Antimicrobial Agents

Nontoxic Membrane-Active Antimicrobial Arylamide Oligomers**

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Non-natural polymers can adopt specific secondary and tertiary structures that resemble those of naturally occurring biopolymers.[1–4] As a result of their stability towards enzymatic degradation and their virtually unlimited diversity, non-natural polymers and oligomers are excellent candidates for therapeutic biomimetics. We previously designed a series of easily synthesized aryl amide oligomers[5] that mimic the properties of membrane-interactive antimicrobial peptides such as the magainins and cecropins. Although these oligomers are highly active, they have significant toxicities toward human cells, as assessed from the hemolytic activities of the compounds. Herein, we describe the fine-tuning of the

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[**] We thank the NIH BECON program and PolyMedix. Inc. for financial support.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200352791

physicochemical properties of these oligomers to enhance their potency while eliminating their toxicity.

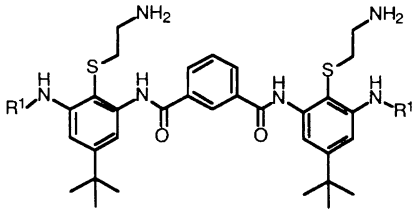
Antimicrobial peptides act on the membranes of their targets,[6–9] and bacteria appear to develop resistance to these surface-active peptides less easily than to antibiotics that act on more-specific enzymatic targets. The peptides appear to differentiate bacterial from mammalian membranes by virtue of the fact that bacterial membranes present a greater density of negatively charged phospholipids and lack cholesterol. Although these peptides differ in sequence and in their detailed secondary structure, they all adopt amphiphilic conformations with spatially segregated hydrophobic and positively charged surfaces. Upon binding to a membrane, these peptides cause the formation of pores and disruption of the integrity of the bilayer.[9–11]

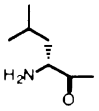
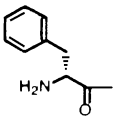
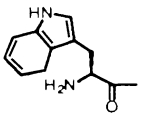
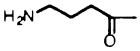
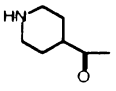
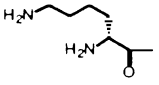
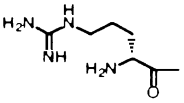
Several research groups have recently reported antimicrobial oligomers that act by this mechanism, such as β-peptides,[12–14] cyclic D,L-peptides,[15] diastereomeric peptides,[9] peptoids,[16] and cyclic peptides related to gramicidin S.[17] However, these peptides are rather closely related to traditionally studied peptides and have relatively high molecular weights (for example, the antimicrobial β-peptides are 1379–1719 Da), and hence can be expected to suffer from many of the limitations inherent to this class of compounds. We therefore recently designed a new class of facially amphiphilic aryl amide oligomers[5] related to compound **1** (see Table 1). However, although these oligomers have potencies comparable to those of host defense peptides, they are toxic towards human red blood cells at comparable concentrations.

The selectivity of natural antimicrobial peptides for lysing bacterial rather than mammalian cells depends on the appropriate balance of conformational rigidity, hydrophobicity, and the distribution of charged side chains in the peptide.[14,17,18] If the compounds are too polar they have little affinity for bacterial membranes; if they are too hydrophobic they fail to discriminate between mammalian and bacterial targets.

The short triaryl amide **1** served as the starting point for optimization of the selectivity of the aryl amide series of antimicrobial oligomers, whose amphiphilic structure is stabilized by weak hydrogen bonds between the sulfur

Table 1: Antibacterial activity and selectivity.



