Synthetic Antimicrobial Peptidomimetics with Therapeutic Potential

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A series of synthetic antimicrobial peptidomimetics (SAMPs) have been prepared and found to be highly active against several Gram-negative and Gram-positive bacterial strains. These derivatives comprise the minimal structural requirements for cationic antimicrobial peptides and showed high selectivity for Gram-negative and/or Gram-positive bacteria compared to human red blood cells. We have found that SAMPs share many of the attractive properties of cationic antimicrobial peptides inasmuch that a representative SAMP was found to insert into the bilayers of large unilamellar vesicles, permeabilized both the outer and cytoplasmic membrane of *Escherichia coli* ML-35p, and displayed an extremely rapid bacterial killing for *Staphylococcus aureus*. However, while antimicrobial peptides are prone to proteolytic degradation, high in vitro stability in human blood plasma was shown for SAMPs. A combination of high antibacterial activity against methicillin-resistant staphylococci and low toxicity against human erythrocytes makes these molecules promising candidates for novel antibacterial therapeutics.

Introduction

The number of infections caused by bacterial strains resistant to conventional antibiotics has been rising steadily, and infections caused by methicillin resistant *Staphylococcus aureus* (MRSA,[a] also denoted multidrug-resistant *S. aureus*) previously only associated with hospitalized patients have started to spread into community-acquired infections.[1] A recent report on the in vivo evolution of multidrug resistance in *S. aureus* revealed the passage of a mere 2.5 months from the initial treatment of an MRSA infection with vancomycin to the identification of isolates with a considerably decreased susceptibility.[2] The resistance resulted from 18 sequential point mutations, also leading to the development of resistance toward daptomycin, which had never been administered to the patient.[2]

As a consequence of resistance development, the search for novel antibiotics has attracted an enormous effort in the pharmaceutical industry and by research groups in academia.[3] In recent years, several cationic antimicrobial peptides (CAPs) have been investigated clinically as potential future antibiotics. Antimicrobial peptides (AMPs) are widespread in nature and have been isolated from essentially all species investigated, ranging from insects to humans. It has been well established that these compounds play an important role in innate immunity (for reviews, see refs 4–6). At present, more than 500 ribosomally synthesized antimicrobial peptides (sometimes referred to as RAMPs) are listed in the antimicrobial peptide database (APD),[7] and although they are structurally very different, some common features can be identified. In general, CAPs are fairly large molecules, ranging from 12 to 50 residues[5] (average length in APD is 28 residues),[7] and they carry a net positive charge (average charge in APD is +4.5)[7] and contain about 50% hydrophobic residues. CAPs are proposed to act by associating with the negatively charged components of the cytoplasmic membrane of bacteria, thereby increasing the permeability to ions and solutes, events that may lead to cell death. Several mechanisms have been proposed to describe the peptide−lipid interactions,[8–12] but it is still a matter of controversy whether the final killing event of CAPs is due to a damaged cytoplasmic membrane or a combination of membrane permeabilization and peptide interaction with intracellular targets.[13,14] In addition, a number of antimicrobial peptides have also been found to induce an immune response in vivo.[15] Seemingly, CAPs utilize an array of means to kill bacteria, and in addition, CAPs show an unusually broad antimicrobial activity spectrum (for a recent review, see ref 16). Thus, CAPs constitute an attractive class of compounds for further development of novel therapeutics against infections caused by bacterial strains resistant to the antibiotics of today.[4] In spite of all their attractive features, few (if any) CAPs have made it through clinical trials. This may at least partly be connected to the poor drugability of large peptides.

For a number of years our research group has focused on the development of extremely short CAPs (for a recent review, see ref 17). In our endeavors we have elucidated a pharmacophore for short cationic antimicrobial peptides that is surprisingly small.[18] For antistaphylococcal activity the presence of two cationic units and two lipohilic and bulky units approximately the size of a phenyl group is required. We have previously shown that replacing tryptophan residues with large noncoded aromatic amino acids can result in a dramatic increase in antibacterial activity,[19–23] and in this respect β-(2,5,7-tri-*tert*-

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[a] Abbreviations: ATCC, American Type Culture Collection; CAP, cationic antimicrobial peptide; CCUG, culture collection of the University of Gothenburg; CENTA, (6*R*,7*R*)-3-[[(3-carboxy-4-nitrophenyl)thio]methyl]-8-oxo-7-[(2-thienyl-acetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; GISA, glycopeptide intermediate resistant *S. aureus*; LUV; large unilamellar vesicles; MIC, minimal inhibitory concentration; MLV, multilamellar vesicles; MRSA, methicillin resistant *S. aureus*; MRSE, methicillin resistant *S. epidermidis*; ONPG, 2-nitrophenyl-β-D-galactopyranoside; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocolin; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; RP-HPLC, reversed phase high performance liquid chromatography; SAMP, synthetic antimicrobial peptidomimetic; Tbt, β-(2,5,7-tri-*tert*-butylindol-3-yl)alanine.

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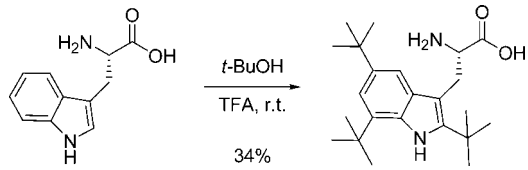
Scheme 1. Alkylation of Trp To Produce Tbt[25]



butylindol-3-yl)alanine[24,25] (Tbt; see Scheme 1) has proven to be especially useful.

In a drug development perspective, it is vital that most of the attractive properties of CAPs are still contained in our series of extremely short synthetic CAPs. Even though these molecules contain amide bonds, they can hardly be regarded as antimicrobial peptides in the classical sense. In consequence of this we propose to use the term synthetic antimicrobial peptidomimetics (SAMPs) to describe this class of compounds.

Thus, the focus of the present work has been to establish whether SAMPs share the same properties as CAPs. The interaction with large unilamellar vesicles (LUVs) of different lipid composition has been used as a model system for an initial investigation of the bactericidal mechanism. The time-course of bacterial killing and the effects of a representative SAMP on the integrity of the outer and cytoplasmic membranes of *E. coli* ML-35p have also been investigated. To further assess their potential as novel antibiotics, in vitro toxicity has been assessed by measuring the lytic activity against human red blood cells, antibacterial activity against a range of Gram-positive and Gram-negative bacteria has been measured, and proteolytic stability in human blood plasma, which is an inherent problem for CAPs and naturally occurring peptides, has been investigated for selected SAMPs.

