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Conjugation Approach To Produce a *Staphylococcus aureus* Synbody with Activity in Serum

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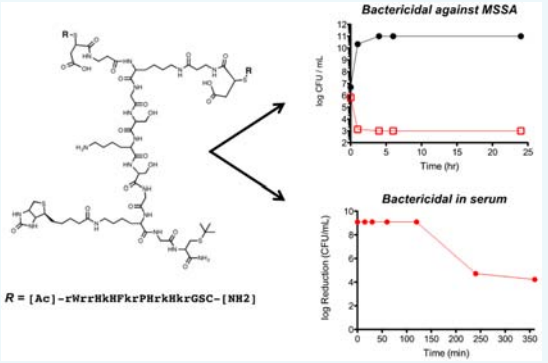
Supporting Information

ABSTRACT: Synbodies show promise as a new class of synthetic antibiotics. Here, we explore improvements in their activity and production through conjugation chemistry. Maleimide conjugation is a widely used conjugation strategy due to its high yield, selectivity, and low cost. We used this strategy to conjugate two antibacterial peptides to produce a bivalent antibacterial peptide, called a synbody that has bactericidal activity against methicillin resistant *Staphylococcus aureus* (MRSA). The synbody was prepared by conjugation of a partially D-amino acid substituted synthetic antibacterial peptide to a bis-maleimide scaffold. The synbody slowly degrades in serum, but also undergoes exchange reactions with other serum proteins, such as albumin. Therefore, we hydrolyzed the thiosuccinimide ring using a mild hydrolysis protocol to produce a new synbody with similar bactericidal activity. The synbody was now resistant to exchange reactions and maintained bactericidal activity in serum for 2 h. This work demonstrates that low-cost maleimide coupling can be used to produce antibacterial peptide conjugates with activity in serum.



INTRODUCTION

There is an urgent need for new antibiotics to combat the international rise of multidrug resistant bacteria.[1−4] The severity of the problem has led the Infectious Diseases Society of America to propose an initiative to deliver ten new antibiotic drugs by the year 2020.[5,6] Despite this push, many of the antibiotics in clinical development are designed from existing scaffolds that have common mechanisms of action that are prone to develop antimicrobial resistance.[2] The low rate of approval of new antibiotics has led many groups to investigate new classes of antibiotics. Natural and synthetic antimicrobial peptides (AMP) are potential alternatives for conventional antibiotics as they differ in terms of structure as well as mode of action, with most AMPs functioning via membrane disruption.[7,8] This mode of action is rapid and lethal to a large spectrum of pathogens, and it severely reduces the possibility for antimicrobial resistance. To date, more than 2500 natural and synthetic antimicrobial peptides have been characterized.[9] However, their development into viable therapeutics has been limited due to low potency, low protease stability, and high toxicity for human cells.[10,11]

While there are many widely used methods for improving protease stability of therapeutic peptides,[12] the low price of antibiotics and small numbers of patients infected with antimicrobial-resistant bacteria[13] renders many of these approaches uneconomical. For example, while many unnatural amino acids, such as β-amino acids, can significantly increase protease resistance, half-life, and pharmacokinetics of a peptide therapeutic,[14] their cost can be 10 to 100 times the cost of the corresponding L-amino acid. Therefore, any modification strategy must fit within the economics of the antibiotics market.

Another strategy that has been widely used to improve both protease stability and potency is to produce multivalent AMPs.[15,16] Multivalency is typically achieved via synthesis off a branched dendrimer or through conjugation to a scaffold.[15] Recent works have utilized the Cu(I)-catalyzed alkyne−azide cycloaddition (CuAAC) reaction to prepare multivalent AMPs with improved potency.[17−19] Maleimide−thiol conjugation is a widely used technique that has been extensively explored for the production of a large number of bioconjugates.[20] This chemistry is rapid, inexpensive, and highly selective, and is frequently used to prepare antibody drug conjugates (ADCs) including two approved for oncology.[21,22] While there have been several reports of the use of maleimides for immobilization of AMPs to surfaces,[23−28] there has been only a single report of the use of maleimide conjugation for AMP therapeutic development.[29]

Despite the use of maleimide conjugation in commercial products, maleimide conjugates can undergo exchange reactions with other thiol containing species *in vivo*.[30−34] While the exchange reaction is often slower than the *in vivo* lifetime of the conjugate, the loss of the drug from the conjugate can reduce activity and impart higher toxicity.[33] The exchange reaction can be problematic, but it has been shown