Compound	R^1	MIC [μg mL^{-1}]		HC50 [μg mL^{-1}]	Selectivity (HC50/MIC)		Relative hydrophobicity (log K_{OW})[a]
		E. coli	*S. aureus*		*E. coli*	*S. aureus*	
1	H	12.5	50	12	0.96	0.24	3.51
2		6.25	12	40	6.4	3.3	3.12
3		6.25	6.25	9	1.4	1.4	3.74
4		6.25	6.25	7	1.1	1.1	3.86
5		25	50	790	32	16	1.45
6		25	100	1230[b]	49	12	2.99
7		50		370	7.4		0.33
8		6.25	12.5	715	110	57	−1.71
MSI-78		12.5		120	9.6		

[a] K_{OW} = *n*-octanol/water partition coefficient. [b] The HC50 value (concentration required for 50% cell lysis) was obtained by extrapolating the fitted curve to 50% lysis.

atoms and neighboring amide groups.[5,19] Amino acids appeared to be good candidates for appendage to this template; their amino groups introduce an additional positively charged center, while their side chains provide a ready source of diversity. Examination of a small collection of amino acids revealed two means of increasing the activity of the peptide: Compounds **2**–**4**, which have increasingly hydrophobic substituents, show good activity against both Gram-negative and Gram-positive bacteria (minimal inhibitory concentration (MIC) 6–12 μg mL^{-1} against both *Escherichia coli* and *Staphylococcus aureus*; Table 1). However, these compounds are also toxic towards human red blood cells, and the toxicity increases as a function of the hydrophobicity of the side chain. By contrast, the introduction of more polar substituents to give **5**–**8** led to oligomers with significantly lower toxicity towards erythrocytes. Compound **8**, which contains the dibasic Arg substituent, was the most active of this series. This compound displays antibacterial activity similar to that of the potent magainin analogue MSI-78,[8] and has significantly greater selectivity. Encouraged by these results, we introduced an additional polar, positively charged aminoalkyl substituent onto the central isophthaloyl group. This substitution enhanced the selectivity of the compounds without greatly altering their potency, irrespective of the amino acid component present (Table 2). The Arg-containing analogue **12** did not lyse red blood cells at concentrations as high as 800 μM.

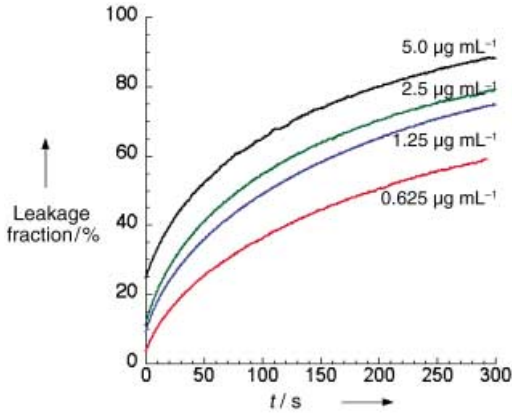
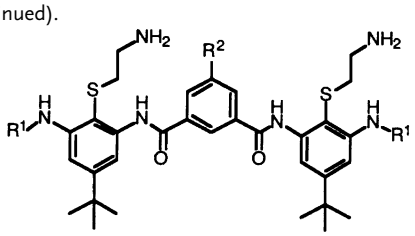
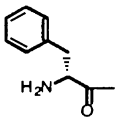
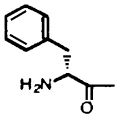
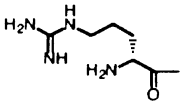
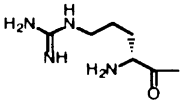


Figure 1. Amphiphilic oligomer **8** induces vesicle leakage at the oligomer concentrations indicated.

To confirm the mechanism of action of this series of compounds, we examined the ability of **8** to induce leakage of the fluorescent dye calcein from large unilamellar vesicles composed of 90% stearolyloleoyl-phosphatidylcholine and 10% stearolyloleoyl-phosphatidylserine.[14] Lysis occurred in a concentration-dependent manner and began at oligomer

Table 2: Antibacterial activity and selectivity (continued).



Compound	R^1	R^2	MIC [μg mL^{-1}] *E. coli*	MIC [μg mL^{-1}] *S. aureus*	HC50 [μg mL^{-1}]	Selectivity (HC50/MIC) *E. coli*	Selectivity (HC50/MIC) *S. aureus*	Relative hydrophobicity (log K_{OW})
1	H	H	12.5	50	12	0.96	0.24	3.51
9	H		25	25	110[a]	4.4	4.4	2.61
10			50	200	400	8.0	2.0	1.53
3		H	6.25	6.25	9	1.4	1.4	3.74
11			12.5	12.5	61	4.9	4.9	2.84
8		H	6.25	12.5	715	110	57	−1.71
12			12.5	12.5	>800	>64	>64	−2.61
MSI-78			12.5		120	9.6		

[a] The HC50 value was obtained by extrapolating the fitted curve to 50% lysis.

concentrations as low as 0.625 μg mL^{-1}. At oligomer concentrations near the MIC value for inhibition of bacterial growth, the vesicle leakage is close to 90% after 300 s (Figure 1).

