Effect of Size and N-Terminal Residue Characteristics on Bacterial Cell Penetration and Antibacterial Activity of the Proline-Rich Peptide Bac7

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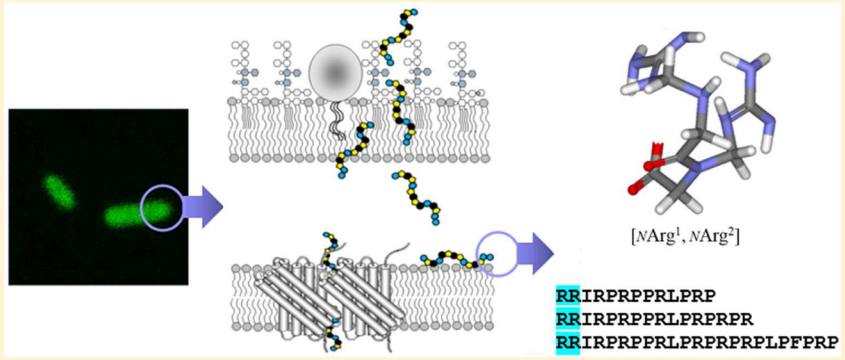
Supporting Information



ABSTRACT: Bac7 is a proline-rich antimicrobial peptide, selective for Gram-negative bacteria, which acts intracellularly after membrane translocation. Progressively shortened fragments of Bac7 allowed determining the minimal sequence required for entry and antimicrobial activity as a 16-residue, N-terminal fragment, while further shortening led to a marked decrease in both functions. Furthermore, two N-terminal arginine residues were required for efficient translocation and activity. Analogues in which these residues were omitted, or where the side chain steric or physicochemical characteristics were systematically altered, were tested on different *Escherichia coli* strains, including a mutant with a destabilized outer membrane and one lacking the relevant SbmA membrane transport protein. H-bonding capacity, stereochemistry, and charge, in that order, played a determining role for efficient transit through both the outer and cytoplasmic membranes. Our studies allowed building a more detailed model for the mode-of-action of Bac7, and confirming its potential as an anti-infective agent, also suggesting it may be a vehicle for internalization of other antibiotic cargo.

INTRODUCTION

Antimicrobial peptides (AMPs) are a fundamental component of host defense in multicellular organisms.[1] They comprise very diverse, gene-encoded, innate immune effectors that help to prevent or combat microbial infections. Most AMPs, such as the ubiquitous cationic, α-helical peptides and different types of defensins, interact with the microbial surface and act mainly by interfering with the cell wall and/or microbial membranes.[2,3] This "lytic" mechanism generally results in a quite broad spectrum of activity, and while there is some selectivity for microbial with respect to host cells primarily due to distinctive features of the respective membranes, AMPs acting in this manner are to some extent cytotoxic for eukaryotic cells.[4]

Proline-rich AMPs (PR-AMPs) do not conform to this paradigm. They comprise a varied group of linear, cationic peptides of widespread natural origin that share an uncommonly high proline content, associated with the presence of numerous arginine residues. They have a nonlytic mode-of-action that is selective for some types of Gram-negative bacteria.[5−8] Furthermore, unlike "lytic" AMPs, for which the mode of action is generally not stereospecific (i.e., all-D enantiomers tend to display similar activities to the natural all-L counterparts), all-D PR-AMPs show a significant loss of activity.[9,10] As they were shown to translocate across the bacterial membrane without damaging it,[8,9] this suggested a two-stage mechanism of action initially involving a membrane transport system and then inhibition of specific intracellular target(s), with either or both of these processes requiring stereospecific interactions. This is consistent with their quite narrow activity spectrum, as the target bacterial cell has to express specific transport system(s) and internal target(s), and also with their exceptionally low toxicity toward host cells.[8]

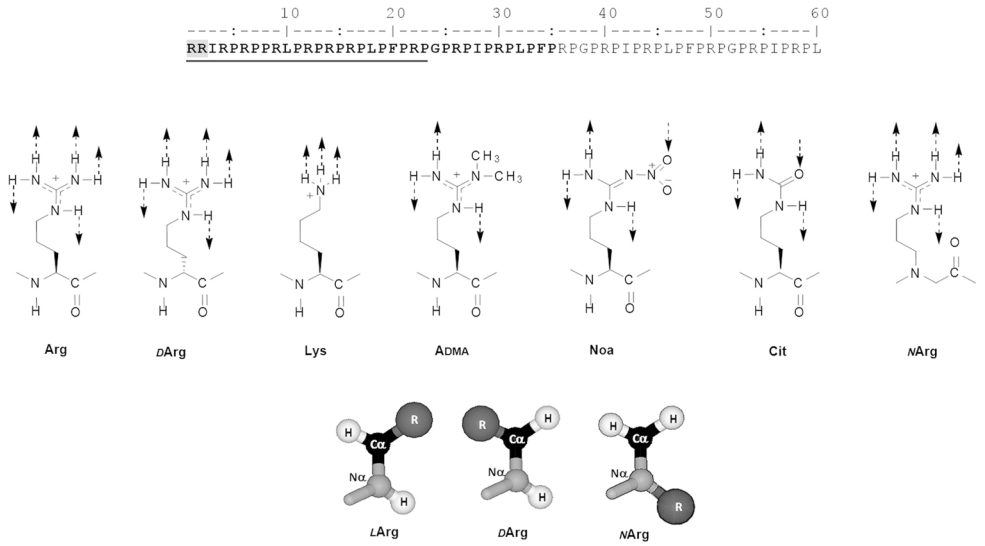


Figure 1. Sequence of Bac7 and residues used to replace Arg^1 and/or Arg^2 in Bac7(1−23). Sequence of the fully active fragment Bac7(1−35) is shown in bold in the full peptide sequence (above). N-Terminal residues that were replaced in the active fragment Bac7(1−23) (underlined) are shaded gray. The replacement residues are shown in the middle row; DArg is the D-enantiomer of arginine, ADMA is the asymmetric dimethylarginine, Noa is nitroarginine, Cit is citrulline, and *N*Arg is the *N*-(3-guanidinopropyl)glycine peptoid residue. The capacity to participate in H-bonding as donor or acceptor is indicated by the arrows. The variation in stereochemistry of the D- and L-enantiomers of arginine and of the peptoid arrangement are schematically shown in the bottom row.

