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Substitution of the Arginine/Leucine Residues in Apidaecin Ib with Peptoid Residues: Effect on Antimicrobial Activity, Cellular Uptake, and Proteolytic Degradation

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Several aspects of the mechanism of action of Pro-rich antimicrobial peptides, together with their low toxicity in mammalian cells, make them good candidates for the development of new antibiotic agents. We investigated the effect induced in the insect antimicrobial peptide apidaecin Ib by the replacement of a single arginine/leucine residue with a N-substituted glycine. The resulting peptoid−peptide hybrids are more resistant to proteolysis and devoid of any significant cytotoxic activity, but moving the [*N*Arg]residue from the N- to the C-terminal end of the molecule progressively reduces the antibacterial activity. Cell uptake experiments in *E. coli* cells suggest that the loss of antibacterial activity of [*N*Arg^{17}]apidaecin is a consequence of its inability to translocate into bacterial cells. Conversely, apidaecin and its peptoid−peptide hybrids are able to cross the plasma membrane in eukaryotic cells and to diffuse in the cytosol, although their translocating ability is far less effective than that of other known cell permeant peptides.

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Introduction

Small cationic antimicrobial peptides (AMPs[a]) are evolutionarily ancient components of the host defense system of many different unicellular and pluricellular organisms, from bacteria to plants, insects, fish, amphibians, birds, and mammals, including humans.[1] In spite of their highly diverse sequences and structural motifs, most of them show a tendency to assume amphiphilic structures in membrane environments. This feature correlates with their ability to permeabilize the bacterial membranes, eventually leading to lysis of the microbial cells.[2] In addition to this membrane damaging mechanism, a minority of AMPs, such as those belonging to the Pro-rich group of insects and mammals,[3] are able to kill bacteria without any apparent membrane destabilization. They can efficiently translocate inside both prokaryotic[4,5] and eukaryotic cells,[6−8] and accumulate in the cytoplasm or in other subcellular compartments. The mechanism of cellular uptake is not yet fully understood. However, the structural similarity of these peptides to the arginine-rich cell-penetrating peptides from protein transduction domains[9] and solid experimental evidence suggest a common translocation mechanism for eukaryotic cells, via endocytic pathways.[8] Much less is known of the mechanism of penetration in bacterial cells, although a more specific translocation machinery has been hypothesized, involving a bacterial permease/membrane transporter.[10] In this respect, it has been recently shown that mutations in the *sbmA* gene, coding for a protein predicted to be part of an inner membrane ABC uptake transporter, cause a decreased susceptibility of *Escherichia coli* cells to Pro-rich AMPs, including the bovine Bac7 and the honeybee apidaecin 1b.[11] These mutant cells show a decreased ability to internalize the Pro-rich peptides, suggesting that the SbmA protein is necessary for their transport inside the bacterial cells. These findings indicate that the mechanism of penetration of these peptides in prokaryotic and eukaryotic cells may be different, in general depending on uptake transporters in the former cells and in membrane internalization mechanisms (e.g., macropinocytosis) in the latter.

The small size of apidaecin (only 18 residues), its low toxicity,[12] and its activity directed against different bacterial targets,[10] which is expected to slow the appearance of resistance phenomena, suggest that this AMP could be a good candidate for developing novel antibiotic drugs. Moreover, the ability of the Pro-rich peptides to penetrate bacterial and host cells suggests that these peptides, or their transduction domain, could be useful in delivering cargo molecules and diagnostic probes inside the cells.

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[a] Abbreviations: AMPs, antimicrobial peptides; Bod, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl (BODIPY) *N*-(2-(aminoethyl)maleimide; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; Fluo, 5(6)-carboxyfluorescein; Fmoc, 9-fluorenylmethoxycarbonyl; Dde, 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione; HATU, *O*-[7-azabenzotriazolyl]tetramethyluronioum hexafluorophosphate; HBTU, 2-[1*H*-benzotriazole-1yl]-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MIC, minimum inhibitory concentration; *N*Arg, *N*-(3-guanidinopropyl)glycine; *N*Har, *N*-(3-guanidinobutyl)glycine; *N*Leu, *N*-isobutylglycine; *N*Nar, *N*-(3-guanidinoethyl)glycine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; SDS, sodium dodecyl sulfate; TB, Trypan blue; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TIS, triisopropylsilane; Trt, trityl.

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Table 1. Name, Sequence, and Net Charge of the Peptides Used in This Study

compd	peptide	sequence[a]	net charge[b]
0	apidaecin Ib	GNNRPVYIPQPRPPPHPRL	+4
1	[*N*Arg[17]]apidaecin	GNNRPVYIPQPRPPPHP***R***L	+4
2	[*N*Nar[17]]apidaecin	GNNRPVYIPQPRPPPHP***nR***L	+4
3	[*N*Har[17]]apidaecin	GNNRPVYIPQPRPPPHP***hR***L	+4
4	[*N*Arg[12]]apidaecin	GNNRPVYIPQP***R***PPHPRL	+4
5	[*N*Nar[12]]apidaecin	GNNRPVYIPQP***nR***PPHPRL	+4
6	[*N*Har[12]]apidaecin	GNNRPVYIPQP***hR***PHPRL	+4
7	[*N*Arg[4]]apidaecin	GNN***R***PVYIPQPRPPHPRL	+4
8	[*N*Arg[4,12,17]]apidaecin	GNN***R***PVYIPQP***R***PPHP***R***L	+4
9	[*N*Leu[18]]apidaecin	GNNRPVYIPQPRPPPHPR***L***	+4
10	des-Pro[14]-apidaecin	GNNRPVYIPQPRPHPRL	+4
11	des-Pro[5,14]-apidaecin	GNNRVYIPQPRPHPRL	+4
12	Fluo-apidaecin	Fluo-GNNRPVYIPQPRPRHPRL	+2
13	Cys(*S*-Bod)-apidaecin	C(*S*-Bod)GNNRPVYIPQPRPRHPRL	+4
14	Fluo-[*N*Nar[17]]apidaecin	Fluo-GNNRPVYIPQPRPRHP***R***L	+2
15	Cys(*S*-Bod)-[*N*Nar[17]]apidaecin	C(*S*-Bod)GNNRPVYIPQPRPRHP***R***L	+4
16	Fluo-[*N*Nar[12]]apidaecin	Fluo-GNNRPVYIPQP***R***PRHPRL	+2
17	Fluo-Tat_{47-57}	Fluo-YGRKKRRQRRR	+6

[a] ***R***: *N*Arg [$NH_2C(NH)NH(CH_2)_3NHCH_2COOH$]. ***nR***: *N*Nar [$NH_2C(NH)NH(CH_2)_2NHCH_2COOH$]. ***hR***: *N*Har [$NH_2C(NH)NH(CH_2)_4NHCH_2COOH$]. ***L***: *N*Leu [$(CH_3)_2CHCH_2NHCH_2COOH$]. [b] Approximate charge at pH 7.4.

