Antimicrobial Peptides: Synthesis and Antibacterial Activity of Linear and Cyclic Drosocin and Apidaecin 1b Analogues†

Marina Gobbo,*,‡ Laura Biondi,‡ Fernando Filira,‡ Renato Gennaro,§ Monica Benincasa,§ Barbara Scolaro,‡ and Raniero Rocchi‡

Institute of Biomolecular Chemistry, C. N. R., Department of Organic Chemistry, University of Padova, via Marzolo, 1, I-35131 Padova, Italy, and Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, via Giorgieri, 1, I-34127 Trieste, Italy

Received February 25, 2002

Drosocin and apidaecin Ib are two insect antimicrobial peptides showing a significant sequence homology and a common mechanism of action, which includes stereoselective elements but is devoid of any pore-forming activity. A substantial difference between the two peptides is the presence in the drosocin sequence of an *O*-glycosylated threonine residue, which is important for its antimicrobial activity. Through the synthesis of a series of differently glycosylated drosocin analogues, we have shown that the antimicrobial activity against several Gram-negative bacteria appears to be modulated by the sugar moiety (Gal vs GalNAc) and the type of glycosidic linkage (α-*O*-, *β*-*O*-, or α-*C*-). The insertion of a glycosylated threonine residue in the apidaecin Ib sequence improves the sequence homology with drosocin but reduces the antimicrobial activity. To gain information on the possible bioactive conformation of these peptides, we synthesized an unglycosylated cyclic analogue of drosocin, containing an intrachain disulfide bond, and the head-to-tail cyclic analogues of drosocin and apidaecin, as well as their corresponding cyclic dimers. Only the large cyclic dimer of apidaecin partially retained the antimicrobial activity, suggesting that a bending of the peptide chain, in particular in the middle of the molecule, is not a structural element characteristic of the bioactive conformation of drosocin and apidaecin. Experiments aimed at testing the effect of selected drosocin and apidaecin peptides on biological membranes showed that some peptides display a moderate hemolytic activity and that a dissociation between antibacterial activity and cytotoxicity to eukaryotic cells can be achieved in differently glycosylated peptide analogues.

Introduction

The remarkable resistance of insects to bacterial infection is at least in part explained by their ability to rapidly synthesizing a battery of small-sized cationic antibacterial peptides.[1−4] The large number of inducible antibacterial peptides/polypeptides isolated from a number of insect species was tentatively grouped into four families. Two of these, the cecropins and insect defensins, are relatively homogeneous, while the families of the proline-rich and of the glycine-rich peptides are quite heterogeneous.[5]

A remarkable feature of some proline-rich antibacterial peptides from insects, such as drosocin,[7] pyrrhocoricin,[8] formaecins,[9] diptericin,[10] and lebocins,[11] is the presence of an *O*-glycosylated substitution on a conserved threonine residue. Glycosylation with one, two, or three glycan residues has been described.[7−11] Drosocin, the first characterized *O*-glycosylated antibacterial peptide,[7] consists of 19 amino acid residues, nearly one-third of which are prolines, and contains three characteristic Pro-Arg-Pro motifs. Drosocin is glycosylated on Thr 11 by either a monosaccharide (2-acetamido-2-deoxy-D-galactopyranosyl (GalNAc) → Thr, drosocin MS) or a disaccharide (D-galactopyranosyl (Gal) → GalNAc → Thr, drosocin DS), and is active against Gram-negative bacteria at concentrations in the low micromolar range. Previous studies showed that drosocin bearing the disaccharide was the most active glycoform, whereas the activity of the unglycosylated drosocin was significantly lower than that of the glycosylated peptides.[12] The integrity of the carbohydrate side chain is also necessary for maximum antimicrobial activity of other antibacterial peptides such as formaecin 1[9] and diptericin.[10] On the contrary, unglycosylated pyrrhocoricin appears to be more potent than the native glycosylated peptide.[13] The significance of the carbohydrate moiety is still unknown, and only hypotheses can be made. On the basis of conformational NMR studies on drosocin and on its unglycosylated derivative, MacManus et al.[14] suggested that the presence of the disaccharide in the middle of the molecule may open the turn comprising residues 10−13 to a more extended conformation, thus helping drosocin to assume the proper orientation to bind to its putative intracellular target.

The relatively slow-killing kinetics of drosocin and the observation that an all-D analogue is 50−150-fold less active than the native isomer suggested that the peptide may act through a nonpore-forming mechanism, involving interaction with a stereospecific target, which is thus

* To whom correspondence should be addressed. Tel: +39049 8275741. Fax: +39049 8275239. E-mail: marina.gobbo@unipd.it.

† Presented in part at the 26th European Peptide Symposium, Montpellier, France, September 2000 (Abstracts of Communications P64) and at the 2nd International Peptide Symposium−17th American Peptide Symposium, San Diego, CA, June 2001 (Abstracts of Communications P468).

‡ University of Padova.
§ University of Trieste.

10.1021/jm020861d CCC: $22.00
Published on Web 08/27/2002

markedly dissimilar to that of lytic peptides such as cecropins.[12] A similar killing mechanism[15,16] was originally proposed for the apidaecins, a family of 18 residue, Pro-rich peptides isolated from Hymenoptera and predominantly active against Gram-negative species.[17] Apidaecins contain up to 33% of proline residues with characteristic Pro-Arg-Pro or Pro-His-Pro motifs and show a significant sequence homology with drosocin, pyrrhocoricin, and formaecins but lack the *O*-glycosylated threonine residue.

The search for novel antibacterial agents and the attempt to modify and improve the features of the currently available chemotherapeutic agents are in high demand as a consequence of the rapid spread of multidrug resistant bacterial pathogens. It was recently reported that glycosylated drosocin was completely inactive when intravenously injected into mice infected with a lethal dose of *Escherichia coli*.[14] The lack of in vivo activity was attributed to an unusually high degradation rate of the peptide in mouse serum in contrast to the considerable stability in insect hemolymph.

In an attempt to improve the drosocin features and to make it suitable for drug development, a structure−activity relationship study was undertaken. The role of the carbohydrate component was investigated by varying the monosaccharide as well as the type of sugar−peptide linkage. In particular, the glycosylated Thr 11 was either substituted by alanine or (Galα)Ala (Table 1). For comparison, the unglycosylated drosocin, the (Galα)Thr-drosocin, and the (Gal*β*)Thr-drosocin were also synthesized. In addition, cyclic and semicyclic analogues were synthesized to test the importance of an extended conformation occurring in the middle of the molecule, as suggested by the NMR studies. Cyclic drosocin (Table 2) was prepared by head-to-tail cyclization of the linear, side chain-protected, unglycosylated peptide. A semicyclic analogue was prepared by replacing both Ser 7 and Ser 12 with Cys, followed by oxidation to form [Cys^{7,12}]drosocin with an intramolecular disulfide bond, a posttranslational modification relatively common in antimicrobial peptides.[6] An attempt was also made to emphasize the sequence similarity between drosocin and apidaecin Ib by inserting a threonine residue, either glycosylated or not, in the proper position of the apidaecin sequence, or by removing the threonine residue from the drosocin sequence. For structure−activity relationship studies, the head-to-tail cyclic apidaecin Ib was also synthesized. The conformational properties of the synthetic peptides and glycopeptides were examined by CD, and their antibacterial activity was tested against several bacterial pathogens, including the encapsulated *E. coli* O18:K1:H7, a common cause of neonatal bacteremia and meningitis. The ability of the drosocin and apidaecin analogues to permeabilize natural and artificial phospholipid membranes was also tested.