Results

Fluoresence Spectroscopy. In order to shed some light on the bactericidal mechanism of SAMPs, fluorescence emission spectroscopy was used to study the interactions of RTbtR-OMe **7** (Figure 1) with large unilamellar vesicles of different lipid composition (Figure 2). The liposomes were prepared using only negatively charged phospholipids with a phospoglycerol headgroup (POPG), only zwitterionic phosphlipids with a phosphocolin headgroup (POPC), or a mixture of the two phospholipids. An inactive tripeptide, RWR-OMe was also included in order to investigate whether active and inactive tripeptides show different peptide−lipid interactions.

The excitation maxima for RWR-OMe (inactive peptide) and RTbtR-OMe **7** were found to be 291 and 282 nm, respectively, and both compounds showed fluorescence emission in buffer with maxima at 355 and 353 nm, respectively. The fluorescence emission maximum of RTbtR-OMe **7** was blue-shifted in the presence of POPC, POPG, and POPC/POPG (3:1) vesicles (Figure 2A), commonly associated with the embedding of tryptophan residues into the hydrophobic environment of the phospholipid bilayers.[26] RWR-OMe demonstrated a 14 nm blue shift in fluorescence emission when the LUVs contained only negatively charged phospholipid head-groups (Figure 2B), whereas a 28 nm blue shift was observed for RTbtR-OMe **7** in POPG relative to buffer. RWR-OMe displayed a 7 nm blue shift in POPC/POPG (3:1), but no change in fluorescence emission maximum was detected in the presence of liposomes composed solely of POPC. RTbtR-OMe **7** displayed 26 and 12 nm blue shifts in the fluorescence emission maximum in these liposome systems, respectively.

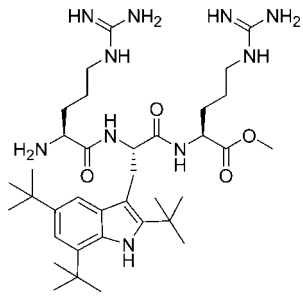


Figure 1. Structure of tripeptide methyl ester RTbtR-OMe (**7**).

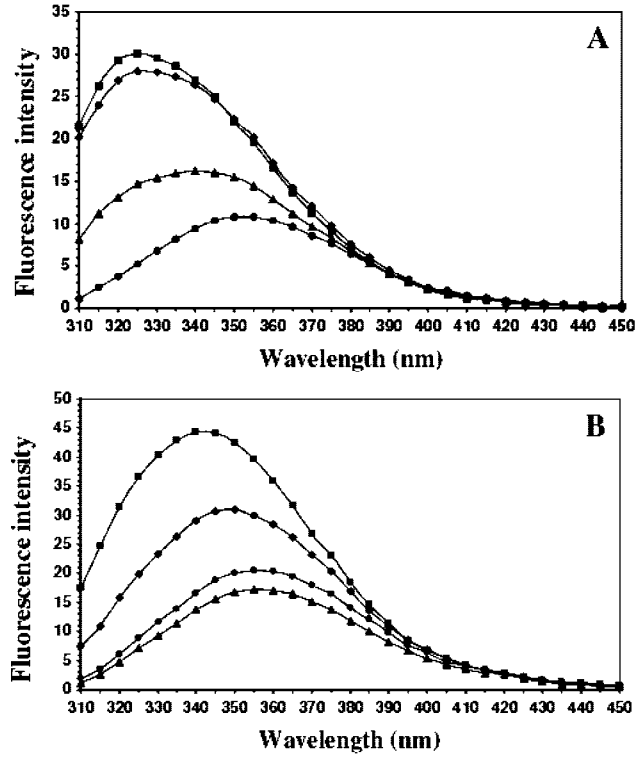


Figure 2. Fluorescence spectra of RTbtR-OMe (A) and RWR-OMe (B) recorded in buffer (●), POPC (▲), POPC/POPG (3:1) (◆), and POPG (■).

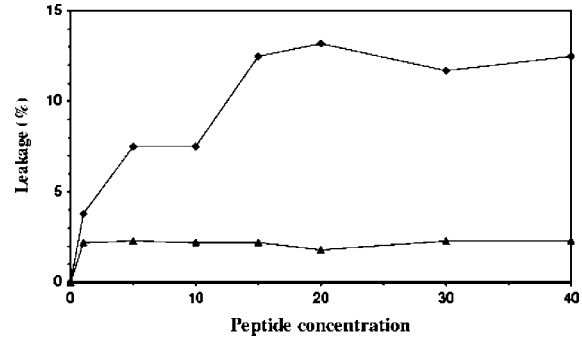


Figure 3. Leakage of calcein from POPG vesicles induced by RTbtR-OMe (◆) and RWR-OMe (▲). Peptide concentration is in μg/mL. Concentration of liposomes is 0.3 mg/mL.

Calcein Release. In order to measure membrane permeabilization caused by the peptides, we examined the induced leakage of calcein by RTbtR-OMe **7** from vesicles of various lipid compositions (Figure 3). A moderate, yet concentration dependent leakage of calcein from POPG vesicles induced by RTbtR-OMe **7** was observed, whereas RWR-OMe did not display any significant leakage (Figure 3). No leakage of calcein

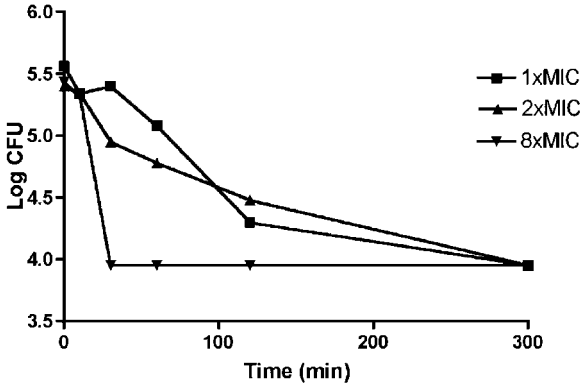


Figure 4. Bacterial kill-kinetics for **7** against *S. aureus*.

from POPC vesicles was observed upon exposure to either RWR-OMe or RTbtR-OMe **7**.

Bacterial Kill-Kinetics. Exposure of *S. aureus* cultures to SAMP **7** at concentrations corresponding to 1× and 2× the measured MIC values gave a slow onset of antibacterial activity and the minimum cfu/mL was read after 300 min (Figure 4). At a peptide concentration corresponding to 8× the MIC value (Figure 4), a rapid onset of bacterial killing was observed and after 30 min the number of bacteria had dropped from 10^6 to 10^4 cfu/mL.

