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Structure-activity relationship study of anoplin

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Abstract: Anoplin is a decapeptide amide, GLLKRIKTLL-NH_2 derived from the venom sac of the solitary spider wasp, *Anoplius samariensis*. It is active against Gram-positive and Gram-negative bacteria and is not hemolytic towards human erythrocytes. The present paper reports a structure-activity study of anoplin based on 37 analogues including an Ala-scan, *C*- and *N*-truncations, and single and multiple residue substitutions with various amino acids. The analogues were tested for antibacterial activity against both *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, and several potent antibacterial analogues were identified. The cytotoxicity of the analogues against human erythrocytes was assessed in a hemolytic activity assay. The antibacterial activity and selectivity of the analogues against *S. aureus* and *E. coli* varied considerably, depending on the hydrophobicity and position of the various substituted amino acids. In certain cases the selectivity for Gram-positive and Gram-negative bacteria was either reversed or altogether eliminated. In addition, it was generally found that antibacterial activity coincided with hemolytic activity.

Keywords: anoplin; antibacterial peptide; mean hydrophobicity; mean hydrophobic moment

INTRODUCTION

The widespread and indiscriminate use of antibiotics since their discovery in the 1930s has necessitated a dramatic change in how we treat what once were controllable diseases, but which now require renewed attention. Drug resistance is quickly becoming a world issue on many fronts, requiring extensive research to develop more advanced and inevitably costly antibacterial agents which give no long term guarantee against the emergence of resistant pathogens (e.g. viral, bacterial, fungal, protozoa, etc.) [1,2]. The pressing search for antibiotic/antibacterial agents has generated over the past 70 years approximately 150 drugs, limited to a handful of target sites and a small family of antibiotics dominated by β-lactams, tetracyclines, aminoglycosides, macrolides, sulfonamides and quinolones [3–5]. Though some success has been achieved in circumventing and deactivating resistance mechanisms [6,7], eventually the flexibility and adaptability of pathogens will make these new drugs obsolete [8], unless restrictive measures are taken [9,10]. Our main goal therefore should be to extend the life of existing antibacterial drugs, as well as to continue research to improve our understanding of the relationship to these potential pathogens [11]. Therefore, the search for alternative modes of action which could slow down the alarming trend of resistance is vital. With a better understanding of drug resistance mechanisms, as well as an appreciation of the biological cost of antibacterial resistance modifications [12,13], alternate modes of action begin to emerge based on more fundamental and primitive innate defence mechanisms in the form of cationic antimicrobial peptides [14,15], originally discovered as the main defence mechanism of insects [16,17]. These relatively low-molecular weight peptides were later found to be present in all living organisms, sometimes in surprisingly large quantities freely circulating or sequestered in inducible compartments throughout the organism [18]. These peptides are generally classified into four major groups according to sequence and secondary structure. They generally possess a net positive charge, are composed of 10–40 amino acids and are amphipathic. They are often remarkably active against a large number of different pathogens; Gram-positive and Gram-negative bacteria, fungi [19], viruses [20], parasites [21] and cancer cells [22].

Despite their small size, cationic antimicrobial peptides have demonstrated antibacterial activity, as well as involvement in many physiological processes [14]. In addition to being important components of the innate immune response, antimicrobial peptides also

Abbreviations: ACTH, adrenocorticotropic hormone; ATCC, American type culture collection; ATR-FTIR attenuated total reflectance Fourier transform infrared spectroscopy; Boc, *tert*-butyloxycarbonyl; CFU, colony forming unit; CPD, citrate-phosphate-dextrose; DIEA, diisopropylethylamine; DIPCI, *N*,*N*′-diisopropylcarbodiimide; EC_{50}, concentration required for 50% hemolysis; GRAVY, grand average of hydrophobicity; $\langle H \rangle$, mean hydrophobicity; HOBt, 1-hydroxybenzotriazole; LC-MS, liquid chromatography mass spectroscopy; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMP, *N*-methyl-2-pyrrolidone; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SM, sphingomyelin; TBTU, *N*-[(1*H*-benzotriazol-1-yl) (dimethylaminomethylamino) methylene]-*N*-methyl methanaminium tetrafluoroborate *N*-oxide; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TIS, triisopropylsilane; Trt, triphenylmethyl; $\langle \mu_H \rangle$, Eisenberg mean hydrophobic moment.

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mediate the adaptive immune response by, for example, inducing mast cell degranulation, or acting as receptor mediated chemo-attractants [14,23], and they have the ability, for example, to block the biological effects of a lipopolysaccharide induced inflammatory response [24,25]. The classical view of antimicrobial peptides possessing an indiscriminate membrane detergent mode of action is replaced with a better understanding of the complex interactions of peptides with membrane environments, and the factors contributing to their activity [26].

The perpetual search for novel cationic antimicrobial peptides as lead structures obtained from natural sources has recently revealed interesting candidates from wasp venom [27,28]. Anoplin, GLLKRIKTLL-NH_2 is one of the major components isolated from the venom sac of the solitary spider wasp, *A. samariensis*, and is the smallest linear α-helical antimicrobial peptide found to date, with only 10 residues. Though no crystal structure of anoplin is yet available, it has been possible to determine a plausible solution structure from the CD spectrum, which indicates a high α-helix content in 50% trifluoroethanol; this is supported by molecular modeling based on homology with crabrolin [27] and mastoparan-X [29]. The amphipathic character of anoplin can also be shown by representing it as a helical wheel (Figure 1). Like crabrolin and mastoparan-X, anoplin is a potent mast cell degranulator, and shows a broad spectrum of antimicrobial activity in low-salt media against both Gram-positive and Gram-negative bacteria. Furthermore, the hemolytic activity of anoplin towards human erythrocytes is very low.