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The use of peptides as therapeutic agents has some serious drawbacks such as their poor bioavailability, rapid excretion through liver and kidneys, and low metabolic stability to proteolysis. In particular, trypsin-like enzymes attack proteins at basic residues, which are a common characteristic of this class of antimicrobial compounds. Several solutions have been proposed to extend their stability in vivo, including the use of non-natural amino acids (such as D-amino acids, β-amino acids, peptoid residues), backbone modifications, and glycosylation. In a previous study, we synthesized cyclic and glycosylated analogues of the insect peptide apidaecin[13] and observed that both modifications dramatically reduce the antimicrobial activity. In addition, the all-D analogues of the apidaecin family are completely devoid of antibacterial activities.[14] However, substitution of the C-terminal leucine, an essential residue for activity, with its enantiomer does not result in such a dramatic effect.[10] Considering that basic residues represent a preferred attack site for trypsin-like enzymes and that arginine side chains are important for the antibacterial activity of apidaecin,[10] we attempted to improve the peptide's half-life in biological fluids by replacing each of three arginines by the corresponding peptoid residue, *N*-(3-guanidinopropyl)glycine (*N*Arg), in which the typical amino acid side chain is shifted from the α-carbon to the amide nitrogen.[15] The same modification was performed on the C-terminal leucine, which was replaced by a *N*-isobutylglycine (*N*Leu) residue (Table 1). The stability of all these analogues to trypsin degradation and their antimicrobial and hemolytic activity were investigated, as well as their conformational preferences in different environments. Moreover, the ability of apidaecin and of its peptoid–peptide hybrids to penetrate into bacterial and mammalian cells was compared, following the translocating properties of the corresponding fluorescently labeled analogues.

Results and Discussion

Synthesis of Peptoid–Peptide Hybrids. In the solid-phase synthesis of peptoid–peptide hybrids, two different approaches can be used to introduce an *N*-alkylglycine (peptoid residue) on the growing peptide chain: (i) the N-substituted glycine derivative, suitably protected at the tertiary nitrogen atom, can be separately prepared and directly utilized as building block in the solid phase procedure (monomer method),[15] or (ii) the peptoid residue is built during the peptide chain elongation by a combination of two submonomers, an α-haloacetic acid and a primary amine (submonomer method).[16] In both cases to achieve a *N*Arg peptoid residue, a suitable N^G-protected-3-guanidinopropanamine has to be synthesized in advance.[17] To speed up the synthesis of *N*Arg containing peptoid–peptide hybrids, we optimized a procedure based on the submonomer method, which makes possible a direct assembling of the functionalized peptoid residue starting from commercially available reagents (Scheme 1). Briefly, the peptide chain was first assembled on the resin by the standard Fmoc/HBTU protocol[18] until the peptoid residue position. Bromoacetic acid was then coupled to the NH_2-peptide resin in the presence of *N*,*N*′-diisopropylcarbodiimide (DIC), and the halogen was displaced with a large excess of 1,3-diaminopropane. The resulting *N*-(3-aminopropyl)glycine (a precursor of *N*Arg) was then selectively protected at the side chain amino function by reaction with 2-acetyl-5,5-dimethyl-1,3-cyclohexanedione (Dde-OH),[19] which is known to leave unaffected secondary amines even when used in a large excess.[20] Further elongation of the peptide chain was carried out according to the standard protocol, and the last residue was introduced as *N*-Boc-derivative, which is orthogonal to the Dde group. This one was removed from the peptoid side chain by treatment with 2% hydrazine in DMF, and the resulting δ-amino function was on-resin guanidinylated with *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine.[21] Simultaneous deprotection and cleavage of peptides from the resin gave the peptoid–peptide hybrids in high yield (70–80%). Following the same procedure and by use of a suitable diamine in the displacement reaction of the halogen, we could easily introduce in the apidaecin peptide chain lower and higher homologues of *N*Arg, respectively: *N*-(3-guanidinoethyl)glycine (*N*Nar) in hybrids **2** and **5**, and *N*-(3-guanidinobutyl)glycine (*N*Har) in hybrids **3** and **6** (Table 1).

Apidaecin analogues containing multiple peptoid residues and the fluoresceinated peptoid–peptide hybrids were synthesized by the monomer method, starting from a preformed and suitably protected *N*Arg residue (Fmoc-*N*Arg(Pmc)-OH), prepared in advance.[17]

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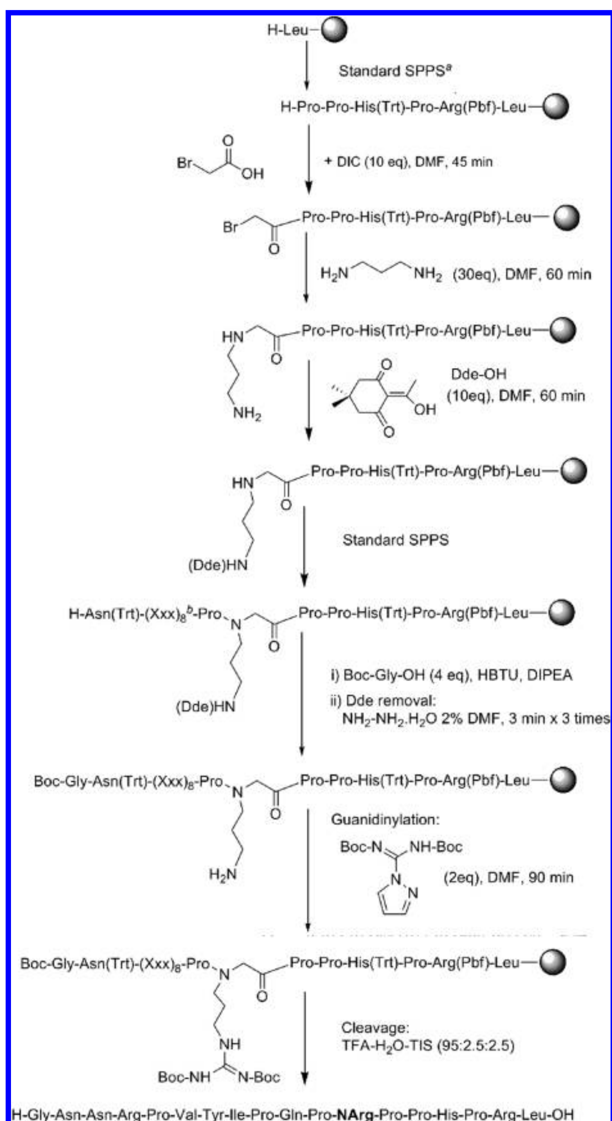
Scheme 1. Solid-Phase Synthesis of [*N*Arg[12]]Apidaecin (**4**)[c]



[a] Standard SPPS refers to peptide synthesis based on Fmoc chemistry and HBTU/HOBt as coupling reagents. [b] $(Xxx)_8$ = -Asn(Trt)-Arg(Pbf)-Pro-Val-Tyr(*t*Bu)-Ile-Pro-Gln(Trt)-. [c] Peptoid–peptide hybrids **1–3** and **5–7** were synthesized according to this general procedure.

