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# Synthesis and Antibacterial Activity of Trivalent Ultrashort Arg-Trp-based Antimicrobial Peptides (AMPs)

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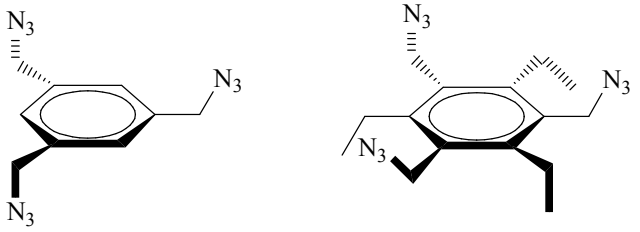
Multivalent display of identical ultrashort (only 2-3 amino acids long) antimicrobial peptides (AMPs) was used in order to create potential new antimicrobial agents. A series of small synthetic arginine and tryptophan containing peptides was synthesized and covalently bound to two different trivalent scaffold molecules using the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction. The effect of steric preorganization of AMPs on the antibacterial activity was studied using an 1,3,5-tris(azidomethyl)benzene and an 1,3,5-tris(azidomethyl)-2,4,6-triethylbenzene substituted scaffold. The comparison of these two scaffolds showed that preorganisation leads to twice as active compounds. We further more obtained a synergistic effect and could show, that the presence of a certain number of amino acids in a close proximity is more important than their relative spatial orientation.

## Introduction

Microorganisms, especially bacteria, are known for their enormous ability to adapt to their environment: resistant strains appear rapidly under antibiotic pressure.[1] Because of this, antibiotics have a limited lifespan of utility, especially in the hospital environment.[2] One way to delay this rapid occurrence of resistance is to develop antibiotics that are less prone to suffer from the development of resistance by bacterial strains. Considering these aspects, host-defense and antimicrobial peptides (AMPs) which target the bacterial membrane specifically have been proclaimed as potential new antibacterial agents.[3] AMPs are present in virtually all classes of higher organisms like plants, insects, and animals and fend off a wide range of different pathogens.[4] Naturally occuring AMPs are typically amphipathic peptides with a positive net charge and approximately 50% hydrophobic residues and consisting of 12 up to 50 amino acids.[3,5] A major disadvantage of natural AMPs is their relatively large size, which leads to high production or isolation cost[6] as well as inactivation by proteolytic degradation.[7] Because of this, synthetic antimicrobial peptides (synAMPs), have attracted considerable attention, since chemists can modify the peptides in many ways. The broad range of resulting compounds includes peptides which contain D-amino acids,[9] lipidated peptides[10] and metal-complexes.[8] Among synAMPs, those based on arginine (Arg or R) and tryptophan (Trp or W) are of special interest as these peptides are among the smallest peptides which show significant antibacterial activity.[3,11] It was shown, that different modifications of this sequence can tune the antibacterial activity of these peptides. For example the addition of metallocene substituents to the N-terminus of the peptide[8,12,13] or lipidation of a C- or N-terminal lysine residue can increase the activity.[10] We have shown that an L- to D-substitution scan can lead to peptides with significantly reduced hemolytic activity.[14] Alternative approaches to increase activity of AMPs are peptide dendrimers;[15] Otherwise inactive ultrashort lipidated peptides could be turned into antifungal constructs by a trivalent presentation.[16] For this trifold display three ultrashort peptides were attached to a scaffold molecule that had three amino-groups. Other examples have emerged recently, and it has been observed in several instances that multivalent representation can increase the activity for different AMPs.[15-18] In most of these studies, however, the effect of preorganization on the activity has not been assessed. This is rather unfortunate since the anticipated target of many AMPs, and proven target for these short Arg-Trp based AMPs, is the bacterial membrane. Having the AMPs on one side of a scaffold molecule should result in enhanced activities.