A number of substitution and truncation analogues of anoplin were synthesized and tested for antibacterial and hemolytic activity. From the experimental data obtained, an attempt was made to determine whether there were correlations between the empirical data observed (CD spectra, MIC activity and hemolysis) and the semi-empirical data (hydrophobicity and amphipathicity) which could help to explain the activity observed [30].

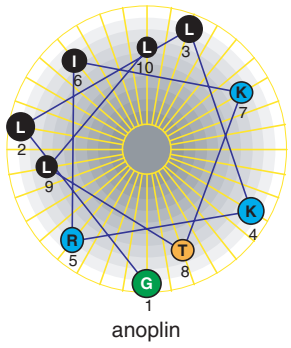

Figure 1 Helical wheel representation of anoplin emphasizing the amphipathic character of this α-helical peptide with segregation of the hydrophobic (L, I) and hydrophilic (R, K, T) amino acids.

MATERIALS AND METHODS

HPLC

Analytical HPLC was performed using a Waters C_{18}-reverse-phase column (Delta-Pak 100 Å 15 µm, Millipore) on a Waters 600E system equipped with Millennium software. Samples were chromatographed at a flow-rate of 1.5 ml/min starting with 0.1% aqueous TFA (buffer A) for 10 min and increasing over 45 min to 0.1% TFA in CH_3CN/H_2O (9 : 1, buffer B), detection at 220 nm.

Preparative HPLC was done on a Waters C_{18}-reverse-phase column (SymmetryPrep™, 7 µm, Millipore). Samples were initially chromatographed at a flow-rate of 1 ml/min starting with buffer A/buffer B (9 : 1) for 10 min and increasing over 60 min to buffer A/buffer B (4 : 6) and finally to buffer B over an additional 8 min. Flow-rate was 4 ml/min, detection at 220 nm.

Chemicals

TentaGel S RAM resin (loading 0.24 meq/g) from RAPP Polymers (Tübingen, Germany); TFE from ACROS (Geel, Belgium); DIEA, α-cyano-*p*-hydroxycinnamic acid and MTT from Aldrich (Steinheim, Germany); HOBt, TBTU and protected amino acids were purchased from PerSeptive Biosystems (Hamburg, Germany), Novabiochem (Läufelfingen, Switzerland) and Bachem (Bubendorf, Switzerland); phenol, piperidine, anhydrous ampicillin and TIS were obtained from Fluka (Buchs, Switzerland); L-α-aminobutanoic acid, BSA, Triton X-100, ACTH and Substance P were obtained from Sigma (St Louis, USA); phenylisothiocyanate from Pierce (Rockford, USA); TFA from Merck (Schuchardt, Germany). All starting chemicals were used without further purification.

MALDI-TOF-MS and LC-MS

MALDI-TOF-MS was done on a VG T of Spec E Fisons instrument, using α-cyano-*p*-hydroxycinnamic acid as matrix. Substance P and ACTH were used as calibrants.

LC-MS was done on a Bruker Esquire Mass Spectrometer. The LC part consisted of a HP 1100 equipped with a Vydac C18 column (cat. #238MS215) and a diode array detector. Samples were chromatographed at a flow-rate of 0.25 ml/min starting with 0.1% aqueous TFA (buffer A) and increasing over 25 min to 0.1% TFA in CH_3CN/H_2O (4 : 1, buffer B), finally increasing to buffer B over 10 min, detection at 220 nm.

Amino Acid Analysis

Amino acid analysis was performed on a Waters PicoTag [31] analyser, after samples were hydrolysed with 6 M aqueous HCl and 0.1% phenol at 110 °C and derivatized with phenylisothiocyanate. The concentration of each antibacterial solution was determined by including a standard, L-α-aminobutanoic acid.

Materials

Sterile 96-well polypropylene plates were from COSTAR, Corning Incorporated, (Corning, USA); sterile 96-well polystyrene microtiter plates were from Nunc (Roskilde, Denmark); Mueller-Hinton broth was from Fluka (Buchs, Schwitzerland).

Freshly drawn human erythrocytes in CPD (citrate-phosphate-dextrose) buffer was obtained from Rigshospital Blood bank, (Copenhagen, Denmark).

Peptide Synthesis

Peptide syntheses were performed using standard Fmoc-chemistry procedures on 50 mg Tenta Gel S RAM resin. The following side-chain protections of amino acid derivatives were used: Pbf for Arg; Trt for Asn; Bu^t for Thr; Boc for Trp and Lys. The amino acid (3 equiv.) together with TBTU (2.9 equiv.), HOBt (3 equiv.) and DIEA (6 equiv.) in NMP were coupled for 40 min followed by a 20 min second coupling. Following synthesis, the peptidyl-resin was washed with NMP, EtOH and ether and dried *in vacuo*. The resin was treated with TFA/H_2O/TIS (95 : 2.5 : 2.5, 2 ml) for 2 h, filtered, and washed with neat TFA (2 ml). TFA was removed by evaporation and the product precipitated in ether. After filtration the product was washed with ether (3 × 2 ml), dried and lyophilized from 10% aqueous acetic acid.

Purification and Characterization of Peptides

Following synthesis, the products were purified by preparative HPLC, lyophilized and the masses were verified by MALDI-TOF-MS or LC-MS as described in the general section. Stock solutions of the peptides were dissolved in 1% DMSO to a concentration of approximately 1 mg/ml. The exact concentration was determined by amino acid analysis. These stock solutions were used for antibacterial testing, hemolytic activity studies and CD spectroscopy.