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that hydrolysis of the succinimide ring produces a stable conjugate.[30−33,35−38] The hydrolysis reaction is often slow,[31,32] but can be accelerated through changes to the maleimide linker[36,38] or pH in the vicinity of the conjugation site.[33]

Our group recently developed a specific bivalent peptide, called a synbody, that inhibited the growth of *Staphylococcus aureus*.[39] The synbody **ASU001** was constructed from a 20-amino-acid (aa) peptide that specifically bound *S. aureus* linked to a second 20-aa peptide that inhibited *S. aureus* growth. The resulting synbody had a narrow spectrum of antibacterial activity with an MIC of 14 μM for *S. aureus*. However, as this synbody is composed of L-amino acids and produced by solid-phase peptide synthesis (SPPS), we explored methods to improve the *S. aureus* activity and protease resistance while using synthetic approaches that kept cost-of-goods low. We subsequently developed several variants using two well-known strategies to increase protease resistance of a peptide therapeutic: acetylation of terminal and primary amines,[12,40−43] and selective substitution of D-amino acids.[44−47] Selective substitution of Arg with D-Arg and Lys with D-Lys along with N-terminal acetylation greatly increased the protease stability (unpublished results). However, this molecule was produced by SPPS with low yield. Therefore, we explored other approaches to produce bivalent variants of the synbody.

Here we demonstrate chemical conjugation and modification methods to improve the synbody class of peptide antibiotics. We prepared a new bivalent synbody **ASU007** through conjugation of two copies of the D-Arg, D-Lys substituted version of the inhibitory peptide[39] followed by conjugation to a bis-maleimide peptide scaffold to produce a synbody with improved protease stability that was bactericidal for a community acquired strain of methicillin resistant *S. aureus* (MRSA). We then used a mild hydrolysis reaction to produce the hydrolyzed synbody **ASU008** that maintained bactericidal activity even when incubated in serum for 2 h at 2× MIC. This study demonstrates that it is feasible to use maleimide−thiol conjugation for the production of new multivalent antibacterial peptides.

RESULTS AND DISCUSSION

Conjugation of Ly to Produce Bactericidal Synbody. While the original *S. aureus* synbody was bacteriostatic and protease sensitive, our goal was to modify the synbody in order to produce a protease stable, bactericidal synbody. We constructed a new synbody **ASU007** composed of two copies of the D-Arg, D-Lys substituted inhibitory peptide from **ASU001**[39] that were conjugated via maleimide−thiol conjugation to a bivalent peptide scaffold (Figure 1). The scaffold was based on that of **ASU001** that used the N-terminal amine and the ε-amine for synthesis of each *S. aureus* peptide.[39] The new scaffold was prepared by SPSS (Sigma Custom Peptide, The Woodlands, TX) and incorporated 3-maleimido-propionic acid onto each amine of Lys, followed by Gly-Ser-Lys-Ser-Gly-Lys(biotin)-Gly-Cys(tBu). The additional amino acids were incorporated into the scaffold in order to increase the molecular weight to aid in detection by MALDI-MS.

To prepare **ASU007**, we added 2.2 equiv of peptide to 1 equiv of scaffold in 1× PBS pH 7.0 and incubated the mixture for 24 h at 25 °C. The synbody was then purified by HPLC, lyophilized and analyzed by HPLC and MS (SI Figure S1). We measured the MIC against a MSSA and MRSA (USA300) strain and found that **ASU007** had similar MIC values for both *S. aureus* strains (Table 1). The compound was also active against *Staphylococcus epidermidis*, but not against the Gram-negative pathogen *Pseudomonas aeruginosa*. Additionally, **ASU007** was slightly hemolytic with 22% hemolysis observed at 500 μM. A therapeutic index[48] was calculated to measure the selectivity of the synbodies for *S. aureus* over mammalian cells and **ASU007** had a TI = 40 indicating selectivity.

