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Expedient Synthesis of SMAMPs via Click Chemistry

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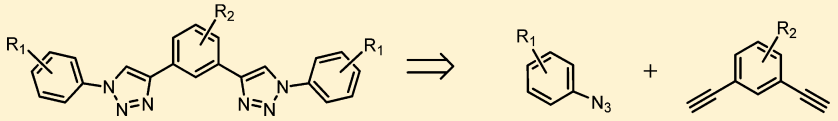
Supporting Information



ABSTRACT: A novel series of synthetic mimics of antimicrobial peptides (SMAMPs) containing triazole linkers were assembled using click chemistry. While only moderately active in buffer alone, an increase in antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* was observed when these SMAMPs were administered in the presence of mouse serum. One compound had minimum inhibitory concentrations (MICs) of 0.39 μg/mL and 6.25 μg/mL, respectively, and an HC_{50} of 693 μg/mL. These values compared favorably to peptide-based antimicrobials. A correlation between the net positive charge and SMAMP antimicrobial activity was observed. The triazole linker, an amide surrogate, was found to provide better antimicrobial activity against both *S. aureus* and *E. coli* when compared to other analogues.

KEYWORDS: *Synthetic mimics of antimicrobial peptides, click chemistry, antibiotics, Gram-positive and Gram-negative bacteria, 1,2,3-triazoles*

Infections caused by bacteria are an increasing threat to human safety and well-being. Every year a large number of deaths are caused by bacterial infections.[1] Traditional antibiotics used to treat these bacterial infections, such as β-lactams, are becoming ineffective as these bacteria develop resistance to them. While bacteria are developing resistance at an alarming rate, the development of new antibiotics has slowed considerably, with many pharmaceutical companies abandoning their antibiotic research, resulting in fewer and fewer antibiotics approved by the FDA (Food and Drug Administration).[2] Antimicrobial peptides (AMPs) represent a class of antibiotics currently being investigated as an alternative to traditional antibiotics.[3] They are peptides that show broad-spectrum antimicrobial activity against various pathogens. Unlike traditional antibiotics, AMPs do not target bacterial enzymes, which can mutate easily. Although the exact mechanisms of how AMPs operate are not completely clear, their ability to permeabilize the bacterial cell membrane[4−7] has been identified along with several other targets.[8,9]

Since their initial discovery, hundreds of AMPs have been catalogued by scientists around the world, and web-based databases have been established to keep track of the vast number of AMPs.[10,11] Despite this, there has been little success of developing AMPs into FDA-approved drugs. One of the very few examples is pexiganan, also known as MSI-78, which reached phase III clinical trials but was not approved by the FDA due to the fact that it was no more effective than existing treatments.[12] A major difficulty in AMP drug development is production.[13] AMPs are typically isolated from nature in minute amounts. Peptide synthesis methodologies are too expensive for manufacturing AMPs on the scale necessary for clinical testing. Using genetic engineering to produce AMPs in yeast or bacteria has proven to be ineffective due to the toxicity of AMPs toward their expression host.

These disadvantages of AMPs thus motivated the development of synthetic mimics of antimicrobial peptides (SMAMPs), synthetic molecules designed to capture the active structural features of AMPs, such as amphiphilicity. SMAMPs can exhibit improved antimicrobial activity and are easier to synthesize than AMPs due to their simplified structure. Examples of SMAMPs include peptoids,[14] β-peptides,[15−17] short linear[18,19] and cyclic peptides,[20] synthetic polymers,[21−24] oligo-acyl lysines,[25,26] ceragenins,[27] and aromatic oligomers.[28−33] In our research group, we previously synthesized SMAMPs possessing triaryl scaffolds linked with urea,[29] amide,[30] and acetylene[34] moieties. Of the three, SMAMPs with amide linkers proved to be the most active, and one of these has completed a phase II clinical trial for pan-Staph infections in humans. 1,2,3-Triazoles are commonly used as bioisosteres of amides[35,36] in medicinal chemistry, and replacing the amide functionality with a triazole can lead to improved activities.[37−39] Therefore, it seemed reasonable to determine if SMAMPs containing triazole linker would be more active as well. Additionally, 1,2,3-triazoles are formed via click chemistry, which is highly reliable, regiospecific, and functional group tolerant. To the best of our knowledge, the use of click chemistry to create a SMAMP scaffold has not been demonstrated before. Hence, we set out

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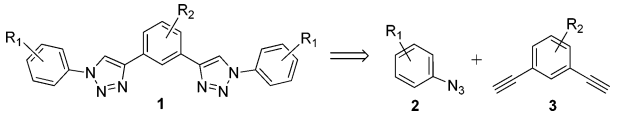
to synthesize SMAMPs of the general structure **1** (Scheme 1) and characterize their antimicrobial properties.