PR-AMPs were among the first antimicrobial peptides to be reported in the late 1980s independently in honeybees[5] and cattle.[6] AMPs with motifs enriched in proline and arginine residues, often arranged in recurring patterns,[8] have thus evolved independently in vertebrates and invertebrates, which suggests that they represent an effective defense system with a common mode-of-action. The mammalian PR-AMPs belong to the cathelicidin family of innate immune effectors, which are defined by a conserved, cathelin-like pro-region followed by a highly variable C-terminal domain corresponding to the AMP itself.[7] Most of what is known about their mechanism of action comes from studies with overlapping peptide fragments covering the whole sequence of the natural peptides to dissect the role of different regions. One of the best-studied, bovine Bac7, could be substantially shortened from the C-terminus without significantly impairing antimicrobial activity.[9,11−13] On the other hand, residues at the N-terminus (the so-called N-cap) were important for full potency. The active domain was therefore located in the N-terminal portion of the peptide and the residue stereochemistry appeared to be important, as all-D enantiomers of active Bac7 fragments were significantly less active.[9]

A genetic approach has allowed the identification of some components of the bacterial transport system for Bac7, which in *Escherichia coli* comprises a homodimeric cytoplasmic membrane protein, SbmA, and an outer membrane lipoprotein YaiW.[14−17] All bacterial species in which the *sbmA* gene, or its homologue *bac*A, are present (Enterobacteriaceae and Alphaproteobacteria) are highly susceptible to Bac7 as well as other PR-AMPs,[18,19] while those lacking homologues, such as the Gram-negative *Pseudomonas aeruginosa* or Gram-positive *Staphylococcus aureus*, are considerably less susceptible.[8] Furthermore, deletion or mutation of either SbmA or YaiW markedly decreased the capacity of Bac7 to internalize and significantly reduced susceptibility to it. The biological roles played by these membrane proteins in *E. coli* are currently unknown, although the BacA protein is reported to be essential to establish chronic intracellular infections by Alphaproteobacteria.[20]

The question remained as to whether the structural requirements for Bac7 activity are dictated principally by its translocation through bacterial membranes, or by its interaction with internal targets, or in some measure by both these processes. To answer this, rationally altered analogues of native Bac7 were prepared and experiments were designed that attempted to take into account several different aspects of the peptide's trajectory from bulk solution into the cell. A method combining the use of fluorescently labeled peptide fragments of decreasing size with that of a cell impermeant quencher[21] allowed correlating the minimum internalization sequence with the minimum active one. Nonproteinogenic arginine analogues with altered bulk, charge, H-bonding capacities, or stereochemistry were instead used to probe the functional characteristics of N-cap residues. *E. coli* mutants with altered outer membrane stability or lacking the SbmA transporter were respectively used to study factors affecting transit through the outer and cytoplasmic membranes. Our results lead to a better understanding of an unusual and fascinating AMP family, which apart from combining a potent activity against susceptible Gram-negative species with a particularly low host cytotoxicity,[22] represent a rare example of efficient cell penetration peptides into bacteria, potentially capable of delivering other antibiotic cargo into susceptible pathogens.

RESULTS AND DISCUSSION

Peptide Design. A series of peptides were derived from the sequence of Bac7(1−35), a fully active fragment of Bac7 (Figure 1), by systematically shortening from the C-terminus to generate the (1−23), (1−16), (1−15), (1−13), and (1−8) fragments to determine the minimum size necessary for both efficient internalization and antimicrobial activity. To monitor internalization, a cysteine residue was added at the C-terminus of each of these fragments, to which the fluorescent probe BODIPY (BY) was covalently linked. This is indicated as the

Table 1. Peptides Used in This Study

peptide	abbreviation	sequence	charge	MW_{calc} [a]	MW_{meas}
[Cys^{n+1}BY] Bac7(1−*n*) Series					
[Cys^{36}BY] Bac7(1−35)	(1−35)BY	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFPC-BY	11	4724.7	4724.5
[Cys^{24}BY] Bac7(1−23)	(1−23)BY	RRIRPRPPRLPRPRPRPLPFPRPC-BY	9	3415.1	3416.0
[Cys^{17}BY] Bac7(1−16)	(1−16)BY	RRIRPRPPRLPRPRPRC-BY	8	2594.1	2593.6
[Cys^{16}BY] Bac7(1−15)	(1−15)BY	RRIRPRPPRLPRPRPC-BY	7	2437.9	2438.0
[Cys^{14}BY] Bac7(1−13)	(1−13)BY	RRIRPRPPRLPRPC-BY	6	2184.6	2184.6
[Cys^{9}BY] Bac7(1−8)[b]	(1−8)BY	RRIRPRPRC-BY	5	1623.8	1623.4
[X^1,X^2] Bac7(1−23) Series[c]					
Bac7(1−23)	(1−23)	RRIRPRPPRLPRPRPRPLPYPRP	9	2897.6	2898.0
Bac7(3−23)	(3−23)	IRPRPPRLPRPRPRPLPYPRP	7	2585.2	2585.0
[Cys^{24}BY] Bac7(3−23)	(3−23)BY	IRPRPPRLPRPRPRPLPYPRPC-BY	7	3102.7	3102.3
[Cit^{1}Cit^2] Bac7(1−23)	[Cit^{1}Cit^2]	XXIRPRPPRLPRPRPRPLPYPRP X = Cit	7	2899.5	2899.2
[Cit^{1}Cit^{2}Cys^{24}BY] Bac7(1−23)	[Cit^{1}Cit^2]BY	XXIRPRPPRLPRPRPRPLPYPRPC-BY X = Cit	7	3417.1	3417.0
[DArg^{1}DArg^2] Bac7(1−23)	[DArg^{1}DArg^2]	XXIRPRPPRLPRPRPRPLPYPRP X = DArg	9	2897.6	2897.9
[Lys^{1}Lys^2] Bac7(1−23)	[Lys^{1}Lys^2]	XXIRPRPPRLPRPRPRPLPYPRP X = Lys	9	2841.5	2841.8
[ADMA^{1}ADMA^2] Bac7(1−23)	[ADMA^{1}ADMA^2]	XXIRPRPPRLPRPRPRPLPYPRP X = ADMA	9	2953.6	2953.4
[ADMA^1] Bac7(1−23)	[ADMA ^{1}]	XRIRPRPPRLPRPRPRPLPYPRP X = ADMA	9	2925.6	2925.6
[ADMA^2] Bac7(1−23)	[ADMA ^{2}]	RXIRPRPPRLPRPRPRPLPYPRP X = ADMA	9	2925.6	2925.5
[Noa^1] Bac7(1−23)	[Noa^1]	XRIRPRPPRLPRPRPRPLPYPRP X = Noa	8	2942.5	2942.2
[*N*Arg^1*N*Arg^2] Bac7(1−23)	[*N*Arg^1*N*Arg^2]	XXIRPRPPRLPRPRPRPLPYPRP X = NArg	9	2897.6	2987.2

[a]The mass was calculated using the Peptide Companion software (Coshisoft). [b]Bac7(1−8) has Pro^8 replaced with Arg to maintain a charge of +5. [c]In Bac7(1−23) and all its analogues, Phe^{20} was replaced by Tyr to improve the accuracy of quantification. This has no effect on the mode of action or antimicrobial potency.