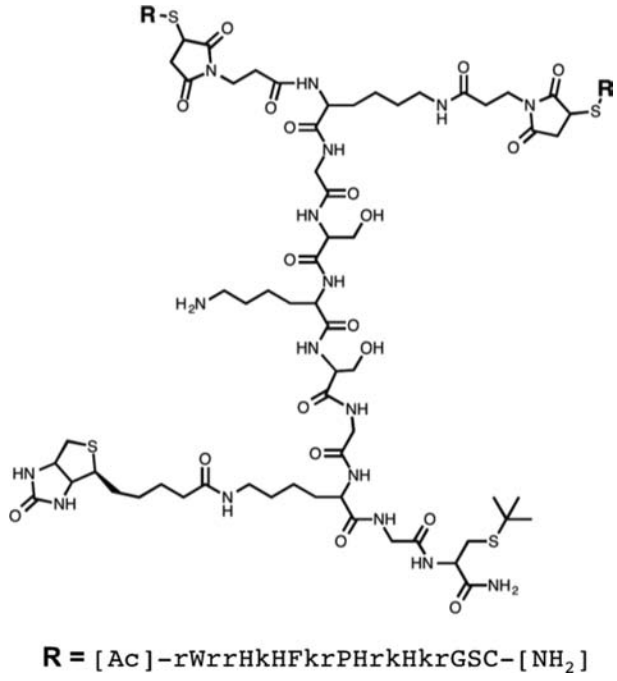

Figure 1. Chemical structure of synbody **ASU007**.

Table 1. Minimum Inhibitory Concentration (MIC) and Hemolysis for ASU007 and ASU008

	ASU007	ASU008
S. aureus UAB637 (MSSA)	12.5 μM	12.5 μM
S. aureus HI-168 (MRSA − USA300)	6.25 μM	12.5 μM
S. epidermidis (ATCC 29886)	6.25 μM	6.25 μM
P. aeruginosa (PAO1)	n.i.	n.i.
Hemolysis (H_{50})	>500 μM	>500 μM

We next tested the bactericidal activity of **ASU007** against early and late exponential phase cultures of MSSA (Figure 2A), as others have found growth phase dependent effects of AMPs against *S. aureus*.[49] We found that **ASU007** was bactericidal (>3 $\log_{10}$ reduction relative to starting culture) at 2× MIC for MSSA in both growth phases. This demonstrates that **ASU007** has potent bactericidal activity. To better understand the kinetics of bactericidal activity, we performed a time kill experiment at 0.5×, 1×, and 2× MIC and found that **ASU007** was rapidly bactericidal at 2× MIC. The rapid cell death observed is consistent with other AMPs that rapidly permeabilize the bacterial membrane leading to cell death.[50]

Hydrolysis of Succinimide Ring Does Not Affect Bactericidal Activity. Maleimide−thiol chemistry is a highly effective conjugation method; however, it has been documented that maleimide conjugates can undergo exchange reactions with other thiol containing species.[30−34] We observed this reaction for **ASU007** and for a second synbody constructed through maleimide−thiol conjugation. Each synbody undergoes exchange with other serum proteins, such as albumin (SI Figure S2). Since these exchange reactions can have deleterious

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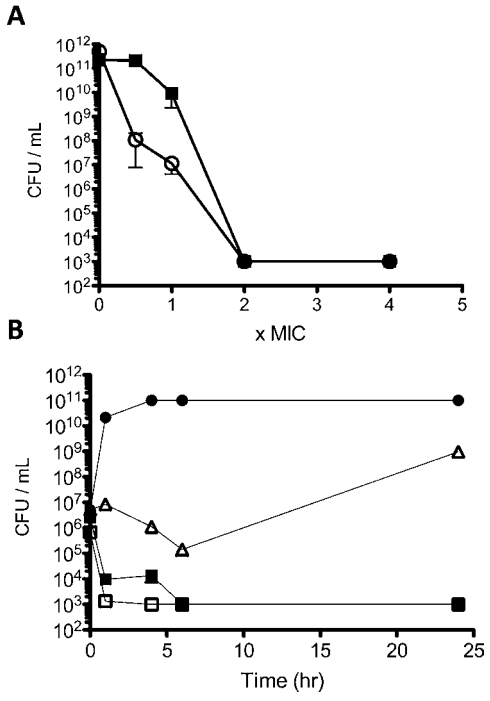