Antibacterial Activity

Strains used for determining antibacterial activity included the two American Type Culture Collection (ATCC) strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. The MIC of each peptide was determined using a broth micro-dilution assay modified from the method of Hancock [32]. Further dilutions of the stock solution to ten times the required test concentration were made to reach a final concentration of 0.2% bovine serum albumin and 0.01% acetic acid. Serial two-fold dilutions of the peptides were made in 0.2% bovine serum albumin and 0.01% acetic acid in sterile 96-well polypropylene microtiter plates. To each well was added 100 µl of the test bacteria in Mueller-Hinton broth to a final concentration of $\sim 2 \times 10^5$ cfu/ml and 100 µl of the peptide in the different concentrations. Following the addition of MTT [33] (10 µl, 3 mM), the MIC of each peptide was read as the lowest concentration of peptide that inhibited visible growth of the bacteria after 24 h of incubation at 37 °C. All MIC determinations were performed in duplicate, and are the average of three independent determinations with ampicillin (*S. aureus* MIC = 2–4 µg/ml and *E. coli* MIC = 8–16 µg/ml) as control. All MIC values were corrected following amino acid analysis concentration determination of the stock peptide solution.

Hemolytic Activity Study

Freshly drawn human erythrocytes in CPD solution were washed three times (3000 rpm) with a cold solution of PBS (0.15 M phosphate buffer/0.15 M NaCl (pH 7.0)). The erythrocytes were diluted to a final concentration of 0.5% in PBS. Serial two-fold dilutions of the peptide (75 µl) were added to each well of a polypropylene microtiter plate followed by addition of 75 µl of human erythrocytes in PBS to final peptide concentrations ranging from 128 mM to 2 mM. The microtiter plates were allowed to incubate at 37 °C for 1 h and centrifuged 10 min at 4000 rpm. The supernatant (60 µl) of each well was then transferred to a new polystyrene microtiter plate and the absorbance was read at 414 nm on an ELISA-reader. The blank is evaluated from PBS (A_{PBS}) and 100% hemolysis in the presence of 0.1% Triton X-100 ($A_{TritonX}$). The hemolysis percentage was calculated as follows [34]: $[(A_{peptide} - A_{PBS})/(A_{TritonX} - A_{PBS})] \times 100$. All hemolysis determinations were performed in duplicate and are the average of two independent determinations. Peptide concentrations causing 50% hemolysis (EC_{50}) were determined from the dose-response curves.

CD Spectroscopy

CD spectra were recorded at 25 °C on a Jasco J10 spectropolarimeter. Peptides were dissolved to a final concentration of 62 µM in either 10 mM phosphate buffer pH 7.0 or 10 mM phosphate buffer pH 7.0 containing 50% TFE. Scans between 280 nm–195 nm were made in a 0.1 cm cell. Following baseline correction, the observed ellipticity θ (mdeg) was converted to the mean residue ellipticity [Θ] (deg cm^2/dmol), using the relationship $= 100\theta/(lcn)$, where l is the path length, c is the molar concentration and n the number of residues in the peptide [28]. The percentage α-helicities of the peptides were determined from the mean residue ellipticity [Θ] at 222 nm according to the relation % α-helix $= -100(\Theta_{222} + 3000)/33\,000$ [35].

RESULTS AND DISCUSSION

Peptide Design and Synthesis

The evaluation of individual amino acid contributions to the overall structure, antibacterial activity and selectivity was feasible due to the shortness of anoplin. Standard alanine-positional scanning, as well as *C*- and *N*-terminus truncation analogues were synthesized and tested for activity (Table 1). Based on the MIC values obtained compared with the parent peptide, and helical wheel prediction of amphipathicity, a first generation of analogues were synthesized to evaluate the individual amino acid contributions responsible for the observed MIC values. A total of 38 peptides were synthesized (including the Ala-scan and truncations) by standard Fmoc solid phase peptide chemistry [36]. The crude peptides were purified by preparative RP-HPLC and identities of the final products were confirmed by MALDI-TOF MS. A stock solution of each peptide was made and its exact concentration determined by amino acid analysis for MIC determinations, toxicity against human erythrocytes and CD spectroscopy using 50% TFE as the structure-inducing co-solvent.