It is known that diketopiperazine formation easily occurs as side reaction in the solid phase peptide synthesis when benzyl ester type peptide-resin linkages are used and residues such as glycine, proline, a D-amino acid, or an *N*-alkylamino acid are present in the C-terminal dipeptide.[22] However this side reaction did not occur during the synthesis of [*N*Arg[17]]apidaecin and of its fluorescent analogues, and both compounds were obtained with yields comparable to those of all other peptoid–peptide hybrids. For the synthesis of the [*N*Leu[18]]apidaecin analogue we preferred to use a trityl-type resin, which is known to prevent diketopiperazine formation during the synthesis of peptides containing a C-terminal proline.[23] Nevertheless, an extensive removal of the C-terminal dipeptide [Arg(Pbf)-*N*Leu] occurred during the Fmoc deprotection step, and this peptoid–peptide hybrid was obtained only in 30% yield.

Circular Dichroism (CD) Studies. The conformational properties of apidaecin and the peptoid–peptide hybrids were investigated by CD spectroscopy in Tris buffer (pH 7.4), 90% trifluoroethanol (TFE), or micellar sodium dodecyl sulfate (SDS). The CD spectrum of apidaecin in water is characterized by a broad negative band around 200 nm, characteristic of unordered structures.[13] The proline content in apidaecin is quite high (6 out of 18 residues), and some authors suggested the presence of a significant population of conformers adopting an ordered left handed polyproline type II structure, whose CD curves are similar to those of unordered peptides.[24,25] Peptoid residues, lacking the hydrogen of the peptide secondary amide, are expected to increase the flexibility of the peptide chain.[15] Nevertheless, the CD spectra of all apidaecin peptoid–peptide hybrids in aqueous environment are very similar to that of the native peptide (Figure 1A). In 90% aqueous TFE (Figure 1B), the spectra showed a red-shifting and a broadening of the negative band, with reduction of the CD band intensity. This pattern, more pronounced in the [*N*Arg[4]]apidaecin and [*N*Leu[18]]apidaecin spectra, can be correlated to an increase of the β-turn percentage in the conformational mixture.[26] Only the trisubstituted peptoid–peptide hybrid, which is lacking three amide hydrogens in comparison with the natural peptide, remained largely unstructured even in organic solvent. Micellar SDS has been used as a model of the negatively charged bacterial membrane. In this environment, apidaecin and its analogues show very similar CD spectra (Figure 1C), suggesting that the interaction with the micelles is mainly electrostatic. The CD spectra of a homologue series of peptoid–peptide hybrids (**4**, **5**, **6**) does not significantly differentiate, showing that a small change in the size of the peptoid residue does not affect the conformer population in different environments (see Supporting Information). Analogously, the CD spectra of the fluoresceinated derivatives indicate that the fluorescent moiety at the peptide N-terminal end does not modify the peptide conformational preferences (see Supporting Information).

In summary, the replacement of a single amino acid by the corresponding peptoid residue in the Pro-rich AMP apidaecin does not significantly affect the conformer populations in different environments.

Enzymatic Degradation Studies. One of the aims for the arginine/peptoid residue substitution was to increase the apidaecin stability to proteolytic degradation. Owing to the presence of several basic residues, cationic antimicrobial peptides are in fact very sensitive to the action of trypsin-like enzymes. In apidaecin, in particular, enzymatic cleavage of the C-terminal Arg-Leu bond produces analogues with no or very low activity.[10,27] Peptoid residues hamper the action of proteases, and no significant degradation of the apidaecin peptoid–peptide hybrids was detected after incubation for 24 h with trypsin, when the peptoid residue occupied either the P1 site (Arg) or the P1′ site (Leu)[28] (Figure 2B and Figure 2C). On the contrary, the Arg-Leu bond in unmodified apidaecin was completely cleaved by the enzyme in less than 30 min (Figure 2A). Therefore, the introduction of a peptoid residue at the trypsin-sensitive Arg-Leu bond could improve considerably the enzymatic stability of apidaecin in biological fluids.

Antimicrobial Activity. Similar to the natural peptide,[29] the peptoid–peptide hybrids of apidaecin Ib were active against Gram-negative bacteria,[30] with a potency that strongly depends on the position of the non-natural amino acid within the peptide sequence (Table 2). A *N*Arg residue at position 4 or 12 caused only a slight reduction of the antibacterial activity with respect to the natural peptide,

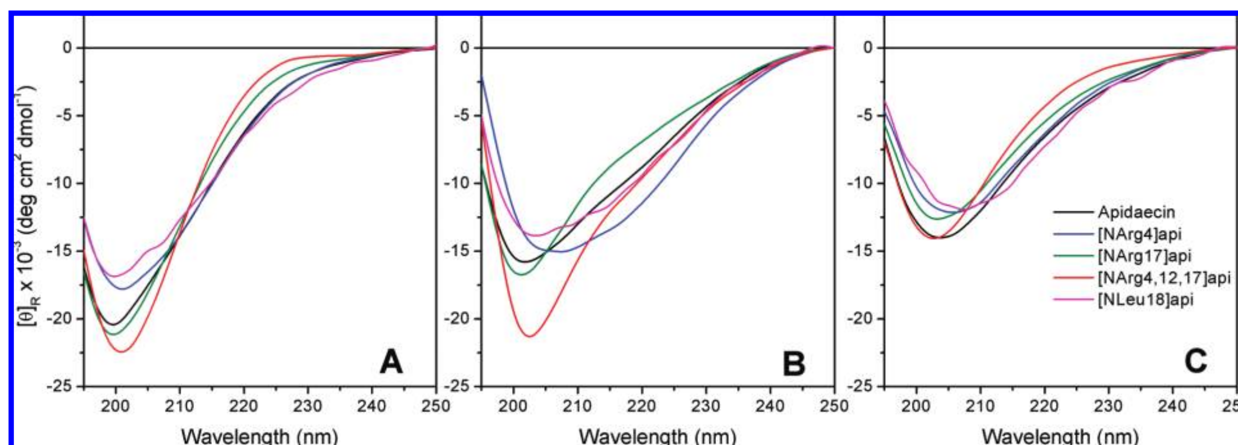


Figure 1. CD spectra of apidaecin and its peptoid−peptide hybrids **1**, **7**, **8**, and **9** in Tris buffer (pH 7.4) (A), aqueous 90% TFE (B), and 30 mM SDS in Tris buffer (C).