***E. coli* Membrane Destabilization.** In order to explore further the ability of SAMP **7** to cause membrane destabilization, its action on *E. coli* ML-35p bacteria was investigated. For the ML-35p strain of *E. coli* the integrity of the outer and the cytoplasmic membrane could be assessed directly by measuring the amount of substrate hydrolysis for the periplasmic enzyme β-lactamase and the cytoplasmic enzyme β-galactosidase, respectively.[27] It was found that outer membrane and inner membrane integrity was lost at a SAMP **7** concentrations of 128 and 256 μg/mL, respectively, whereas the MIC of SAMP **7** against *E. coli* ML-35p was found to be 32 μg/mL.

Peptide Hydrophobicity. The retention times obtained from RP-HPLC analyses of the peptides are compiled in Table 1. All peptides were analyzed in triplicate, and the deviation in measured retention time was less than 0.15 min between the parallels. The dipeptides were found to have a higher effective hydrophobicity, i.e., displayed longer retention times than the corresponding tripeptide derivatives, and di- and tripeptides containing an unsubstituted C-terminal amide were found to be less hydrophobic than the corresponding peptide methyl esters. Furthermore, the peptide derivatives with a C-terminal benzylamide were found to be the most hydrophobic.

To further investigate the configurational dependence of the measured hydrophobicity of diastereoisomeric dipeptides **2** and **3** and diastereoisomeric tripeptides **7** and **8**, the hydrophobicity parameter log k_w was determined for these four peptides.[28] From the retention times obtained from analyses of peptides at various isocratic gradients a series of log k values were calculated for each peptide in the different mobile phase compositions. An extrapolation to 100% water gave the log k_w values (data not shown). Also here both di- and tripeptides containing the L-enantiomer of Tbt were found to have higher log k_w values than their diastereoisomers, i.e., displayed higher effective hydrophobicity.

Antibacterial Activity. The antibacterial activities of the peptides against the Gram-negative *E. coli* and *P. aeruginosa* and the Gram-positive *S. aureus*, the two methicillin resistant staphylococci strains (MRSA and MRSE), and the *S. aureus* strain intermediate resistant to vancomycin (GISA) are shown in Table 1. The antibacterial activity of compounds **1**, **2**, and **4** against *E. coli* and *S. aureus* has been reported previously,[22] and even though they were found to be overall less active than the tripeptides, the most active dipeptides showed MIC values as low as 2.5−5 μg/mL against the various staphylococci strains. The dipeptides showed higher antibacterial activity against the Gram-positive strains than against the Gram-negative bacteria. Moreover, the dipeptide benzylamide derivative TbtR-NHBn **4** was found to be the overall most active dipeptide, and notably this dipeptide derivative displayed MIC values of 10 and 7.5 μg/mL against *E. coli* and *P. aeruginosa*, respectively. The tripeptides were also found to be somewhat more active against the methicillin resistant strains than against the normal *S. aureus*. Furthermore, the MRSE strain was found to be the most susceptible bacterium to all peptides, of which both SAMPs RTbtR-OMe **7** and RTbtR-NHBn **9** displayed MIC values of 1.0 μg/mL. The tripeptide derivatives were also active against *E. coli* and *P. aeruginosa*, although the MIC values were considerably higher against these Gram-negative bacteria than those observed against the Gram-positive strains. As was found for the dipeptides, the benzylamide derivative had the highest antibacterial activity of the tripeptides, and RTbtR-NHBn **9** showed MIC values between 7.5 and 1.0 μg/mL against the different bacterial strains.

Hemolytic Activity. The concentrations of the tested di- and tripeptides that induced 50% hemolysis of human erythrocytes (EC_{50}) were found to range between 60 to above 1000 μg/mL and are compiled in Table 1. The dipeptides displayed the lowest EC_{50} values, and among the tripeptide derivatives, SAMP **9** was the most hemolytic.

Plasma Stability. Incubation of SAMP **7** with human blood plasma resulted in a degradation of the peptide to produce one main metabolite. After treatment of RTbtR-OMe **7** with an excess of LiOH in water, the obtained tripeptide carboxylic acid was found to coelute with the metabolite. The half-life ($t_{1/2}$) of RTbtR-OMe **7** was found to be between 2 and 4 h. In contrast, RTbtR-NH_2 **5** and RTbtR-NHBn **9**did not show any degradation, even after incubation for up to 4 days. No attempts at assessing if any enzymes were still active after 4 days were performed.

Discussion

In a previous study we have shown that tetrapeptides composed of tryptophan and arginine are efficient antibacterials[18] and that these peptides constitute the lower limit as to how short antimicrobial peptides composed of only naturally occurring amino acids can be made. In the present study we report the high antibacterial activity of a series of synthetic antimicrobial peptidomimetics (SAMPs), which have been prepared by further elaboration of our previously reported pharmacophore[18] for antimicrobial peptides. In earlier communications we have identified a supertryptophan residue, β-(2,5,7-tri-*tert*-butylindol-3-yl)alanine[24,25] (Tbt; see Scheme 1) that when combined with arginine derivatives gave rise to highly active antimicrobial dipeptide derivatives.[22] Attachment of a second cationic amino acid to produce derivatives containing three cationic charges and a superbulky moiety led not only to a further increase in antibiotic potency but also to a decrease in hemolytic activity (see Table 1).

The overall cationic charge of CAPs is a requisite for their efficient electrostatic interaction with the negatively charged bacterial surface and thereby also their antimicrobial activity. Thus, to eliminate the counterproductive negative charge[17] on the C-terminal and to ensure a high net positive charge of the SAMPs of this study, we decided to mask the carboxylic acid

Table 1. Antibacterial and Hemolytic Activity of SAMPs

SAMP[a]		minimal inhibitory concentration (MIC)[b]						hemolysis[c] (EC_{50})	t_R (min)[d]
		E. coli	*P. aeruginosa*	*S. aureus*	MRSA	MRSE	GISA		
TbtR-NH_2	1	Na[e]	30 (57)	15 (28)	15 (28)	7.5 (14)	30 (57)	305 (578)	13.85
TbtR-OMe	2	30 (55)	30 (55)	5 (9.2)	5 (9.2)	2.5 (4.6)	7.5 (14)	245 (451)	19.15
TbtR-OMe	3	Na[e]	50 (92)	15 (28)	15 (28)	7.5 (14)	30 (55)	273 (503)	13.45
TbtR-NHBn	4	10 (16)	7.5 (12)	2.5 (4.0)	2.5 (4.0)	2.5 (4.0)	5 (8.1)	60 (97)	23.88
RTbtR-NH_2	5	60 (88)	30 (44)	7.5 (11)	7.5 (11)	2.5 (3.7)	15 (22)	Nh[f]	11.05
KTbtR-NH_2	6	70 (107)	50 (76)	15 (23)	15 (23)	2.5 (3.8)	30 (46)	Nh[f]	10.11
RTbtR-OMe	7	20 (29)	15 (21)	5 (7.2)	2.5 (3.6)	1.0 (1.4)	7.5 (11)	705 (1008)	15.69
RTbtR-OMe	8	50 (72)	40 (57)	5 (7.2)	5 (7.2)	2.5 (3.6)	15 (21)	Nh[f]	9.97
RTbtR-NHBn	9	7.5 (9.7)	5 (6.5)	2.5 (3.2)	2.5 (3.2)	1.0 (1.3)	2.5 (3.2)	90 (116)	19.52

