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Improving the biological activity of the antimicrobial peptide anoplin by membrane anchoring through a lipophilic amino acid derivative

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ABSTRACT

The lipophilic amino acid, (*S*)-2-aminoundecanoic acid, was synthesized and incorporated at a number of specific positions within the peptide sequence of anoplin. These lipophilic anoplin analogs showed to be more active against *Escherichia coli* and *Staphylococcus aureus* compared to native anoplin, while the EC_{50}-value of hemolysis was at least one order of magnitude lower than the MIC values. This was in sharp contrast to the N-acylated anoplin derivative, where a gain in activity also led to a complete loss of selectivity. Thus, the incorporation of a lipophilic amino acid residue into anoplin enhanced the antimicrobial activity, while selectivity towards microbial membranes was retained.

There is a growing need for the development of novel antibiotics, or for improving upon existing ones, as established antibiotic compounds continue to lose ground in the struggle against resistant bacteria. Of particular concern are the Methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecium* (VRE) infections that continue to rise. Clearly compounds active against these resistant classes are urgently required.[1]

Host-defensive antimicrobial peptides (AMPs), have considerable promise as novel antibacterial agents.[2] Their mode of action is (target-unspecific) permeabilization of bacterial membranes which induces little stable resistance, since it is very difficult for a bacterium to change its membrane composition in order to counteract the activity of AMPs. This might explain the great potential of AMPs as lead compounds for the development of the next generation peptide-based antibiotic drug molecules, like the FDA-approved lipopeptides Caspofungin **1**[3] and Daptomycin **2**,[4] which are active against fungal and bacterial infections, respectively (Fig. 1).

Lipopeptides form a subclass of antimicrobial peptides in which a lipophilic alkyl chain acts as a membrane anchor. The length of the alkyl chain is highly important for the bioactivity, since analogs with a truncated alkyl chain show a dramatic decrease in antimicrobial activity.[5,6] Acylation of the N-terminal α-amino functionality is a well-known approach to increase membrane affinity.[7–12] However, such N-acylation results in a non-charged amino terminus, while a positively charged N-terminus, in combination with a peptide sequence that is rich in arginine and lysine residues, is often important for activity and selectivity.[10] This is especially the case when the peptide is meant to interact with negatively charged bacterial membranes, and not with overall neutral mammalian membranes.

To increase the membrane affinity for membrane-acting antimicrobial peptides, without sacrificing these important positively charged backbone/side chain functionalities, a lipophilic amino acid derivative ((*S*)-2-aminoundecanoic acid) has been designed and synthesized that can be incorporated at any position of the peptide sequence. Herein, we report that the antimicrobial decapeptide anoplin, H-Gly^{1}-Leu^{2}-Leu^{3}-Lys^{4}-Arg^{5}-Ile^{6}-Lys^{7}-Thr^{8}-Leu^{9}-Leu^{10}-NH_2, was modified at residues Leu^{2}, Ile^{6}, and Leu^{10}, with the lipophilic amino acid residue, and that these anoplin derivatives were found to be ten times more active compared to native

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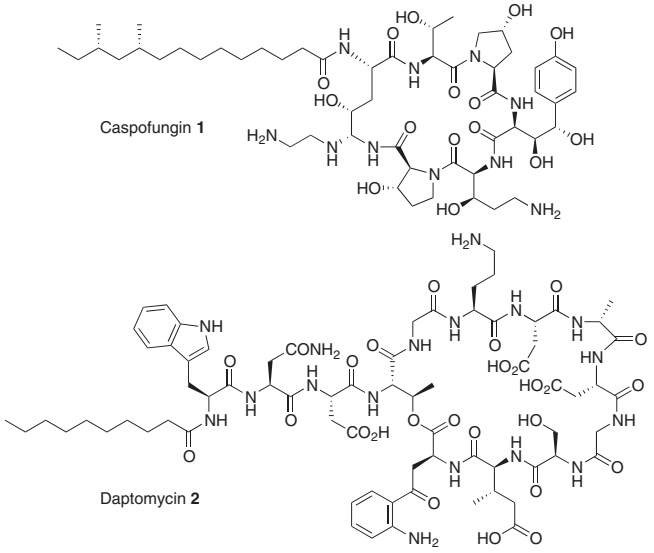