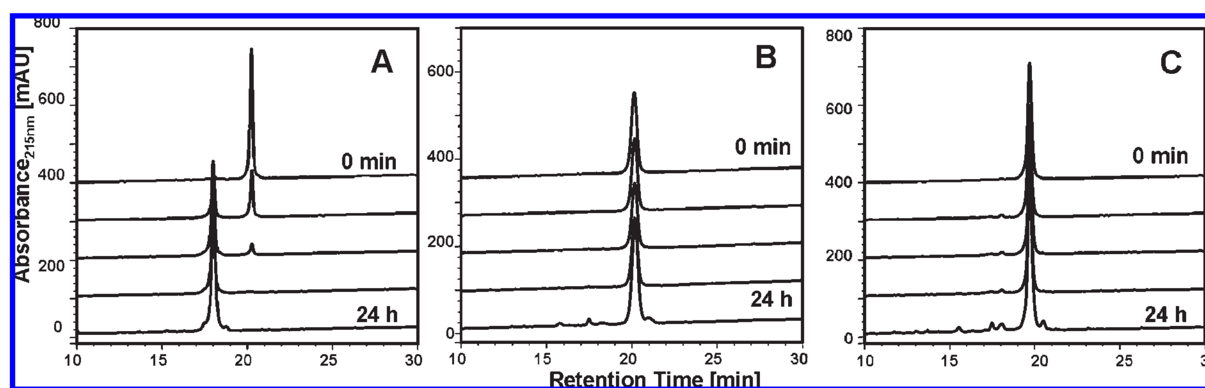


Figure 2. HPLC chromatograms of aliquots from incubation of apidaecin (A), [*N*Arg[17]]apidaecin (B), or [*N*Leu[18]]apidaecin (C) with trypsin at 37 °C. Aliquots were sampled at the following incubation times (from top to bottom): 0 min, 10 min, 20 min, 1 h, and 24 h.

Table 2. Antimicrobial and Hemolytic Activity of Apidaecin Ib and of Its Analogues

	MIC[a]							
peptide	*E. coli* ML35	*E. coli* O18K1H7	*E. coli* ATCC 25922	*K. pneumoniae* 22	*S. enteritidis* D5	*S. enteritidis* PD1	*S. typhimurium* ATCC 14028	% HA[b]
apidaecin Ib	8	8	8	64	8−16	8−16	4−8	1.19
[*N*Arg[17]]apidaecin	> 128	> 128	> 128	> 128	> 128	> 128	> 128	0.44
[*N*Arg[12]]apidaecin	16	16	16	> 128	16−32	32	16	2
[*N*Arg[4]]apidaecin	16	16	16−32	> 128	32−64	32−64	16	0.06
[*N*Arg[4,12,17]]apidaecin	> 128	> 128	> 128	> 128	> 128	> 128	> 128	0.06
[*N*Nar[17]]apidaecin	> 128	> 128	> 128	> 128	> 128	> 128	> 128	0.62
[*N*Nar[12]]apidaecin	16−32	32	32	> 128	64	64−128	32	0.87
[*N*Har[17]]apidaecin	> 128	> 128	> 128	> 128	> 128	> 128	> 128	0.81
[*N*Har[12]]apidaecin	16−32	32	32−64	> 128	64	64−128	32	1.31
[*N*Leu[18]]apidaecin Ib	> 128	nd[c]	> 128	> 128	> 128	> 128	> 128	nd[c]
des-Pro[14]-apidaecin	nd[c]	nd[c]	128	> 128	nd[c]	nd[c]	> 128	nd[c]
des-Pro[5,14]-apidaecin	nd[c]	nd[c]	> 128	> 128	nd[c]	nd[c]	> 128	nd[c]

[a] The minimum inhibitory concentration (MIC) value is expressed in μM. Results are the mean of at least three independent determinations with a divergence of not more than 1 MIC value. [b] Hemolytic activity (HA) against red blood cells, determined using a peptide concentration of 300 μM. Results are the mean of two independent experiments performed in duplicate. [c] nd: not determined.

and the minimum antimicrobial activity values (MICs) of the corresponding analogues (**7** and **4** in Table 1) increased from 2- to 4-fold at most. Conversely, a dramatic decrease in activity was induced by modifying the apidaecin C-terminal region, and [*N*Leu[18]] and [*N*Arg[17]] hybrids proved to be inactive, showing MIC values higher than 128 μM against all the strains tested. A similar lack of activity was shown by the [*N*Arg[4,12,17]] trisubstituted analogue (**8**). Considering that previous studies have established that a basic amino acid in position 17 is necessary to retain the antimicrobial activity,[10] it turns out that not only the side chain functional group but also its arrangement in the backbone is important for apidaecin activity. Also, the [*N*Leu[18]]apidaecin analogue **9** proved to be inactive, although other apidaecin analogues containing glycine or L- or D-amino acids in the same position[10,31] retained at least part of the antibacterial activity. These results agree with previous findings showing that another analogue,

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[Pro^{18}]apidaecin, which is structurally very close to the peptoid−peptide hybrid **9**, displayed a negligible antibacterial activity.[31] These data suggest that the amide protons in the C-terminal portion of apidaecin can play an important role, inducing the backbone to adopt a conformation that is essential for the engagement with bacterial targets. In this regard, Dutta et al.[27] have recently hypothesized that in a bacterial environment, the apidaecin C-terminal domain assumes a bent structure, promoting its entry in the bacterial membrane.

In an attempt to improve the antibacterial activity of these proteolitically stable analogues, we synthesized new hybrids containing peptoid residues with an alkyl side chain shorter or longer than arginine, corresponding to *N*-norarginine (*N*Nar) and *N*-homoarginine (*N*Har), respectively. Similar to their parent peptide, the analogues modified at position 17 (**2** and **3**) were inactive at the tested concentrations (MIC > 128 μM). The peptoid−peptide hybrids modified at position 12 (**4**−**6**) showed an increase of the antibacterial potency in the order [*N*Arg^{12}]apidaecin > [*N*Nar^{12}]apidaecin > [*N*Har^{12}]apidaecin, indicating that the size of the peptoid residue influences the antimicrobial activity and three methylene groups represent the optimal side chain length.

During the synthesis of these new analogues, we incidentally obtained two peptides missing Pro^{14} (**10**) or Pro^{5} and Pro^{14} (**11**), respectively. The MIC values of ≥128 μM displayed by these analogues against the strains tested (Table 2) confirm the importance of proline residues in apidaecin peptides.[10,31]

N-Terminal labeling of short proline-rich peptides often results in a strong reduction of the antibacterial activity, as a consequence of the loss of a positive charge.[32] This effect is even more remarkable in peptides modified with 5(6)-carboxyfluorescein, which decreases the net positive charge of the molecule by 2 (Table 1), as shown by the lack of activity of the fluoresceinated apidaecin analogues (MIC > 128 μM) against all the strains tested (data not shown). To keep unchanged the overall net positive charge of the native peptide also in the dye-labeled analogues, a cysteine residue was added to the N-terminus of the synthesized peptides and, subsequently, modified at the thiol function with the neutral boron dipyrrin (BODIPY) dye through a 2-aminoethylmaleimide spacer. BODIPY-labeled apidaecin retained most of its antimicrobial activity against *E. coli* ATCC 25922, with an approximately 4-fold decrease in potency with respect to the unlabeled peptide (data not shown). As expected, BODIPY-labeled [NArg^{17}]apidaecin did not show any activity with a MIC value higher than 128 μM against *E. coli* ATCC 25922 cells (data not shown).

