affinity iron uptake systems consisting of a small organic chelators, termed siderophores, that are released into the environment and recaptured following iron chelation. These siderophores form highly stable coordination complexes with ferric iron and can exhibit very high affinity for their cognate receptors on the bacterial membrane.^{2,3} In the case of the pathogenic bacterium, *Staphylococcus aureus* (*S. aureus*), the HtsA receptor specifically binds the polycarboxylate siderophore staphyloferrin A (Figure 1).⁴ The binding affinity of iron-complexed staphyloferrin A to the HtsA receptor has been demonstrated to be in the low nM range.⁵ Importantly, this highly selective association between *S. aureus* and staphyloferrin A can be exploited to deliver attached therapeutic and imaging agents to bacteria *in vivo* for treatment and localization of infections.^{5,6}

Very recently, we and others have further utilized siderophores as immutable ligands for the capture and identification of their cognate pathogens.^{7,8} In the course of this research, we failed to find an efficient method for the synthesis staphyloferrin A. Thus, although a method for its bacterial fermentation was reported⁹, the resulting yield was poor and prompted us to search for a more efficient chemical synthesis of the pathogen's siderophore. Herein, we describe an efficient chemical synthesis of staphyloferrin A and demonstrate that the resulting siderophore successfully captures pathogenic *S. aureus* on a gold chip (Scheme 1).¹⁰

Commercially available Fmoc-D-Ornithine(Alloc)-OH, **3**, was loaded on StratoSpheresTM PL-Wang resin, **2**, by Mitsunobu reaction conditions using triphenylphosphine and diisopropylazodicarboxylate (DIAD) as reagents in a mixture of DCM and THF. The loading efficiency of **3** onto the resin was determined by using a reported method involving quantification

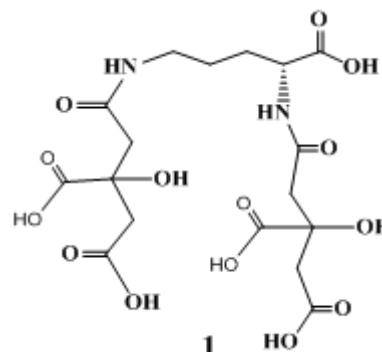
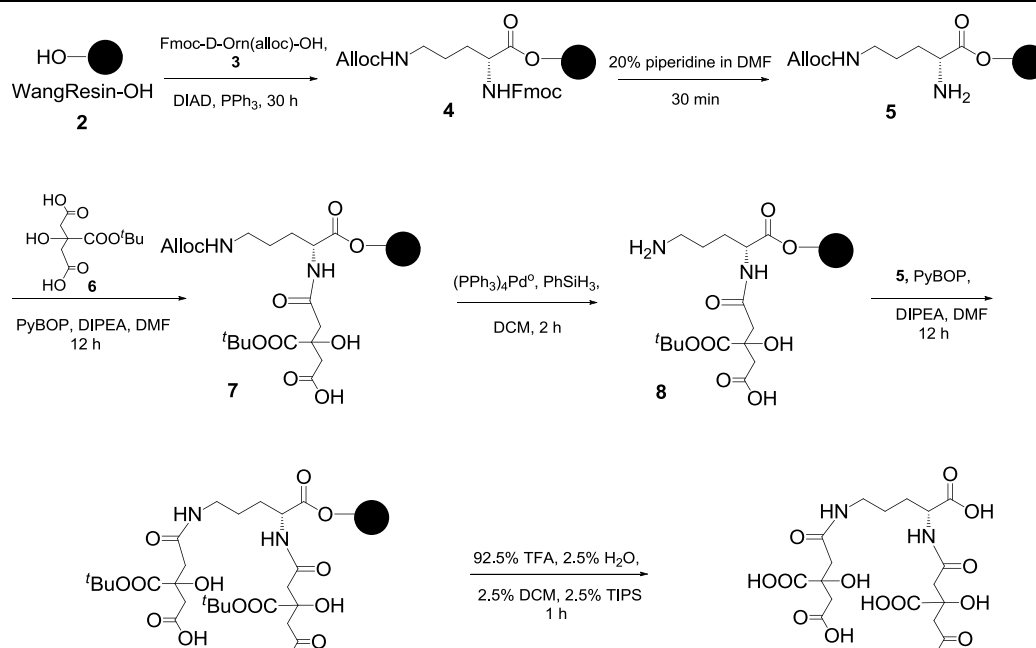