Results and Discussion

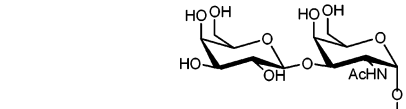
Peptide Synthesis. The synthesis of linear peptides and glycopeptides, shown in Table 1, was carried out by the solid phase methodology, using preformed glycosylated amino acids, either *O*-acetylated or unprotected at the sugar moiety. Yields, after high-performance liquid chromatography (HPLC) purification, and physicochemical properties of the synthesized peptides are shown in Table 3.

Head-to-tail cyclic peptides **XII^{b}** and **XIII^{b}** (Table 2) were obtained by cyclization in solution of the protected linear peptides **XII^{a}** and **XIII^{a}** (see Experimental Section), previously assembled by the solid phase procedure. For the sake of comparison, cyclization reactions were carried out under high-dilution conditions, in dimethylformamide (DMF) or in dichloromethane (DCM), by using different coupling reagents [2(1H-benzotriazole-1-yl)-1,1,3,3,tetramethyluronium tetrafluoroborate (TBTU)/hydroxybenzotriazole (HOBt), 1-benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP), or diphenylphosphoryl azide (DPPA)]. In all cases, cyclization occurred but the reaction was faster by using the uronium-based reagents instead of DPPA (1−2 h vs 20 h). In the presence of PyBOP as the condensing agent, cyclization of the linear peptides was over in about 20 min in DMF but it took 2 h in DCM. The choice of the solvent also affected the regiochemistry of the cyclization reaction involving the apidaecin-protected analogue **XIII^{a}**. A surprisingly high formation of the cyclic dimer, as compared to that of the cyclic monomer, occurred in DCM but not in DMF. Cyclodimerization and oligomerization are well-documented side reactions occurring during the synthesis of short homodetic cyclic peptides.[20−21] These side reactions depend on a number of factors, such as the sequence and the concentration of the linear peptide, the coupling reagent, and the solvent. The high formation of the apidaecin cyclodimer in DCM could be attributed to an uneffective solvation of the linear peptide, which favored an antiparallel arrangement of the peptide chain prior to cyclization.

Selective formation of the intramolecular disulfide bond in the [Cys^{7,12}]drosocin analogue (**VIII**) was achieved by air oxidation of highly diluted buffered solutions (pH 8.0). Addition of a small amount of dimethyl sulfoxide (10%) to the reaction mixture[22] sped up the formation of the product (**XIV**) (about 6 h vs 40 h) but made the following workup of the reaction mixture more difficult. The physicochemical properties of the drosocin and apidaecin cyclic analogues are shown in Table 3.

Circular Dichroism (CD) Study. The conformational properties of drosocin and apidaecin analogues were investigated by CD spectroscopy in water, in 2,2,2-trifluoroethanol (TFE) and in micellar sodium dodecyl sulfate (SDS, 30 mM). As an example, the CD spectra of apidaecin and its cyclic analogue in different solvents are shown in Figure 1A,B, respectively. A conformational CD study on drosocin and its unglycosylated analogue, in water and in TFE, was previously reported.[12] As shown in Figure 1A for apidaecin, the CD spectrum in water of linear peptides, either glycosylated or not, was characterized by a broad negative band around 200 nm, characteristic of unordered structures. Cyclization did not restrict significantly the conformational freedom of the peptide chain and the CD curve, in water, of peptides **XII^{b}** and **XIII^{b}** was similar to that of the corresponding linear peptide, as shown in Figure 1B for cycloapidaecin. In TFE, linear and cyclic analogues of drosocin exhibited a CD pattern qualitatively

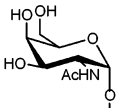
Table 1. Amino Acid Sequence of Native Drosocin DS, Unglycosylated Drosocin (**I**), (des Thr[11])drosocin (**II**), (GalNAcα-*O*-Thr[11])drosocin (**III**), (Galα-*O*-Thr[11])drosocin (**IV**), (Gal*β*-*O*-Thr[11])drosocin (**V**), [Ala[11]]drosocin (**VI**), [Galα-*C*-Ala[11]]drosocin (**VII**), [Cys[7,12]]drosocin (**VIII**), Apidaecin Ib (**IX**), [Endo Thr[13a]]apidaecin Ib (**X**), and [Endo GalNAcα-*O*-Thr[13a]]apidaecin Ib (**XI**)



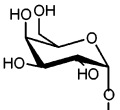
Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Thr-Ser-His-Pro-Arg-Pro-Ile-Arg-Val **Drosocin DS**
1 19

Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Thr-Ser-His-Pro-Arg-Pro-Ile-Arg-Val **I**
1 19

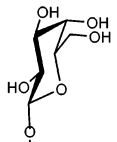
Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Ser-His-Pro-Arg-Pro-Ile-Arg-Val **II**
1 18



Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Thr-Ser-His-Pro-Arg-Pro-Ile-Arg-Val **III** **Drosocin MS**
1 19

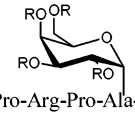


Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Thr-Ser-His-Pro-Arg-Pro-Ile-Arg-Val **IV**
1 19



Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Thr-Ser-His-Pro-Arg-Pro-Ile-Arg-Val **V**
1 19

Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Ala-Ser-His-Pro-Arg-Pro-Ile-Arg-Val **VI**
1 19

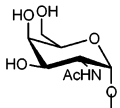


Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Ala-Ser-His-Pro-Arg-Pro-Ile-Arg-Val **VII[a]** R=Ac
1 19 **VII[b]** R=H

Gly-Lys-Pro-Arg-Pro-Tyr-Cys-Pro-Arg-Pro-Thr-Cys-His-Pro-Arg-Pro-Ile-Arg-Val **VIII**
1 19

Gly-Asn-Asn-Arg-Pro-Val-Tyr-Ile-Pro-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu **IX** **Apidaecin Ib**
1 18

Gly-Asn-Asn-Arg-Pro-Val-Tyr-Ile-Pro-Gln-Pro-Arg-Pro-Thr-Pro-His-Pro-Arg-Leu **X**
1 19



Gly-Asn-Asn-Arg-Pro-Val-Tyr-Ile-Pro-Gln-Pro-Arg-Pro-Thr-Pro-His-Pro-Arg-Leu **XI**
1 19

similar to type C spectra,[23] characterized by two negative bands at about 225 and 203 nm, respectively, and a positive band at 195 nm (data not shown). This pattern, previously reported also for the native drosocin,[12] has been assigned to the presence of type I (III) *β*-turns or *β*-turn mixtures with significant type I character. The structure promoting effect of TFE was apparently less marked on apidaecin and its elongated analogues **X** and **XI**, whose CD spectra (Figure 1C) were characterized by a more or less pronounced shoulder at 218 nm and an intense negative band at 200 nm, indicative of a significant presence of unordered structures, particularly in the case of apidaecin and its glycosylated analogue **XI**. On the contrary, cyclic apidaecin exhibited, in the same solvent, a characteristic type C CD spectrum (Figure 1B) similar to that previously described for drosocin and its analogues.

