On the Antimicrobial and Hemolytic Activities of Amphiphilic β-Peptides

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KEYWORDS:

amphiphiles · antibiotics · antifungal agents · helical structures · peptides

β-Peptides, such as the oligomers **1** – **4** containing the triad of β-homo (β-H) amino acids β-HAla-β-HLys-β-HPhe, have been designed to be capable of forming an amphiphilic 3_{14} helix (Figure 1).[1] Indeed, we have shown that the β-nonapeptide **1** inhibits an intestinal cholesterol-transporting receptor protein, and thus mimics natural peptides having amphiphilic helical structures.[2]

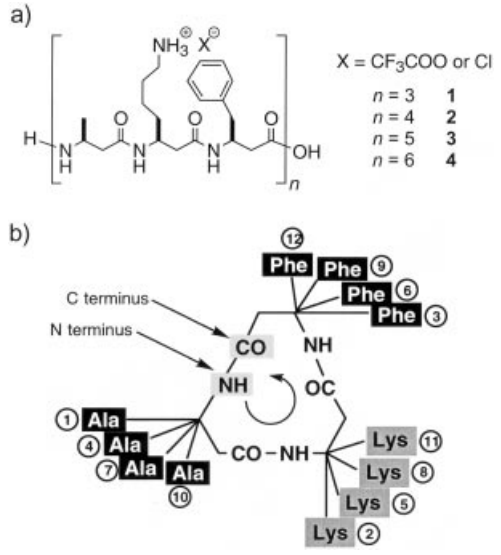

Figure 1. *a) Molecular formula of the investigated β-peptides* ***1*** *–* ***4****. b) Schematic helical-wheel-type representation of the dodecapeptide* ***2*** *in the* 3_{14}*-helical conformation, illustrating its amphiphilic character.*

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Peptides like **1** – **4**, with hydrophobic side chains on one side of the helix and cationic ones on the other, are known to be membrane-active (cf. naturally occurring toxins and antibiotics like melittin and magainins);[3] their physicochemical properties, and not the precise sequence or sense of chirality, are imperative for activity. DeGrado et al. have reported that amphiphilic β-peptides constructed from the triads β-HLeu-β-HLys-β-HLeu and β-HVal-β-HLys-β-HLeu, which are similar to those in **1** – **4**, have antibacterial activity toward *Escherichia coli*, while also exhibiting hemolytic activity.[4, 5] In view of the outstanding stability of β-peptides against all kinds of peptidases,[6] and their resulting potential use as drugs (cf. cholesterol transport inhibition,[2] somatostatin-type[7] and antiproliferative activities[8]), it is necessary to determine whether β-peptides in general, and our amphiphilic peptides in particular, are also antimicrobial and cytotoxic.

Initially, we screened for antimicrobial activity by incubating β-peptides of various concentrations (10, 20, 50 μg) with 13 different eukaryotic and prokaryotic microorganisms. To test whether the trifluoroacetate counterions of the ammonium groups[9] contribute to the antibacterial activity, we chose to make a comparison between the trifluoroacetate salts and the corresponding hydrochloride salts. The results of this study are presented in Table 1.

The β-dodecapeptide **2** inhibited growth of two eubacteria, *Escherichia coli* (Gram-negative) and *Actinomycetes* (Gram-positive), while the nonapeptide **1** displayed activity only against *Actinomycetes*. None of the longer-chain peptides showed antimicrobial activity in this initial study. Interestingly, all β-peptides showed induction of aerial mycelium formation and fructification of *Cladosporium elatum* and *Penicillium claviformae*, and most β-peptides also stimulated growth of *Aspergillus niger*. This surprising observation suggests that eukaryotic fungi somehow utilize β-peptides as an energy source, in accordance with our ongoing studies on the biodegradability of β-peptides. Furthermore, it is evident from the data in Table 1 that there are no differences between the TFA and the HCl salts, as far as antimicrobial activity is concerned.