Hemolytic Activity and Membrane Permeabilization Ability. As a measure of cytotoxicity of apidaecin peptoid−peptide hybrids, we determined their hemolytic activity and their ability to permeabilize cholesterol containing zwitterionic phospholipid bilayers, which can be considered a model of the eukaryotic cell membrane. On human erythrocytes, the lytic effect of all the tested peptides was negligible (≤2%) at either 30 or 300 μM (Table 2). Consistent with this low hemolytic activity, neither apidaecin nor its analogues induced leakage of an entrapped fluorescent dye from single unilamellar vesicles, even at high peptide-to-lipid ratio (see Supporting Information). These results indicate that the peptoid−peptide hybrids maintain the poor cytotoxic effect of the parent peptide, a feature that is shared by other Pro-rich peptides of both vertebrate and invertebrate origin,[3] and support the notion that apidaecin and its active peptoid analogues kill bacteria with a nonlytic mechanism.

Internalization Efficiency of Apidaecin Analogues in Cells. Several authors have emphasized the similarity between nonlytic antimicrobial peptides and cell-penetrating peptides[33] that translocate across the eukaryotic cell membrane via endocytic pathways and/or by a physical, energy-independent mechanism. In particular, arginine-rich peptides are highly effective as molecular transporters, and the substitution of the arginine residues by guanidinium-containing unnatural amino acids (e.g., D-arginine, β-amino acid, and peptoids) does not compromise the cellular uptake.[34] Some antimicrobial peptides have been able to translocate efficiently across cell membranes,[8,35] and in particular, amphibian buforin 2 could deliver a large cargo inside the cell.[35] In peptides belonging to the Pro-rich AMPs, such as the bovine Bac7 and insect pyrrhocoricin, the presence of a module with cell penetrating activity has been recognized.[6−8] Much less is known on the mechanism of penetration in bacterial cells, although a more specific traslocation machinery, involving a bacterial permease/membrane transporter, has been hypothesized.[10] By use of radiolabeled mutants of an isoform of apidaecin (Ho+), Castle et al.[4] showed that mutations of the C-terminal leucine led to full specific uptake of the peptide but largely reduced its killing activity. Moreover they demonstrated that proper peptide uptake of apidaecins is a necessary but not sufficient step for full antibacterial activity.

To verify whether the peptoid−peptide hybrids of apidaecin keep the translocation ability of the natural peptide, we investigated the cellular uptake of their fluorescent derivatives in bacterial and mammalian cells.

Uptake into Bacterial Cells. Although the peptides labeled with 5(6)-carboxyfluorescein lose most of their antibacterial activity, their penetrating capacity was investigated by confocal microscopy, after incubation of *E. coli* ATCC 25922 cells with peptides for 30 or 60 min. Less than 1% of the cells was stained by the fluorescent derivatives of either apidaecin or peptoid−peptide hybrids used at a concentration of 30 μM (data not shown). Quantitative data on the cellular uptake of apidaecin and [*N*Arg^{17}]apidaecin, obtained by flow cytometric analysis, confirmed the results of confocal microscopy. The cell-associated fluorescence of cells treated with fluoresceinated apidaecin was almost completely removed after extensive washing of bacteria with a solution at high salt concentration, showing that most of the peptide was not retained by the cells (Figure 3A). The residual fluorescence was efficiently abolished in the presence of the extracellular quencher Trypan blue (TB),[11] suggesting that the peptide was loosely associated with the bacterial surface (Figure 3B). Considering that the ineffective cellular uptake of our fluoresceinated peptides could be due to a reduction of the overall positive charge in the molecule, we prepared BODIPY-labeled derivatives of apidaecin and of [*N*Arg^{17}]apidaecin that retain the same net charge of the native peptide at pH 7. Cytofluorimetric analysis showed that BODIPY-labeled apidaecin was easily washed from the *E. coli* cells; nevertheless, the remaining cell-associated fluorescence was higher than with the fluoresceinated peptide, as a consequence of a stronger electrostatic interaction with the outer membrane (Figure 3C). Moreover a significant portion of Trypan blue resistant signal, due to BODIPY-labeled apidaecin, remains associated with the *E. coli* cells also after extensive washings (Figure 3D), consistent

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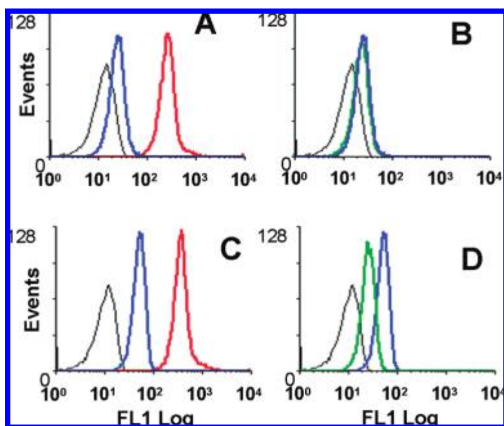

Figure 3. Binding and uptake of dye labeled apidaecin to *E. coli* ATCC 25922. The fluorescent label was 5(6)-carboxyfluoresceine (A, B) or BOPIPY (C, D). Bacterial cells (1×10^6 CFU/mL) were incubated with 10 μM peptide for 60 min and then analyzed by flow cytometry without washing (red lines), after washing with high-salt solution (blue lines), and after washing and incubation for 10 min with 1 mg/mL of the extracellular quencher TB (green lines). Black histograms represent the untreated bacterial cells.

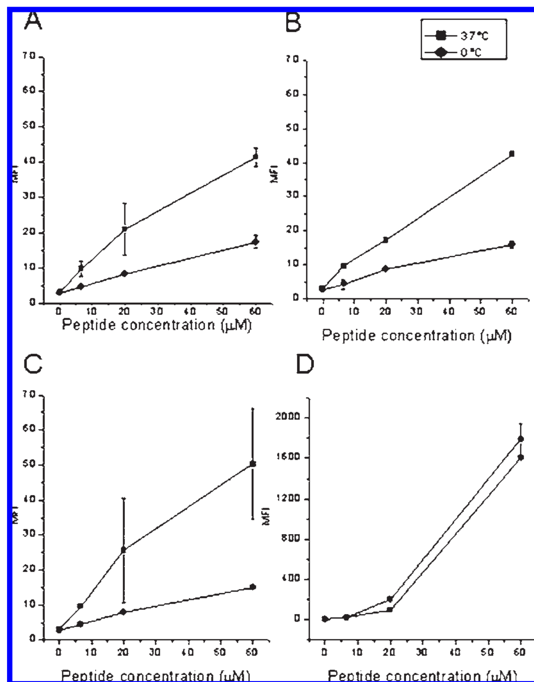

Figure 4. Dose-dependent uptake of the fluoresceinated derivatives of apidaecin (A), [*N*Arg[12]]apidaecin (B), [*N*Arg[17]]apidaecin (C), and HIV Tat_{47-57} peptide (D) in HeLa cells. HeLa cells were incubated at 37 or 0 °C for 1 h with increasing concentrations of each peptide. Data are from an experiment representative of two, run in triplicate, and bars represent ranges.

with the retention of antimicrobial activity observed for this labeled peptide. Data obtained for the BODIPY-labeled [*N*Arg[17]]apidaecin were, on the contrary, very similar to those of the fluoresceinated peptide (data not shown), and this observation again correlates with the loss of antimicrobial activity of such a derivative.