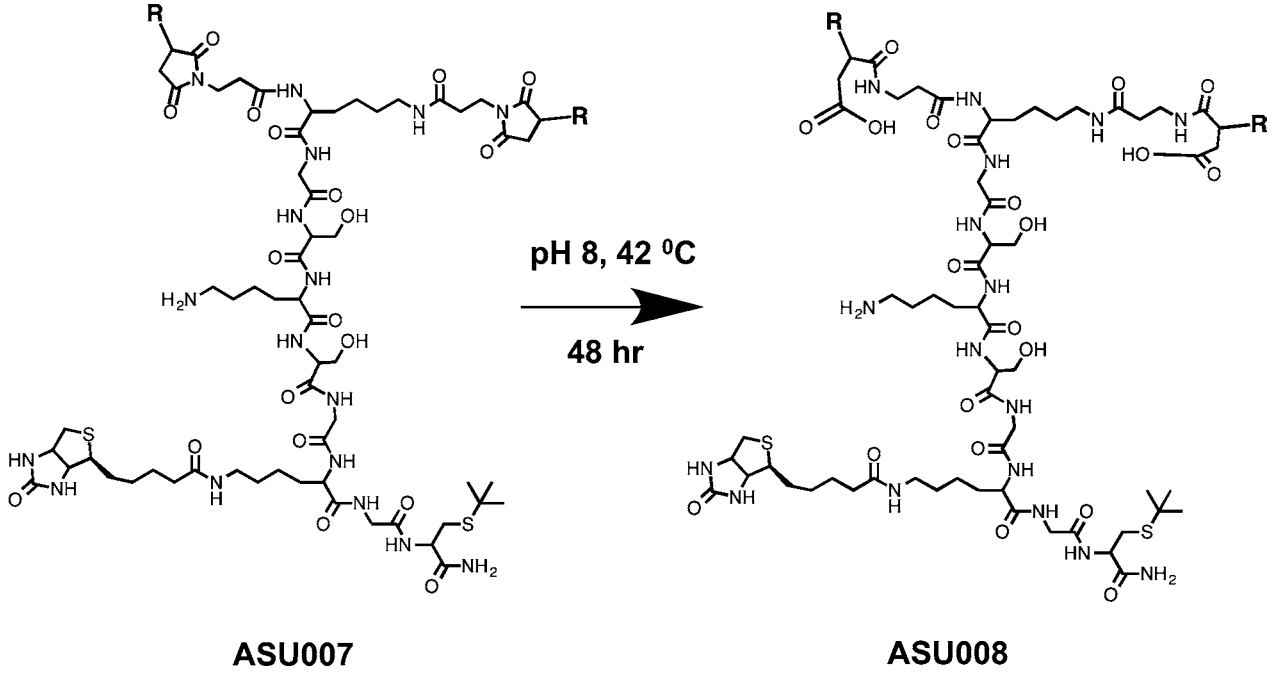
Figure 2. Bactericidal activity of **ASU007**. (A) Bactericidal activity of **ASU007** against MSSA. Bacteria were added to a 96 well plate at either 10^6 CFU/mL (circles) or 10^8 CFU/mL (squares) and treated with 0.5, 1, 2, or 4× MIC of **ASU007** for 22 h. After overnight incubation, samples were plated and viable colonies were counted. The average viable colonies from two independent runs are shown. The limit-of-detection in this assay is 10^3 CFU/mL. (B) Time-kill kinetics study of **ASU007** added to 10^6 CFU/mL culture of MSSA. Bacteria were incubated for 1, 2, 4, 6, or 24 h with either 0.5× MIC (triangles), 1× MIC (solid squares), or 2× MIC (open squares) of **ASU007** and then plated.

effects on bioconjugate performance *in vivo*, it has been shown that succinimide ring-opening prevents exchange reactions and greatly increases the stability of maleimide conjugates.[31−33,36,37] The hydrolysis reaction proceeds slowly under neutral conditions and can take from hours to days.[32,51] These mild reaction conditions were compatible with the solution phase conjugation reaction used to prepare **ASU007**; therefore, we chose a strategy (Scheme 1) in which **ASU007** was prepared and the reaction mixture was hydrolyzed prior to HPLC purification of the hydrolyzed product, **ASU008**. This protocol eliminates one purification step and subsequent sample loss associated with purification. After conjugation, the pH of the reaction was adjusted to 8 and incubated at 42 °C for 48 h. This slow hydrolysis rate is similar to that reported for maleimidocaproyl linked ADCs.[37] We performed the hydrolysis under these mild conditions as preliminary experiments indicated that the synbody degraded at high pH (unpublished results). The conversion of **ASU007** to the ring-opened form was monitored by HPLC and MALDI. After completion, the mixture was purified by HPLC, lyophilized, and the mass characterized by mass spectrometry (SI Figure S3).