We also tested the β-peptides **1** – **4** for hemolytic activity, and the results are shown in Table 2: In almost all cases, the activity is low ($\leq$5%), with the exception of dodecapeptide **2** (15%).[10] Once again, no clear-cut difference between the TFA and HCl salts is detected.[11]

After the initial screening of β-peptides **1** – **4** we carried out quantitative antimicrobial tests with six selected bacterial strains (Table 3). The dodecamer **2** and the pentadecamer **3** have comparable activities toward most of the tested bacteria; the measured IC_{50} values for *E. coli* are in the same range (when recalculated to μM) as those reported by DeGrado et al. for similar β-peptides.[4]

Although encouraging, IC_{50} values are, however, not reliable when judging antimicrobial activity as they are known to vary widely with experimental conditions.[12] The standard measurement of antibacterial potency of a compound is the minimum inhibitory concentration (MIC), required for *complete* inhibition of growth. The MIC values for the β-peptides **1** – **4** toward the six selected bacterial strains are shown in Table 4: Only the

Table 1. *Initial screening of the antimicrobial activity of the amphiphilic β-peptides.*[a]

Microorganism taxonomy	strain	Antibiotic tetramycin	nystatin	β-Peptide[b] 1 HCl	1 TFA	2 HCl	2 TFA	3 HCl	3 TFA	4 HCl	4 TFA
Geotrichum sp.	F7	0	−	0	0	0	0	0	0	0	0
Mycotypha	F135	0	−	0	0	0	0	0	0	0	0
Cladosporium elatum	89001201	−	−	+	+	+	+	+	+	+	+
Penicillium claviformae	23408	0	−	+	+	+	+	+	+	+	+
Aspergillus niger	7281	0	−	+	+	+	+	+	0	0	0
Candida albicans	2869	0	−	0	0	0	0	0	0	0	0
Bacillus subtilis	ATCC6633	−	0	0	0	0	0	0	−	0	0
Saccharomyces cerevisiae	YSD1	0	0	0	0	0	0	0	0	0	0
Staphylococcus aureus	2104	−	0	0	0	0	0	0	0	0	0
Escherichia coli	3914	−	−	0	0	−	−	0	0	0	0
Comomonas sp.	4157	−	0	0	0	0	0	0	0	0	0
Actino sp.	4955	−	0	−	−	−	−	0	0	0	0
Paecilomyces variotii	Tü137	0	−	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa		0	0	0	0	0	0	0	0	0	0

[a] 0 = no inhibition or growth observed; + = growth observed; − = inhibition observed. [b] HCl = hydrochloride salt, TFA = trifluoroacetate salt.

Table 2. *Hemolytic activity of the β-peptides, determined with human erythrocytes.*

β-Peptide	Hemolysis [%][a] HCl salt	TFA salt
1	0.5	0.7
2	10.1	14.9
3	6.9	3.8
4	1.6	5.3

[a] At 300 μM concentration. Controls for 0% hemolysis and 100% hemolysis were determined in phosphate-buffered saline (PBS; 0.01 M phosphate, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) and 1% (w/v) SDS, respectively.

Table 3. *IC_{50} values ($\mu g\,mL^{-1}$), defined as the lowest β-peptide concentration causing at least 50% reduction of bacterial growth.*[a]

Bacterial test strain	β-Peptide 1	2	3
Enterococcus faecalis NB 04001	> 128	64	8
Staphylococcus aureus NB 01001	> 128	128	64
Streptococcus pneumoniae NB 07001	> 128	32 – 64	32
Escherichia coli NB 27001	> 128	16	64
Klebsiella pneumoniae NB 29001	> 128	32 – 64	> 128
Pseudomonas aeruginosa NB 52001	> 128	64	64

[a] All β-peptides were tested as HCl salts. The standard antibiotic tetracycline was run in parallel as a positive control (data not shown).

Table 4. *Minimal inhibitory concentration (MIC, in $\mu g\,mL^{-1}$) defined as the lowest concentration of β-peptide required for complete inhibition of growth of bacterial test strains.*[a]

Bacterial test strain	Tetra-cycline	β-Peptide 1	2	3	4
Enterococcus faecalis NB 04001	32	> 128	128	> 128	> 128
Staphylococcus aureus NB 01001	0.5	> 128	128	> 128	> 128
Streptococcus pneumoniae NB 07001	0.25	> 128	128	> 128	> 128
Escherichia coli NB 27001	2	> 128	32	> 128	> 128
Klebsiella pneumoniae NB 29001	32	> 128	32 – 64	> 128	> 128
Pseudomonas aeruginosa NB 52001	16	> 128	128	> 128	> 128

[a] All β-peptides were tested as HCl salts. The standard antibiotic tetracycline was run in parallel as a positive control.

dodecapeptide **2** has any measurable antibacterial activity in this test. Furthermore, the detectable activity is limited to only two of the investigated microorganisms (*E. coli* and *Klebsiella pneumoniae*, both Gram-negative).