Micellar SDS has been used as a model of the negatively charged bacterial lipid membranes, with which highly cationic peptides first interact. In this environment, the CD pattern of the cyclic peptides **XII[b]** and **XIII[b]** was similar to that observed in TFE (Figure 1B), suggesting that the cyclic analogues interact with the hydrophobic core of the micelle. In SDS, the spectrum of linear peptides was characterized by a broad negative band at around 200 nm, far less intense than that observed in water (Figure 1A), suggesting a mainly

Table 2. Amino Acid Sequence of Cyclo^{N-C}Drosocin (**XII**[b]), Cyclo^{N-C}Apidaecin Ib (**XIII**[b]), Cyclo^{S-S} [Cys^{7,12}]drosocin (**XIV**), Cyclo^{N-C}Dimer of Unglycosylated Drosocin (**XII**[c]), and Cyclo^{N-C}Dimer of Apidaecin Ib (**XIII**[c])

Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg (1) / Val-Arg-Ile-Pro-Arg-Pro-His-Ser-Thr-Pro (19)	**XII**[b]
Gly-Asn-Asn-Arg-Pro-Val-Tyr-Ile-Pro (1) / Leu-Arg-Pro-His-Pro-Pro-Arg-Pro-Gln (18)	**XIII**[b]
Gly-Lys-Pro-Arg-Pro-Tyr-Cys(S)-Pro-Arg-Pro-Thr-Cys(S)-His-Pro-Arg-Pro-Ile-Arg-Val (1–19)	**XIV**
Gly-Lys-Pro-Arg-Pro-Tyr-Ser-Pro-Arg-Pro-Thr-Ser-His-Pro-Arg-Pro-Ile-Arg-Val (1) / Val-Arg-Ile-Pro-Arg-Pro-His-Ser-Thr-Pro-Arg-Pro-Ser-Tyr-Pro-Arg-Pro-Lys-Gly (38)	**XII**[c]
Gly-Asn-Asn-Arg-Pro-Val-Tyr-Ile-Pro-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu (1) / Leu-Arg-Pro-His-Pro-Pro-Arg-Pro-Gln-Pro-Ile-Tyr-Val-Pro-Arg-Asn-Asn-Gly (36)	**XIII**[c]

Table 3. Physicochemical Properties of Linear and Cyclic Analogues

compd no.	peptide	yield (%)	$[\alpha]_D^{20°}$ (°)[a]	HPLC[b] (t_R, min)	MALDI-TOF MS $[M + H]^+$
I	(Thr^{11})drosocin	62	−178.7	13.74	2199
II	(des Thr^{11})drosocin	76	−126.1	13.75	2099
III	(GalNAcα-O-Thr^{11})drosocin	56	−102.0	13.23	2402
IV	(Galα-O-Thr^{11})drosocin	56	−88.0	13.53	2362
V	(Galβ-O-Thr^{11})drosocin	62	−103.0	13.38	2362
VI	[Ala^{11}]drosocin	69	−170.6	13.66	2171
VII[b]	[Galα-C-Ala^{11}]drosocin	62	−144.5	13.63	2333
VIII	[Cys^{7,12}]drosocin	65	−147.9	15.43	2230
IX	apidaecin Ib	56	−119.8	16.54	2108
X	[endo Thr^{13a}]apidaecin Ib	63	−144.1	16.20	2210
XI	[endo GalNAcα-O-Thr^{13a}]apidaecin Ib	44	−120.6	10.54	2412
XII[b]	cyclo $^{N-C}$drosocin	59	−104.3	13.86	2181
XIII[b]	cyclo $^{N-C}$apidaecin Ib	43	−109.8	16.96	2093
XII[c]	cyclo $^{N-C}$(drosocin)$_2$	5−10	−106.8	15.82	4363
XIII[c]	cyclo $^{N-C}$(apidaecin Ib)$_2$	5−20	−132.6	18.38	4185
XIV	cyclo ^{SS}[Cys^{7,12}]drosocin	48	−64.1	14.72	2228.2[c]

[a] Optical rotations in water at $c = 1$. [b] See Experimental Section. [c] ESI MS.

electrostatic peptide/micelles interaction without clear effects on the peptide conformation.

Antibacterial Activity. The antibacterial properties of the drosocin and apidaecin synthetic analogues were investigated against an array of Gram-negative and Gram-positive microorganisms and compared with those of the [GalNAcα-Thr^{11}]drosocin (**III**) and apidaecin Ib (**IX**). As already observed for the native peptides,[12,17] the activity of the synthetic analogues, when present, is directed against Gram-negative bacteria (Table 4). The three new synthetic glycoforms of drosocin, bearing a galactose moiety linked to residue 11 through a α-*O*- (**IV**), *β*-*O*- (**V**), or a metabolically stable α-*C*-glycosidic bond (**VII**[a] and **VII**[b]), were all in general more active than the unglycosylated peptide **I**, in the order **III** > **IV** ≈ **V** > **VII**[a] ≈ **VII**[b] ≥ **I**, but less active than the native glycoform **III**. Some bacterial strains, e.g., *Klebsiella pneumoniae* and especially *E. coli* D21, were highly susceptible to the native glycopeptide **III**.

Conversely, *E. coli* D22, a strain derived from *E. coli* D21 and with a barrier defect in the outer membrane due to mutation of the *env*A gene, was susceptible to all glycosylated analogues. The bacterial elements responsible for these striking differences in activity are not yet known. One possibility could be that one of the putative steps involved in the bacterial inactivation might require a specific recognition of the sugar residue. To address this question, the antimicrobial activity of **III** was evaluated in the presence of *N*-acetyl-D-galactosamine, D-galactose, or isopropyl *β*-D-thiogalactopyranoside, a nonmetabolizable galactose analogue, added at high concentrations (10 mM) in the culture medium to compete for a possible receptor of the glycosylated peptide. However, no inhibitory effect was observed as the minimum inhibitory concentration (MIC) values remained substantially unchanged (data not shown). In summary, bacteria appear to be susceptible to the different glycoforms, and as previously reported,[12,13,24] glycosylation is necessary for maximal antimicrobial activity, which nevertheless does not depend on a specific interaction with the sugar. On the contrary, modifications in the drosocin peptide sequence, as in