For this study of preorganized presentation of three ultrashort synAMPs on the activity of the constructs, we chose two 1,3,5-tri(azidomethyl)benzene scaffolds for copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC): one with only hydrogen-atoms on the 2,4,6 positions (**a**) and one with ethyl-groups (**b**) (Fig 1).




a b

**Fig. 1** Structure of the two 1,3,5-tris(azidomethyl)benzene scaffolds.

By using these two seemingly very similar scaffolds with three-fold symmetry, we want to address the effect of preorganisation of AMPs towards a bacterial membrane on the antibacterial activity. As the crystal structure in Fig. 2 shows, all substituents occupy alternating positions, and thereby the three functional substituents point in the same direction in scaffold **b**, while this is likely not the case in the less sterically demanding derivative **a**.

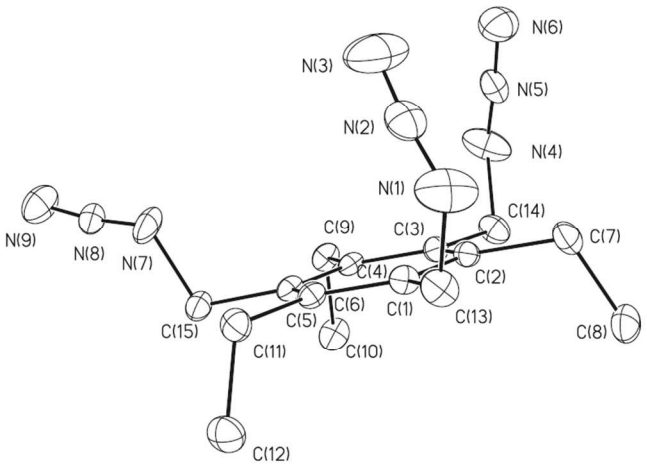



**Fig. 2** ORTEP plot of scaffold **b**. Thermal allipsoids are shown at 20% probability, and hydrogen atoms are omitted for clarity. Further details can be found in the ESI, and were deposited in the CCDC no. 1025189.

Although the degree of preorganization of alternating substitutes on one side of the hexasubstituted ring will eventually depend on several factors – an important one being the size of the attached groups[19] several successful applications of this principle are known.[20-22] In any case, the least thing we can expect is that the hexasubstituted benzene-ring is less flexible than the trisubstituted version. Thus, we would be able to assess the effect of flexibility of our constructs on the antibacterial activity.

## Results and discussion

### Synthesis of short modified RW-peptides

For the conjugation of the synAMPs to the azide-containing benzene cores, the peptides had to be functionalized with an acetylene moiety. As shown before, these peptides are most active when a free N-terminus is present,[3] therefore we introduced the acetylene moiety at the C-terminus (scheme 2). Although resin-based methods for the direct intoduction of C-terminal acetylene moieties are known,[23-25] we pursued two procedures in which propargylamine was attached to a protected amino acid at the beginning of the synthesis sequence, or as the last step to amidate a protected peptide acid. Using either solution-phase peptide chemistry or a solid-phase resin with a high loading sufficiently large quantities of the acetylene-functionalized peptides could be obtained.

First, N-terminally and side chain-protected arginine and tryptophan residues were coupled to 2-propargylamine using 1 equiv. isobutylchloroformate (ICF) and 1 equiv. *N*-methylmorpholine (NMM) in tetrahydrofuran (THF) (Yields: 47 - 54%). After Fmoc-deprotection (20% $Et_2NH$ in dichloromethan (DCM)), alkyne-functionalized amino acids **1** and **2** were coupled to either Fmoc-Trp(Boc)-OH or Fmoc-Arg(Pbf)-OH. From these dipeptides, alkyne-functionalized peptides **5** and **6** were obtained after Fmoc-removal (Yields after purification: 2.2 - 45%).