These experiments show that the peptide N-terminal modification with 5(6)-carboxyfluorescein seriously compromises the cell-penetrating activity of both apidaecin and its [NArg[17]]analogue, while N-terminal BODIPYlation is

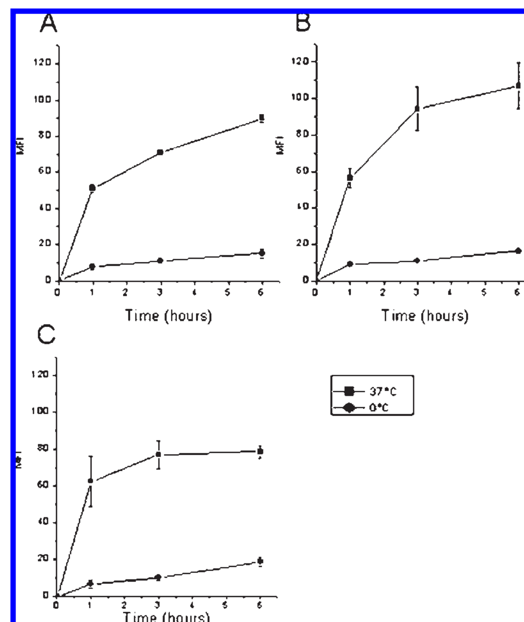

Figure 5. Time-dependent uptake in HeLa cells of fluoresceinated analogues of apidaecin (A), [*N*Arg[12]]apidaecin (B), and [*N*Arg[17]]apidaecin (C). HeLa cells were incubated at 37 or 0 °C for 1, 3, or 6 h with 60 μM of each peptide. Data are from an experiment representative of two, run in triplicate, and bars represent ranges.

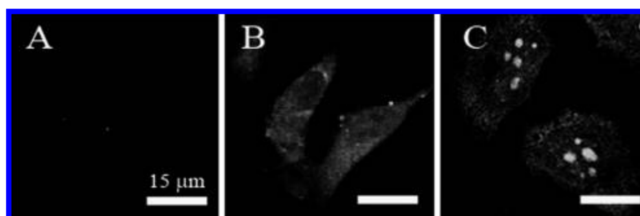

Figure 6. Confocal microscopy images of untreated HeLa cells (A) or after treatment for 16 h with 30 μM fluoresceinated derivatives of [*N*Arg[17]]apidaecin (B) or HIV Tat_{47-57} (C).

less functionally detrimental. As a consequence, using this fluorescence tag, we could establish that the loss of antimicrobial activity of [*N*Arg[17]]apidaecin is likely due to its inability to enter bacterial cells.

Translocation of Dye-Labeled Peptide in Mammalian Cells. The ability of the fluorescent derivatives of apidaecin and of its hybrid analogues, [NArg[12]]- and [NArg[17]]apidaecin, to enter HeLa cells was evaluated by flow cytometry, after incubation for 60 min at 37 °C, and compared to that of the known cell permeant peptide HIV Tat_{47-57}.[36] As shown in Figure 4, the amount of cell-associated apidaecin, as well as of the peptoid−peptide hybrids, was very low compared to the Tat peptide. Interestingly, at 0 °C, when energy-dependent cellular processes are blocked, the association of both apidaecin and its analogues to the cells was significantly reduced. In contrast, Tat association was essentially temperature-independent. The dose-dependent peptide association to cells was roughly linear and only slightly saturable at either 37 or 0 °C, in agreement with the lack of specific apidaecin receptors on HeLa cells. Competition experiments showing that an excess of unlabeled peptide did not affect the cell fluorescence due to labeled peptides confirmed these observation (see Supporting Information). Kinetics experiments showed that the cell-association

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efficiency of peptoid–peptide hybrids and of the natural peptide was the same (Figure 5). Confocal microscopy demonstrated that labeled apidaecin and its peptoid–peptide hybrids distribute in the whole cellular body and do not accumulate at the level of the plasma membrane or of any cytoplasmic structure. Conversely, the Tat peptide accumulates at the level of nucleoli (Figure 6), as already reported.[37]

The lack of saturation in the association to cells of apidaecin and its hybrids and the lack of competition are compatible with a conventional passive diffusion across the nonpolar interior of the plasma membrane.[34] Such diffusion may be preceded by the association of the side chain guanidinium groups to cell surface structures bearing a complementary charge, followed by a rapid translocation into the cell cytosol, which improves moving from 0 °C to physiological temperatures. Our data do not provide any insight into the reason for this temperature dependence. One possibility is that other mechanisms (e.g., endocytosis, a process inhibited at low temperatures) improve peptide cellular uptake. The need for a proper membrane fluidity or the involvement of energy-dependent peptide carriers cannot be excluded, and further studies are needed to clarify the mechanism of apidaecin translocation into the cytosol of eukaryotic cells.

In conclusion, the reported data are consistent with a receptor-independent but temperature-dependent crossing of the plasma membrane barrier by apidaecin and, with a similar efficacy, by its peptoid–peptide hybrids, followed by the translocation of the peptides in the cell cytoplasm.

Conclusion

We have shown that the synthesis of peptoid–peptide hybrids involving a side chain functionalized *N*Arg residue can be greatly facilitated by assembling the N-protected aminoalkylglycine residue first, followed the on-resin guanidinylation of the final peptoid–peptide hybrid. The replacement of some amino acids, recognized by specific proteases, by the corresponding peptoid residue significantly increases the stability to enzymatic degradation of the resulting apidaecin hybrids but can impair their antimicrobial activity. This effect is strongly related to the position of the peptoid residue in the apidaecin sequence. A *N*Arg residue at position 4 or 12 only slightly reduces the peptide antibacterial activity, but peptoid residues in the C-terminal part of the molecule (*N*Arg[17] or *N*Leu[18]) dramatically decrease the activity.

Considering that apidaecin kills bacteria with undetectable membrane permeabilization and by acting on cytoplasmatic targets, the step of peptide translocation across the bacterial membrane has a fundamental role on the whole mechanism of action. Uptake experiments in *E. coli* cells suggest that the inactivity of the [*N*Arg[17]]apidaecin is a consequence of its inability to translocate into the cell, further supporting the involvement of a membrane transporter in the translocation mechanism, as already shown for Bac7.[11]

By use of human cervical carcinoma HeLa cells, it has been shown that apidaecin and its peptoid–peptide hybrids can cross the cell membrane and enter the cytoplasm, although with a translocating efficiency far lower than that of the cell permeant Tat peptide. They do not exhibit any hemolytical activity on human red blood cells even at 300 μM peptide concentration, resulting even less cytotoxic than Tat peptides.[38] Compared to other highly cationic guanidinium-carrier systems (oligoarginines, peptoids, etc.),[34] apidaecin peptoid–peptide hybrids also contain a significant number of prolines, which could contribute to the translocation in eukaryotic cells, in analogy with other proline-rich peptide,[6,39] and influence the intracellular localization of the peptide vector. This hypothesis is supported by the confocal microscopy studies with apidaecin derivatives that showed a diffuse, nonstructured signal in the cell cytoplasm, while peptoidic guanidinium-carrier systems accumulate preferentially in the nucleus.[40]

The significant stability of the apidaecin peptoid–peptide hybrids and their virtual absence of cytotoxic activity indicate them as a possible model to design new carrier systems for bioactive molecules.