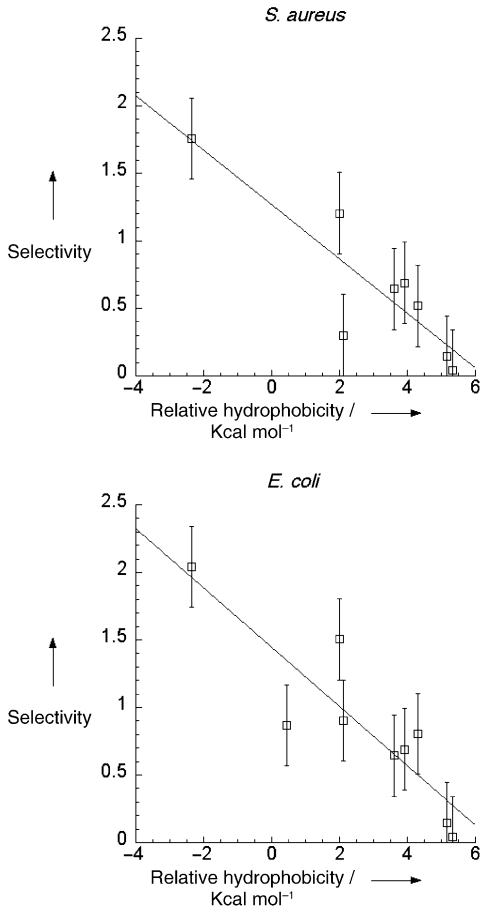


Figure 2. Relative hydrophobicity (measured as $\log K_{OW}$) and selectivity (HC50/MIC) of the aryl amides.

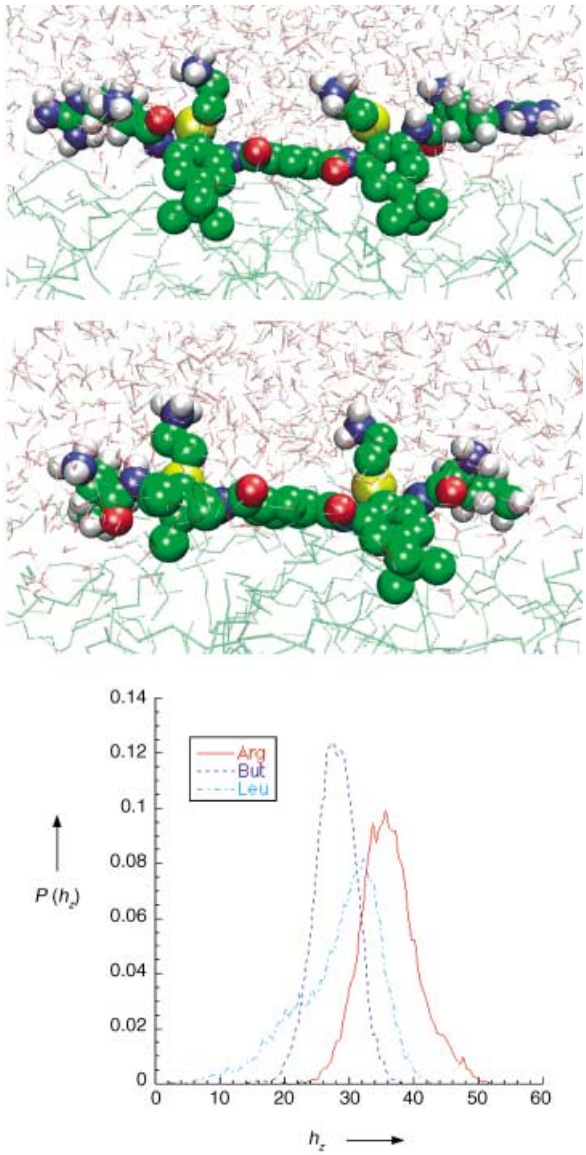


Figure 3. Snapshots showing aryl amide with D-Arg (**8**, top) and D-Leu (**2**, middle) end groups at the *n*-octane/water interface; hydrophobic moment distributions for **2**, **5**, and **8** (bottom).