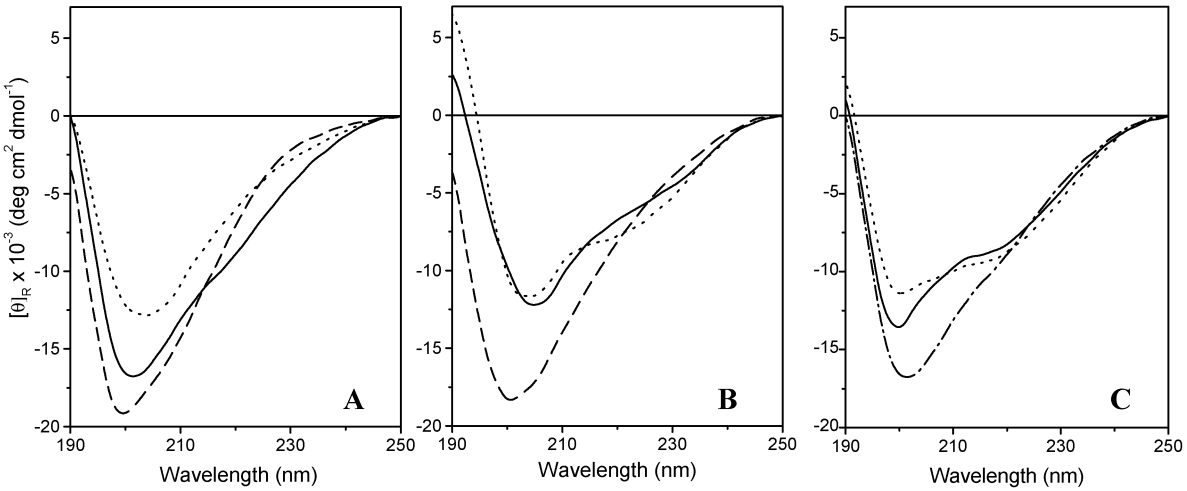


Figure 1. (A) CD spectra of apidaecin Ib (**IX**) in water (- - -), 95% TFE (—), and 30 mM SDS (· · ·). (B) CD spectra of cyclic apidaecin (**XIII**[b]) in water, 95% TFE, and 30 mM SDS (legend as in panel A). (C) CD spectra of linear apidaecin analogues in 95% TFE (— · —, apidaecin Ib (**IX**); —, peptide **XI**; and · · ·, peptide **X**).

Table 4. Antibacterial Activity of Linear and Cyclic Analogues of Drosocin and Apidaecin Ib

	MIC[a] (μM)															
strain	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**[a]	**VII**[b]	**IX**	**X**	**XI**	**XII**[b]	**XII**[c]	**XIII**[b]	**XIII**[c]	**XIV**
E. coli ML-35	2	32	**1**	2	1	16	4	2	**4**	>128	32	>128	>64	64	16	>64
E. coli ATCC 25922	4	64	**1**	2	4	64	8	4	**4**	>128	32	>128	>64	>64	16	>64
E. coli D21	>128	>64	**8**	>64	>64	>128	>128	>128	>**64**	>64	>64	>64	>64	>64	>64	>64
E. coli D22	>64	>64	**1**[b]	2	2	>64	8	>64	**4**	>64	32	64	>64	64	16	>64
E. coli O18:K1:H7	8	64	**1**	2	2	16	4	8	**4**	>128	64	>128	>64	32	32	>64
S. typhimurium ATCC 14028	32	>64	**2**	8	8	32	16	16	**8**	>128	64	>128	>64	>64	8	>64
S. enteritidis D5	16	32	**1**	2	4	8	8	4	**4**	>128	64	>128	>64	>64	64	>64
S. enteritidis PD1	8	>64	**2**	4	4	16	8	16	**4**	>128	>64	>128	>64	>64	64	>64
P. aeruginosa ATCC 27853	>128	>128	>**128**	>128	>128	>128	>128	>128	>**128**	>128	>128	>128	>64	>128	>128	>64
A. baumannii 118A	>128	>64	>**128**	>128	>128	>128	>128	>128	>**128**	>128	>128	>64	>64	>128	>128	>64
K. pneumoniae 22	128	>128	**4**	32	16	>128	32	64	**32**	>128	>128	>128	>64	>64	32	>64
K. pneumoniae 79	64	>128	**8**	32	8	>128	64	64	**32**	>128	>128	>128	>64	>64	>64	>64
S. aureus G	>64	>64	>**64**	>64	>64	>64	>64	>64	>**64**	>64	>64	>64	>64	>64	>64	>64
S. epidermidis 4	>64	>64	>**64**	>64	>64	>64	>64	>64	>**64**	>64	>64	>64	>64	>64	>64	>64

[a] MIC was defined as the lowest peptide concentration that prevented visible bacterial growth after 18 h of incubation at 37 °C in Mueller−Hinton broth, with approximately 1.5−2.0 × 10^5 colony forming units/mL. Results are the mean of at least three independent determinations with a divergence of not more than one MIC value. [b] For the native glycoforms of drosocin, drosocin MS (corresponding to our peptide **III**), and drosocin DS (see Table 1), antibacterial activities (expressed as IC_{50}) of 1 and 0.2 μM, respectively, have been previously reported.[12]

analogues **VI** (Thr 11 replaced by Ala) and **II** (deletion of Thr 11) (Table 4), or N- or C-terminal truncation of the peptide chain,[12,13] strongly reduced the antimicrobial activity.

The insertion of an extra threonine residue in the native apidaecin **IX** improved the sequence homology with drosocin, but the resulting peptide (**X**) was completely devoid of antimicrobial activity. This result was not unexpected, when considering that in apidaecin type peptides the C-terminal stretch of eight amino acids is strictly conserved.[25] Surprisingly, glycosylation of this extra threonine residue (compound **XI**) partially restored the antimicrobial activity. This result suggests that glycosylation of peptide **X** somehow reduces the possible perturbation induced by the extra Thr residue on the peptide chain structure, but no significant conformational differences between apidaecin and its extended analogues were shown by CD analysis.

Time-killing experiments were carried out to follow the kinetics of inactivation of *E. coli* O18:K1:H7 by apidaecin Ib (**IX**), the cyclodimer of apidaecin Ib (**XIII**[c]), and drosocin, either glycosylated (**III**) or not (**I**), and to establish whether these peptides are bacteriostatic or bactericidal. The results show that the killing kinetics of **IX** and **XIII**[c] is relatively rapid, even at a peptide concentration corresponding to the MIC value (Figure 2A,B) and resembles that of other cationic peptides.[19] Conversely, the drosocin analogues **I** and **III** act more slowly, as already reported for the native diglycosylated drosocin.[7] The former peptide, at 1−2-fold the MIC value, is essentially bacteriostatic, and the latter is fully bactericidal only at peptide concentrations of 2−4-fold the MIC value (Figure 2C,D). The different killing kinetics could be ascribed to a different mode of action of these peptides. The multistep mechanism, recently proposed for apidaecin,[16] involves an initial nonspecific encounter of the peptide with a component of the outer membrane, followed by invasion of the periplasmatic space and by a specific and essentially irreversible engagement with a receptor/docking molecule, most likely a component of the permease type transporter system. In the final step, the peptide is translocated into the interior of the cell where it meets its ultimate target, one or more components of the protein synthesis machinery. Otvos et al.[26] proposed a similar pathway of bacterial cell entry, for drosocin and pyrrhocoricin, and