[a] Underlined residues are of D-configuration. [b] Minimal inhibitory concentration of peptides in μg/mL; values in parentheses are in μM. [c] Peptide concentration that induces 50% hemolysis of human erythrocytes, in μg/mL; values in parentheses are in μM. [d] Hydrophobicity measured as retention time (t_R) on a linear 1% acetonitrile/min gradient. [e] Na: no antibacterial activity within concentration range, i.e. up to 150 μg/mL. [f] Nh: no hemolytic activity within concentration range, i.e. up to 1000 μg/mL.

as a primary amide or as a methyl ester. We have previously found that the C-terminal offers an excellent attachment point for modulation of hydrophobicity, and with this in mind a benzylamide derivative was also included.[18]

The mode of bacterial killing of CAPs is generally believed to involve the bacterial membranes, and in a recent review the mode of membrane permeabilization was collectively denoted the Shai−Matsuzaki−Huang mechanism.[4] However, the effects imposed on various membrane systems by CAPs are vastly different, and intracellular targets have been established as possible targets for antibiotic action (for a recent review, see ref 29). There has yet to be established a consensus over the mechanism of action, and most likely this varies among different peptides, some acting solely by membrane disruption and others by several mechanisms also involving immuno modulator properties.[15,30]

To determine if peptide−lipid interactions also are crucial for the antibiotic activity of SAMPs, the interactions of a representative SAMP (RTbtR-OMe **7**) with large unilamellar vesicles, a liposome system that can be made to mimic the properties of various phospholipid bilayers, were investigated. For comparison an inactive tripeptide was also included in these studies. Both compounds demonstrated a blue-shifted fluorescence maximum in the presence of the negatively charged POPG vesicles compared to buffer alone, an effect that is commonly associated with the embedding of the tryptophan residues into the bilayers.[26] The SAMP had a higher propensity to partition into phospholipid bilayers than the inactive peptide, and the inactive peptide did not associate with the zwitterionic POPC membranes, whereas RTbtR-OMe **7** did. The association to POPC is presumably a hydrophobic effect, as it has been shown that association to zwitterionic membranes compared to negatively charged membranes is more governed by the lipophilicity of peptides than their charge.[31] The higher blue shift of the Tbt-containing SAMP compared with the inactive tripeptide containing tryptophan in all liposome systems indicated an overall higher ability of the SAMP to associate to phospholipid membranes, a feature that is essential for membrane active peptide. However, for the POPC model system, a higher fraction of the SAMP apparently remains unbound in the buffer solution. Furthermore, the higher blue shift of Tbt in POPG and POPC/POPG (3:1) compared to POPC suggests a deeper burial of Tbt than tryptophan into membranes containing negatively charged phospholipids. A deeper location of the Tbt residue would lead to a more effective disruption of the phospholipid packing, and furthermore, since the side chain of Tbt is 2.5 times larger than indole itself,[20] this may be an explanation of why derivatives containing Tbt were antibacterial whereas the corresponding short peptides containing tryptophan did not display any antibacterial activity (vide infra).

Liposomes can also be used as a model system to study the potential permeability the exposure to CAPs can induce in a phospholipid bilayer. The encapsulation of fluorescent dyes into liposomes and the subsequent measurement of their release as a function of CAP concentration may be used for this purpose. In our case, we found that RWR-OMe was not able to induce leakage of the fluorescent dye calcein from POPG vesicles, which indicated that an association of the peptide to the membrane is not enough to affect membrane permeabilization. On the other hand, SAMP **7** was found to induce a concentration dependent, albeit relatively low, release of calcein from the negatively charged liposomes. Seemingly, the presence of only one tryptophan residue in a peptide is not sufficient to induce membrane destabilization, whereas the presence of a Tbt residue fulfills the structural requirements (i.e., provides hydrophobic bulk) previously identified as a pharmacophore for antibacterial activity and renders the SAMPs sufficiently hydrophobic to induce destabilization of the vesicle bilayer. It should be noted that RTbtR-OMe **7** and calcein are of comparable size, and this may diminish the ability of the SAMP to render the liposomes permeable to the fluorescence probe. As could be expected from the low ability to interact with POPC liposomes, no leakage of calcein from the zwitterionic POPC vesicles was observed by treatment with RTbtR-OMe **7**, indicating a selective action on negatively charged bacteria-like membranes compared to zwitterionic mammalian-like membranes.

One of the attractive features of CAPs as a novel class of antibiotics is their fast bacterial killing kinetics, which in most cases is superior to commercially available antibiotics. To establish whether this ability also is inherent to SAMPs, the time-course of bacterial killing was studied by exposing *S. aureus* to various concentrations of derivative **7** (Figure 4).

At 8 MIC, which is a therapeutically relevant concentration, most of the bacteria were killed within 30 min, an extremely rapid bactericidal effect often also seen for CAPs.[32,33] The fast bacterial killing suggests that at this concentration the antibacterial effect is mediated through a significant permeabilization or lysis of the bacterial membranes.[33] Killing of the bacteria through the interaction of SAMPs with intracellular targets would lead to a much slower onset, and consequently intracellular targets cannot be reached before the bacteria have disintegrated. It should be noted that some extent of membrane permeabilization is required for the compounds to reach additional intracellular targets, and for CAPs the kinetics of bacterial killing and the rate of membrane permeabilization are not necessarily well correlated.[32] At 2 MIC and 1 MIC the

maximum bacterial killing is not achieved before 300 min have passed. Clearly, at high concentrations, SAMPs share the exceptionally fast bacterial killing kinetics of CAPs, but whether SAMPs also can benefit from the multitarget mechanism of action of many CAPs remains to be elucidated.