Scheme 1. General Synthetic Design of SMAMPs Containing Triazoles Utilizing Click Chemistry



Previous studies showed that the central aromatic core and charge density were important parameters in optimizing SMAMP activity.[33] As a result, three groups of SMAMPs were targeted in this endeavor. The first group (SMAMPs **4**–**5**) consists of SMAMPs with a central phenyl ring flanked by two phenyl groups and a net positive charge of two or four (Figure 1). The second group (SMAMPs **6**–**10**) consists of SMAMPs

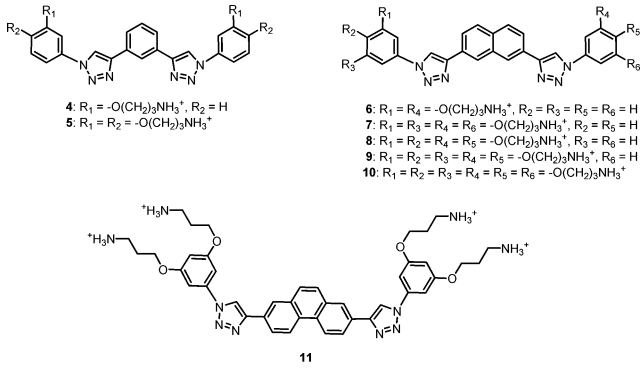


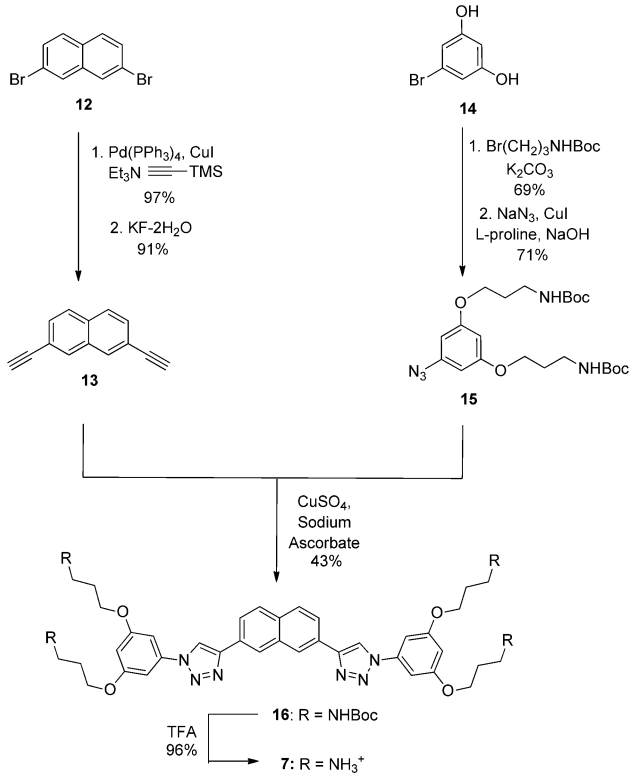
Figure 1. Three groups of SMAMPs investigated for their antimicrobial activity.

with a central naphthyl ring flanked by two phenyl groups and a net positive charge ranging from two to six. The third group consists of SMAMP **11**, with a central phenanthryl ring flanked by two phenyl groups and a net positive charge of four.

These SMAMPs were synthesized by reacting two equivalents of azide with one equivalent of bis-alkyne. A representative procedure for the synthesis of SMAMP **7** is depicted in Scheme 2. Starting with dibromide **12**, a Sonogashira reaction[40] with trimethylsilylacetylene gave the bis-trimethylsilylalkyne intermediate in excellent yields. Removal of the trimethylsilyl groups with KF furnished bis-alkyne **13**. The azide fragment was prepared by the alkylation of diphenol **14** with $Br(CH_2)_3NHBoc$, followed by the conversion of the intermediate bromide to azide **15** via procedures developed by Zhu and Ma.[41] Standard 1,3-dipolar cycloaddition conditions developed by Sharpless et al.,[42] using $CuSO_4$ as the copper source and sodium ascorbate as the reductant, gave **16** in 43% yield. The remainder of the mass balance consisted of the product in which bis-alkyne **13** reacted with only one equivalent of azide **15** and additional heating to 50 °C was required for **13** to react completely with two equivalents of **15**. This difference in reactivity of the two alkyne moieties enabled the synthesis of asymmetrical SMAMPs such as **9**. Finally, deprotection of **16** with trifluoroacetic acid (TFA) provided SMAMP **7** as its TFA salt. Further details for the synthesis of all SMAMPs can be found in the Supporting Information.