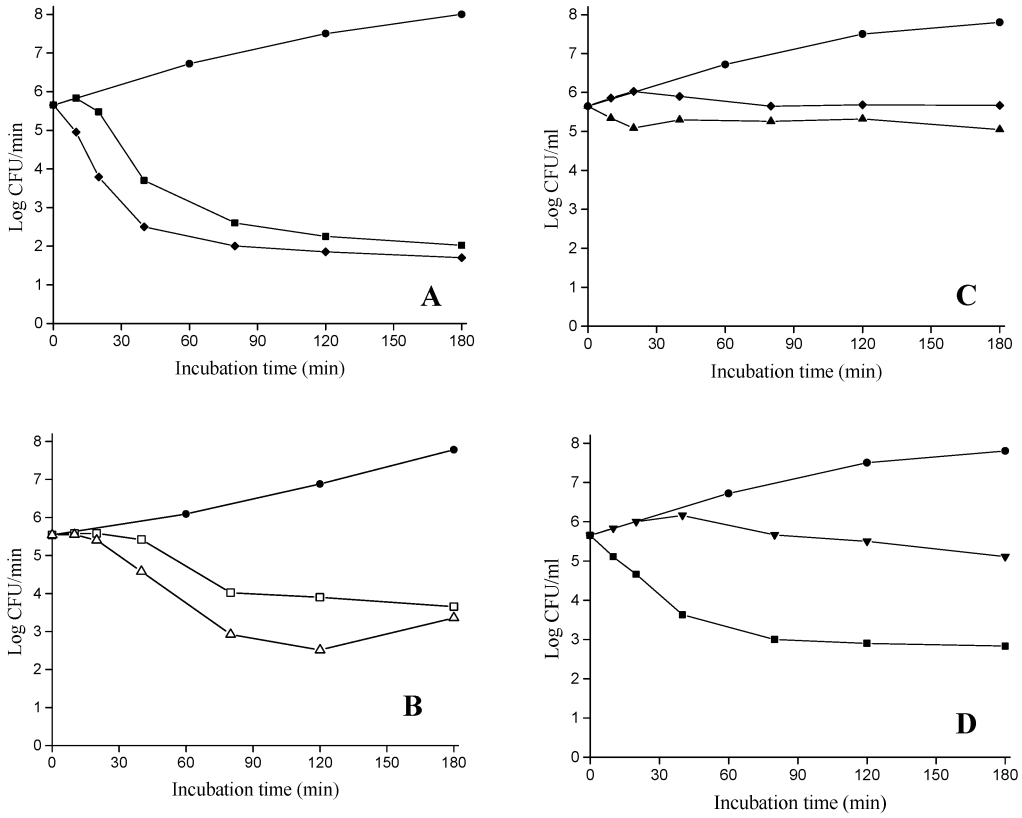


Figure 2. Kinetics and dose−response effect of the bactericidal activity of (A) apidaecin Ib (**IX**), (B) the cyclic dimer of apidaecin Ib (**XIII**^{c}), (C) unglycosylated drosocin (**I**), and (D) drosocin MS (**III**) on mid-log-phase cultures of *E. coli* O18:K1:H7. Controls (●); peptide concentrations: 1 μM (▼), 4 μM (■), 8 μM (◆), 16 μM (▲), 32 μM (□), and 64 μM (△).

identified some biopolymers involved in this cascade, i.e., the 70 kDa heat shock protein DnaK and the 60 KDa chaperonine GroEL. Moreover, they suggested that these peptides kill bacteria by inhibiting chaperonine-assisted protein folding rather than protein synthesis, as hypothesized for apidaecin.[27]

To better define the structural features required for an effective binding to their putative final target, we attempted to restrict the ligand conformational freedom by introducing some conformational constraints by cyclization.[28] The head-to-tail cyclic analogues of drosocin and apidaecin (**XII**^{b} and **XIII**^{b}), the semicyclic drosocin analogue **XIV**, and the drosocin cyclodimer **XII**^{c} did not show any antimicrobial activity up to a concentration of at least eight times the MIC value of the respective parent peptide. It was recently reported[29] that the head-to-tail cyclization of the antimicrobial peptide pyrrhocoricin caused the loss of activity in comparison with the linear peptide. However, the enlargement of the ring size, by repeating an internal octapeptide fragment, produced a cyclic analogue highly active against both Gram-negative and Gram-positive strains. Similarly, the apidaecin cyclic dimer (**XIII**^{c}) was active against some Gram-negative bacterial strains, at concentrations similar or higher than the MIC value of native apidaecin. Taken together, these data suggest that constrained cyclic analogues do not represent a good model of the bioactive conformation of these antimicrobial peptides. More flexible cycles, such as peptide **XIII**^{c}, can preserve some antimicrobial activity probably because they maintain an extended domain in the central part of the peptide. This structural hypothesis is supported by NMR conformational studies recently reported for drosocin and pyrrhocoricin.[4]

Membrane Permeabilization Assays. The complete lack of membrane permeabilization and the loss of antimicrobial activity in the all-D-analogues support the hypothesis that apidaecin and drosocin act through a killing mechanism that involves stereoselective targets and does not depend on pore-forming activity. To investigate whether the same mechanism can be proposed for the new synthetic analogues, their ability to permeabilize lipid membranes was tested by using *E. coli* cells, human erythrocytes, and phospholipid vesicles. Permeabilization of the bacterial inner membrane was determined on the mutant ML-35 strain of *E. coli*[30] by following the extent of unmasking of cytoplasmic β-galactosidase activity. Neither the monoglycosylated drosocin **III** nor the antibacterial active peptide analogues **I**, **VI**, **VII**^{a}, and **VII**^{b} promoted any hydrolysis of the impermeant β-galactosidase substrate *o*-nitrophenyl β-D-galactopyranoside (ONPG), even at 200 μM concentration ($\geq$100 times the MIC value) (Figure 3). On the contrary, the membrane active peptide SMAP-29,[18] used as a positive control, caused a rapid membrane permeabilization at 1 μM. It was reported[18] that SMAP-29 adopts an amphipathic, α-helical conformation in structure-promoting solvents, which is a requisite for an effective insertion of the peptide into microbial membranes and subsequent permeabilization. Our CD measurements showed that the drosocin and apidaecin analogues, as well as the parent peptides, are largely unstructured even in 90% aqueous TFE, a solvent in which there are only evidences of subpopulations of

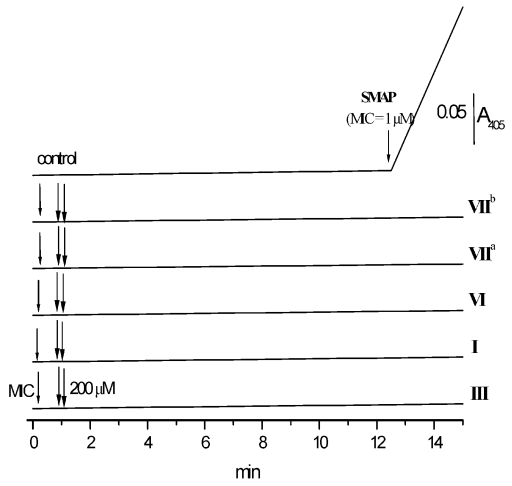


Figure 3. Kinetics of permeabilization of *E. coli* ML-35 inner membrane. Peptides: **III**, (GalNAcα-O-Thr[11])drosocin; **I**, unglycosylated drosocin; **VI**, [Ala[11]]drosocin; **VII[a]**, [Gal(Ac)$_4$α-C-Ala[11]]drosocin; and **VII[b]**, [Galα-C-Ala[11]]drosocin. A single arrow indicates the addition of peptide at the MIC value; double arrows correspond to a 200 μM peptide concentration. SMAP-29 was used as a positive control.