The mechanism of action for CAPs in many cases involves a direct destabilizing effect on the bacterial membranes,[34] and the ability of SAMPs to interact with phospholipid membranes was demonstrated in the liposome studies (vide supra). In order to gain some insight into the interaction with bacterial membranes, the membrane integrity of a strain of *E. coli* when exposed to SAMP **7** was investigated. With an intact outer membrane, bacteria of the *E. coli* ML-35p strain are only able to cause a minimal β-lactamase induced hydrolysis of the chromogenic cephalosporin CENTA and are also unable to hydrolyze 2-nitrophenyl β-D-galactopyranoside (ONPG), which is a substrate for the cytoplasmic β-galactosidase enzyme.[27] We found that outer and inner membrane integrity was lost at a SAMP **7** concentration of 128 and 256 μg/mL, respectively, indicating that SAMPs may have a direct bacterial killing effect through membrane lysis at high concentrations. The relatively large difference in SAMP concentration required for bacterial killing and for loss of membrane integrity may indicate that at concentrations closer to the MIC value mechanisms other than membrane permeabilization contribute to the antimicrobial activity. On the contrary, the bacterial membranes may well be permeable enough to cause a lethal effect at concentrations lower than those where efficient hydrolysis of the chromogenic substrates was observed.

Clearly the super-tryptophan residue Tbt confers high antibacterial activity for both the di- and tripeptidomimetics (Table 1) and fulfills the lipophilic bulk requirements of the pharmacophore by itself. By comparison, the tryptophan analogues of peptides **2** and **7** (see Figure 1), WR-OMe and RWR-OMe, both showed MIC values above 500 μg/mL against *E. coli* and *S. aureus* (data not shown).

All the SAMPs prepared in this study were found to be equally active against the MRSA strain and the normal *S. aureus*. The MRSE strain proved to be the most susceptible bacterium, and the GISA strain was somewhat less sensitive the SAMPs. Normally bacterial resistance against vancomycin is caused by a change in the peptide side chain of the peptidoglycan, which in resistant strains ends in D-Ala-D-Lac rather than the D-Ala-D-Ala, an alteration that makes the binding affinity of vancomycin a 1000-fold lower (for a review, see ref 35). This reprogramming of the vancomycin target is not expected to influence the antibacterial activity of the SAMPs against this bacterium. Apparently, the difference in the lowest obtained MIC value against each of the four staphylococci strains stems from susceptibility variations between the bacteria rather than being connected to antibiotic resistance.

Most of the antibacterial active SAMPs were found to be bactericidal, as the minimal bactericidal concentrations (MBC) generally were found to be identical to or one titer-step higher than the MIC values obtained (data not shown). However, in some cases and mainly for derivatives displaying relatively high MIC values, the MBC values against the Gram-negative bacteria *E. coli* and *P. aeruginosa* were several titer-steps higher than the MIC values. Whether the additional barrier afforded by the outer membrane of Gram-negative bacteria causes this difference or if this variance is inherent in the method for determination of antibacterial activity remains to be elucidated. In addition both di- and tripeptides generally showed higher antibacterial activity against the Gram-positive strains than against the Gram-negative bacteria, presumably because of the additional barrier afforded by the outer membrane of *E. coli* and *P. aeruginosa*.

In general, the tripeptidomimetics showed higher antibiotic potency relative to the corresponding dipeptidomimetics, which is most likely attributable to their higher net positive charge. Higher net charge is expected to result in a stronger initial interaction of the SAMPs with bacterial surfaces, which is a crucial step in the bacterial killing mechanism. We have chosen to use arginine as the cationic residue for construction of highly active SAMPs because this amino acid is preferred over lysine,[36] possibly because of the more dispersed positive charge of the guanidine compared to the amine and the possibility of multiple hydrogen bond formation of the former.[26] As expected, SAMP **6** containing a N-terminal lysine residue showed lower antibacterial activity than SAMP **5** containing a N-terminal arginine. Interestingly, the lysine containing SAMP is also less hydrophobic (see Table 1).

To evaluate the in vitro toxicity of the SAMPs, their hemolytic activity against human red blood cells was measured (Table 1). In general, tripeptidomimetic derivatives were found to be overall less hemolytic than the dipeptidomimetic derivatives. It thus seems that a higher net positive charge disfavors interaction with the zwitterionic membranes of erythrocytes. The ability of CAPs to discriminate between bacterial surfaces and the surfaces of our own cells is determined by the overall hydrophobicity of the peptides, and highly hydrophobic CAPs are often found to be toxic also to human cells. This was also found to be the case for the SAMPs presented in this study and was in accordance with Kondejewski et al.,[37] who also reported a correlation between hydrophobicity and hemolytic activity.

The two sets of diastereomeric compounds, SAMPs **2** and **3** and SAMPs **7** and **8**, showed different biological activity (Table 1), and changing the chirality of the Tbt residue dramatically affected the RP-HPLC retention times.[38] Also for longer CAPs it has been shown that introduction of D-amino acids can alter the hydrophobicity and biological activity as a result of the structural changes induced.[31] We have also recently reported a good correlation between the antibacterial activity and RP-HPLC retention times for tripeptide derivatives containing novel biaryl amino acids.[23] A similar hydrophobicity−antibacterial activity relationship was found for all bacterial strains. Furthermore, the least hydrophobic diastereoisomers of TbtR-OMe **2** and TbtR-OMe **3** and of RTbtR-OMe **7** and RTbtR-OMe **8** were also found be less hemolytic. Apparently, also for SAMPs the different configuration of the diastereoisomers influences both their interactions with the C_{18}-matrix of the RP-HPLC column and their interactions with bacteria and human erythrocytes. Moreover, it is noteworthy that the MIC and EC_{50} values are differently affected by configurational changes in the SAMPs, an observation that opens up the possibility of fine-tuning the biological activity by manipulating the configuration.