Table 1 Anoplin and Analogues

Peptide	Sequence	Calc. MW	Obs. MW	MIC (μg/ml)		EC_{50} (μg/ml)	Mean hydrophobicity[b] ⟨H⟩	Mean hydrophobic moment ⟨μ_H⟩
				S. aureus	*E. coli*			
Anoplin	G L L K R I K T L L-NH_2	1152.5	1154.3	13	26	—[a]	−0.113	0.366
ano-A1	**A** L L K R I K T L L-NH_2	1166.5	1167.8	>21	>21	—	−0.104	0.358
ano-A2	G **A** L K R I K T L L-NH_2	1110.4	1110	>43	>43	—	−0.141	0.346
ano-A3	G L **A** K R I K T L L-NH_2	1110.4	1111.6	>38	>38	—	−0.141	0.351
ano-A4	G L L **A** R I K T L L-NH_2	1095.4	1096	28	16	—	0.022	0.248
ano-A5	G L L K **A** I K T L L-NH_2	1067.4	1067.9	10	5	161	0.088	0.373
ano-A6	G L L K R **A** K T L L-NH_2	1110.4	1112	>41	>41	—	−0.161	0.318
ano-A7	G L L K R I **A** T L L-NH_2	1095.4	1097.7	11	11	132	0.022	0.375
ano-A8	G L L K R I K **A** L L-NH_2	1122.5	1122.5	8	16	97	−0.07	0.325
ano-A9	G L L K R I K T **A** L-NH_2	1110.4	1112	>31	49	—	−0.141	0.354
ano-A10	G L L K R I K T L **A**-NH_2	1110.4	1112	>48	>48	—	−0.141	0.343
ano-10	G L L K R I K T L-NH_2	1039.4	1040	>43	>43	—	−0.184	0.361
ano-9-10	G L L K R I K T-NH_2	926.2	928	>23	>23	—	−0.274	0.375
ano-8-10	G L L K R I K-NH_2	825.1	826	>28	>28	—	−0.287	0.403
ano-7-10	G L L K R I-NH_2	696.9	698	>38	>38	—	−0.152	0.493
ano-6-10	G L L K R-NH_2	583.8	585	>31	>31	—	−0.328	0.467
ano-1	L L K R I K T L L-NH_2	1095.5	1096	>60	60	—	−0.143	0.421
ano-1-2	L K R I K T L L-NH_2	982.3	982.7	>31	>31	—	−0.228	0.429
ano-1-3	K R I K T L L-NH_2	869.1	871.6	>61	>61	—	−0.336	0.444
ano-5V	G L L K **V** I K T L L-NH_2	1095.5	1096.2	3	3	60	0.117	0.383
ano-5L	G L L K **L** I K T L L-NH_2	1109.5	1111	2	2	13	0.116	0.383
ano-5I	G L L K **I** I K T L L-NH_2	1109.5	1110.7	2	2	29	0.136	0.391
ano-5F	G L L K **F** I K T L L-NH_2	1143.5	1142.9	2	2	77	0.124	0.386
ano-5N	G L L K **N** I K T L L-NH_2	1110.4	1111	69	34	—	−0.001	0.356
ano-5W	G L L K **W** I K T L L-NH_2	1182.5	1183.7	2	2	7	0.1	0.377
ano-5K	G L L K **K** I K T L L-NH_2	1124.5	1125	22	16	—	−0.047	0.356
ano-8V	G L L K R I K **V** L L-NH_2	1150.5	1151.6	22	22	310	−0.041	0.297
ano-8L	G L L K R I K **L** L L-NH_2	1164.6	1164	7	13	81	−0.042	0.298
ano-8I	G L L K R I K **I** L L-NH_2	1164.5	1164.4	14	9	119	−0.022	0.279
ano-8F	G L L K R I K **F** L L-NH_2	1198.6	1200.3	8	8	98	−0.034	0.291
ano-8N	G L L K R I K **N** L L-NH_2	1165.5	1167.8	12	22	381	−0.159	0.41
ano-8W	G L L K R I K **W** L L-NH_2	1237.6	1238	4	2	98	−0.058	0.313
ano-8K	G L L K R I K **K** L L-NH_2	1179.6	1181	5	10	—	−0.205	0.455
ano-1K	**K** L L K R I K T L L-NH_2	1223.6	1224.2	>24	24	312	−0.239	0.473
ano-5V8V	G L L K **V** I K **V** L L-NH_2	1093.5	1094	14	28	32	0.189	0.344
ano-1K5F	**K** L L K **F** I K T L L-NH_2	1214.6	1215.2	7	3	231	−0.002	0.440
ano-1K8K	**K** L L K R I K **K** L L-NH_2	1250.7	1250.8	28	37	369	−0.331	0.564
ano-1K5V8K	**K** L L K **V** I K **K** L L-NH_2	1193.7	1195	9	6	231	−0.101	0.438

[a] Indicates no hemolysis was observed within the concentration range tested.
[b] Eisenberg consensus scale was used to calculate both mean hydrophobicity and hydrophobic moment.

Antibacterial Studies

The antibacterial activity of the peptides was tested on *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 by the micro-dilution method [32]. The MIC values obtained are listed in Table 1. The MIC values for the lead structure were 13 μg/ml for *S. aureus* and 26 μg/ml for *E. coli*, demonstrating a clear 2-fold selectivity for *S. aureus*.

Alanine-positional Scanning

The Ala-scan identified 6 of 10 residues (ano-A1, ano-A2, ano-A3, ano-A6, ano-A9, ano-A10) with MIC values >21–48 μg/ml for both bacterial strains which were important for maintaining activity comparable to the lead structure. Improved MIC values were observed for analogues: ano-A5, ano-A7 and ano-A8 ranging over 8–11 μg/ml for *S. aureus* and 5–16 μg/ml for *E. coli*

(improved MIC values were observed for ano-A4 against *E. coli*). This can be explained by the increase in the Eisenberg mean hydrophobicity ⟨H⟩ which measures the average hydrophobicity of amino acids in a peptide segment [37] (see Table 2 and Figure 2) compared with anoplin. Interestingly, there is a certain degree of selectivity reversal in the case of ano-A4 with a 1.5-fold improvement for *E. coli* and a 2-fold worsening for *S. aureus* with respect to anoplin. Selectivity reversal was also observed for ano-A5 with a minor improvement for *S. aureus* and a 5-fold improvement for *E. coli*. Peptide ano-A7, on the other hand abolished selectivity for either bacterium with a 2.3-fold improvement for *E. coli* and little improvement for *S. aureus* compared with anoplin.