Experimental Section

Materials and Methods. All chemicals were commercial products of the best grade available. Fmoc-Leu-Wang resin and 2-chlorotrityl resin were purchased from Novabiochem (Merck Biosciences). Trypsin from bovine pancreas (≥10000 BAEE units/mg protein), Trypan blue, 5(6)-carboxyfluorescein, Fmoc-amino acids, and all other chemicals for the solid phase synthesis were supplied by Sigma-Aldrich. 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl (BODIPY FL) *N*-(2-aminoethyl)maleimide was from Invitrogen (Molecular Probes). Analytical HPLC separations were carried out on a Dionex Summit dual-gradient HPLC, equipped with a four-channel UV–vis detector, using a Vydac 218TP54 column (250 mm × 4.6 mm, 5 μm, flow rate of 1.5 mL/min, W. R. Grace and Co.). The mobile phases A (aqueous 0.1% TFA) and B (90% aqueous acetonitrile containing 0.1% TFA) were used for preparing binary gradients. All analyses were carried out under gradient conditions (10–50% B in 20 min except otherwise indicated). All crude peptides were purified for analytical and other experimental purposes. Semipreparative HPLC was carried out on a Shimadzu series LC-6A chromatographer, equipped with two independent pump units, a UV–vis detector, and a Vydac 218TP1022 column (250 mm × 22 mm, 10 μm, flow rate of 15 mL/min). Elutions were carried out by the same mobile phases described above. All the purified peptides (≥95% purity established by HPLC analysis) were characterized by mass spectrometry (MS). Mass spectral analyses were carried out on a Mariner API-TOF workstation (PerSeptive Biosystems Inc.) operating in positive mode. CD measurements were carried out on a Jasco-715 spectropolarimeter, using a quartz cell of 0.02 cm path length. The spectra were recorded at 298 K and were the average of a series of six scans made at 0.1 nm intervals in the 250–190 nm region. Sample concentrations in 10 mM Tris buffer (pH 7.4), aqueous 95% TFE, and aqueous 30 mM SDS were in the range 0.11–0.15 mM, as determined by quantitative UV measurements (ε_{Tyr} = 1420 M^{-1} cm^{-1} at 275 nm). Ellipticity is reported as mean residue ellipticity $[\theta]_R$ (deg cm^2 $dmol^{-1}$).

Synthesis of Peptoid–Peptide Hybrids. Peptides were synthesized at 0.10 mmol scale, using an Advanced Chemtech 348Ω peptide synthesizer, starting from Fmoc-Leu-Wang (substitution 0.6 mmol/g resin) or, only for compound **9**, 2-chlorotrityl resin (substitution 1.5 mmol/g resin). The *tert*-butyl and 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) groups were used to protect tyrosine and arginine side chains, respectively, and the trityl (Trt) group was used for masking the asparagine, histidine, and cysteine side chains. Fmoc deprotection was achieved with 20% piperidine in DMF (5 + 15 min). Couplings were performed in the presence of HBTU/HOBt/DIPEA (reaction time of 45–60 min), using an excess of 4 equiv of the carboxyl component. HATU was used as coupling reagent instead of HOBt to acylate peptoid residues. Preformed Fmoc-*N*Arg(Pmc)-OH and Fmoc-*N*Leu-OH were prepared according to the literature[17,41] and used in the synthesis of peptoid–peptide hybrids **8** and **9** as well as of the

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fluoresceinated derivatives. In all other cases, the peptoid residue was introduced on the growing peptide chain by the submonomer method,[16] according to the modified procedure outlined below.

(a) For on-resin assembling of N^{κ}-protected *N*-aminoalkylglycine residue, a 1 M solution of bromoacetic acid in DMF (10 equiv) and DIC (10 equiv) was added to the N^{α}-deprotected peptide resin. After the mixture was stirred for 45 min, the resin was washed with DMF (6 times) and a solution of the selected diamine (1,3-diaminopropane, 1,2-diaminoethane, or 1,4-diaminobutane, 30 equiv) in DMF was added. After 2 h of reaction, the resin was collected by filtration and washed with DMF. The primary amino group on the resulting peptoid residue was protected by reaction with a 0.5 M solution of Dde-OH (10 equiv, 90 min) in DMF, and the assembling of the peptide chain was resumed. The N-terminal amino acid residue was introduced as Boc derivative. Different from the Fmoc group, the Boc is stable to the basic conditions required to remove the Dde group.

(b) For on-resin guanidinylation of peptoid−peptide hybrids, the peptoid−peptide hybrid, still attached to the solid support, was repetitively treated with 2% hydrazine in DMF (3 times for 3 min) to remove the Dde group from the peptoid side chain. After the usual washing cycles, the resulting *N*-aminoalkylglycine residue was guanidinylated by reaction with 2 equiv of *N*,*N'*-bis-Boc-1-guanylpyrazole (0.03 M in DMF); the reaction was performed at 35 °C and was complete in less than 2 h.

Cleavage of peptides from the resin and removal of the acid labile protecting groups were simultaneously achieved by treatment of the final peptide−peptoid hybrid resin with a TFA−H_2O−triisopropylsilane (TIS) mixture (95:2.5:2.5 by volume) for 90−120 min at room temperature. Peptides were precipitated by addition of cold diethyl ether and dried overnight under vacuum. Crude peptides were obtained in 70−80% yield except compound **9** (33% yield), whose synthesis suffered extensive diketopiperazine formation. After purification by semipreparative HPLC (linear gradient of 15−45% B in 20 min), the peptides where analyzed by HPLC and ESI-MS (Supporting Information Table 1).

Synthesis of Labeled Peptide Conjugates. The peptide, free at the N^{α}-terminal amino group and still anchored to the resin, was reacted overnight with 5(6)-carboxyfluorescein in the presence of DIC/HOBt (2.5 equiv/2.5 equiv), as coupling reagent. To prevent an overincorporation of carboxyfluorescein, two 30 min treatments with 20% piperidine−DMF were carried out before cleavage of the peptide from the resin.[42] Cleavage was carried out as previously described, and the fluoresceinated peptide was purified by semipreparative HPLC (linear gradient 25−45% B in 20 min) and analyzed by HPLC and ESI-MS (Supporting Information Table 1).

To conjugate the BODIPY dye through an aminoethylmaleimido spacer to peptides, Cys-apidaecin and Cys-[*N*Arg[17]]apidaecin were synthesized on solid-phase according to the general procedure previously described. Peptides were cleaved from the resin with a mixture of TFA−H_2O−1,2-ethanedithiol−TIS (94:2.5:2.5:1 by volume), precipitated, and repeatedly washed with ether, dried in vacuo, and stored under argon. Labeling was performed by adding aliquots of the lyophilized peptide to a 60 μM solution of BODIPY *N*-(2-aminoethyl)maleimide in 25% CH_3CN in phosphate buffer (60 mM, pH 7.2). The final peptide/dye ratio was 1/5. The reaction mixture was stirred at room temperature for 3 h in the dark, under nitrogen bubbling, and overnight at 4 °C. The unreacted dye was quenched by addition of 10-fold excess of cysteine. After 1 h, the reaction mixture was diluted with 0.1% aqueous TFA to a final concentration of 15% CH_3CN and eluted from a semipreparative column with a linear gradient from 15% to 50% B in 40 min. Purity and identity of the conjugated peptides were confirmed by analytical HPLC and ESI-MS (Supporting Information Table 1).