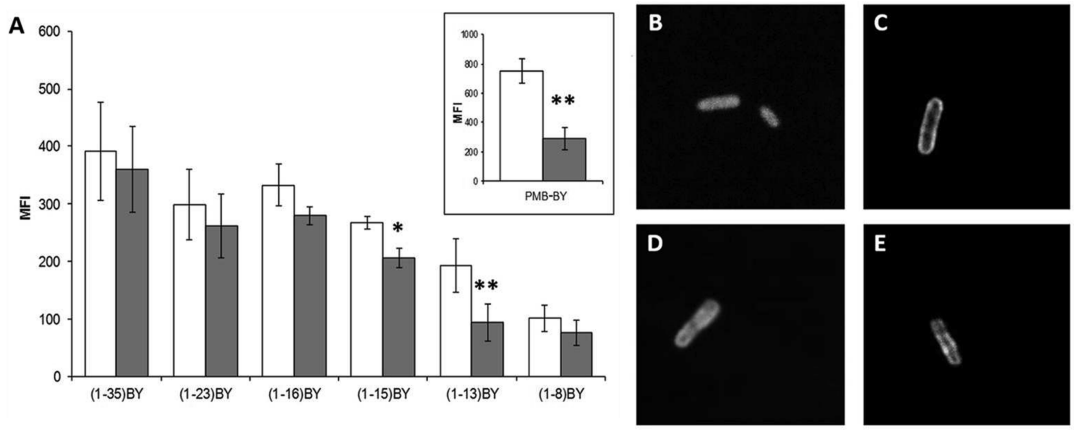


Figure 2. Flow cytometric and confocal microscopic analyses of peptide internalization. (A) Uptake of fluorescent derivatives [Cys^{n+1}BY] Bac7(1−*n*) (0.25 μM) into *E. coli* ATCC 25922 cells, after 10 min incubation. White bars are total fluorescence intensity (membrane bound and internalized peptide), gray bars are fluorescence from internalized peptide only, after quenching with Trypan blue (TB). The same experiment was carried out with BODIPY labeled polymyxin-B (PMB-BY, inset), which is known to remain surface-bound and is not internalized.[21] Mean fluorescent intensities (MFI) are the mean of at least three independent experiments, and the error bars indicate the SD. Statistical significance for the decrease between TB-treated and untreated samples was determined using the Student−Newman−Keuls multiple comparisons test, ANOVA; *$p < 0.01$ **$p < 0.001$. (B−E) Confocal microscopy carried out in the presence of (B) (1−35)BY, (C) (1−13)BY, (D) (1−15)BY, and (E) PMB-BY, (0.25 μM, 10 min incubation). All of the images are representative sections from the middle of the bacterial cell. Many fields were examined, and for each experiment over 95% of the cells displayed the same behavior as the respective representative cells shown here. The images were excised from a wider field using Paint Shop Pro V9.0 but were not otherwise manipulated.

[Cys^{n+1}BY] Bac7(1−*n*) series (Table 1). Aliquots of the peptides without Cys-BY, or with alkylated cysteine without BY, were also prepared as controls.

The active fragment Bac7(1−23) (Figure 1, underlined) was then used as the lead peptide to study the role of first two residues in the N-cap. These two arginine residues were either omitted or replaced with lysine or nonproteinogenic analogues of arginine (the DArg enantiomer, the *N*Arg peptoid residue, nitro-Arg, asymmetric dimethyl-Arg, or citrulline) (Figure 1), to systematically vary key structural or physical features. This is the [X^1,X^2] Bac7(1−23) series (Table 1). Substitution by Lys keeps the charge unaltered but significantly reduces bulk and H-bond donor capacity. Substitution with ADMA increases bulk and reduces H-bond donor capacity to a lesser extent. Citrulline or nitroarginine substitutions neutralize the charge and introduce H-bond acceptor capacity. Substitution with DArg or *N*Arg changes the direction of the side chain without altering other characteristics (see Figure 1, bottom row).

Internalization of Shortened Bac7 Fragments into *E. coli* Cells and Antimicrobial Activity. *E. coli* ATCC 25922 cells were incubated with the C-terminally BODIPY-labeled fragments, washed, and analyzed by flow cytometry in the

absence or presence of the impermeant quencher Trypan blue (TB), used to selectively quench fluorescence from residual surface-bound peptides.[21] This allowed distinguishing total fluorescence from the fluorescence deriving only from internalized peptides (Figure 2).

For BY-labeled fragments with ≥16 residues, a strong increase of the mean fluorescence intensity (MFI) of cells was observed after 10 min incubation (Figure 2A), and this was unaffected by subsequent treatment with TB quencher, indicating a fast and extensive internalization. For (1−15)BY and (1−13)BY, the total fluorescence was lower, and it significantly decreased on treatment with TB, suggesting that internalization was less efficient. For (1−8)BY, the total MFI was close to the lower limit of detection, so that a statistically relevant difference on TB quenching could not be evaluated. BODIPY-labeled polymyxin B (PMB-BY), which is known to bind tightly to the surface of bacteria but not to internalize, was quenched by TB in a manner similar to (1−13)BY (Figure 2A, inset). Increasing the time of exposure to labeled fragments (up to 60 min) did not significantly increase the MFI, independently of length, nor did it affect the TB quenching efficiency (not shown). A length of 16 or more residues from the N-terminus thus ensures rapid and efficient internalization of Bac7 fragments into susceptible bacteria. Furthermore, these cells were not permeable to propidium iodide (PI) (not shown), indicating that the bacterial membrane was undamaged, consistent with a nonlytic internalization of the peptides.

Confocal microscopy, carried out with the same samples used for flow cytometry, allowed direct visualization of internalization of the peptides and confirmed its length dependence (Figures 2B−E). Thus, (1−35)BY internalized efficiently, as shown by the diffuse fluorescence of the cytoplasm (Figure 2B), while (1−13)BY remained on the membrane surface (Figure 2C), resembling the negative control PMB-BY (Figure 2E). In agreement with flow cytometric analyses, (1−15)BY showed an intermediate behavior, with some fluorescence in the cytoplasm but more on the bacterial surface (Figure 2D).

The capacity to translocate into the bacterial cytoplasm correlated with the antimicrobial activity, as measured in terms of MIC values against two *E. coli* strains (Table 2). However, it should be taken into account that introduction of the BODIPY moiety required addition of a C-terminal cysteine on which to anchor it, both of which could affect activity. To account for this, the corresponding unlabeled Bac7(1−*n*), and alkylated [Cys^{n+1}]Bac7(1−*n*) fragments were tested in parallel. All peptides with $n \geq 16$ had a potent antimicrobial activity (MIC of 0.5−1 μM), irrespective of the presence or absence of Cys or Cys-BY. However, while unlabeled Bac7(1−15) was poorly active (MIC of 16−64 μM), addition of Cys^{16} slightly improved activity (MIC of 8−16 μM) and further addition of BODIPY improved it to levels comparable to those of longer active fragments (MIC of 1−2 μM). This is consistent with the partial capacity of (1−15)BY to internalize into bacteria, as shown by flow cytometry. Unlabeled (1−13) was relatively inactive, and addition of Cys^{14} did not affect this, while BY labeling resulted in some improvement in activity (MIC of 8−16 μM), albeit not to the same extent as for (1−15)BY.