Finally, ano-A8 demonstrated a 1.6-fold improvement over anoplin for both *E. coli and S. aureus* while maintaining the same selectivity as anoplin; this may be explained by the similarity and slightly increased mean hydrophobicity compared with anoplin (⟨H⟩ = −0.070 vs−0.113, respectively). Generally, improved MIC activity compared with anoplin had a larger beneficial effect against *E. coli* than *S. aureus*, clearly demonstrating membrane affinity for the peptides according to the mean hydrophobicity as one of the determining factors. Additional information is obtained from the correlation ($r^2 = 0.906$) of RP-HPLC retention times with mean hydrophobicity for the Ala-scan analogues (see Table 3). As a general observation, substitution of hydrophobic residues resulting in Ala analogues with ⟨H⟩ = ≤ −0.104 did not demonstrate any MIC activity over the test concentrations used.

Table 2 Ala-Scan Peptides of Interest

Peptide	Amino acid substituted	MIC μg/ml		Mean hydrophobicity ⟨H⟩
		S. aureus	*E. coli*	
ano-A4	Lysine	28	16	0.022
ano-A5	Arginine	10	5	0.088
ano-A7	Lysine	11	11	0.022
ano-A8	Threonine	8	16	−0.07

Table 3 Anoplin Ala-scan Correlation of Retention Time and Mean Hydrophobicity

Peptide	Retention time (min)	Mean hydrophobicity ⟨H⟩
ano-A5	17.4	0.088
ano-A7	17.2	0.022
ano-A4	16.9	0.022
ano-A8	16.2	−0.07
ano-A1	14.9	−0.104
ano-A9	14.8	−0.141
ano-A3	14.4	−0.141
ano-A10	14.3	−0.141
ano-A2	14.1	−0.104
ano-A6	14.1	−0.161

C- and N-Terminus Truncations

The next part of the study of anoplin was the *C*- and *N*-terminus truncations. All truncated analogues gave MIC values >23–61 μg/ml for both classes of bacteria (see Table 1). Based on the MIC values obtained, it was decided that for the purposes of this study, the size of the peptide analogues would be restricted to 10 amino acids. The lack of any activity or selectivity for either bacterial or mammalian cells is correlated with the low mean hydrophobicities for all truncated peptides compared with anoplin. The lack of activity observed for all membrane types by these short peptides can be explained by their inability to form a secondary α-helix structure.

First Generation Analogues: Single Amino Acid Substitutions

Based on the above results, a series of first generation substituted analogues was designed. The focus of this first study was directed towards residues, Arg^5 and Thr^8 based on the improved antibacterial activity of

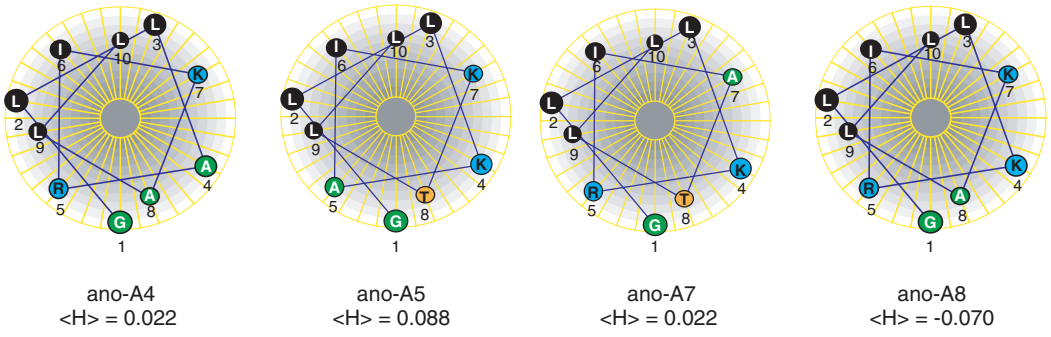

Figure 2 Helical wheel representation of first generation peptides, ⟨H⟩ = mean hydrophobicity.

peptides ano-A5 and ano-A8 compared with anoplin. The amino acid substitutions were chosen to study the range of effects possible by substituting more and less hydrophobic amino acids than alanine.

A good correlation was observed between the RP-HPLC retention times and the mean hydrophobicities ($r^2 = 0.855$) of Arg^5 analogues (see Table 4). The MIC values for analogues ano-5I, ano-5F, ano-5V, ano-5L and ano-5W were improved by comparable amounts (ranging from MIC = 2–3 μg/ml) despite the difference in hydrophobicity of the substituted amino acids (see Table 5) [38], while at the same time completely eliminating the selectivity for *S. aureus* compared with anoplin. In this case, it can be deduced that a reduced overall charge and an increased amount of hydrophobicity was a factor in increasing membrane activity.

There was no obvious correlation between hydrophobicity and MIC values observed for the Thr^8 residue substitutions. On the other hand, there was more variation in activity ranging from 4–22 μg/ml for *S. aureus* and 2–22 μg/ml for *E. coli* compared with Arg^5 substitutions. The position of Thr, viewed on a helical wheel (see Figure 2) is expected to greatly influence the hydropathic moment depending on the type of amino acid substituted. This is clearly demonstrated by comparing the mean hydrophobic moments $\langle\mu_H\rangle$ [37] of Arg^5 substitutions (ranging from $\langle\mu_H\rangle = 0.36–0.39$) compared with similar substitutions for Thr^8 (ranging from $\langle\mu_H\rangle = 0.29–0.46$). In addition to displaying a large range of MIC values, the selectivity also undergoes significant changes. A 2-fold preference for *S. aureus* by ano-8L and ano-8K, and an almost 2-fold preference for *E. coli* by ano-8I and ano-8W were observed, while loss of selectivity was observed for ano-8V and ano-8F. The best MIC values for this series were observed for the two aromatic side groups (ano-8F and ano-8W) which are known to impart an additional anchoring effect within lipid membranes [39,40] and the additional selectivity for prokaryotic cells contributed by the increased overall charge from ano-8K. Little change was observed for Asn residue substitution compared with the anoplin. A possible explanation may be that ano-8N, as the only other amino acid with an uncharged polar-side chain probably interacts similarly to Thr residue in the parent peptide. A similar explanation can be applied to ano-8K which displays the same selectivity compared with anoplin, though in this case with an improved MIC.