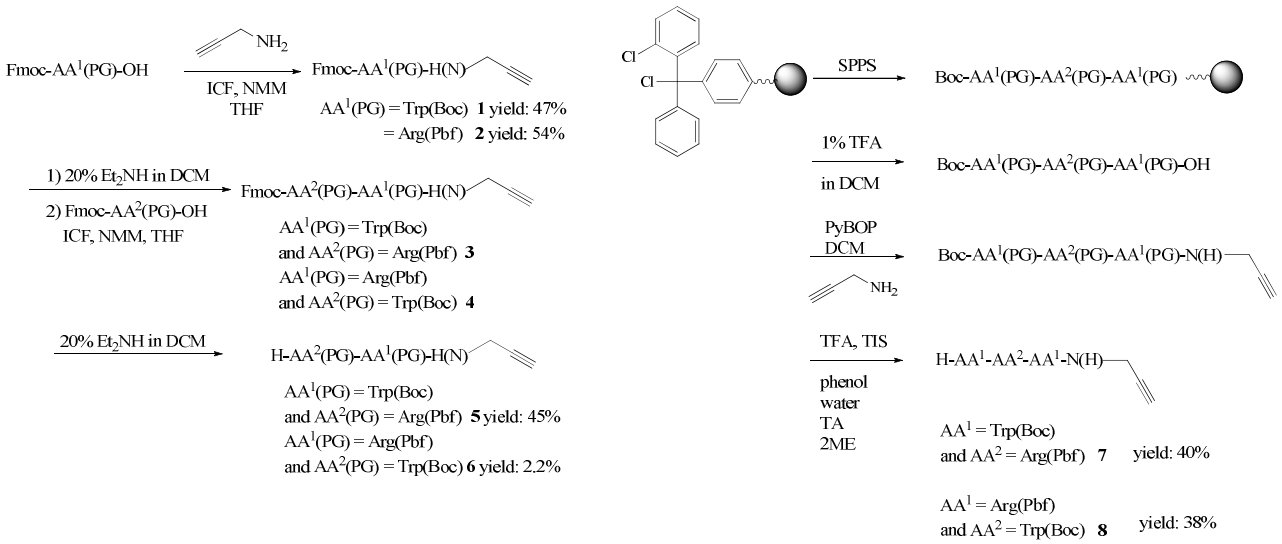
In view of the troublesome purification of dipeptides **5** and **6** a slightly different procedure was used for the synthesis of the acetylene-functionalized tripeptides. The peptides were synthesized by solid phase peptide synthesis (SPPS), using a tritylchloride resin that yields a C-terminal carboxylic acid after cleavage. The N-terminus was protected with a Boc-group, so that after cleavage the peptide could be coupled to propargylamine in solution using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as coupling reagent (scheme 2). Finally, complete deprotection was realized using a mixture of trifluoroacetic acid (TFA) (79%), triisopropylsilane (TIS) (1%), phenol (5%), water (5%), thioanisol (TA) (5%) and 2-mercaptoethanol (2ME) (5%) to yield peptides **7** and **8** (Yields after purification: 38 - 40%).

### Synthesis of trivalent di- and tripeptides

For the central scaffold molecule of the trivalent peptides two compounds with azido groups were selected: the flexible 1,3,5-tris(azidomethyl)benzene and the preorganized 1,3,5-tris(azidomethyl)-2,4,6-triethylbenzene (scheme 1).[14] In principle, these two compounds should allow us to investigate the effect of flexibility on the activity.

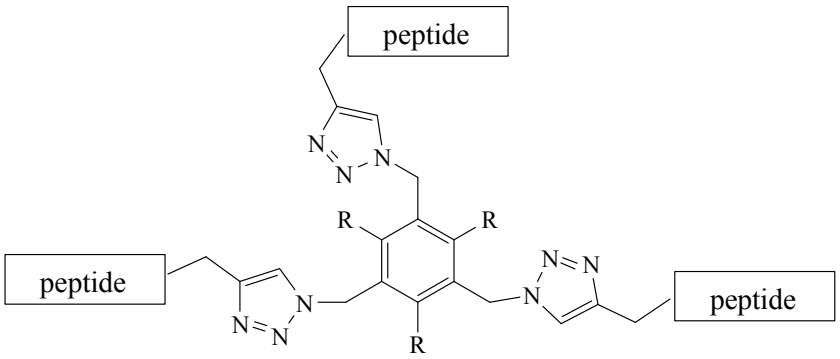
For the dipeptides **5** and **6**, side-chain protected peptides were used in the Cu(I) catalyzed alkyne-azide cycloaddition. The reaction was carried out in THF, to which CuI (0.3 eq.) and *N*,*N*-diisopropylethylamine (D*i*PEA) were added. The reaction mixture was stirred at room temperature for 1 day; when the color of the reaction mixture changed from colorless to intense blue, thus indicating that a significant amount of $Cu^{2+}$ had formed, an additional amount of CuI was added to ensure continuation of the reaction and the mixture was stirred at room temperature for an additional day. Afterwards, the mixture was concentrated, the residue dissolved in acetonitrile and water, and the products were purified by HPLC . Lastly the side-chain protection-groups were removed using a mixture of TFA (79%), TIS (1%), phenol (5%), water (5%), thioanisole (5%)