Taken together, results indicate that there is a good correlation between peptide internalization and antimicrobial activity, and that a minimum size is required for both functions. The charge of active peptides varied from +8 to +11, so that size appeared to be more important than electrostatic

Table 2. Antimicrobial Activity of Bac7 Fragments

	MIC (μM)[a]	
	E. coli	
peptide	ATCC 25922	HB101
(1−23)	0.5−1	0.5
(1−16)	0.5−1	0.5
(1−16)BY	0.5−1	0.5
(1−15)	32−64	16
[Cys^{16}] Bac7(1−15)	8−16	8−16
(1−15)BY	1−2	1
(1−13)	>128	64−128
[Cys^{14}] Bac7(1−13)	>128	128
(1−13)BY	16	8
Bac7(1−8)	>128	>128
[Cys^{9}] Bac7(1−8)	>128	>128
(1−8)BY	>128	>128

[a]MIC values were determined using the broth microdilution susceptibility test according to the CLSI (Clinical and Laboratory Standards Institute) guidelines in Muller−Hinton (MH) broth on (1−5) × 10^5 CFU/mL. They were defined as the lowest peptide concentration that prevented visible bacterial growth after incubation for 18 h at 37 °C.

attractions, and this was confirmed by C-terminal amidation of the poorly active (1−15) and (1−8), so as to increase their charge respectively to +8 and +6, which did not improve their MIC (results not shown). Finally, the fact that active Bac7 fragments can efficiently transport molecules such as BODIPY (as well as other dyes[22]) into bacterial cells suggests they may be used to internalize other types of molecular cargo.[23] They represent a relatively rare example of Cell Penetrating Peptides (CPPs) directed at Gram-negative bacteria.

The question remains as to why native Bac7 should have 60 residues, making it a rather long antimicrobial peptide, when only 16−35 N-terminal residues are sufficient for efficient antimicrobial activity. The porcine orthologue PR-39 only has 39 residues and is a potent antimicrobial. Cloning of the Bac7 gene has shown that the C-terminal part was inserted into an ancestral gene resembling that of PR-39, after the porcine/bovid divide.[24] The function of this C-terminal part is unknown. It may improve the antimicrobial activity by increasing the membranolytic effect of the peptide, so that it can access this second mode of action at lower concentrations, or increases the speed of bacterial inactivation. Otherwise, it may be required for the peptide to access other activities related to host defense such as modulation of host cells involved in immunity or healing.

Role of N-Cap Residues on the Antimicrobial Activity. Having defined the size restraints on Bac7 activity, (1−23) (Table 1, charge +9) was selected as the lead sequence for studies aimed at probing the role of N-Cap residues. Arg^1 and Arg^2 were either omitted from the sequence or systematically substituted with amino acids selected for specific variations in charge, steric features, hydrophobicity, or H-bonding capacity (see Figure 1).

The effect of substitutions on the activity of the [X^{1}X^2] Bac7(1−23) series of analogues was evaluated by observing both variations in MIC values (Table 3) and the capacity to inhibit short-term bacterial growth (Figure 3). This allows the determination of IC_{50} values for growth inhibition, which are more sensitive in picking up functional differences in closely related peptides then MIC values. Effectively all modifications

Table 3. Antimicrobial Activity of Bac7(1-23) Variants against Different *E. coli* Strains

	MIC (μM)[a]			
	E. coli[b]	*E. coli* BW25113[b]		
peptide	ATCC 25922	*wt*	Δ*waaG*	Δ*sbmA*
(1–23)	1	1	0.5	8
(3–23)	8	8	4	32
[Cit^{1}Cit^2]	32	32	8	>64
[D-Arg^{1}D-Arg^2]	4	4	2	16
[Lys^{1}Lys^2]	4	4	2	16
[ADMA^{1}ADMA^2]	8	8	2	16
[ADMA^1]	2	2	0.5	8
[ADMA^2]	4	4	1	8
[Noa^1]	4	4	1	8
[*N*Arg^1*N*Arg^2]	1	0.5–1	0,5	8

[a]MIC values were determined using the broth microdilution susceptibility test according to the CLSI (Clinical and Laboratory Standards Institute) guidelines in Muller–Hinton (MH) broth on (1–5) × 10^5 CFU/mL. They were defined as the lowest peptide concentration that prevented visible bacterial growth after incubation for 18 h at 37 °C; [b]ATCC 25922, (full O-antigen); BW25113, a strain derived from the K12 strain, in which the LPS is lacking the O-antigen; Δ*waaG*, a deletion mutant of BW25113 with altered LPS core oligosaccharide; Δ*sbmA*, deletion mutant lacking the SbmA protein transporter in the cytoplasmic membrane.

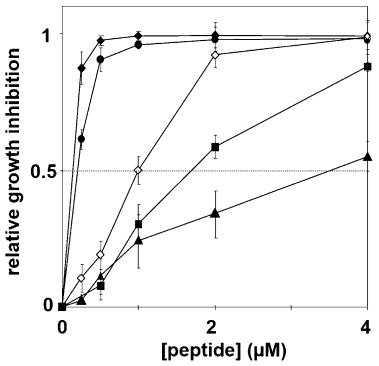


Figure 3. Inhibition of the growth of *E. coli* BW25113 by Bac7(1–23) N-cap variants. Bacteria were treated with Bac7(1–23) (●), Bac7(3–23) (■), [Cit^{1}Cit^2]Bac7(1–23) (▲), [D-Arg^{1}D-Arg^2]Bac7(1–23) (◇), and [*N*Arg^1*N*Arg^2]Bac7(1–23) (◆). Inhibition was determined in the presence and absence of peptide, calculated as $1 - (A^P/A^0)$, where A^P and A^0 are the absorbance of the bacterial suspension at 600 nm after 3.5 h incubation, respectively, in the presence and absence of peptides, starting with 1 × 10^6 bacteria/mL. Data points are the mean from at least three independent experiments carried out in duplicate. The standard deviation is shown as error bars. The concentration of peptide causing a 50% reduction in growth kinetics (dashed line) is the IC_{50} value.

to Arg^1 and/or Arg^2 residues were detrimental to activity, confirming that these residues play a key role in the mode-of-action of Bac7. The only exception was substitution with two peptoid residues (*N*Arg), for which activity was equal, if not better, than the lead peptide. The largest decrease in activity was not observed, as might be expected, by omitting the two N-terminal residues, as in (3–23) (charge +7), but rather when these were replaced by isosteric but neutral citrulline residues, in [Cit^{1}Cit^2] (+7). This may indicate that interaction with the transporter and/or target involves the whole peptide sequence but that residues at the N-terminus are particularly important. For (3–23), Arg^4 now moves to position two, as in the parent peptide, and this appears to be more beneficial than having citrulline in positions one and two.