Most naturally occurring antimicrobial peptides are rather large molecules, and therefore, many do not have the physiochemical properties that are desirable for new therapeutics. Peptides are also prone to enzymatic degradation in vivo and thus may require encapsulation for systemic use; alternatively, use of antimicrobial peptides may be limited to topical indications. One class of antimicrobial peptides that shows promising proteolytic stability are the cyclic D,L-α-peptides, which are proposed to assemble into tubular structures upon interaction with bacterial cell membranes.[39] Presumably, this unique architecture and the alternating D- and L-amino acids provide the stability of these peptides in vivo. In addition, aggregation of peptide monomers is also reported to increase the stability

against proteases of other antimicrobial peptides.[31] In any case, the in vivo biological activity is often affected by proteolytic hydrolysis, and this is an issue that needs to be resolved before these compounds can enter the clinic.[30] In this respect, a stability study of SAMPs **5**, **7**, and **9** in human blood plasma was undertaken in order to assess their potential as novel drug candidates. Incubation of RTbtR-OMe **7** with human blood plasma resulted in degradation producing one main metabolite, RTbtR-OH; thus, it would appear that the SAMP was subject to cleavage by esterases in blood plasma. The antibacterial activity of the main metabolite was found to be >100 and 75 μg/mL against *E. coli* and *S. aureus*, respectively (data not shown); i.e., metabolism of RTbtR-OMe **7** renders it considerably less active. Even though the carboxylic acid derivative has the same net positive charge as the dipeptidomimetics, the antibacterial activity was considerably lower. The negative charge of the carboxylate seemingly disfavors interactions with bacteria, an observation that underscores the importance of a high net positive charge as a structural element in highly active SAMPs, and clearly demonstrates the importance of avoiding zwitterionic structures when designing novel SAMPs. The amide RTbtR-NH_2 **5** and the benzylamide derivative RTbtR-NHBn **9** did not show any enzymatic degradation in human blood plasma. These results clearly demonstrate that it is possible to design SAMPs that may be pharmacologically useful. The Tbt residue used in the present study has previously been found to induce proteolytic protection to peptides in which it has been incorporated.[40]

Binding to albumin, as has recently been reported for a series of related compounds,[41] will influence systemic antibacterial activity but will likely also contribute to a reduced clearance. Further studies into the pharmacokinetics of this novel class of compounds will establish their potential for clinical use; however, the ability to modulate their stability in blood plasma gives further promise to the progression of this class of compounds toward new antibacterial drugs.

Conclusions

In conclusion, we have prepared novel antibiotic compounds, SAMPs, that display high specificity toward bacteria relative to eukaryotic red blood cells and show antibacterial activity in the low micromolar range against several clinically relevant pathogens. As for cationic antimicrobial peptides, from which SAMPs have been derived through systematic investigations of structure−activity relationships, both antibacterial activity and toxicity toward human cells are modulated by hydrophobicity. Our preliminary investigations into the antibiotic mechanism suggest that peptidomimetic−lipid interactions, mediated through the presence of a supertryptophan residue, play an important role in the mechanism of action of the SAMPs reported. Our results show that the attractive features of CAPs can be condensed to produce SAMPs, compounds that can give the additional proteolytic stability that is often desired for clinical use and thus provide superior drugability compared to antimicrobial peptides. Further evaluation of these compounds in relevant biological systems will be reported in due course.

Experimental Section

Amino acids were purchased from Bachem (Bubendorf, Switzerland), and coupling reagents, DMF, DIPEA, and TFA were purchased from Fluka (Buchs, Switzerland). The β-lactamase substrate CENTA was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany), and the β-galactosidase substrate ONPG was obtained from Sigma-Aldrich (Schnelldorf, Germany).

β-(2,5,7-tri-*tert*-Butylindol-3-yl)alanine (Tbt).[25] A mixture of H-Trp-OH (19.14 g, 94 mmol) and *t*-BuOH (74.18 g, 1.0 mol) in trifluoroacetic acid (290 mL) was stirred at room temperature for 69 h (Scheme 1). The resulting dark solution was evaporated to a black oil under reduced pressure, and water (100 mL) was added. To the resulting suspension was added potassium hydrogen carbonate until the pH was neutral. Constant trituration of the oily material using a spatula during the addition of base resulted in the formation a pinkish gumlike material. The aqueous layer was decanted off, and the gumlike material was crystallized from 50% ethanol in water. The first crop of crystalline material (21.48 g) was recrystallized from 50% ethanol in water to give the title compound as a colorless crystalline solid (11.74 g, 34%). Analytical data were in accordance with literature data.[25]

Boc-Tbt-OH. H-Tbt-OH was Boc protected following a standard procedure.[42] To a solution of H-Tbt-OH (3.41 g, 9.15 mmol) in dioxane/water, 1:1 (80 mL), was added sodium hydrogen carbonate (2.34 g, 27.85 mmol), and the resulting mixture was stirred at room temperature overnight. The reaction mixture was evaporated to a solid residue under reduced pressure and separated between ethyl acetate and a 5% aqueous solution of potassium hydrogen carbonate. The aqueous layer was extracted twice more with fresh ethyl acetate, and the combined organic phases were washed with a 5% aqueous solution of potassium hydrogen carbonate and a saturated aqueous solution of sodium chloride before it was dried over anhydrous magnesium sulfate. After removal of the drying agent by filtration and evaporation under reduced pressure the title compound was isolated as a light-yellowish foam (4.14 g, 95% crude yield). The crude material was used without any further purification. Analytical data were in accordance with literature data.[43]

General Procedure for Peptide Couplings. Boc-Tbt-OH (0.474 g, 1.0 mmol), H-Arg-OMe dihydrochloride (0.276 g, 1.06 mmol) and 1-HOBt (0.165 g, 1.22 mmol) were dissolved in DMF (4 mL) in a vial. DIPEA (0.755 mL, 4.41 mmol) was added and the solution cooled in ice/water. HBTU (0.458 g, 1.21 mmol) was added, and the solution was cooled in ice/water for another 10 min before the vial was agitated on an orbital shaker for 1.5−2 h at room temperature. The reaction mixture was diluted with ethyl acetate (21 mL) and extracted with 2 × 8 mL of citric acid in aqueous sodium chloride, 2 × 8 mL of sodium bicarbonate in aqueous sodium chloride, and 20 mL of saturated aqueous sodium chloride. All extractions were performed using an automated Myriad ALLEX (Mettler-Toledo, U.K.) liquid−liquid extractor. The ethyl acetate phase was dried over $MgSO_4$, filtered, and evaporated to yield 0.63 g of a colorless solid. The Boc protecting group was removed by treatment with 10 mL of TFA−H_2O (95:5) by stirring in the dark for 2.5 h. The oil obtained after evaporation was treated with a solution of *p*-toluenesulfonic acid (2.5 equiv) in diethyl ether, and the resulting colorless solid material was triturated again before it was dried in vacuo.

Purification. Peptide crude products were either taken directly to the next coupling step or purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a C_{18}-column (Delta-Pak™ C18, 100 Å, 15 μm, 25 mm × 100 mm, Waters Corp., Milford, MA) with a mixture of water and acetonitrile (both containing 0.1% TFA) as mobile phase and UV detection at 254 nm. The homogeneity of the purified peptides was analyzed by RP-HPLC on an analytical C_{18}-column (Delta-Pak C18, 100Å, 5 μm, 3.9 mm × 150 mm, Waters Corp., Milford, MA), and all peptides were found to be >96.5% pure. Correct peptide masses (Table 1) were confirmed by positive ion electrospray ionization mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, U.K.).