Figure 1. Structure of staphyloferrin A

of fluorenylmethyloxycarbonyl (Fmoc) present in solution after cleavage from 10 mg of resin. We calculated a 90% loading efficiency onto the resin based on the UV-Vis absorbance of Fmoc at 300 nm.¹¹

Fmoc deprotection of **4** was carried out using 20% piperidine in DMF for 30 min and completion of the deprotection reaction was confirmed by a positive Kaiser test result, indicating the presence of a primary amine on the resin. First, coupling between amine **5** and acid **6** was carried out using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and diisopropylethylamine (DIPEA) in DMF for 12 h. A negative Kaiser test result was obtained to verify reaction completion.

The mono-protected derivative of citric acid, **6**, was prepared in three steps by a reported method.¹² After completion of the reaction, the allyloxycarbonyl (alloc) moiety was removed from compound **7** by treating the resin with phenylsilane (PhSiH₃) and tetrakis(triphenylphosphine)palladium(0) [(Ph₃P)₄Pd₀] in DCM for 2 h. Alloc deprotection was confirmed by a positive Kaiser test. Coupling of amine **8** and acid **6** was performed in a similar manner as the first coupling reaction of acid **6** with the ornithine backbone, and reaction completion was confirmed by a negative Kaiser test. The peptide, **9**, was cleaved from the resin using a mixture consisting of 92.5% TFA, 2.5% DCM, 2.5% water, and 2.5% triisopropylsilane (TIPS). The cleavage mixture was concentrated to ca. 2 mL using high vacuum without heating to avoid intramolecular imide formation between the amide bonds and the tertiary carboxylic



Scheme 1. Solid phase synthesis of staphyloferrin A¹⁰

acid groups of the citric acid moieties.¹³ Staphyloferrin A, **1**, was precipitated by adding diethyl ether to the concentrated mixture. The crude precipitate exhibited 91% purity, which was confirmed by liquid chromatography-mass spectrometry (LCMS). High performance liquid chromatography (HPLC) purification yielded two separable fractions containing staphyloferrin A diastereomers observed as adjacent peaks with identical mass characterization data. Diastereomer 2 was identical with the authentic staphyloferrin A as confirmed by ¹H NMR and ¹³C NMR. Diastereomeric mixture of staphyloferrin A was utilized in pathogen detection experiments.

In order to examine the capacity of staphyloferrin A to bind *S. aureus*, we attached the siderophore to a solid support, which involved conjugating the siderophore to bovine serum albumin (BSA) and subsequently adhering the complex to a gold substrate. To achieve this, the ferric iron chelate was first prepared by reacting the purified siderophore with FeCl₃. The addition of ferric iron to the system serves to limit the chemical reactivity of citrate-based carboxylic acids that are involved in chelation. This enabled us to specifically conjugate the siderophore to BSA, via the ornithine carboxylate. According to a recently published staphyloferrin A-HtsA co-crystal structure, utilizing the ornithine carboxylate as a conjugation site does not appear to interfere with HtsA binding while the receptor is in the open conformation.⁵ As a result, we formed a BSA-staphyloferrin A conjugate via peptide coupling at a molar ratio of 4 siderophores for each molecule of BSA.¹⁴ BSA-conjugated staphyloferrin A was then stamped in a specific pattern on a gold substrate using methods developed in our laboratory with slight modifications.¹⁵⁻¹⁷

BSA-staphyloferrin A-patterned chips were incubated with 1 x 10⁶ CFU/mL *S. aureus*¹⁷ and the pattern of capture was examined using darkfield microscopy (Fig. 2). Captured bacteria appeared