Steric features seem more important than the local charge. This is supported by the fact that [Lys^{1}Lys^2] and [ADMA^{1}ADMA^2] variants, which have the same charge as Arg in the parent peptide, but respectively a decreased and increased bulk, as well as a different capacity to form H-bonds (see Figure 1), also show an appreciable decrease in activity. Even single Arg substitutions, such as Arg^1 → Noa/ADMA or Arg^2 → ADMA, had an observable effect, so that the steric/physicochemical requirements of the N-cap for full activity are quite stringent.

The observed variations in bacterial growth in the presence of selected analogues (Figure 3) are consistent with the MIC determinations (Table 3), and the inhibiting capacity of the peptides over a 3.5 h period follows the same clear trend [*N*Arg^1*N*Arg^2] > Bac7(1–23) > [D-Arg^{1}D-Arg^2] > (3–23) > [Cit^{1}Cit^2]. Taken together, these results suggest that a correct H-bonding capacity is particularly important for the two N-terminal residues, followed by steric characteristics, while the charge is less important. By comparison, several nonproteinogenic amino acids, including some of the arginine analogues used here, have been introduced in derivatives of the Pro-rich peptide oncocin without affecting or increasing their antimicrobial activity due to improved serum stability.[25,26] In those cases, however, the modifications were all made at the C-terminus. In the case of Bac7(1–23), only the [*N*Arg^1*N*Arg^2] modification seems acceptable at the N-terminus, and it will be interesting to determine if this also confers increased serum stability.

The dependence of activity on the steric characteristics of the two N-cap residues is clearly shown by the contrasting behavior of the enantiomeric [D-Arg^{1}D-Arg^2] and peptoid [*N*Arg^1*N*Arg^2] variants. The arginine side chain is unaltered but respectively shifted to the other side of the α-carbon or onto the α-nitrogen, as schematically shown in Figure 1. However, only the former modification decreases activity. To try to understand this behavior, a conformational search was performed on model dipeptides with the three arrangements of side chains (D,D L,L, and *N*,*N*). This started from extended or helix conformations for the D,D and L,L peptides, while a Monte Carlo search was carried out on the *N*,*N* derivative, as its peculiar structure does not allow to reach stable minima starting from standard peptide conformations. As shown in Figure 4, the lowest energy structures for the all-D dipeptide has the side chains facing in opposite directions (this is regardless of the starting conformation), while the lowest energy structures for the all-L dipeptide and dipeptoid have both side chains pointing roughly in the same directions. This may allow them to interact in a similar manner with proteins involved in transport and/or with their final targets, processes that both likely involve H-bonding.

Role of N-Cap Residues in Outer and Inner Membrane Translocation and Antimicrobial Activity. N-Cap residue variations in Bac7(1–23) analogues can affect their activity at different points of their trajectory from bulk solution to the intracellular target(s). This includes modifying their capacity (i) to overcome the outer membrane (OM) barrier of the Gram-negative bacteria so as to reach the cytoplasmic membrane and/or (ii) to be transported across the cytoplasmic membrane so as to reach intracellular target(s) and/or (iii) to interact with and inactivate these target(s).

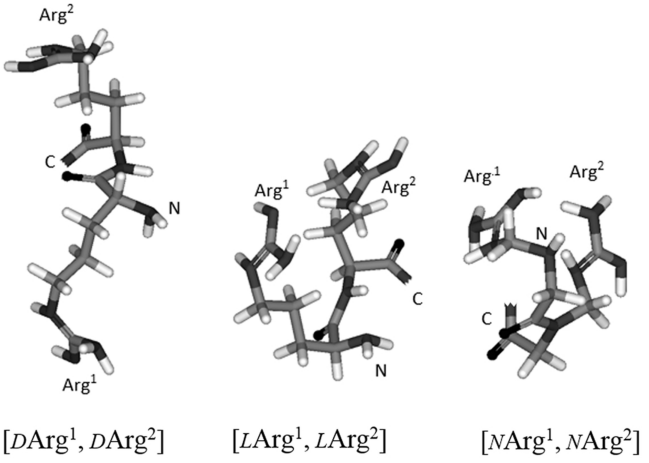


Figure 4. Lowest energy conformations of dipeptides with different Arg side chain stereochemistry. Peptides were constructed using the Spartan 14 software, starting from either extended (β-sheet) or helical conformations. They were then subjected to local optimization with the MMFF94 force field. The peptoid conformation was obtained from a Monte Carlo search. The final structures were then visualized with the DS Visualizer 2.0. (Accelrys) that was used to prepare the figure.

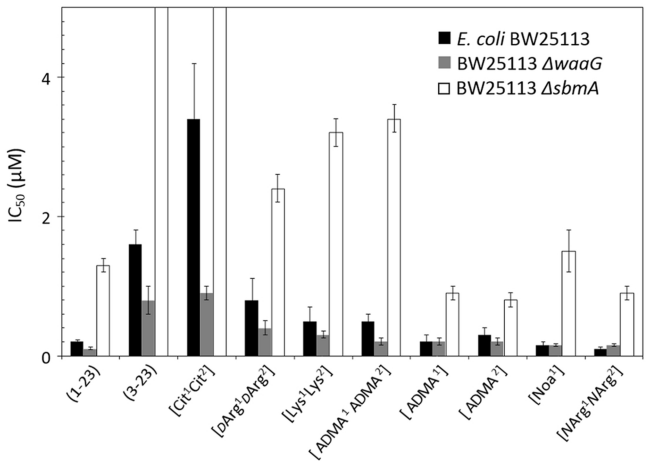


Figure 5. Bacterial growth inhibition by "N-cap" variants. IC_{50} values were determined from growth inhibition plots, as shown in Figure 3. Each value represents the mean of at least three independent experiments carried out in duplicate, starting with 1×10^6 cfu/mL bacteria. BW25113 is a K12 derived strain, devoid of O-antigen, Δ*waaG* is a deletion mutant with altered LPS core oligosaccharide and destabilized OM, while Δ*sbmA* is a deletion mutant lacking the sbmA protein transporter.