Substituting an additional basic group for Gly^1, ano-1K (see Figure 3), resulted in a more than 2-fold increase in MIC against *S. aureus* and essentially no change against *E. coli*. An additional positive charge at the *N*-terminus and a resulting $\langle H\rangle = -0.239$ clearly has a negative effect on the peptide activity.

Table 4 Retention Time and Mean Hydrophobicity Correlation of Arg^5 Substitution Analogues

Peptide	Retention time (min)	Mean hydrophobicity ⟨H⟩
ano-5L	19.3	0.116
ano-5I	19.2	0.136
ano-5V	19	0.117
ano-5F	16.4	0.124
ano-5W	16.4	0.1
ano-5N	15.8	−0.001
ano-5K	12.3	−0.047

Table 5 Hydrophobicities of Arg^5 Analogues

Peptide	MIC μg/ml		Mean hydrophobicity ⟨H⟩
	S. aureus	*E. coli*	
ano-5I	2	2	0.136
ano-5F	2	2	0.124
ano-5V	3	3	0.117
ano-5L	2	2	0.116
ano-5W	2	2	0.1
ano-A5	10	5	0.088
ano-5N	69	34	−0.001
ano-5K	22	16	−0.047

Multiple Amino Acid Substitutions

Substitutions for Gly^1 and/or Arg^5 and Thr^8 resulted in significant changes in MIC and selectivity compared with anoplin. Substituting both Arg^5 and Thr^8 with Val (ano-5V8V) had only a slight worsening effect (compared with anoplin) and resulted in regenerating the 2-fold *S. aureus* selectivity compared with individual substitutions with Val (ano-5V and ano-8V). Although substituting $Gly^1 \rightarrow$ Lys (ano-1K) alone resulted in a loss of activity against *S. aureus*, the activity can be completely regained by substitution of $Arg^5 \rightarrow$ Phe or Val (ano-1K5F and ano-1K5V8K) which is the same pattern observed for the single substitutions mentioned above, where Arg^5 alone was substituted (see Table 1).

Substituting Gly^1 and Thr^8 with Lys (ano-1K8K) resulted in a worsening of *E. coli* and only a slight improvement for *S. aureus* compared with the single substitution ano-1K. On the other hand, if Arg^5 is also substituted (ano-1K5V8K), activity is greatly improved. The calculated overall charge at pH 7 of ano-1K8K is +4.91 while that of ano-1K5V8K and ano-1K is +3.91. In addition to the difference of charge, the large difference in mean hydrophobicity (ano-1K8K = −0.331, ano-1K = −0.239 and ano-1K5V8K = −0.101) must also account for the activity observed. The charge difference and its distribution could explain the difference in activity between these two analogues (see Figure 3).

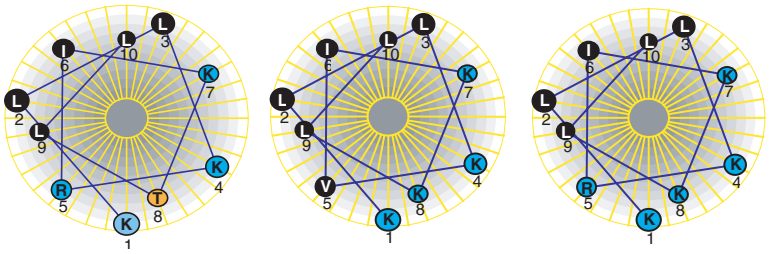

Figure 3 Helical wheel of ano-1K, ano-1K5V8K and ano-1K8K.

Hemolytic Studies

As previously described, anoplin was found not to be hemolytic. The hemolysis results for the Ala-scan demonstrated a similar tendency except for instances where polar groups were replaced by Ala-residues. Peptides, ano-A5, ano-A7 and ano-A8 (replacement of Arg^5, Lys^7, or Thr^8 with Ala) resulted in 50% hemolysis at 161 μg/ml, 132 μg/ml and 97 μg/ml peptide, respectively. As a generalized observation, increased hydrophobicity and in this case reduced positive charge, reduces the selectivity of antimicrobial peptides for negatively charged bacterial membranes (PG/PE) over the more neutral (zwitterionic) human erythrocytes (PC/SM/cholesterol) [19,41]. Truncated analogues from either the *N*-terminus or the *C*-terminus demonstrated no hemolytic activity. Clearly the truncated analogues resulted in overall decreased hydrophobicities and hence selectivity for negatively charged membranes. For this antibacterial peptide at least, a minimum requirement of 10 amino acids is necessary for both antibacterial and hemolytic activity. Substitution of Arg^5 by hydrophobic amino acids (Val, Leu, Ile, Phe or Trp) resulted in significant hemolytic activity, from 7–77 μg/ml while substitution by polar groups (Asn or Lys) at this position resulted in the complete loss of hemolytic activity. A similar pattern was observed for substitutions of Thr^8 with the same amino acid substitutions. When overall charge is increased by substitution with a positively charged amino acid (Lys or Arg), hemolysis was generally reduced.

The general tendency observed is for very active analogues against Gram-positive and Gram-negative bacteria with large mean hydrophobicities to be also hemolytic, while ineffective antimicrobial peptides with low mean hydrophobicities are not hemolytic [42]. The exceptions are ano-8K, ano-1K5F and ano-1K5V8K, all of which have low MIC values, low hemolytic activity and relatively low mean hydrophobicities.