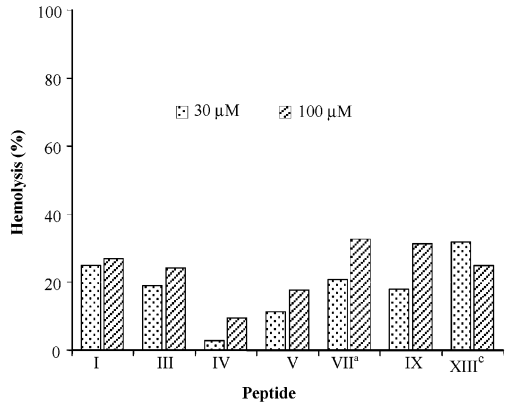


Figure 4. Hemolytic activity of peptides **I**, **III**−**V**, **VII[a]**, **IX**, and **XIII[c]** on human erythrocytes. The assays were performed by incubating 0.5% (vol/vol) suspensions of erythrocytes with the peptide concentrations indicated for 60 min at 37 °C. The results are the mean of two independent experiments run in duplicate and with an absorbance at 415 nm differing by less than 10% from the mean value.

molecules presenting elements of local secondary structure (β-turns). The inability of these peptides to adopt highly ordered amphipathic structures could explain the lack of membrane permeabilization.

The effect of selected drosocin and apidaecin peptides on biological membranes was also tested on 0.5% (vol/vol) suspensions of human erythrocytes. Interestingly, unglycosylated (**I**) and (GalNAcα-*O*-Thr[11])drosocin (**III**) at 30 and 100 μM concentrations lysed between 20 and 30% of the red blood cells (Figure 4). Similar results were obtained with the C-glycosylated analogue **VII[a]**, that showed the highest activity at 100 μM concentration among the peptides tested. This is likely due to acetylation that increases the hydrophobicity of the peptide. Conversely, peptides **IV** and **V** showed a decrease in hemolytic activity, which was particularly marked for the former peptide (2.9 and 9.4% of hemolysis at, respectively, 30 and 100 μM concentration). At present, we do not have a rational explanation of this unexpected result. However, it indicates that the cytotoxic activity of antibacterial peptides against eukaryotic cells can be modulated while leaving the antibacterial activity virtually unchanged. As for the apidaecin peptides, the parent peptide **IX** showed a hemolytic activity similar to that of peptides **I** and **III**. This activity significantly increases for the apidaecin cyclodimer **XIII[c]** at least at 30 μM concentration. The decrease observed at 100 μM concentration is likely due to peptide aggregation. The above results are quite unexpected. Previous studies on the hemolytic activity of drosocin (apidaecin peptides have not been tested in this respect) indicated that diglycosylated drosocin had no hemolytic activity on 0.5% bovine red blood cells up to 40 μM concentration.[12] Similar results were obtained with pyrrhocoricin that was not lytic to 1% suspensions of sheep erythrocytes up to 256 μM.[29] These contrasting results are at least in part explained by the lower susceptibility of bovine and particularly sheep erythrocytes to lytic agents, as compared to human red blood cells, as previously shown for the SMAP-29 peptide[18] and for several other lytic agents,[41] including melittin.

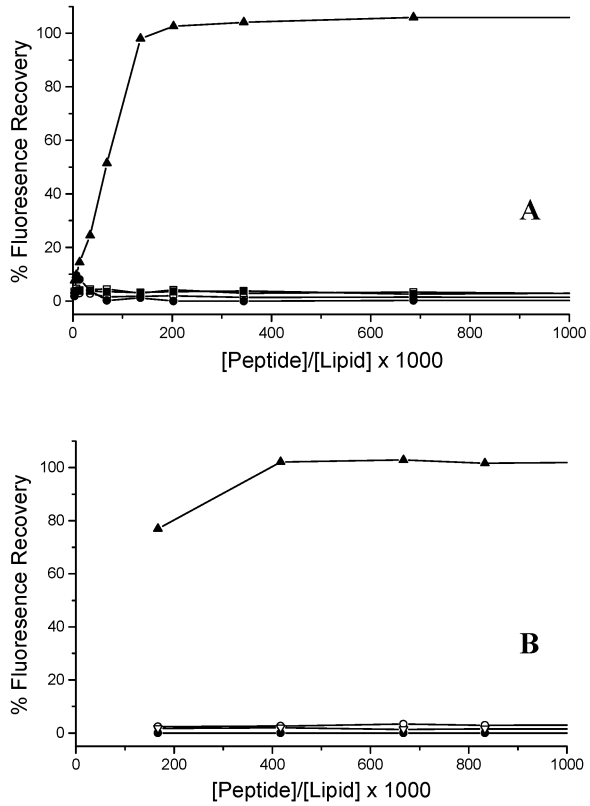


Figure 5. Profiles of the CF leakage of peptides **III** (●), **IX** (■), **XII[b]** (○), **XIII[b]** (□), and **XIV** (△). (A) PC−CH (7:3) and (B) DPPC−DPPG (3:1). The lipopeptaibol trichogin GA IV (▲) was used for comparison.

To evaluate if the phospholipid membrane composition can affect the peptide-induced permeabilization process, we examined the leakage of an entrapped fluorescent dye from zwitterionic (L-α-phosphatidylcholine (PC)−cholesterol (CH), 3:1) and anionic (dipalmitoyl-D,L-3-phosphatidylcholine (DPPC)−dipalmitoyl-D,L-3-phosphatidylglycerol (DPPG), 3:1) phospholipid vesicles as membrane models. As shown in Figure 5 for peptides **III**, **IX**, **XII[b]**, **XIII[b]**, and **XIV**, both linear and cyclic drosocin and apidaecin analogues show negligible leakage activity, even at a high peptide/lipid ratio.

Conclusions

As already pointed out, deglycosylation significantly reduces the antimicrobial activity of drosocin. The synthesis of a series of differently glycosylated peptide analogues showed that the antimicrobial activity against several Gram-negative bacteria is affected by the type of sugar and the type of glycosidic linkage, particularly in the case of *E. coli* D21 and the *K. pneumoniae* strains. According to previously reported results,[12] all glycosylated analogues were more potent than unglycosylated drosocin. Modifications of the drosocin peptide chain at the glycosylation site, by substitution or deletion of the threonine residue at position 11, significantly reduced the antimicrobial activity. The observation that despite the improved sequence homology with drosocin, the [endo Thr^{13a}]apidaecin analogue (**X**) is fully inactive and that glycosylation of the Thr residue led to a partial recovery of the antimicrobial activity suggests that inactivation depends on the extra amino acid residue, rather than on the sugar moiety. In addition, we have shown that the introduction of conformational constraints in the peptide chain of apidaecin and drosocin, as a consequence of head-to-tail cyclization or the formation of an intrachain disulfide bond, abolished the antimicrobial activity and that only the more flexible cyclic dimer of apidaecin (**XIII^c**) was partially active against some bacterial strains. These findings support the structural hypothesis, recently reported for pyrrhocoricin, that the bioactive conformation of drosocin and apidaecin Ib requires an extended domain in the central part of the peptide. Finally, experiments aimed at testing the cytotoxicity toward human red blood cells of the drosocin and apidaecin analogues endowed with antibacterial activity have shown that some peptides display a moderate hemolytic activity at 30 μM concentration and in the presence of a low cell density (0.5% by volume). This activity is however negligible for (Galα-O-Thr^{11})drosocin (**IV**) that maintains an antibacterial activity comparable to that of the parent peptide **III**, indicating that a dissociation between antibacterial activity and cytotoxicity to eukaryotic cells can be achieved in differently glycosylated peptide analogues.