SMAMPs **4**–**11** were tested against five different bacteria strains: Gram-positive *Staphylococcus aureus* (ATCC 27660)

Scheme 2. Synthesis of SMAMP 7 via Click Chemistry



and *Enterococcus faecalis* (ATCC 29212), and Gram-negative *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 10145), and *Klebsiella pneumoniae* (ATCC 13883). Their antimicrobial activity was expressed in terms of minimum inhibitory concentration (MIC). These values were determined according to Hancock's method for cationic antimicrobial peptides, which is a slight modification of the classical microbroth dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI), and they were measured either in the absence or presence of 40% mouse serum.[43,44] The upper limit of mouse serum was 40%, as the bacteria had difficulty growing when the percentage was increased further. The results are shown in Table 1. SMAMPs **4**–**11** possessed a variable spectrum of activity, generally showing lower MIC values toward *S. aureus* and *E. coli* than *E. faecalis*, *P. aeruginosa*, and *K. pneumoniae*. Interestingly, SMAMP **10** showed modest MIC values against *P. aeruginosa*, which is a difficult bacteria to kill.

The hemolytic activity of SMAMPs **4**–**11** toward mammalian cells was also evaluated, where the ability to induce lysis in human erythrocytes was measured as an HC_{50} value, the lowest concentration that causes 50% lysis of the red blood cells. Surprisingly, SMAMPs **4**–**11** all displayed higher antimicrobial activity (lower MIC values) toward *S. aureus* in the presence of 40% mouse serum. This serum effect was less pronounced toward *E. coli* but was still observed for the majority of SMAMPs. This result was unexpected because it is well-known that antibiotics are generally less effective when administered in the presence of serum, as the binding of antibiotics to serum proteins usually deactivates the antibiotic.[45] Svenson et al. had previously studied the binding of cationic peptides to albumin, and the results indicated that the binding of peptides to albumin lowers the effective concentration of peptides needed to combat bacteria.[46] Hence, the MIC values of the peptides

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Table 1. Antimicrobial Activities with and without Mouse Serum and Hemolysis of the SMAMPs

	MIC (μg/mL)							
SMAMP	SA[a]	SA+40%MS[b]	EC[c]	EC+40%MS[d]	EF[e]	PA[f]	KP[g]	HC50 (μg/mL)
4	50	12.5	25	25	25	25	>50	Nd[h]
5	12.5	0.39	>50	6.25	125.5	50	>50	>1000
6	>50	12.5	>50	>50	50	>50	>50	Nd[h]
7	12.5	0.39	>50	6.25	25	>50	50	693
8	25	0.19	>50	3.13	25	50	50	81
9	12.5	0.39	50	25	25	50	>50	94
10	12.5	0.19	25	25	50	12.5	>50	828
11	25	0.39	>50	12.5	50	25	>50	109
pexiganan[i]	4	>256	8	128				120

[a]*S. aureus* (ATCC 27660). [b]*S. aureus* (ATCC 27660) in the presence of 40% mouse serum. [c]*E. coli* (ATCC 25922). [d]*E. coli* (ATCC 25922) in the presence of 40% mouse serum. [e]*E. faecalis* (ATCC 29212). [f]*P. aeruginosa* (ATCC 10145). [g]*K. pneumoniae* (ATCC 13883). [h]Not determined due to low antimicrobial activity. [i]MIC and HC_{50} of pexiganan were reported previously: Ge et al. *Antimicrob. Agents Chemother.* **1999**, *43*, 782−788.

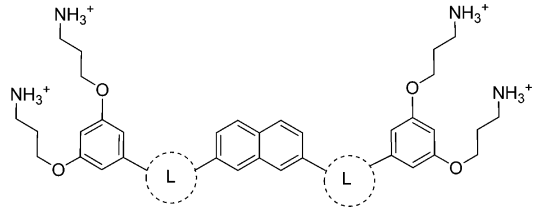
increased in the presence of albumin. There are only a limited number of examples[47−49] in which an increase in antimicrobial activity was observed when an antibiotic was administered in the presence of serum. The exact reasons for this phenomena are not completely clear. It has been previously suggested that changes in the pH has lowered the MIC.[50] In addition, the presence of other antibacterial peptides[51] in serum or unidentified synergistic host factors[52] could contribute to the observed lower MIC. Another explanation is that serum increases the solubility or internalization of the antibiotic.