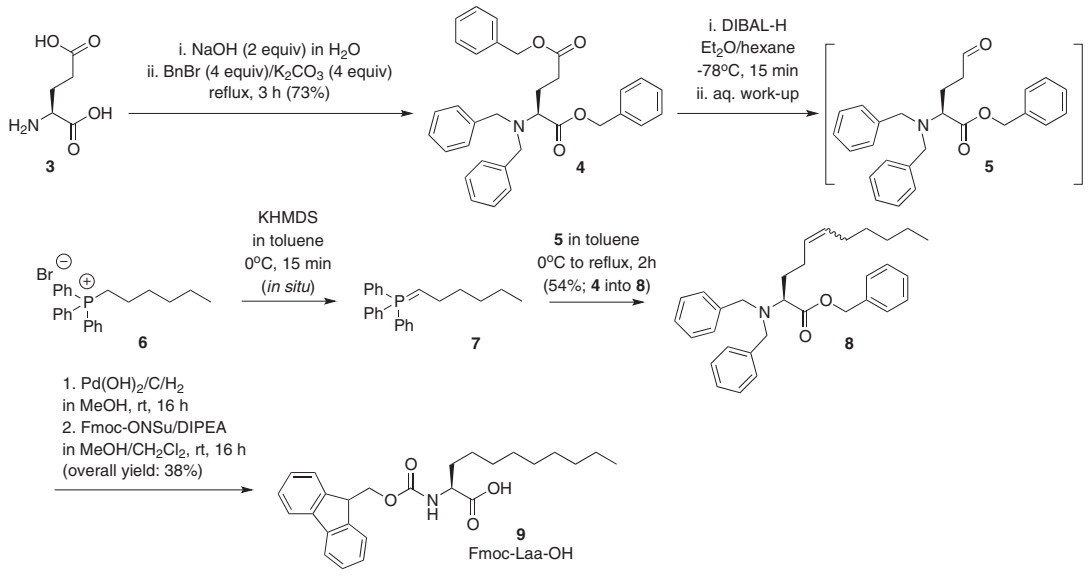
Figure 1. Structural formula of Caspofungin **1** and Daptomycin **2**.

anoplin, while their selectivity towards microbial membranes remained unaffected.

The lipophilic amino acid derivative (*S*)-2-aminoundecanoic acid was synthetically accessible from L-glutamic acid **3** in only four steps, as shown in Scheme 1.[13,14] To this end, **3** was treated with benzyl bromide under basic conditions[15] to obtain the *N*,*N*,*O*,*O*-perbenzylated Bzl-*N*(Bzl)Glu(OBzl)-OBzl derivative **4** in a good yield of 73%. Then, diester **4** was subjected to a controlled DIBAL-H mediated reduction, according to the method of Martín and co-workers,[16] to afford aldehyde **5**, which was used without further purification, in the subsequent Wittig reaction.[17] In a separate flask, hexyltriphenylphosphonium bromide **6** was treated with hexamethyldisilazane potassium salt (KHMDS) in toluene to generate in situ ylide **7** and after 15 min at 0 °C, aldehyde **5** was added, and the reaction mixture was heated at reflux temperature for 2 h to give alkene **8** in an overall yield of 54% after purification by column chromatography. In the original literature procedure,[16] the Wittig reaction was performed at −78 °C (and gradually raising the temperature to 0 °C), however, it turned out that for a complete conversion of the starting materials, reaction at reflux temperature was required. In the next step, hydrogenation of the double bond and the simultaneous removal of the benzylic protecting groups of compound **8** was initially attempted with 10%-Pd on activated charcoal in a hydrogen atmosphere in *tert*-BuOH/H_2O as the solvent. Under these conditions the reaction did not go to completion, even at 55 psi (3.8 bar) hydrogen pressure. Gratifyingly, hydrogenation in the presence of Pearlman's catalyst (10%-Pd$(OH)_2$ on activated carbon) in MeOH, a complete conversion was obtained. The desired (*S*)-2-aminoundecanoic acid was difficult to isolate due to the amphiphatic character in its zwitterionic form. Therefore, protection of the α-amino group was performed in MeOH/CH_2Cl_2 (after removal of the catalyst by filtration) in the presence of Fmoc-ONSu and DIPEA as base. Finally, *N*-α-(9-fluorenylmethyloxycarbonyl)-(*S*)-2-aminoundecanoic acid (Fmoc-Laa-OH) **9** was obtained in a modest yield of 38% (62% per step) after aqueous work-up and purification by column chromatography.[18]

A previously reported structure–activity relationship study of anoplin performed by Hansen and co-workers[19] indicated that any substitution of the lysine or arginine residues by alanine or modification of the α-amino terminus resulted in anoplin derivatives with an increased hemolytic activity. Furthermore, antimicrobial activity was completely lost when the leucine residues at position 2 and 10, or the isoleucine at position 6, were replaced by alanine (see Fig. 2A for amino acid numbering). These data indicate that the overall charge of +4 is essential for selectivity, while hydrophobicity is required for activity. Therefore, we hypothesized that incorporation of (*S*)-2-aminoundecanoic acid on position 2, 6, or 10 might increase activity while selectivity remained unaffected. The rationale for this design strategy is illustrated in Figure 2B, since the sites of modification form a hydrophobic patch as indicated by the helical wheel representation of the anoplin sequence. To test this hypothesis, anoplin derivatives **11**–**13** were synthesized, and their activity/selectivity profile was analyzed and compared to anoplin **10** and N-decanoylated anoplin **14** (Fig. 2).[20]

The peptides **10**–**14** were tested for their potency to inhibit bacterial growth, expressed as their minimal inhibition concentration (MIC), of a Gram-negative (*E. coli*) as well as a Gram-positive (*S. aureus*) bacterium. Selectivity against bacterial membranes was measured by exposing red blood cells to the peptides in a hemoglobin leakage assay (EC_{50}). Anoplin **10** was active against



Scheme 1. Synthesis of *N*-α-(9-fluorenylmethyloxycarbonyl)-(*S*)-2-aminoundecanoic acid (Fmoc-Laa-OH) **9** from L-glutamic acid **3**.