and 2-mercaptoethanol (5%). This resulted in pure deprotected peptides **9a**, **9b**, **10a**, and **10b**, which were suitable for testing against bacteria (Yields: 54 - 97%).




**Scheme 1** Synthesis of acetylene-functionalized peptides **5**-**8**.

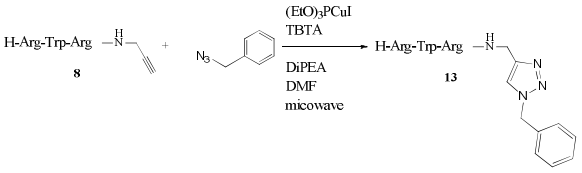
For the tripeptides slightly different reaction conditions needed to be used. Using the protected tripeptides the desired products could not be obtained under conditions such as $CuSO_4$/Na-acetate/acetonitril or CuI/D*i*PEA/THF at room temperature or under reflux or $(EtO)_3PCuI$/D*i*PEA in dimethylformamide (DMF). However using deprotected tripeptides and $(EtO)_3PCuI$/D*i*PEA in DMF the desired products could eventually be obtained, albeit only after prolonged heating (6 hours) at 60 °C using a microwave. To prevent oxidation or disproportionation of copper(I) tris((1-benzyl-1*H*-1,2,3-triazolyl)methyl)amine (TBTA)[26] was added as stabilizer. During this time, additional Cu(I) catalyst and TBTA were added after 4.5 hrs, and the peptides (4 eq. in total) were added in three steps, i.e. at $t$ = 0 min (2 eq.), $t$ = 3 h (1 eq.) and $t$ = 4.5 h (1 eq.) to force the formation of the trivalent construct to completion. After the microwave treatment the mixture was concentrated and the products were purified by preparative HPLC (Yields: 25 – 36%).




| sequence: | R = H | R = Et |
|---|---|---|
| Arg Trp | **9a** | **9b** |
| Trp Arg | **10a** | **10b** |
| Arg Trp Arg | **11a** | **11b** |
| Trp Arg Trp | **12a** | **12b** |

**Fig. 3:** Structure of the trivalent peptide molecules **9**-**12**. Amino acids are denoted in three letter code.

To investigate the hypothesis that trivalent constructs of AMPs were more active than monovalent molecules, one of the alkyne-functionalized tripeptides was also clicked to azidomethylbenzene as a control compound. The reaction was carried out in the microwave under the same reaction conditions as used for the trivalent scaffold, which leads to compund **13** with a yield of 54% after purification (scheme 4).




**Scheme 4** Synthesis of monovalent compound **13**.

**Table 1** Minimal inhibitory concentrations (μM) of the trivalent peptide compounds **9, 10, 11,** and **12** and the monovalent peptide compound **13**. Also MIC-values for the H-$(RW)_3$-$NH_2$ sequence that we studied earlier are shown (these were obtained using identical conditions); 'n.a.' means 'not active'(MIC > 100 μM). For the calculations of the MIC values in μM, molecular weights of the peptides together with one TFA-counterion for each basic amino acid residue were used.