To evaluate the relevance of the OM barrier, peptides were tested against *E. coli* strains with different outer membrane characteristics. MIC and IC_{50} values for growth inhibition were similar against the ATCC 25922 strain, in which LPS molecules have a fully developed O-antigen, and the BW25113 which lacks this (Tables 3), so that the presence of the O-antigen does not markedly affect transit through the OM. The BW25113 Δ*waaG* deletion mutant (formerly known as Δ*rfaG*) lacks a key glycosyltransferase required for complete assembly of the core oligosaccharide, resulting in a deep-rough phenotype and significantly destabilized OM.[27] As expected, this mutant was more susceptible to all the tested (1−23) variants, especially the least active ones, (3−23), [$ADMA^1ADMA^2$], and [Cit^1Cit^2] (Table 3, Figure 5). This difference in behavior provides an insight into OM translocation. (1−23) is active against the wild-type strain at about 1 μM, a concentration at which it does not permeabilize either the outer or cytoplasmic membranes (see Supporting Information, Figure S1). It is therefore unlikely that it crosses the OM by a "self-promoted" uptake mechanism, as do membranolytic helical cathelicidins such as SMAP-29 (used as positive control in Supporting Information, Figure S1). This means there must be some other forms of translocation. On the other hand, when the OM is destabilized, as in the Δ*waaG* strain, all peptides can translocate more easily through the membrane, thus reducing the need for another form of transport.

The outer membrane lipoprotein YaiW has been reported to be involved in the internalization of Bac7(1−35)[17] so that uptake decreases for the Δ*yaiW* mutant in a similar manner to the Δ*sbmA* mutant lacking the cytoplasmic membrane transporter[16] (see below). This suggests that OM transport may be protein-assisted. The wide range of MIC values (0.5−32 μM) of variants against the wild-type strain (see Table 3) further suggests that N-cap characteristics are relevant to this process. The residual difference observed in MIC values (0.5−8 μM) of variants against the Δ*waaG* strain may be due to translocation through the cytoplasmic membrane transporter or intracellular target inactivation. For longer peptides (such as the full-length Bac7), especially in low salt conditions, OM permeabilization may however become a relevant part of the translocation mechanism at higher concentrations[28] so that the dual mechanism of action proposed for the cytoplasmic membrane may also apply to the outer one.

Labeled (3−23)BY and [Cit^1Cit^2]BY allowed monitoring of the internalization into *E. coli* BW25113 or its Δ*waaG* mutant, as shown in Figure 6. Uptake at 1 μM was much lower than for (1−23)BY and did not increase after extended incubation (up to 3.5 h, Figure 6A). Under these conditions, both peptides entered the bacterial cytoplasm at 4 μM concentration and internalization was significantly higher for Δ*waaG* mutant with respect to the *wt* strain (Figure 6B), but while (1−23) showed a significant killing activity against both the *wt* and mutant

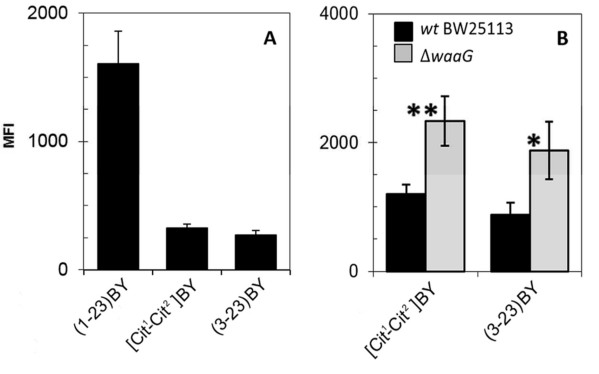


Figure 6. Flow cytometric analysis of N-cap variant internalization. Uptake of the fluorescent derivatives (1−23)BY, [Cit^1Cit^2]BY, and (3−23)BY by *E. coli* BW25113 cells (black bars) and its Δ*waaG* deletion mutant (gray bars). (A) Incubation with 1 μM peptide, and (B) incubation with 4 μM peptide for 3.5 h, washing, and treatment with TB quencher for 10 min. Values are derived from the mean of at least five independent experiments. The standard deviation is shown as error bars. Statistical significance for the difference between *wt* and mutant strain was determined using the Student−Newman−Keuls multiple comparisons test, ANOVA; $*p < 0.001$, $**p < 0.0001$.

strains, (3−23) and [Cit[1]Cit[2]] were at best bacteriostatic (see Figure S2 in Supporting Information). Thus, the parent peptide could rapidly and efficiently overcome both the outer and cytoplasmic membranes and inactivate bacteria at low concentrations, while these two variants required significantly higher concentrations and exposure times for internalization and even higher ones for bacterial inactivation.

As expected, the Δ*sbmA* BW25113 mutant lacking the protein transporter responsible for cytoplasmic membrane translocation[14−16] was less susceptible to all tested peptides, with a 4−8-fold increase in MIC values for most variants (Table 3), which also had a markedly reduced inhibitory effect (IC_{50}) on bacterial growth kinetics (Figure 5). Significantly, however, their activity was not completely abrogated. MIC values of 8 μM for the more active peptides, with corresponding IC_{50} values of 1−2 μM, are still quite appreciable and well below the concentration reportedly required for a membranolytic killing mechanism to kick in.[9] Cytofluorimetric experiments in which *E. coli* BW25113 was treated with up to 32 μM (1−23) for 60 min in the presence of propidium iodide (PI) confirmed this, as no uptake of the dye was observed (not shown), consistent with a lack of membrane permeabilization (see Figure S1 in Supporting Information). This suggests that an additional translocation system is present that allows entry into bacteria in the absence of SbmA, but with a higher K_M for transport, resulting in MIC values of around 8 μM. The MIC values of variants like (3−23) and [Cit[1]Cit[2]] remain significantly higher also against this mutant, which may indicate the N-cap residues are relevant for transport by this putative supplementary transporter and/or for internal target inactivation. This hypothesis needs to be confirmed.

Taken together, our data provides an insight into several factors affecting the trajectory of Bac7 peptides from bulk solution to their intracellular targets and indicate that the N-terminal residues have an extensive and quite a complex role in mediating transit through both the outer and cytoplasmic membranes of *E. coli*, and probably also subsequent interaction with the cytoplasmic target(s). Only the wild-type residues [Arg[1]Arg[2]] and peptoid residues [*N*Arg[1]*N*Arg[2]] have the correct steric and physical characteristics to allow efficient passage through both membranes, which may respectively involve specific interactions with outer and cytoplasmic membrane protein transport systems, allowing the peptides to internalize and inactivate bacteria at low to submillimolar concentrations. Residual differences in the activities of variants observed when the known cytoplasmic transporter is absent indicate that these residues play a role also in transit through a putative alternative transporter and/or affect interaction with the intracellular target(s). In this respect, one possible target identified for Bac7 as well as other proline rich AMPS is the chaperone DnaK,[29−31] and a second recently identified one are ribosome subunits.[32,33] At significantly higher concentrations, other mechanisms of action may also become relevant, such as membrane permeabilization[9] or interference with membrane located protein machinery involved in energy production, transport, or cell wall biosynthesis.[3]

■ CONCLUSIONS

Our studies with Bac7 analogues of varying length or with different N-cap characteristics, together with recently published data on the antibacterial mechanism of Bac7,[9,14−17] have allowed us to formulate a scheme for the mechanism of internalization, which is illustrated in Figure 7. Active Bac7