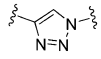
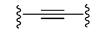
Several trends were observed in the antimicrobial activity in the presence of mouse serum and hemolysis of SMAMPs **4**−**11**. Regardless of the size of the central ring in SMAMPs **4**−**11**, the antimicrobial activity increased dramatically when the net positive charge was increased from two (SMAMPs **4** and **6**) to four (SMAMPs **5** and **7**). Further increases in net positive charge to five or six did not seem to impact the antimicrobial activity significantly. The size of the aromatic core appeared to have a proportional effect on hemolytic activity but not on MIC values. When comparing SMAMPs **5** and **8**, both with a net positive of four, there was approximately a 12-fold increase in hemolytic activity with the addition of one phenyl moiety in the aromatic core. The hemolytic activity also varied with the substitution pattern as the *ortho*-substituted SMAMP **8** had a much higher hemolytic activity than its *meta*-substituted counterpart, SMAMP **7**. The effect of charge and hydrophobicity had been previously described by Strøm et al. in the context of short cationic peptides.[53] These peptides required a minimal charge of +2 for antistaphylococcal activity, whereas our SMAMPs **4**−**11** required a minimal charge of +4. The presence of a larger hydrophobic group gave lower MIC values in Strøm's peptides; however in this report increasing the size of the central aromatic ring did not have a significant effect on MIC values.

Out of all of these SMAMPs, **5**, **7**, and **10** were among the most potent and showed the highest selectivity even compared with pexiganan, the most thoroughly studied AMP to date. For example, SMAMP **5** is much more potent than pexiganin and is approximately 10 times less hemolytic giving selectivity values (HC_{50}/MIC) of >2500 and >160 for *S. aureus* and *E. coli*, respectively. When **5**, **7**, and **10** were compared to various SMAMPs in the literature, it was found that **5**, **7**, and **10** possessed better antimicrobial activity against *S. aureus* than most peptoids, β-peptides, short linear and cyclic peptides, synthetic polymers, and oligo-acyl lysines, which typically possessed MIC values of 2−15 μg/mL.

As an effort to showcase the importance of the 1,2,3-triazole linker used in this study, the activity of SMAMP **7** was compared to analogues previously synthesized in our laboratory (Table 2, SMAMPs **17** and **18**). When the 1,2,3-triazole linker

Table 2. Effect of the Linker Moiety on the Antimicrobial and Hemolytic Activities of SMAMPs



SMAMP	L	MIC(μg/mL)		HC_{50} (μg/mL)
		SA+40%MS[a]	*EC+40%MS*[b]	
7		0.39	6.25	693
17	None	1.56-3.13	25	194
18		3.13	12.5	3.4

[a]*S. aureus* (ATCC 27660) in the presence of 40% mouse serum. [b]*E. coli* (ATCC 25922) in the presence of 40% mouse serum.

was removed from the scaffold (SMAMP **17**[33]), there was a 4−8-fold decrease in antimicrobial activity toward *S. aureus* and a 4-fold decrease toward *E. coli* in the presence of mouse serum. Additionally, the hemolytic activity was approximately four times higher. When the 1,2,3-triazole linker was replaced with an acetylene moiety (SMAMP **18**), again a significant decrease in antimicrobial activity toward *S. aureus* in the presence of mouse serum was observed as well as a lower HC_{50} value. Overall, the presence of the 1,2,3-triazole linker enabled SMAMP **7** to outperform analogues with other linkers in terms of both antimicrobial and hemolytic activities.

In conclusion, the first example of utilizing click chemistry to generate a SMAMP scaffold was reported. The size of the aromatic core and the number and substitution pattern of the ammonium groups were varied; the effects of these variables on SMAMP antimicrobial and hemolytic activities were measured. All SMAMPs had higher antimicrobial activities when

administered in the presence of mouse serum and exhibited higher antimicrobial activity toward *S. aureus* over *E. coli*. SMAMPs **5**, **7**, and **10** possessed comparable antimicrobial activities toward *S. aureus* as the most potent SMAMPs reported in the literature while maintaining lower hemolytic activity. The importance of the linker was also investigated by comparing **7** to **17**−**18**, and a pronounced difference in antimicrobial and hemolytic activities was observed, thus validating the use of the 1,2,3-triazole moiety. As a result of this study, we have identified three SMAMPs, **5**, **7**, and **10**, that outperformed pexiganan, and they are currently being investigated for use as new antibiotics.

■ ASSOCIATED CONTENT

Supporting Information

Procedure for the synthesis of **7**, characterization data for **4**−**11**, and procedures for determining antimicrobial and hemolytic activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

T-h.F. and Y.L. contributed equally.

Notes

The authors declare no competing financial interest.

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