| | compound | sequence | MIC (μM) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Gram-negative | | Gram-positive | | |
| | | | *E. coli DSM30083* | *A. baumannii DSM 30007* | *B. subtilis 168* | *S. aureus DSM 20231* | *S. aureus (MRSA) ATCC 43300* |
| 1 | **9a** | RW | 60 | 60 | 7.6 | 30 | 15 |
| 2 | **9b** | RW | 15 | 15 | 2.2 | 15 | 7.3 |
| 3 | **10a** | WR | 60 | 30 | 3.8 | 30 | 15 |
| 4 | **10b** | WR | 60 | 15 | 1.8 | 7.3 | 7.3 |
| 5 | **11a** | WRW | 27-13 | 54 | 13-6 | 27 | 13 |
| 6 | **11b** | WRW | 26-13 | 26 | 6-3 | 13 | 13 |
| 7 | **12a** | RWR | 24-12 | n.a. | 6-3 | n.a. | 24-12 |
| 8 | **12b** | RWR | 16 | 32 | 1 | 4 | 2 |
| 9 | **13** | RWR | n.a. | n.a. | 35 | n.a. | 70 |
| 10[8] | | $(RW)_3$ | 21 | 21 | 1.3 | 11 | 5.3 |
| 11 | Ciprofloxacin | | 1.5 | 24 | 24 | 12 | 12 |

**Antibacterial activity**

The antibacterial activity of the clicked compounds was assessed by determining their minimal inhibitory concentration (MIC) in a microdilution assay as described previously[10]. This value represents the lowest concentration of compound that is needed to inhibit growth of the bacteria.[27] For this, two Gram-negative and three Gram-positive bacterial strains were used (Tabel 1). A molecule is usually designated as antimicrobial, if the MIC value is in the low micromolar range. In general, the investigated compunds are more active against Gram-positive than against Gram-negative bacteria, although in four cases, **9b**, **11a**, **11b**, and **12a** the difference in activity against Gram-positive and Gram-negative are negligible. Since the monovalent compound **13** shows no activity against gram-negative bacteria, and is poorly active against Gram-positive bacteria, it can be concluded, that trivalency increases the antibacterial activity significantly. Against Gram-positive bacteria all compounds show good to high activity. Especially compound **10b** has MIC values (7.3–1.8 μM) that are as good as the linear hexapeptide H-RWRWRW-$NH_2$ $(RW)_3$ (5.3–1.3 μM)[8] which was the lead structure for the trivalent peptide conjugates. As it was shown before making the linear peptide longer than six amino acids does not necessarily lead to a better activity.[11] Therefore, no longer linear peptides were investigated herein. In fact, comparing the activities of all peptides that have six amino acid residues (entries 1-4, 10) we can conclude that the presence of six amino acids in a close proximitry is more important than their relative spatial orientation. Compairing the two different scaffolds used, the less flexible scaffold 1,3,5-tris(azidomethyl)-2,4,6-triethylbenzene leads to compounds that are at least twice as active as the more flexible 1,3,5-(trisazidomethyl)benzene-based constructs. It is also noteworthy that lipophilicity alone does not seem to be the one decisive factor for activity: For example, the more lipophilic compound **11a** is actually less active than the more hydrophilic one **12a**.

A non-linear effect could be observed comparing the monovalent compound **13** (entry 9) to the trivalent peptides on a scaffold (entries 7 and 8). Trivalency increases the activity 5-10 fold when trivalent compounds **12a** and **12b** are compared with monovalent control compound **13**.

## Conclusions

The synthesis of trivalent, preorganized RW containing AMPs by utilizing the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction is described. Eight different trivalent AMPs were synthesized in high purity and acceptable yields, using two different benzene scaffolds: one that is highly flexible and one that has reduced flexibility. Nine new compounds were tested for their antimicrobial activity. They were moderately active against gram-negative bacteria but some showed good activity against Gram-positive bacteria. The results of the MIC tests show, that both the total number of arginine and tryptophan residues and their relative spatial assembly are relevant parameters for activity. Importantly, we show that both of the trivalent scaffolds can be used to generate very active antibacterial conjugates that are based on otherwise inactive ultrashort peptides (only 2-3 amino acids long). The trivalent constructs based on the less flexible scaffold 1,3,5-trisazidomethyl-2,4,6-triethylbenzene showed a higher activity than the compounds consisting of the more flexible scaffold, at least against the Gram-positive bacteria. Together with the existing methods to modulate AMPs, these and other dendrimer scaffolds form a molecular toolkit that can be used to prepare AMP conjugates that are very active against bacteria, and may find clinical applications.

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## Notes and references

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† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/b000000x/

‡ Experimental procedures (preparation of new compounds) and characterizing data can be found in the Electronic Supplementary Information.

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