H-Arg-Tbt-Arg-NH_2 (5). ^{1}H NMR (400 MHz, methanol-d_3) δ: 7.84 (1H, d, J = 8.2 Hz), 7.44 (1H, d, J = 1.5 Hz), 7.17 (1H, d, J = 1.5 Hz), 4.60 (1H, dd, J = 10.6, 5.1 Hz), 4.26−4.19 (1H, m), 4.01 (1H, app t, J = 6.1 Hz), 3.49 (1H, dd, J = 14.2, 10.6 Hz), 3.29 (3H, t, J = 7.1 Hz), 3.18−3.09 (2H, m), 2.06−1.95 (2H, m), 1.89−1.65 (3H, m), 1.56 (9H, s), 1.52 (9H, s), 1.40 (9H, s),

1.56−1.40 (3H, m). ESMS Calcd for $C_{35}H_{61}N_{11}O_3$ (average isotope composition) 683.93 [M]+. Found 684.7. HPLC: t_R = 14.51 min, 100%.

H-Lys-Tbt-Arg-OMe (6). ^{1}H NMR (400 MHz, methanol-d_3) δ: 7.81 (1H, d, J = 8.4 Hz), 7.44 (1H, d, J = 1.5 Hz), 7.17 (1H, d, J = 1.5 Hz), 4.59 (1H, dd, J = 10.8, 5.0 Hz), 4.26−4.18 (1H, m), 3.97 (1H, t, J = 6.48 Hz), 3.49 (1H, dd, J = 14.2, 10.8 Hz), 3.34−3.30 (1H, m), 3.18−3.10 (2H, m), 3.06−3.00 (2H, m), 2.03−1.89 (2H, m), 1.89−1.70 (3H, m), 1.63−1.53 (5H, m), 1.56 (9H, s), 1.52 (9H, s), 1.41 (9H, s). ESMS Calcd for $C_{35}H_{61}N_9O_3$ (average isotope composition) 655.92 $[M]^+$. Found 656.5. HPLC: t_R = 14.26 min, 99.1%.

H-Arg-Tbt-Arg-OMe (7). ^{1}H NMR (400 MHz, methanol-d_3) δ: 7.60−7.55 (1H, d, J = 8.0 Hz), 7.32 (1H, d, J = 1.2 Hz), 7.12 (1H, d, J = 1.2 Hz), 4.59 (1H, dd, J = 10.5, 5.3 Hz), 4.32−4.24 (1H, m), 4.01 (1H, app t, J = 6.1 Hz), 3.44−3.36 (1H, m), 3.40 (3H, s), 3.32−3.26 (3H, m), 3.14−3.08 (2H, m), 2.06−1.94 (2H, m), 1.88−1.65 (3H, m), 1.62−1.53 (3H, m), 1.56 (9H, s), 1.53 (9H, s), 1.40 (9H, s). ESMS Calcd for $C_{36}H_{62}N_{10}O_4$ (average isotope composition) 698.94 $[M]^+$. Found 700.0. HPLC: t_R = 15.55 min, 96.9%.

H-Arg-D-Tbt-Arg-OMe (8). ^{1}H NMR (600 MHz, H_2O/D_2O, 9:1) δ: 8.77 (1H, d, J = 7.2 Hz), 8.36 (1H, s), 7.66 (1H, d, J = 6.0 Hz), 7.14 (2H, bs), 7.08 (1H, s), 6.82−6.79 (1H, m), 6.65−6.34 (5H, bs), 4.48−4.42 (1H, m), 3.95−3.93 (1H, m), 3.70−3.67 (1H, m), 3.54 (3H, s), 3.25−3.17 (1H, m), 3.16 (1H, dd, J = 14.7, 5.1 Hz), 3.09−3.00 (2H, m), 2.72−2.66 (2H, m), 1.80−1.71 (2H, m), 1.50−1.45 (2H, m), 1.34 (9H, s), 1.31 (9H, s), 1.25−1.18 (1H, m), 1.22 (9H, s), 0.92−0.85 (1H, m), 0.73−0.65 (2H, m). ESMS Calcd for $C_{36}H_{62}N_{10}O_4$ (average isotope composition) 698.94 $[M]^+$. Found 700.0. HPLC: t_R = 14.02 min, 97.1%.

H-Arg-Tbt-Arg-NHBn (9). ^{1}H NMR (400 MHz, methanol-d_3) δ: 7.48 (1H, d, J = 1.4 Hz), 7.33−7.21 (3H, m), 7.19 (1H, d, J = 1.4 Hz), 7.08 (2H, d, J = 7.4 Hz), 4.70 (1H, dd, J = 10.1, 5.3 Hz), 4.38−4.30 (1H, m), 4.17−4.08 (1H, m), 3.99 (1H, app t, J = 5.8 Hz), 3.94−3.87 (1H, m), 3.49 (1H, dd, J = 14.4, 10.1 Hz), 3.33−3.29 (1H, m), 3.29−3.21 (2H, m), 3.18−3.05 (2H, m), 2.05−1.91 (2H, m), 1.89−1.65 (3H, m), 1.64−1.51 (3H, m), 1.58 (9H, s), 1.53 (9H, s), 1.41 (9H, s). ESMS Calcd for $C_{42}H_{67}N_{11}O_3$ (average isotope composition) 774.05 $[M]^+$. Found 774.0. HPLC: t_R = 19.52 min, 98.4%.

For analytical data on all compounds see Supporting Information.

Preparation of Liposomes. Large unilamellar vesicles (LUVs) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) ammonium salt, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Genzyme Pharmaceuticals, Switzerland), and a mixture of POPC/POPG (3:1) were prepared as previously described.[44] Briefly, a defined amount of lipid was dissolved in a chloroform/methanol solvent mixture (2:1 v/v). The solvents were evaporated in vacuo at 40 °C, forming a thin lipid film that was hydrated with a buffer comprising 10 mM Hepes, 150 mM NaCl, and 1 mM EDTA at pH 7.4. For dye release experiments, the film was hydrated with calcein buffer (25 mM calcein, 10 mM Hepes, 150 mM NaCl, 1 mM EDTA and adjusted to pH 7.4). After evaporation and rehydration, the produced liposome dispersion was frozen and thawed three times. To make LUVs by extrusion, the multilamellar vesicles (MLVs) or frozen and thawed preparations were passed 10 times through Isopore membrane filters with decreasing pore size of 0.8, 0.4, 0.2, 0.1 μm, respectively, using a miniextruder (Avestin, Ottawa, Canada). Sizes of liposomes were determined by light scattering using a Nicomp submicrometer particle sizer (Nicomp Particle Sizing System, Santa Barbara, CA). Monomodal size distribution were fitted in all cases with a diameter between and 62 and 152 nm for empty liposomes and between 150 and 166 nm for liposomes with entrapped calcein.