Experimental Section

Abbreviations. The amino acid residues are of the L-configuration. Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature. *Eur. J. Biochem*. **1984**, *138*, 9−37 and *J. Biol. Chem*. **1987**, *262*, 13−18. Abbreviations: CF, 5(6)-carboxyfluorescein; DIEA, diisopropylethylamine; ESI MS, electrospray ionization mass spectrometry; Gal(Ac)$_4$, 2,3,4,6-tetra-*O*-acetyl-D-galactopyranosyl; GalNAc(Ac)$_3$, 2-acetamido, 3,4,6-tri-*O*-acetyl-2-deoxy-D-galactopyranosyl; HBTU, N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]hexafluorophosphate *N*-oxide; HMP resin, 4-(hydroxymethyl)phenoxymethyl resin; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Sasrin resin, 2-methoxy-4-alkoxybenzyl alcohol resin; TDM, *N*,*N*,*N*′,*N*′-tetramethyl-4-4-diamino-diphenylmethane; TFA, trifluoroacetic acid; t_R, chromatographic retention time.

General Methods. All chemicals were commercial products of the best grade available. Fmoc-amino acids, Fmoc-Gly-Sasrin resin, and Fmoc-Val-HMP resin were from Novabiochem (Laufelfingen, Switzerland). Fmoc-(Galα)Thr-OH,[31] Fmoc-(Galβ)Thr-OH,[31] Fmoc-[GalNAc(Ac)$_3\alpha$]Thr-OH,[32] and Fmoc-Gal(Ac)$_4\alpha$]Ala-OH[33] were synthesized according to the literature. All other chemicals for the solid phase synthesis were supplied by Applied Biosystems (Foster City, CA). Egg Yolk PC (type V-E, 100 mg/mL solution in $CHCl_3$:MeOH, 9:1) and CH were purchased from Sigma, DPPC and DPPG were purchased from Alexis, Hepes buffer was purchased from Aldrich, and Triton X-100 and CF were purchased from Fluka. Optical rotations were determined with a Perkin-Elmer model 241 polarimeter. Amino acid analyses were done with a Carlo Erba model 3A 30 amino acid analyzer interfaced with a Shimadzu C-R4A Chromatopac, after hydrolysis for 22 h at 110 °C in sealed, evacuated vials in constant boiling hydrochloric acid containing 0.2% phenol. Analytical HPLC separations (Aquapore RP-300 column, 222 mm × 4.6 mm, 7 μm, flow rate 1.5 mL/min, Brownlee Laboratories, Santa Clara, CA) were performed on a Perkin-Elmer series 410 liquid chromatograph equipped with a LC-90 UV detector and LC-100 integrator. Eluents A (0.1% TFA in 90% aqueous acetonitrile) and B (aqueous 0.1% TFA) were used for preparing binary gradients; the elution conditions were isocratic 15% A for 2 min, linear gradient 15−60% A in 30 min, isocratic 60% A for 2 min or, only for peptides **VIII** and **XIV**, isocratic 17% A for 2 min, linear gradient 17−45% A in 30 min. Semipreparative HPLC (Vydak C-18 column, 250 mm × 22 mm, 10 μm) was performed on a Shimadzu series LC-6A chromatograph equipped with two independent pump units model LC-8A, a SPD-6A detector, and a CR-6A integrator. Unless otherwise indicated, the elution conditions were those used for the analytical HPLC separations, the load was 10 mg, and the flow rate was 15 mL/min. Solvents were dried and freshly distilled, and evaporations were carried out under reduced pressure, at 25−35 °C, using a rotary evaporator. Yields, after HPLC purification, are based on the weight of vacuum-dried peptides as trifluoroacetates. Molecular weight determinations were made by MALDI-TOF MS carried out on a Bruker Reflex TOF MS instrument (matrix α-cyano-4-hydroxycinnamic acid) or by ESI MS on an Applied Biosystems Mariner System 5220, both operating in positive mode. CD measurements were carried out on a Jasco-715 spectropolarimeter, using a quartz cell of 0.02 cm path length. CD spectra were the average of a series of six scans made at 0.1 nm intervals over the 250−190 nm region, recorded at 298 K. Sample concentrations in water, aqueous 95% TFE, and aqueous 30 mM SDS and were in the range of 0.11−0.15 mM, as determined by quantitative UV measurements ($\epsilon_{Tyr} = 1420$ M^{-1} cm^{-1} at 275 nm)[34] performed on a Perkin-Elmer Lambda 5 UV/VIS spectropolarimeter. Ellipticity is reported as mean residue ellipticity $[\theta]_R$ (deg·cm^2 dmol^{-1}). Fluorescence measurements were performed on a Perkin-Elmer MPF-66 spectrofluorimeter in quartz cells (1 cm × 1 cm, Hellma).

Solid Phase Synthesis of Linear Peptides. Assemblies of peptides **I**, **II**, **VI**, **VIII**, **IX**, and **X** and of glycopeptides **III**, **IV**, **V**, **VII**, and **XI** were performed on the Applied Biosystems model 431 A Peptide Synthesizer, on a 0.25 mmol scale, starting with Fmoc-Val-HMP resin (385 mg, substitution 0.65 mmol/g) or Fmoc-Leu-HMP resin (366 mg, substitution 0.52 mmol/g). Serine and tyrosine were protected on their side chain functions as *tert*-butyl ethers and histidine and cysteines as trityl derivatives. Pmc was used as guanidino protective group for arginines and the ϵ-amino function of lysine was masked as Boc derivative. The FastMoc strategy (HBTU/HOBt/DIEA in *N*-methylpyrrolidone, single coupling protocol) was routinely used throughout all syntheses. Fmoc-(Galα)Thr-OH and Fmoc-(Galβ)Thr-OH were used for the incorporation of the glycosylated threonine residue in **IV** and **V**, respectively. Coupling yields were determined by ninhydrin[35] analysis of an aliquot of peptide resin (4−5 mg) removed automatically after each coupling reaction. The final peptide resin was N$^\alpha$-deprotected with 20% piperidine in *N*-methylpyrrolidone for 20 min, thoroughly washed, and dried. Removal of the side chain protections, with exception of the acetyl groups, and cleavage from the HMP resin were simultaneously achieved on portions (about 250 mg) of peptide resin by treatment with TFA containing 2.5% water and 2.5% triethylsilane (2−3.5 h at room temperature). The acid solution was concentrated to small volume, and cold *tert*-butyl ether was added in excess. The resulting precipitate was collected and dried in vacuo in