Hydrolysis of both succinimide rings in **ASU007** introduces two carboxyl groups, which lowers the net charge of **ASU008** and introduces added flexibility to **ASU008** that could affect antibacterial activity. Additionally, hydrolysis of the ring occurs at either carbonyl, producing multiple isomers.[32] Therefore, we measured the MIC and H_{50} against both MSSA and MRSA-USA300 as before and found that **ASU008** had similar MIC values to **ASU007**, suggesting that the ring-opening did not affect antibacterial activity (Table 1). To confirm this, we repeated the bactericidal activity and time-kill kinetic assay using **ASU008** and found similar bactericidal activity (Figure 3). These results suggest that introduction of additional carboxyl groups did not alter the activity of **ASU008** toward *S. aureus*.

Protease Stability and Activity of ASU008. To evaluate the effect of D-amino acid substitution, acetylation, and

Scheme 1. Hydrolysis of ASU007 to Produce ASU008



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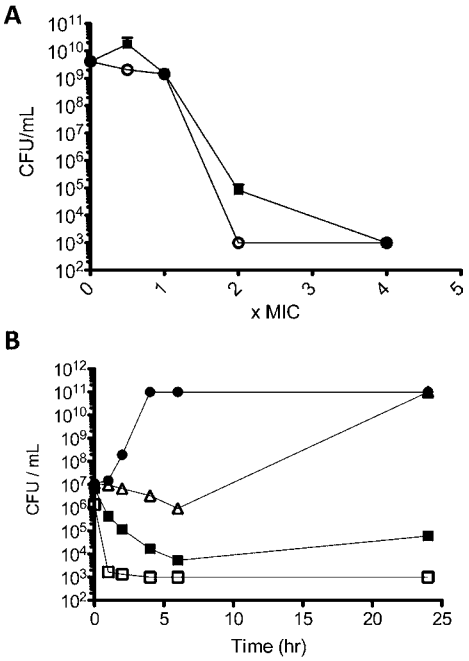

Figure 3. Bactericidal activity of **ASU008**. (A) Bactericidal activity of **ASU008** against MSSA. Bacteria were added to a 96 well plate at either 10^6 CFU/mL (circles) or 10^8 CFU/mL (squares) and treated with 0.5, 1, 2, or 4× MIC of **ASU008**. After overnight incubation, viable colonies were counted. The average viable colonies from two independent runs are shown. The limit-of-detection in this assay is 10^3 CFU/mL. (B) Time-kill kinetics study of **ASU008** added to 10^6 CFU/mL culture of MSSA. Bacteria were incubated for 1, 2, 4, 6, and 24 h with **ASU008** and then plated.

maleimide hydrolysis on the stability of **ASU008** for serum proteases, we incubated **ASU008** in fresh mouse sera for varying amounts of time and detected **ASU008** by the internal biotin tag. Samples were incubated from 15 min up to 6 h in fresh mouse sera, and at the indicated time point, an aliquot was removed and placed in protease inhibitor to stop proteolysis. Streptavidin coated magnetic beads were then added to each sample to capture **ASU008**, the beads were washed, and eluted. Then captured **ASU008** was analyzed by Western Blot using Streptavidin (Figure 4a, SI Figure S4). It can be seen that shortly after incubation in sera, the intensity of **ASU008** begins to decrease indicating degradation by serum proteases. The density of each band was measured using ImageJ and was plotted as a function of time to yield $t_{1/2}$ = 52 min (Figure 4b). While we expected the D-amino acid substituted synbody to have a long half-life in serum, the half-life was shorter than expected. However, this assay relies upon detection of **ASU008** via the biotin tag that is incorporated C-terminal to a Lys, a protease sensitive amino acid. It is possible that **ASU008** is more stable than indicated if the assay simply measures the loss of the biotin tag rather than degradation of the inhibitor peptide arms of **ASU008**.

To test this hypothesis, we designed a modified stability assay in which **ASU008** was incubated in sera at 2× MIC, but now an equal volume of each time point sample was added to a MSSA culture. The samples were incubated overnight, bacteria were plated, and colonies were counted the following day. This assay is a direct measure of the effect of protease degradation on the killing activity of **ASU008** in 50% mouse sera. When **ASU008** was added to fresh mouse sera and protease inhibitor was immediately added to the sample, **ASU008** was bactericidal (SI Figure S6). Bactericidal activity was maintained even after 2 h incubation in serum and **ASU008** was bacteriostatic after 6 h incubation in serum. When plotted as the reduction in bacterial counts relative to the untreated control as a function of time, it is clear that the activity decays at a slower rate than indicated by the Western Blot assay (Figure 4c). This result suggests that the rapid degradation observed in the Western Blot method was caused by the loss of the biotin tag and not by degradation of the D-amino acid containing peptide arms of **ASU008**. This result also demonstrates that the hydrolyzed synbody maintains activity in high concentrations of other thiol containing species, such as albumin.