The presented data suggest that we need not worry that β-peptides in general are dangerous cytotoxic compounds. Moreover, this study supports the general belief that activity and selectivity of amphiphilic peptides and their analogues are critically dependent on the side-chain composition of the helix. Although our study confirms the activity toward *E. coli* initially reported by DeGrado et al., we doubt whether amphiphilic β^3-peptides will be developed into antibiotic agents, considering their apparent limited spectrum of activity, especially when the more stringent MIC test was used. However, other types of β-peptides without conformational backbone restriction might very well show this kind of activity and also selectivities comparable to those reported by Gellman et al.[5] for oligomers of cyclic β-amino acids.

We thank the Swedish Foundation for International Cooperation in Research and Higher Education (STINT) (P.I.A.) and the Deutscher Akademischer Austauschdienst (Hochschulsonderprogramm III) (J.F.) for postdoctoral fellowships.

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[9] Synthetic peptides are normally isolated as their TFA salts after HPLC purification and lyophilization, unless special attention is given to exchanging the counterions, see for example the compounds used in refs. [4, 5].
[10] The behavior of **4** is complex; it has higher hemolytic activity at lower concentration (data not shown), which may be caused by aggregation at higher concentration; cf. ref. [4].
[11] Small differences are likely due to variations in the number of counterions per mole of β-peptide.
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Received: June 5, 2001 [Z 252]

The Large Fragment of *Escherichia coli* DNA Polymerase I Can Synthesize DNA Exclusively from Fluorescently Labeled Nucleotides

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KEYWORDS:

directed evolution · DNA polymerase · fluorescence spectroscopy · nucleotides · transferases

Fluorescence is a highly specific and sensitive property that is frequently used for detecting and identifying certain molecules within synthetic or biological materials. Particularly the augmentation of traditional Sanger sequencing with fluorescent primers or terminators paved the way for the most durable and efficient method of DNA sequencing. With the rapid progress in fluorescence technology, and encouraged by the international race to sequence the human genome as well as the genomes of other model organisms, the idea to combine fluorescent sequencing with single-molecule detection became obvious, and it nourished the hope to simplify and speed up the task of sequencing DNA segments as long as 50 000 bp and joining the sequence information from genome fragments.[1–4] However, single-molecule sequencing requires the complete and faithful tagging of each of the four types of bases of DNA (or RNA) with its own fluorescent compound. In principle, the labeling may be achieved by DNA-polymerase-mediated incorporation of fluorophore-labeled deoxynucleoside triphosphates. In practice, most natural DNA polymerases have been found to discriminate against bulky fluorescent nucleotide analogues which often also exhibit a net charge differing from that of the natural substrates.[5–8] As yet, only few mutant bacteriophage T4 DNA polymerases have been identified that exhibit an increased capability of incorporating modified monomers for the synthesis of long chains of complementary fluorophore-labeled DNA.[9]

We employed the well-documented power of directed evolution[10] to get access to one or more DNA polymerases with the abilities to 1) incorporate a fluorophore-labeled nucleotide, 2) extend the terminus by addition of the next fluorophore-labeled substrate, and 3) retain a sufficient incorporation fidelity. Therefore, we developed a functional screening system that allowed for the assessment of individual clones that show an increased acceptance of fluorophore-labeled nucleotides, and we started the search for the desired enzymatic activity by using the Klenow fragment (KF) of DNA polymerase I (Pol I) of *Escherichia coli*.[11–15] Because elongation of a primer–template is not a single-step reaction requiring simple yes/no decisions, we employed fluorescence correlation spectroscopy (FCS), a highly sensitive method that enables the distinction of chain lengths and fluorescence intensities of individual nucleic acid molecules.[16,17]

The set-up of a fluorescence-based assay for screening active variants within DNA polymerase libraries then revealed that, surprisingly, the wild-type KF retains full activity in the sole presence of artificial deoxynucleoside triphosphates that are labeled with a fluorescent dye of the rhodamine type. The screening procedure that allowed for this discovery included 1) the cloning, individualization, and overexpression of functional KF or its variants, 2) the immobilization and purification of polymerase protein, 3) the polymerase reaction, 4) the immobilization and purification of reaction products, and 5) the spectroscopic distinction of educts and dye-labeled DNA products according to their diffusion times and fluorescence intensities (Figure 1).

The comparative evaluation of individual polymerase variants for activity with unnatural substrates required two purification steps: On the one hand, polymerase mutants that were cloned and expressed in *Escherichia coli* had to be separated from all competing host DNA polymerases, and on the other hand, reaction products, possibly of varying lengths, had to be purified from an excess of fluorescent monomer which could interfere during the FCS measurement. Because extensive purification steps drastically reduce the number of individual mutants that can be tested in a certain period of time (throughput), we employed one-step procedures for solving both problems, and we applied commercially available microwell formats.

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