Stability of Apidaecin Peptoid−Peptide Hybrids to Tryptic Degradation. Each peptide was dissolved in water to a final concentration of 2 mg/mL. A trypsin solution was prepared by dissolving 1 mg of trypsin from bovine pancreas (13 500 units/mg of protein) in 1 mL of 1 mM HCl. A mixture of 15 μL of freshly made trypsin solution, 150 μL of peptide solution, and 150 μL of 0.1 M NH_4HCO_3 buffer (pH 8) was incubated at 37 °C on a rocking platform. Aliquots of 30 μL were sampled at different times, diluted with 200 μL of 2% aqueous formic acid, and analyzed by HPLC (gradient from 2% to 50% B in 40 min) and ESI-MS. Samples without trypsin addition were taken at zero time and after 24 h and used as negative controls.

Bacterial Strains. The bacterial strains used in this study were *Escherichia coli* ML35, ATCC 25922 and O18K1H7, *Klebsiella pneumoniae* 22 (a clinical isolate), *Salmonella enteritidis* D5 and PD1 (two clinical isolates), and *Salmonella enterica* serovar Typhimurium ATCC 14028.

Antimicrobial Activity. MICs of the peptides were determined by the broth microdilution susceptibility test following the guidelines of the NCCLS with mid-log phase cultures. Serial 2-fold dilutions of each peptide were prepared (final volume of 50 μL) in 96-well polypropylene microtiter plates (Sarstedt, Germany) with 50% Muller−Hinton (MH) broth in phosphate-buffered saline (PBS). Each dilution series included a control well without peptide. A total of 50 μL of the adjusted inoculum (approximately 2.5×10^5 cells/mL) in 50% MH broth was then added to each well. To evaluate the MIC, microtiter plates were incubated overnight at 37 °C. The MIC value was defined as the lowest peptide concentration that prevented visible bacterial growth after incubation for 18 h at 37 °C.

Hemolytic Activity. Erythrocytes were prepared from freshly collected human blood, anticoagulated with citrate−dextrose as previously described.[43] The assays were performed with 0.5% (v/v) erythrocyte suspensions in 10 mM phosphate buffer, pH 7.4, containing 139 mM NaCl, at 37 °C. After incubation for 60 min with the peptides at 30 and 300 μM, the reaction was stopped with cold buffer and the supernatant was carefully collected after centrifugation at 10000*g* for 1 min. The hemoglobin released in the supernatant was measured at 415 nm, and the percentage of hemolysis was determined as previously described.[43] The α-helical bee venom toxin melittin was used as positive control.

Evaluation of the Uptake of the Peptides by *E. coli*. Cellular uptake of antibacterial peptides labeled with 5(6)-carboxyfluorescein or BODIPY was determined by flow cytometry,[44] using a Cytomics FC 500 instrument (Beckman-Coulter, Fullerton, CA) equipped with an argon laser (488 nm, 5 mW) and a photomultiplier tube fluorescence detector for green (525 nm) filtered light. All detectors were set on logarithmic amplification. Optical and electronic noises were eliminated by setting an electronic gating threshold on the forward-scattering detector, while the flow rate was kept at a data rate below 300 events per second to avoid cell coincidence. At least 10 000 events were acquired for each sample. Data analysis was performed with the WinMDI software (Dr. J. Trotter, Scripps Research Institute, La Jolla, CA). Mid-log phase bacteria (*E. coli* ATCC 25922) were harvested, diluted to 1×10^6 CFU/mL, and incubated in MH broth with different concentrations of the fluorescently labeled peptides at 37 °C for 60, 120, and 240 min. Treated cells were washed several times with buffered high-salt solution (10 mM sodium phosphate, 400 mM NaCl, 10 mM $MgCl_2$, pH 7.2) and immediately analyzed by the flow cytometer with or without a 10 min preincubation at room temperature with 1 mg/mL of the quencher TB. Cells incubated in the absence of peptides were used as control.

For fluorescence microscopy experiments, *E. coli* cells (2×10^5 CFU/mL) were incubated at 37 °C for 24 h with different concentrations of fluorescein labeled peptides. Treated cells were washed several times with PBS, resuspended in 10 μL of PBS, and placed onto a microscope slide. Cells were examined

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with a Leica 5000 B fluorescence microscope, using the I3 filter cube (λ excitation, 450−490 nm; λ emission, >515 nm). Confocal microscopy images were obtained with cells incubated with the labeled peptides and then immobilized on polylysine-covered microscopy slides. The images were acquired with a Leica TSC SP2 confocal microscope.

Evaluation of the Uptake of the Peptides by HeLa Cells. HeLa cells were maintained in modified Eagle's medium (MEM) (GIBCO BRL) supplemented with gentamicin (50 μg/mL) and 10% (v/v) heat-inactivated FCS. The day before the experiment, semiconfluent HeLa cells were suspended by trypsin−EDTA treatment, seeded onto 24-well culture plates (Falcon) at a density of 6×10^4 cells per well, and incubated for 24 h. The cells were then incubated at 37 or 0 °C for different times and with different concentrations of the 5(6)-carboxyfluorescein-conjugated peptides in MEM medium, supplemented with 10% FCS and 1% gentamicin. Cells were then washed with PBS and incubated for 2 min at 37 °C with 1 mg/mL trypsin to remove surface-bound peptide. Finally, the cells were resuspended in FACS buffer (PBS, 1% FBS) and were scored using a FACScan analyzer (Becton Dickinson) equipped with an argon laser (488 nm, 15 mW) and fluorescence detection for green (525 nm) filtered light. Permeabilized cells were excluded from FACS analysis by adding propidium iodide. Data were processed using the WinMDI software; a minimum of 20 000 events per sample was analyzed.

For microscopy experiments, HeLa cells were incubated or not with 30 μM fluoresceinated peptides for 16 h at 37 °C or at 0 °C. After three washings with PBS, the cells were fixed in ice cold methanol for 5 min and washed three times with PBS; coverslips were mounted in PBS containing 90% glycerol and 3% *N*-propylgallate and were analyzed with a confocal microscope (Bio-Rad MRC1024ES).

Acknowledgment. We thank Dr. Patrizia Polverino de Laureto for MS/MS peptide sequence analysis of des-Pro[5,14]-apidaecin. This research was supported by PRIN 2007 (to R.G.) and institutional grants from the Italian Ministero dell'Istruzione, dell'Università e della Ricerca.

Supporting Information Available: Table of the analytical RP-HPLC and ESI-MS data of the synthesized peptides, additional CD spectra, membrane permeabilization assays, and data from cellular uptake experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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