CD Measurements

The CD spectra of selected anoplin analogues were recorded (data not shown). Anoplin itself and all the analogues examined showed 55%–77% α-helix content (see Table 6) in 50% TFE, but all of them were random-coiled in buffer solution, in agreement with previous reports [43]. The more hydrophobic analogues ano-A5, ano-A7, ano-A8 had almost identical CD spectra and similar α-helix content (72%, 72% and 74%, respectively) [44,45]. Attempts to correlate CD spectra with various properties (RT, MIC, mean hydrophobicity and mean hydropathic moment) of the analogues were inconclusive.

Table 6 Percent Helicity of Representative Peptides

Peptide	Helicity (%)
Anoplin	**77**
ano-A5	72
ano-A7	72
ano-A8	74
ano-5V	72
ano-5W	62
ano-5K	65
ano-8V	55
ano-8N	63
ano-5V8V	70

CONCLUSION

The structure-activity relationship study of the small antimicrobial decapeptide amide anoplin presented here reveals several interesting results. Firstly, *C*- and *N*-terminus truncations indicated that in order to display antibacterial activity towards *E. coli* or *S. aureus* the full decapeptide is required. Secondly, standard alanine-positional scanning showed restrictions on 6 out of the 10 residues, and improved MIC values for the remaining four residues. A series of analogues of peptides ano-A5 and ano-A8 in which Ala was replaced by Val, Leu, Ile, Phe, Asn, Trp or Lys displayed better MIC activities against *E. coli* and *S. aureus* than anoplin (except for ano-5N and ano-8V). Thirdly, there was a definite trend observed when analogues were grouped according to mean hydrophobicity. Generally, an anoplin analogue with a high MIC value will also have high hemolytic activity, and a low

mean hydrophobicity. On the other hand, an anoplin analogue with a low MIC value will have high hemolytic activity, and a high mean hydrophobicity (notable exceptions were ano-8K, ano-1K5F and ano-1K5V8K). Fourthly, replacing a single amino acid may in some cases abolish or reverse selectivity between *E. coli* and *S. aureus*. However, this cannot be explained using mean hydrophobicity nor mean hydrophobic moment calculations. Further investigations are needed, such as interaction with model membranes [46], as well as ATR-FTIR [47] to determine peptide orientation in contact with membranes.

In conclusion, the work presented here demonstrates the power of structure-activity studies of small antibacterial peptides and their usefulness as potent candidates in the search for alternative antibacterial agents.