CONCLUSION

While the challenge of converting antibacterial peptides into effective therapeutics have been extensively documented, there have been numerous developments in the stabilization and pharmacokinetic performance of therapeutic peptides. Yet, the cost sensitive antibiotic market limits the choices for

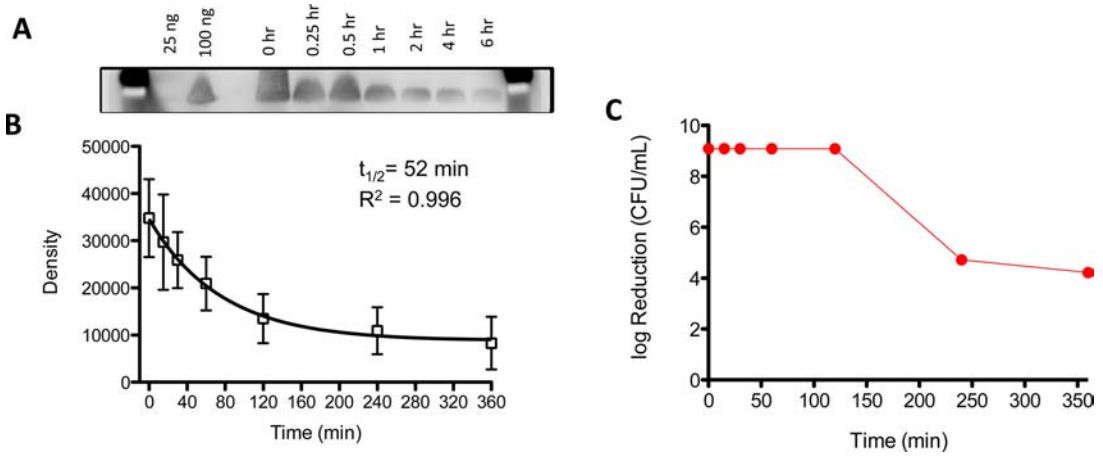

Figure 4. Protease stability of **ASU008**. (A) Western blot of **ASU008** detected by biotin after incubation in fresh mouse sera for increasing amounts of time. Loading controls are shown on the left of the blot. (B) Half-life of **ASU008** determined by densitometry from replicate experiments. (C) Reduction in colonies of MSSA after overnight treatment with 50% sera that had been incubated with **ASU008** for varying amounts of time. Data is plotted as the reduction versus the untreated MSSA sample.

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optimization. However, low cost modifications such as N-terminal acetylation and D-amino acid substitution provide a cost-effective strategy to improve protease stability without loss of activity. The protease stability and half-life improvements needed for an effective antibiotic are much lower than for other therapeutic conditions as many β-lactam antibiotics have half-lives of a few hours or less.

As interest in peptide antibiotics, whether classical AMPs or non-natural mimetics, grows, there is more interest in simple methods to improve the activity of a lead peptide. Preparation of bivalent or multivalent constructs is commonly used to improve activity; however, many reports use SPPS which can have low yields of final product. In contrast, we have found that preparation of bivalent constructs using maleimide chemistry is a facile way to prepare antimicrobial conjugates. Working with purified peptides and a purified maleimide scaffold provides a straightforward method for solution phase conjugation and purification of final products. This method also allows the creation of libraries of synbodies through the use of peptides of different compositions. In this way, rapid medicinal chemistry optimization of a candidate bivalent peptide can be performed.

Similar to ADCs that use maleimide coupling, hydrolysis of the thiosuccinimide ring provides a simple mechanism to stabilize the conjugate and prevent back exchange reactions. In this case, the introduction of additional carboxylate groups from hydrolysis did not adversely affect antibacterial activity, which is often very sensitive to peptide cationic charge. The production of isomeric ring-opened products did not adversely affect antibacterial activity. This might be peculiar to **ASU008** as it has been shown that small antibacterial peptides can kill bacteria through a number of different secondary structures and that physical chemical properties, such as amino acid content and hydrophobicity, are more important for activity.[52] However, if this approach is applied to produce other synbodies, the ring opening could change the secondary structure of the synbody and reduce affinity or activity. Ring opening could also complicate downstream use if single isomer purity is needed.