Fluorescence Spectroscopy. Intrinsic fluorescence of peptides in buffer solution (10 mM Hepes/150 mM NaCl, 1 mM EDTA at pH 7.4) was measured using a Perkin-Elmer luminescence fluorimeter LS-50 B. Individual emission spectra of the peptides with and without lipid vesicles were taken between 310 and 450 nm (both excitation and emission bandwidths were set to 10 nm) with a scan speed of 60 nm/s. The concentrations of the peptides and lipids were 20 μg/mL and 0.3 mg/mL, respectively. In all cases the fluorescence spectra were corrected by subtracting the residual fluorescence of the medium.

Calcein Release. Liposomes (see above) were separated from unentrapped calcein by gel filtration on a Sephadex G 50 (20 cm × 1.5 cm) column (Pharmacia Biotech AB, Sweden) eluting with calcein free buffer (see above). The lipid concentration was determined by quantitative phosphorus analysis.[45] Aliquots of liposome suspension were then diluted in calcein-free buffer to a final concentration of 50 μM lipid and incubated for 5 min with different concentrations of different peptide solutions. Calcein release from LUVs was assessed fluorometrically by measuring the increase in fluorescence intensity resulting from the decrease in the level of self-quenching of calcein (excitation at 489 nm and emission at 520 nm). The fluorescence intensity corresponding to 100% calcein release was determined by addition of a 10% solution (w/v) of Triton X-100. Relative leakage was calculated using the formula $((F_x - F_0) \times 100)/(F_t - F_0)$ where F_x is the intensity measured at a given concentration of peptide, F_0 is the intensity of the liposomes (background), and F_t is the intensity after lysis by Triton X-100.

Time−Kill Studies. *S. aureus* ATCC 25923 was grown in 2% Bacto Peptone water (Difco 1807-17-4) until exponential growth and aliquots of bacterial culture (inoculum of 10^6 cfu/mL) were exposed to peptide **7** at concentrations corresponding to 1×, 2×, and 8× the measured MIC value. The cfu/mL was monitored, and readings were taken at 0, 10, 30, 60, 120, and 300 min. Untreated bacterial culture was used as control.

***E. coli* ML-35p Membrane Integrity.** The outer and cytoplasmic membrane permeabilization assays on *E. coli* ML-35p were performed following a literature procedure with minor modifications.[27] Briefly, the assay mixtures contained 50 μL of 10 mM NaPBS buffer (pH 7.4, with and without 100 mM NaCl), 4×10^6 cfu/mL of *E. coli* ML-35p in 50μL in 10 mM NaPBA buffer (pH 7.4), either 50 μL of the β-lactamase substrate (80 μM in NaBPS buffer) or the β-galactosidase substrate (10 mM in NaPBS buffer) and 50 μL of SAMP **7** in various concentrations. After incubation at 37 °C with shaking every 10 s for 1 h, the absorbance at 420 nm was read. In the present study (6*R*,7*R*)-3-[[(3-carboxy-4-nitrophenyl)thio]methyl]-8-oxo-7-[(2-thienylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (CENTA) and 2-nitrophenyl-β-D-galactopyranoside (ONPG) were used as substrates for the β-lactamase and β-galactosidase, respectively. As control experiments, incubation without bacteria, without SAMP **7**, and with mellitin instead of SAMP **7** was performed.

Hydrophobicity. All peptides were analyzed by RP-HPLC (see above) using a linear gradient ranging from 30% to 60% acetonitrile in water (both containing 0.1% TFA) in 30 min on an analytical C_{18}-column (see above). The column temperature was set to 30 °C for all experiments and the flow rate set to 1 mL/min.

Antibacterial Activity. The bacterial strains *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 33591, methicillin resistant *Staphylococcus epidermidis* (MRSE) ATCC 27626, and glycopeptide (vancomycin) intermediate-resistant *Staphylococcus aureus* (GISA) CCUG 43316 or 43315 (peptide 4 and 9) were grown in 2% Bacto Peptone water (Difco 1807-17-4) until growth was exponential. A standard microdilution technique with an inoculum of 2×10^6 cfu/mL was used. The minimal inhibitory concentration (MIC) of the peptides was determined in 1% Bacto Peptone water after incubation overnight at 37 °C. The concentration range used for the peptides against the Gram-positive strains was 50, 30, 20, 15, 10, 7.5, 5.0, 2.5, 1.0, and 0.5 μg/mL. In addition the concentration series 150, 100, 90, 80, 70, 60, 50, 40, 30, and 20 μg/mL was used against the Gram-negative strains. All peptides were tested at least twice in parallel. Gentamicin was used as a positive control in the antibacterial assay and MIC values of 0.1−1 and <0.05 μg/mL were found for *E. coli* and *S. aureus*, respectively.

Hemolytic Activity. Freshly isolated human red blood cells (RBC), isolated as described by Dathe and co-workers,[46] were incubated for 1 h at 37 °C with 1, 50, 100, 500, and 1000 μg/mL solutions of peptides dissolved in PBS. The samples were centrifuged at 4000 rpm for 5 min before the absorbance of the supernatant was measured at 540 nm by a microtiter plate reader (Thermomax Molecular Devices, NJ). The 0% hemolysis and 100% hemolysis were determined in PBS and 1% Triton X-100, respectively. Peptide concentrations corresponding to 50% hemolysis (EC_{50}) were determined from the dose−response curves.

Plasma Stability Assay. Heparinized blood plasma (0.5 mL) and peptide (20 μL of 1.09−1.25 mg/mL stock solutions of peptide in water) were mixed and incubated at 37 °C. After different time intervals the blood samples were applied onto an Oasis extraction cartridge (Waters Corp., Milford, MA) using 1 mL of water containing 0.1% TFA. After sample application the cartridge was washed with 1 mL of water containing 0.1% TFA, 1 mL of 5% methanol in water containing 0.1% TFA and eluted with 1 mL of 40% acetonitrile in water (both containing 0.1% TFA). External standard was added directly to the eluted solutions. Samples were analyzed by RP-HPLC with UV detection at 280 nm using the same analytical column and data system as above.

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Supporting Information Available: HPLC traces, ^{1}H NMR spectra, analytical data, and structures for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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