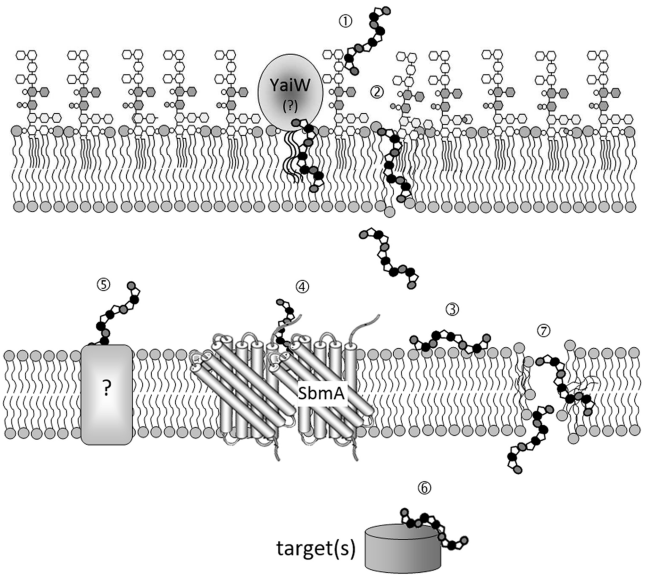


Figure 7. Schematic representation for the proposed mode-of-action of Bac7. Peptides are electrostatically attracted to the anionic surface of the outer membrane (1), which they may cross with the assistance of membrane proteins such as YaiW,[17] and at higher concentrations also by membrane permeabilization (2). The peptides are then attracted to the cytoplasmic membrane (3), where they accumulate and then translocate through to the cytoplasm by a process involving the membrane protein SbmA (4)[14−16] and possibly also a supplementary transporter acting at higher concentrations (5). They subsequently interact with one or more cytoplasmic targets, such as the bacterial chaperone DnaK and ribosomes (6),[29−33] leading to bacterial inactivation. N-terminal residues are important for all these steps. At higher concentrations, peptides can also damage the cytoplasmic membrane (7), adding a membranolytic antibacterial mode, in a size-dependent manner.[9,28]

fragments efficiently enter into susceptible bacteria using a transport system that at their MIC values likely involves both outer and cytoplasmic membrane proteins.[14−17] Our work leads to the following consideration on its mode of action:

(i) A minimum length of 16 residues from the N-terminus is required for both efficient translocation and antibacterial activity.
(ii) The presence of two N-terminal Arg residues in Bac7 is also a requisite for efficient activity. Relevant features of these residues include the H-bonding capacity, stereochemistry, and charge, in that order, and suggest stereospecific interactions are involved.
(iii) These residues are required both for efficient OM translocation and for internalization into bacteria, which depends principally on the SbmA cytoplasmic membrane protein. They may also affect interaction with the cytoplasmic target(s).
(iv) Residual activity in the absence of SbmA suggests the presence of a supplementary transporter that also requires the presence of specific N-terminal residues.

These considerations can now form the basis of new studies aimed at elucidating the molecular details of peptide transport and at identifying the internal target(s). In addition, molecules based on the proline-rich peptide scaffold may be useful lead compounds for the development of novel anti-infective agents or as cell-penetrating peptides for susceptible bacteria to be used as vehicles for carrying antibiotic cargo to the cytoplasm.

EXPERIMENTAL SECTION

Peptide Design. Peptide sequences were based on that of Bac7(1−35), a fully active fragment of native Bac7. The sequence was first shortened down to eight residues from the N-terminus. Subsequently, the fully active Bac7(1−23) was used as parent peptide for substitution of the first two residues with other proteinogenic, nonproteinogenic, or stereochemically altered residues. In the latter case, substitution with the D-enantiomer, or shifting the side chain onto the α-nitrogen in the peptoid *N*Arg, alters the orientation of the side chain (Figure 5). Models of the respective dipeptides were built to evaluate the conformational effect of these substitutions. Calculations were carried out on a workstation equipped with a Dual Opteron ASUS KFSN4-DRE/IKVM/IST motherboard with two AMD Opteron SixCore 2427 2.2 GHz and MB 75W processors. The MMFF94 force field[34] as implemented in the Spartan 14 parallel suite (Wave Function, Inc.) was used in all energy minimizations. Geometry optimizations were driven with a truncated Newton method using a preconditionate conjugate gradient with an exact, sparse, Hessian, down to a convergence limit of 0.0003 kJ/mol. Monte Carlo conformational search was carried out at an initial temperature of 5000 K (the Spartan 14 default), keeping the conformations within a maximum energy range of 40 kJ/mol. The structures with minimum energy were then visualized using DS Visualizer 2.0. (Accelrys).

Peptide Synthesis. All peptides were synthesized in the solid phase using Fmoc chemistry on a CEM Liberty automated microwave peptide synthesizer (Matthews, NC). Fmoc-amino acids were from Novabiochem, including the nonproteinogenic Fmoc-citrulline-OH and Fmoc-Arg(NO_2)-OH. Fmoc-ADMA(Pbf)-OH was prepared as previously described.[35] The synthesis scale was 0.1 mmol using 2-chlorotrityl resin (substitution 0.22 mmol/g), previously loaded manually with 4-fold molar excess of the C-terminal amino acid [Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, or Fmoc-Cys(Trt)-OH] with 6 equiv *N*,*N*′-diisopropylethylamine (DIPEA) in DCM. For successive couplings, protected amino acids were added at a 5-fold excess using *N*-methyl-2-pyrrolidone (NMP) as solvent at 45 °C. Fmoc deprotection was effected with 20% piperidine/0.1 M HOBt in DMF. For [X^1,X^2] Bac7(1−23) series peptides, automated synthesis was interrupted at residue 3 and the appropriate Fmoc-protected arginine substitutes were then coupled.

N-(3-Guanidinopropyl)glycine residues were introduced at positions 1 and 2 using the submonomer approach as described.[36] Briefly, 0.3 mmol (∼30 equiv) of $BrCH_2COOH$ activated with 1 equiv of diisopropylcarbodiimide (DIPCDI) were added to 100 mg of resin bearing Bac7(3−23) (0.012 mmol) and mixed for 15 min, then washed with DMF, and the operation was repeated twice. Then 41 mg (0.1 mmol, ∼10 molar excess) of previously prepared $H_2N(CH_2)_3NH$-C(=NPbf)NH_2 (purity >95% by HPLC) dissolved in 300 μL of DMF were added to the resin and allowed to react for 4 h at 40 °C with periodic mixing. After washing with DMF and then with DCM, the entire procedure was repeated for introduction of the N-terminal *N*Arg residue. The purity of the crude product after deprotection was ∼85% by HPLC.