There are several alternative thiol-selective conjugation strategies in development that could be adopted for the general production of antibacterial synbodies. These include efforts to increase the rate of the hydrolysis reaction through the incorporation of modified maleimide groups that self-hydrolyze.[36−38] Non-maleimide strategies have shown that conjugation through bromoactemide[30] or a variety of sulfone derivatives[53,54] can be used to produce thiol conjugates that have long-term stability in serum or plasma. These approaches are promising but might be limited to therapeutic indications that are less cost-sensitive than antibiotics.

Finally, the bactericidal activity observed with **ASU008** coupled with its low in vitro toxicity suggests that this construct is a promising anti-*Staphylococcal* candidate for future optimization. The linear substitution approach[55] using D-amino acids could be used to identify positions in the inhibitor peptide that increase antibacterial activity and protease stability without increasing toxicity. Variant synbodies could then be prepared via conjugation to quickly produce a library of optimized candidates for evaluation. This systematic approach to new antibiotic development should meet the call for platform technologies for antibiotic development[2] so desperately needed in the face of rising antibiotic resistance.

EXPERIMENTAL PROCEDURES

Chemicals. HPLC grade acetonitrile (ACN), Mueller Hinton broth (MHB), and MH agar were purchased from Thermo Fisher Scientific. Trifluoroacetic acid (TFA) was purchased from Apptec (Louisville, Kentucky). Oxacillin was purchased from Sigma-Aldrich (St. Louis, Missouri).

Bacteria Strains and Growth Conditions. The MSSA strain has been described previously,[39] while the following reagents were obtained through BEI Resources, NIAID, NIH: *Staphylococcus aureus*, Strain F003/HI168, NR-30548. We measured the MIC of this USA300 MRSA strain using the Broth microdilution method[56] for oxacillin (2 μg/mL), ciprofloxacin (0.5 μg/mL), and vancomycin (2 μg/mL). *Pseudomonas aeruginosa* PAO1 and *Staphylococcus epidermidis* (ATCC-29886) were both obtained from ATCC. Bacteria were maintained on Mueller Hinton (MH) Agar plates (Sigma-Aldrich #70191). Working colony plates were prepared by streaking MH agar plates with bacteria from −80 °C glycerol stocks followed by overnight incubation at 37 °C. Single colonies were isolated from working plates for further use.

Synbody Conjugation and Hydrolysis. Peptide **Ly**, [Ac]-rWrrHkHFkrPHrkHkrGSC-[NH_2], and the bivalent maleimide scaffold, **Sc0**, were synthesized by Sigma Custom Peptide and purified to >95% purity. To produce **ASU007**, 2.2 equiv of **Ly** was dissolved in 1× PBS pH 7.0 to a final concentration of 4.4 mM. One equivalent of **Sc0** was dissolved in 1× PBS pH 7.0 to a final concentration of 10 mM. The two solutions were then mixed and incubated overnight at room temperature with shaking. The reaction progress was monitored by MALDI-MS and the sample was purified by preparative HPLC (Agilent 1260). The purification used a Phenomenex Prepex C8 column (250 × 21.2 mm) with a 20 mL/min flow-rate using a gradient from 10% to 50% B in 20 min. The A solvent was water with 0.2% TFA, while the B solvent was ACN with 0.2% TFA. The same procedure was used to prepare **ASU008**; however, after overnight incubation, the pH was increased to ∼8 and the hydrolysis reaction was monitored by MALDI-MS. Upon completion, as evident by the +36 Da increase in molecular weight of **ASU007**, the reaction mixture was purified as before. Analytical HPLC of the final products was performed with an Agilent 1200 HPLC on a Phenomenex Luna C8 column (250 mm × 4.6 mm) using a 50 min gradient from 15% to 50% B with sample detection via Abs_{280}. Electrospray mass spectrometry (microTOFq, Bruker Daltonics) was used to confirm the molecular weight of the purified products.

Determination of MICs and Killing Kinetics. The broth microdilution method[56] was used to determine the MICs for all bacteria reported. Test compounds were prepared in MHB and were added to bacterial suspensions of ∼10^6 CFU/mL in 96-well polypropylene plates. Plates were incubated with shaking for 18 h at 37 °C and the OD_{600} was measured using a SpectraMax M5 microplate reader (Molecular Devices). Visible growth was defined as less than 10% growth relative to the untreated control.