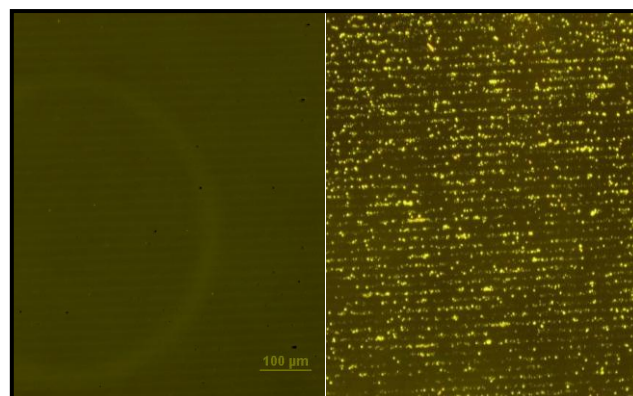


Figure 2. Capture of *S. aureus* with BSA-conjugated staphyloferrin A on gold-coated slides before (left panel) and after incubation (right panel) with 1x10⁶ CFU/mL *S. aureus*.

as gold dots arranged in a parallel line pattern corresponding to the banding pattern on the PDMS stamp employed in printing the BSA-staphyloferrin A on the chip. Capture of *S. aureus* is evident in the image on the right, when comparing to the left image, which was taken prior to bacterial incubation. In order to test the specificity of the siderophore conjugate, we attempted to capture bacteria from other strains, including but not limited to *E. coli*, *P. aeruginosa*, *V. cholera*, and *S. flexneri*, all of which exhibited no capture under the same conditions. More detailed specificity studies are in progress.

It is notable that extensive capture of *S. aureus* was observed at relatively low concentrations. Since the other bacteria examined failed to bind to the chip, the data suggest that a specific interaction exists between staphyloferrin A its cognate transport receptor, HtsA, on the *S. aureus* membrane. Ongoing studies will expand our current understanding of staphyloferrin A specificity with respect to other pathogens and healthy mammalian tissues.

Conclusions

The well-defined and straightforward technique of SPPS was successfully applied to the chemical synthesis of staphyloferrin A, utilizing commercially available starting materials in 6 steps with 79% overall yield. To the best of our knowledge, this report outlines the first chemical synthesis of staphyloferrin A. This synthesis has allowed us to produce larger quantities of the siderophore in less time compared to conventional fermentation/biosynthetic methods. Additionally, our synthetic staphyloferrin A exhibits excellent specificity towards *S. aureus*, opening opportunities for use of the siderophore in the capture and detection of this important human pathogen.