The finding that the amino acid side chain influences the selectivity of the compounds prompted us to examine the relationship between their selectivity ratios (the ratio of the MIC value for a given bacteria to the HC50 value (concentration required for 50% cell lysis) of the compound) and their hydrophobicities. The *n*-octanol/water partition coefficient K_{OW} is a common measure of the hydrophobicity of a molecule and has been successfully used in quantitative structure–activity relationship studies to correlate and predict a plethora of solute properties, including toxicity.[20,21] We chose to use the atom/fragment approach[22] to estimate the value of K_{OW} since the members of this group of oligo(aryl amide)s are structurally similar and all share a rigid and open backbone. Figure 2 shows that the logarithm of the selectivity ratio varies linearly with $\log K_{OW}$. This simple measure thus provides a rapid method to screen for safe compounds. However, although this parameter correlates well with selectivity, it does not explain the antimicrobial potency: the least hydrophobic Arg-containing compound has an antimicrobial activity comparable to that of the most hydrophobic compound.

Calculations of the hydrophobic moment, however, provide an explanation. The atom/fragment approach[22] allows a straightforward definition of the hydrophobic moment.[23] The hydrophobic moment distributions of a few selected aryl amides at an *n*-octane/water interface at 300 K obtained from extensive molecular dynamics simulations of these aryl amides (5–10 ns in each case) are plotted in Figure 3. From the conformational ensembles it is clear that the Arg-containing compound has the largest hydrophobic moment and therefore has the most amphiphilic conformations. Visual inspection of the configurations reveals that the benzene rings on the two sides to which the Arg groups are attached appear to be roughly perpendicular to the interface, with the *tert*-butyl groups sliding into the *n*-octane phase and

the polar side chains exposed in the water phase, a conformation that maximizes both polar and nonpolar interactions. We expect that such a conformation would also enable the molecule to bind favorably to the lipid bilayer/water interface. Thus, hydrophobic moment calculations provide an additional useful measure for predicting potency.

In summary, we have designed a series of oligo(aryl amide)s that have amphiphilic secondary structure. By varying the appending group so as to adjust the overall charge, hydrophobicity, and hydrophobic moment, we obtained molecules with both good activity and selectivity. The oligomers are significantly smaller than comparable antimicrobial oligomers, which might provide significant advantages in terms of tissue distribution, as well as cost of production.

Experimental Section

The synthesis of representative oligomers is described in the Supporting Information. Antibacterial assays, hemolysis assays, and vesicle leakage experiments were carried out as previously described.[5] In the atom/fragment approach,[22] the K_{OW} value is estimated by using the equation: $\log K_{OW} = \Sigma(f_i n_i) + 0.229$, where f_i is the coefficient for fragment I, and n_i is the number of times that fragment i occurs. The molecular dynamics calculations were conducted as previously described.[5] After equilibration, which consisted of short NPT (constant pressure and temperature) and NVT (constant volume and temperature) runs (ca. 100 ps each) and a longer NVE (constant energy) run (ca. 500 ps), snapshots were taken every 0.5 ps, and the hydrophobic moments of the resulting configurations were calculated by using the atom-based method of Eisenberg.[23] The hydrophobic moment is defined as $\boldsymbol{\mu}_H = \Sigma(f_i n_i \mathbf{r}_i)$, where $\mathbf{r}$ is a vector from an origin, which must be specified, to the center of fragment i. We considered only the component of $\boldsymbol{\mu}_H$ normal to the interface, and chose the origin to be in the plane (parallel to the interface) in which the *n*-octane carbon density and water oxygen density profiles cross.

Received: September 4, 2003 [Z52791]
Published Online: January 27, 2004

Keywords: amphiphiles · antimicrobial agents · aryl amides · oligomers · peptides

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