The time kill experiments were conducted using MSSA. After overnight culture in MHB, bacteria were passaged for 2 h at 37 °C and 250 rpm to ensure bacteria were in exponential growth phase. MSSA was then diluted in MHB to an OD_{600} = 0.05 and added to a 96-well polypropylene plate. The synbodies were added to the plate for final concentrations of 50 μM, 25 μM, 12.5 μM, and 6.25 μM. The plate was incubated for the indicated times at 37 °C with shaking and aliquots were

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removed at 1, 2, 4, 6, and 24 h. Bacteria were plated on MH agar plates and incubated at 37 °C overnight. Each experiment was performed in duplicate.

Hemolytic Assay. We adapted our previously published hemolytic assay[39] for use with **ASU007** and **ASU008**. Briefly, blood from female BALB/C mice was collected, centrifuged at 300 g for 5 min, the sera was discarded, red blood cells were rinsed with 1×PBS (v/v − RBC: PBS), and centrifuged at 900 *g* for 15 min. These animal experiments followed an animal use protocol (1099R) that was reviewed and approved by the Arizona State University Institutional Animal Care and Use Committee. The 1×PBS rinse process was repeated three times. The erythrocytes were diluted to 4% in 1× PBS and 50 μL were dispensed into triplicate wells of a 96-well plate. Synbody samples (50 μL) or positive controls were added to each well and the plate was allowed to incubate at 37 °C for 1 h. The plate was centrifuged at 1000 *g* for 5 min and 90 μL of the supernatant from each well was removed and dispensed into a new 96 well titer plate. The absorbance at 414 nm was measured on a Spectramax 190 Plate Reader. The percent hemolysis was calculated using the following formula: percent hemolysis = [Avg. Sample Absorbance/Avg. Control Absorbance] × 100. Hemolysis at 100% was determined in water. Assays were performed in duplicate.

Serum Stability Assay − Pull-Down. Synbody samples were incubated in 1.2 mL of fresh mouse serum (collected from FVBN mice under animal use protocol 13−1287R that was reviewed and approved by the Arizona State University Institutional Animal Care and Use Committee) for the following time points: 0, 15, 30, 60, 120, 240, or 360 min. At each time point, 72 μL of sample was removed and 8 μL of 10× protease inhibitor (Roche Complete, Roche) was added to the aliquot to stop protease activity. Samples were then stored at −20 °C until use. For the pull-down portion of the assay, 30 μL of either M-280 or MyOne C1 (Life Technologies) streptavidin coated beads were added to low-bind microcentrifuge tubes, washed with 1× PBST for 3 times, and then blocked with 1.5 mL of blocking buffer (Superblock, ThermoPierce) overnight at 4 °C with shaking. The following day, blocking buffer was removed and beads were blocked again for 2 h 4 °C with shaking. The beads were then washed three times with 1× PBST and suspended in 30 μL of 1× PBST. Each serum time point sample was diluted to 1 mL with 1× PBST and added to the 30 μL of streptavidin bead slurry. Samples were incubated for 30 min at 4 °C with shaking, beads were washed three times with 1 mL of 1× PBST, and 3 times with 1 mL of 0.5 M citric acid. Beads were then suspended in 40 μL of 1× LDS loading buffer and stored at 4 °C. Samples were analyzed by Western Blot using 1:5000 dilution of Streptavidin AlexaFluor-555 (Life Technologies). Western blots were imaged on a Typhoon (GE Healthcare) and band densities were quantified using ImageJ (NIH).

Serum Stability Activity Assay. Synbody samples were incubated in 1.2 mL of fresh mouse serum for the same time points as before and at each time, 72 μL of sample was removed and 8 μL of 10× protease inhibitor (Roche Complete, Roche) was added to the aliquot to stop protease activity. Samples were then stored at −20 °C until use. After overnight culture in MHB, MSSA was passaged for 2 h at 37 °C and 250 rpm. Bacteria were then diluted in MHB to an OD_{600} = 0.05 and 50 μL per well were added to a 96-well polypropylene plate. We then dispensed 50 μL of each synbody time point in triplicate wells into a 96-well round-bottom plate (Costar, 3879). The 96-well plate was incubated overnight at 37 °C and samples were plated on MH agar plates. After overnight incubation at 37 °C, colonies were counted. Each experiment was performed in duplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00420.

Additional figures illustrating the characterization of **ASU007** and **ASU008**, full Western Blots of protease stability experiments, and bacterial counts of protease stability experiment (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors declare the following competing financial interests: S.A.J. and C.W.D. have a pending patent application for the synbodies described in this manuscript..

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