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10. General procedure for the synthesis of staphyloferrin A: To a cooled solution of Fmoc-D-Orn(Alloc)-OH (0.4930 g, 1.125 mmol) and triphenylphosphine (0.2948 g, 1.125 mmol) in 50% DCM/THF (2.0 mL), DIAD (0.2914 g, 0.393 mL, 2.250 mmol) was added dropwise. The solution was then added to pre-swollen StratoSpheres™ PL-Wang resin, 100-200 mesh (0.25 g, 0.225 mmol) in a SPPS vessel. The resulting solution was bubbled with argon gas for 36 h, the coupling solution was drained, and the resin was washed with DCM (3 x 3 mL), THF (3 x 3 mL), DMF (3 x 3 mL) and DCM (3 x 3 mL). The Fmoc protecting group was removed from **4** by treatment with 20% piperidine in DMF (3 x 5 mL) for 0.5 h, followed by washing with DMF. A positive Kaiser test result was obtained, affirming the presence of primary amines on the resin resulting from successful Fmoc deprotection. To the vessel was then introduced a solution of **6** (0.160 g, 0.680 mmol) in DMF, DIPEA (0.394 μ L, 2.250 mmol), and PyBOP (0.3513 g, 0.680 mmol). The resulting solution was bubbled with Ar for 12 h, the coupling solution was drained, and the resin washed with DMF (3 x 5 mL), methanol (3 x 5 mL) and DCM (3 x 5 mL). A Kaiser test was performed to assess reaction completion. The alloc protecting group removed from **7** by treating the resin with PhSiH₃ (1.11 mL, 9.00 mmol) and (Ph₃P)₄Pd₀ (0.181 g, 0.16 mmol) in DCM (2.5 mL). The resulting solution was bubbled with Ar for 2 h, the solution was drained, and the resin washed with DCM (3 x 5 mL), DCM: DIPEA (19:1, 3 x 5 mL), DMF (5 x 5 mL) and DCM (3 x 5 mL). Positive Kaiser test results ensured deprotection of the primary amine. To the vessel was then introduced a solution of **6** (0.160 g, 0.680 mmol) in DMF, DIPEA (0.394 μ L, 2.250 mmol), and PyBOP (0.3513 g, 0.680 mmol). The resulting solution was bubbled with Ar for 12 h, the coupling solution was drained, and the resin washed with DMF (3 x 10 mL), methanol (3 x 10 mL) and DCM (3 x 10 mL). A Kaiser test was performed to assess reaction completion. The peptide, **9**, was cleaved from the resin in the peptide synthesis vessel using a cleavage mixture consisting of 92.5% TFA, 2.5% DCM, 2.5% water and 2.5% TIPS. Ten milliliters of the cleavage mixture was added to the peptide synthesis vessel, and the reaction was bubbled with Ar for 1 h. The resin was treated with two additional 5 mL quantities of the cleavage mixture for 5 min each. The cleavage mixture was drained and concentrated to ca. 2 mL under high vacuum without heating and diethyl ether was added to induce precipitation. The precipitate was collected by centrifugation, washed with diethyl ether three times, and dried under high vacuum, resulting in the recovery of ca. 85 mg of crude product. The product was further purified by HPLC using an Atlantis HILIC Silica Prep OBD 19 x 150 mm 5 μ m preparative column (Waters). The mobile phase consisted of 10 mM ammonium acetate buffer (pH 7) and acetonitrile using a gradient capable of separating apo-staphyloferrin A diastereomers. Spectroscopic data for the characterization of both diastereomers of staphyloferrin A are given in supporting information.
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14. General procedure for the preparation Staphyloferrin A-BSA conjugate: To a 14 mM solution of staphyloferrin A in water was added 0.2 M aqueous FeCl₃, and the solution was stirred for 5 min then lyophilized. Stamping "ink" was prepared by adding 96 μ mol (15 mg) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to a stirring solution of 28 μ mol (15 mg) staphyloferrin A-Fe chelate in 375 μ L PBS at pH 7.4. After stirring for 30 min at room temperature, a 1 mL solution of 0.9 mmol (60 mg) BSA in PBS was added to the reaction and allowed to stir for 2 h at room temperature. The reaction mixture was then filtered through a 0.45 μ m membrane filter and the filtrate was collected.
15. General procedure for the preparation of BSA-patterned chips: A patterned polydimethylsiloxane (PDMS) stamp with 20 μ m wide raised parallel lines spaced 20 μ m apart was prepared using established UV photolithography techniques. A small volume of ink adsorbed to the tip of a cotton swab was rolled onto the raised portions of the textured stamp. A dry cotton swab was then used to remove any excess liquid from the stamp. The ink-coated surface of the stamp was carefully placed on a sterile 15 x 15 mm gold-coated glass chip, making sure not to allow for any lateral movement. Light pressure (10 KPa) was evenly applied to the stamp for 5 min and promptly removed. The stamp was allowed to sit on the gold chip for an additional 5 h in a sealed, sterile Petri dish at 4°C. After removing the stamp from the gold chip, a 5% (w/w) BSA in PBS (pH 7.4) solution was gently dispensed onto the stamped side of the gold chip, ensuring complete coverage. After 5 min, the solution was removed and the chip was gently rinsed 3 x 5 mL with PBS.
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18. General procedure for the growth of *S. aureus*: *S. aureus* obtained from a frozen stock were cultured overnight in an orbital shaker at 37°C to a concentration of 1 x 10⁸ CFU/mL as determined by OD600. Standard LB broth prepared with 0.2 mM 2,2'-bipyridine was used as a low-iron growth medium.