Peptides were cleaved from the resin using a cocktail of trifluoroacetic acid (TFA), thioanisole, water, 3,6-dioxa-1,8-octanedihiol (DODT), and tri-isopropylsilane (TIPS) (85%,3%, 2%, 8%, 2% v/v), precipitated in *tert*-butyl methyl ether (−20 °C) and then purified by reversed-phase HPLC on a preparative Phenomenex column (Jupiter, C_{12}, 10 μm, 90 Å, 250 mm × 21 mm) using a 10−40% CH_3CN gradient with 0.05% TFA in 60 min at an 8 mL/min flow rate. The correct sequence was verified by ESI-MS (Esquire 4000 Bruker Daltonics). Purity was assessed based on analytical spectra obtained on a reversed-phase Phenomenex Kinetex column (C_{18}, 2.6 μm, 100 Å, 50 mm × 4.6 mm) using a 15−35% CH_3CN gradient with 0.05% TFA in 25 min at an 0.8 mL/min flow rate, so that the *K** value was between 4.8 and 9.8,[37] by integrating areas under the peaks. All peptides had a purity ≥95%.

After lyophilizing twice from 10 mM HCl solutions to remove TFA, the concentration of peptide stock solutions was obtained from accurately weighed peptide, and was confirmed by (i) spectrophotometric determination of the peptide using a ε_{214} calculated as described by Kuipers and Gruppen,[38] or (ii) spectrophotometric determination of Tyr (ε_{280} = 1200 M^{-1} cm^{-1}), that was specifically used to replaced Phe for this purpose in [X^1,X^2] Bac7(1−23) series peptides.

Synthesis of BODIPY-Labeled Peptides. Peptides were synthesized in the solid phase, as described, but introducing a cysteine at C-terminus. After cleavage, 1 equiv of crude peptide was reacted with 5 equiv of BODIPYFL [*N*-(2-aminoethyl)maleimide] (Sigma) in 30% CH_3CN in 10 mM sodium phosphate buffer pH 7.4, (SPB). The reaction was performed under nitrogen bubbling with stirring for 3 h at room temperature and then overnight at 4 °C. The reaction was monitored periodically by analytical RP-HPLC and ESI-MS. Upon completion (at about 24 h), a 10-fold excess of cysteine was added to quench unreacted dye. After a further 60 min, the reaction mixture was diluted with 0.05% TFA in water to a final concentration of 10% CH_3CN and pH 2.5, for purification by RP-HPLC on a semi-preparative Phenomenex column (Jupiter, C_{18}, 5 μm, 300 Å, 100 mm × 10 mm) with a linear 5−30% CH_3CN gradient (0.05% TFA) in 40 min at 2 mL/min rate. Purity was confirmed by ESI-MS. After lyophilization, the concentration of stock solutions was determined spectrophotometrically using the extinction coefficient of BODIPY [ε_{280} = 79000 M^{-1} cm^{-1} in MeOH, according to ref 39].

An aliquot of the peptides was left unlabeled for use as controls, but this required blocking the sulfhydryl group on the C-terminal cysteine residue by alkylation using 2-iodoacetamide to prevent intermolecular dimerization of peptides by disulfide bond formation. The reaction was conducted in 0.5 M Tris, 2 mM EDTA buffer at pH 8.0 (according to ref 40). Iodoacetamide is unstable and light-sensitive, so a 2.2 M solution was prepared immediately before use and the reaction was performed in the dark and under nitrogen, adding 1 mM peptide and 0.5 M ascorbic acid to scavenge traces of iodine. After 2 min, 0.5 M citric acid was added as quencher before purification as described. In this case, peptide quantification was carried out using the extinction coefficient of tyrosine.

Peptide Internalization into Bacterial Cells. Internalization of BODIPY-labeled Bac7 fragments into *E. coli* bacterial cells was quantified using the *E. coli* ATCC 25922 strain (1 × 10^6 CFU/mL) incubated in Muller−Hinton (MH) broth at 37 °C with increasing concentrations of peptide (0.25, 1, or 4 μM) for different times (from 10 min to 4 h). Cells were analyzed using both flow cytometry and confocal microscopy after washing with high-salt solution to remove extracellular and loosely surface bound peptide as previously described.[21,22] Trypan blue (TB) (1 mg/mL) was used as quencher for extracellular fluorescence resulting from residual, surface bound peptide.[22] In each experiment, the permeabilization of cells was monitored in parallel using the propidium iodide (PI, Sigma-Aldrich, St Louis, MO) flow cytometric exclusion assay.[9] BODIPY-labeled polymyxin was used as a negative control (surface-binding only) at the concentration of 0.1 μM to avoid significant membrane lysis. Analyses were performed with a Cytomics FC 500 (Beckman-Coulter, Inc.) equipped with an argon laser (488 nm, 5 mW) and detectors for filtered light set at 525 nm for BODIPY detection and 620 nm for detection of PI-positive cells.

For confocal microscopy, the same batches of treated cells as used for flow cytometry were washed four times with high-salt solution and 10 μL of suspension were placed between two cover glasses to obtain an unmovable monolayer of cells. The unfixed bacterial cells were examined with a Nikon C1−SI confocal microscope using an oil immersion lens. Fluorophores were stimulated at 488 nm. Emission was acquired with a 510 nm band-pass. The image stacks collected by the instrument were analyzed and converted to TIFF format using the EZ-C1 Free Viewer (Nikon Corporation) and ImageJ 1.40g (Wayne Resband, National Institutes of Health, USA) software. Many fields were examined, and for each experiment, over 95% of the cells displayed a similar behavior.

Antimicrobial Activity. The antimicrobial activity of all synthesized peptides was tested in terms of the minimum inhibitory concentrations (MIC), as well as the more sensitive IC_{50} value as determined from inhibition of bacterial growth in the presence of peptides, for several *E. coli* strains: ATCC 25922, HB101, and

BW25113, and the BW25113 Δ*waaG*[27] and Δ*sbmA*[16] deletion mutants. The latter were obtained from the Keio collection of GenoBase (cgsc.biology.yale.edu/index.php) as strains JW3606-1 and JW0368-1, respectively.

MIC determinations were carried out in MH broth on $(1-5) \times 10^5$ bacterial CFU/mL as previously described.[15] The bacterial growth inhibition curves were obtained using 1×10^6 CFU/mL bacteria in MH broth in the presence of increasing peptide concentrations and monitoring the optical density at 600 nm at 37 °C every 10 min for 4 h on a microplate reader (Tecan, Männedorf, Switzerland). IC_{50} values were determined from the degree of growth inhibition calculated as the relation between the absorbance of bacteria in presence and in absence of peptide after 3.5 h incubation.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ADMA, *Nω*,*Nω*-dimethylarginine; AMP, antimicrobial peptide; CFU, colony forming unit; CPP, cell penetrating peptide; IC_{50}, inhibiting concentration for 50% growth rate reduction; MIC, minimum iinhibitory concentration; Noa, *Nω*-nitro-L-arginine; OM, outer membrane; PI, propidium iodide; SPB, 10 mM sodium phosphate buffer pH 7.4; TB, Trypan blue; *wt*, wild-type

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