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REFERENCES

1. Chu DTWP, Jacob J, Leonard K. New directions in antibacterial research. *J. Med. Chem.* 1996; **39**: 3853–3874.
2. Projan SJ. Why is big Pharma getting out of antibacterial drug discovery? *Curr. Opin. Microbiol.* 2003; **6**: 427–430.
3. Coates A, Hu Y, Bax R, Page C. The future challenges facing the development of new antimicrobial drugs. *Nat. Rev. Drug Discov.* 2002; **1**: 895–910.
4. Bryskier A. Novelties in the field of anti-infective compounds in 1999. *Clin. Infect. Dis.* 2000; **31**: 1423–1466.
5. Hancock RE, Knowles D. Are we approaching the end of the antibiotic era? *Curr. Opin. Microbiol.* 1998; **1**: 493–494.
6. Moellering R. New approaches to developing antimicrobials for resistant bacteria. *J. Infect. Chemother.* 2003; **9**: 8–11.
7. Cloete TE. Resistance mechanisms of bacteria to antimicrobial compounds. *Int. Biodeter. Biodegrad.* 2003; **51**: 277–282.
8. Ochman H, Bergthorsson U. Rates and patterns of chromosome evolution in enteric bacteria. *Curr. Opin. Microbiol.* 1998; **1**: 580–583.
9. WHO. *WHO Global Strategy for Containment of Antimicrobial Resistance*. World Health Organization: Geneva, 2001; 1–105.
10. Davies J. Bacteria on the rampage. *Nature* 1996; **383**: 219–220.
11. Matzinger P. The danger model: a renewed sense of self. *Science* 2002; **296**: 301–305.
12. Andersson DI, Levin BR. The biological cost of antibiotic resistance. *Curr. Opin. Microbiol.* 1999; **2**: 489–493.
13. Peschel A. How do bacteria resist human antimicrobial peptides? *Trends Microbiol.* 2002; **10**: 179–186.
14. Yang ODC, Oppenheim JJ. The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. *Cell. Mol. Life Sci.* 2001; **58**: 978–989.
15. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; **415**: 389–395.
16. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* 2003; **55**: 27–55.
17. Bulet CPH, Dimarcq J-L, Hoffmann D. Antimicrobial peptides in insects; structure and function. *Dev. Comp. Immunol.* 1999; **23**: 329–344.
18. Lehrer R, Ganz T. Cathelicidins: a family of endogenous antimicrobial peptides. *Curr. Opin. Hematol.* 2002; **9**: 18–22.
19. Hong SY, Park TG, Lee K-H. The effect of charge increase on the specificity and activity of a short antimicrobial peptide. *Peptides* 2001; **22**: 1669–1674.
20. Belaid MAA, Khelifa R, Trabelsi A, Jemmali M, Hani K. *In vitro* antiviral activity of dermaseptins against herpes simplex virus type 1. *J. Med. Virol.* 2002; **66**: 229–234.
21. Efron L, Dagan A, Gaidukov L, Ginsburg H, Mor A. Direct interaction of dermaseptin S4 aminoheptanoyl derivative with intraerythrocytic malaria parasite leading to increased specific antiparasitic activity in culture. *J. Biol. Chem.* 2002; **277**: 24 067–24 072.
22. Yang N, Rekdal O, Stensen W, Svendsen JS. Enhanced antitumor activity and selectivity of lactoferrin-derived peptides. *J. Pept. Res.* 2002; **60**: 187–197.
23. Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H, Nagaoka I. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology* 2002; **106**: 20–26.
24. Scott MG, Vreugdenhil ACE, Buurman WA, Hancock REW, Gold MR. Cutting edge: cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS binding protein. *J. Immunol.* 2000; **164**: 549–553.
25. Ding L, Yang L, Weiss T, Waring A, Lehrer R, Huang H. Interaction of antimicrobial peptides with lipopolysaccharides. *Biochemistry* 2003; **42**: 12 251–12 259.
26. Cronan JE. Bacterial membrane lipids: Where do we stand? *Ann. Rev. Microbiol.* 2003; **57**: 203–224.
27. Krishnakumari V, Nagaraj R. Antimicrobial and hemolytic activities of crabrolin, a 13-residue peptide from the venom of the European hornet, *Vespa crabro*, and its analogs. *J. Pept. Res.* 1997; **50**: 88–93.
28. Konno K, Hisada M, Fontana R, Lorenzi CCB, Naoki H, Itagaki Y, Miwa A, Kawai N, Nakata Y, Yasuhara T. Anoplin, a novel antimicrobial peptide from the venom of the solitary wasp *Anoplius samariensis*. *Biochim. Biophys. Acta* 2001; **1550**: 70–80.
29. Whiles JA, Brasseur R, Glover KJ, Melacini G, Komives EA, Vold RR. Orientation and effects of mastoparan X on phospholipid bicelles. *Biophys. J.* 2001; **80**: 280–293.
30. Giangaspero A. Amphipathic α helical antimicrobial peptides A systematic study of the effects of structural and physical properties on biological activity. *Eur. J. Biochem.* 2001; **268**: 5589–5600.
31. Cohen SA, Strydom DJ. Review. Amino acid analysis utilizing phenylisothiocyanate derivates. *Anal. Biochem.* 1988; **174**: 1–16.
32. http://cmdr.ubc.ca/bobh/showmethod.Php?methodid = 42
33. Mshana RN, Tadesse G, Abate G, Miorner H. Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide for rapid detection of rifampin-resistant mycobacterium tuberculosis. *J. Clin. Microbiol.* 1998; **36**: 1214–1219.
34. Park Y, Lee DG, Jang S-H, Woo E-R, Jeong HG, Choi C-H, Hahm K-S. A Leu-Lys-rich antimicrobial peptide: activity and mechanism. *Biochim. Biophys. Acta* 2003; **1645**: 172–182.
35. McLean LR, Hagaman KA, Owen TJ, Krstenansky JL. Minimal peptide length for interaction of amphipathic alpha-helical peptides with phosphatidylcholine liposomes. *Biochemistry* 1991; **30**: 31–37.
36. Chan WC, White PD (eds). *Fmoc Solid-Phase Peptide Synthesis*. Oxford University Press: Oxford, 2000.
37. Eisenberg D. Three-dimensional structure of membrane and surface proteins. *Ann. Rev. Biochem.* 1984; **53**: 595–623.
38. Eisenberg D, Weiss RM, Terwilliger TC. The hydrophobic moment detects periodicity in protein hydrophobicity. *Proc. Natl Acad. Sci. USA* 1984; **81**: 140–144.

39. Haug BE, Svendsen JS. The role of tryptophan in the antibacterial activity of a 15-residue bovine lactoferricin peptide. *J. Pept. Sci.* 2001; **7**: 190–196.
40. de Planque MRR, Kruijtzer JAW, Liskamp RMJ, Marsh D, Greathouse DV, Koeppe RE, II, de Kruijff B, Killian JA. Different membrane anchoring positions of tryptophan and lysine in synthetic transmembrane alpha -helical peptides. *J. Biol. Chem.* 1999; **274**: 20 839–20 846.
41. Wieprecht T, Dathe M, Beyermann M, Krause E, Maloy WL, MacDonald DL, Bienert M. Peptide hydrophobicity controls the activity and selectivity of magainin 2 amide in interaction with membranes. *Biochemistry* 1997; **36**: 6124–6132.
42. Dathe M, Schumann M, Wieprecht T, Winkler A, Beyermann M, Krause E, Matsuzaki K, Murase O, Bienert M. Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry* 1996; **35**: 12 612–12 622.
43. Avrahami D, Oren Z, Shai Y. Effect of multiple aliphatic amino acids substitutions on the structure, function, and mode of action of diastereomeric membrane active peptides. *Biochemistry* 2001; **40**: 12 591–12 603.
44. Liu LP, Deber CM. Combining hydrophobicity and helicity: a novel approach to membrane protein structure prediction. *Bioorg. Med. Chem.* 1999; **7**: 1–7.
45. Li S, Deber C. Peptide environment specifies conformation. Helicity of hydrophobic segments compared in aqueous, organic, and membrane environments. *J. Biol. Chem.* 1993; **268**: 22 975–22 978.
46. Zhang L. Interaction of cationic antimicrobial peptides with model membranes. *J. Biol. Chem.* 2001; **276**: 35 714–35 722.
47. Vigano C, Manciu L, Buyse F, Goormaghtigh E, Ruysschaert J. Attenuated total reflection IR spectroscopy as a tool to investigate the structure, orientation and tertiary structure changes in peptides and membrane proteins. *Biopolymers* 2000; **55**: 373–380.