the presence of P_2O_5 and KOH pellets. Deacetylation of the carbohydrate moieties was routinely achieved by adding hydrazine hydrate (5:1 molar excess with respect to each *O*-acetyl group) to a concentrated, methanolic solution of the crude glycopeptide. Only for glycopeptide **XI** deacetylation was achieved by treatment with 0.1 N NaOH as already described.[12] For the synthesis of the cyclic analogues, the linear precurors H-Lys(Boc)-Pro-Arg(Pmc)-Pro-Tyr(*t*Bu)-Ser(*t*Bu)-Pro-Arg(Pmc)-Pro-Thr(*t*Bu)-Ser(*t*Bu)-His(Trt)-Pro-Arg(Pmc)-Pro-Ile-Arg(Pmc)-Val-Gly-OH (**XII**[a]) and H-Asn(Trt)-Asn(Trt)-Arg(Pmc)-Pro-Val-Tyr(*t*Bu)-Ile-Pro-Gln(Trt)-Pro-Arg(Pmc)-Pro-Pro-His(Trt)-Pro-Arg(Pmc)-Leu-Gly-OH (**XIII**[a]), covering the entire amino acid sequence of drosocin and apidaecin Ib, respectively, were performed by the same procedure starting from Fmoc-Gly-Sasrin resin (385 mg, substitution 0.65 mmol/g). Final peptide resin was in the range of 1.01−1.04 g. Cleavage from the resin was achieved on portions (about 300 mg) of the N^{α}-deprotected peptide resin by a repeated, mild acid treatment (10 times, 1% TFA/DCM, 15−20 mL/g peptide resin, 2−3 min, room temperature). After each treatment, the resin was collected by filtration and the filtrate was immediately neutralized with a methanolic 10% pyridine solution. The combined filtrates were concentrated to a small volume, and the side chain-protected peptide was precipitated by addition of cold ether. Average yield of cleavage was 85%.

All synthesized products were purified to homogeneity by semipreparative HPLC before characterization. All synthesized peptides gave the expected amino acid composition.

Cyclic Peptides. Head-to-Tail Cyclic Peptides (XII[b], XII[c] and XIII[b], XIII[c]). Compounds **XII**[b] and **XIII**[b] were prepared from aliquots (50 mg) of the crude precursor, **XII**[a] or **XIII**[a], respectively, using different coupling reagents (TBTU,[36] Py-BOP,[37] or DPPA[38]). Typically, the condensing agent was added to the 10^{-3} M peptide solution in DMF or DCM, and the pH value was kept in the range of 8−9 by adding DIEA. When HPLC monitoring indicated that the reaction was over (usually 20 min in DMF and 2 h in DCM for uronium-based reagents), the solution was concentrated to a small volume and the product was precipitated with ether (yield 87−92%). The crude cyclic peptide was side chain-deprotected with TFA, as described for linear peptides, and isolated by semipreparative HPLC together to a small amount of the corresponding cyclo dimer (**XII**[c] or **XIII**[c]). Only when the cyclization of the apidaecin analogue **XIII**[a] was carried in DCM, we obtained comparable amounts of cyclo monomer and cyclo dimer. The cyclic peptides were characterized as shown in Table 3.

Cyclo^{S-S}[Cys^{7,12}]Drosocin, XIV. The synthesis of the linear precursor H-Gly-Lys(Boc)-Pro-Arg(Pmc)-Pro-Tyr(*t*Bu)-Cys(Trt)-Pro-Arg(Pmc)-Pro-Thr(*t*Bu)-Cys(Trt)-His(Trt)-Pro-Arg(Pmc)-Pro-Ile-Arg(Pmc)-Val-OH (**XIV**[a]) was performed starting with Fmoc-Val-HMP resin (305 mg, 0.21 mmol), and the final peptide resin was 983 mg. Cleavage from the resin and removal of the side chain protecting groups were simultaneously achieved on an aliquot (595 mg) of the peptide resin; yield, 204 mg (63%). For characterization, an aliquot of the crude peptide (20 mg) was purified by semipreparative HPLC (elution conditions: isocratic 19% A for 2 min, linear gradient 19−29% A in 30 min, 29−90% A in 5 min). The reduced peptide (**VIII**) can be stored under argon. The crude linear peptide (102 mg) was dissolved in aqueous 0.1 M NH_4HCO_3, and the solution was allowed to stir in the air. The disulfide bond formation was monitored by the Ellman test[39] and analytical HPLC analysis. After 40 h, the reaction mixture was lyophilized and the crude cyclic, heterodetic peptide was purified by semipreparative HPLC (elution conditions as those used for purification of the linear precursor). Peptides **VIII** and **XIV** were characterized as shown in Table 3.

Antimicrobial Activities. The antimicrobial activity of drosocin and apidaecin and of their analogues was evaluated by the broth microdilution susceptibility test in Mueller−Hinton broth (Difco, Sparks, MD) as previously described,[40] according to the guidelines of the National Committee for Clinical Laboratory Standards. The activity, espressed as MIC and corresponding to the lowest concentration of peptide preventing visible bacterial growth, was determined on clinical isolates and on bacterial strains obtained from the American Type Culture Collection (ATCC). The following strains were used *E. coli* ATCC 25922, D21, D22, ML-35, and the encapsulated O18:K1:H7 Bort strain retrieved from human neonatal spinal fluid, *Salmonella enterica* serovar *Typhimurium* ATCC 14028, two clinical isolates of *Salmonella enterica* serovar *Enteritidis* (strains D5 and PD1), *Pseudomonas aeruginosa* ATCC 27853, two mucoid isolates of *K. pneumoniae* (strains 22 and 79), and a clinical isolate each of *Acinetobacter baumannii*, *Staphylococcus epidermidis*, and methicillin resistant *Staphylococcus aureus* (MRSA). The kinetics of inactivation of the *E. coli* O18:K1:H7 Bort strain by peptides **I**, **III**, and **IX** was performed in phosphate-buffered saline containing 10 mM sodium phosphate and 145 mM NaCl, pH 7.4. Mid-log phase bacteria (approximately 5×10^5 colony forming units/ml) were incubated at 38 °C for up to 180 min in the absence (control) or the presence of the appropriate peptide at a concentration equal to or 2−4-fold higher than the MIC value. Aliquots of the suspension were withdrawn at the selected time intervals, serially diluted in phosphate-buffered saline, and plated in Mueller−Hinton solid medium to allow colony counts.

Hemolytic Activity. Erythrocytes were prepared from freshly collected human blood anticoagulated with citrate-dextrose, as previously described.[18] The assays were performed in 10 mM phosphate buffer, pH 7.4, containing 139 mM NaCl, by incubating 0.5% (vol/vol) erythrocyte suspensions with 30 or 100 μM peptides for 60 min at 38 °C. The reaction was stopped with cold buffer, and the supernatant was carefully collected after centrifugation at 10 000*g* for 1 min. The released hemoglobin was measured at 415 nm, and the percentage of hemolysis was determined as previously reported.[18] Melittin and the CS-5 fragment of fibronectin were respectively used as positive and negative controls.

Preparation of Phospholipid Vesicles. Small unilamellar vesicles of PC−CH (7:3) or DPPC−DPPG (3:1) were prepared for the dye leakage experiments. The lipid (20 mg) was dissolved in chloroform−methanol (2 mL) and then dried by a stream of N_2 gas. The dried lipid was hydrated in the appropriate buffer (5 mL of 1 mM Hepes, pH 7.5, for PC−CH, and 2 mL of 20 mM Tris-HCl buffer, pH 7.4, for DPPC−DPPG), and the suspension was sonicated for 20 min using a Branson 250 Ultrasonic Processor. The CF-entrapping vesicles were prepared according to ref 27. The lipid was hydrated in the appropriate buffer containing 0.1 M CF, as previously described. The CF-entrapped vesicles were separated from free CF by gel filtration using Sephadex G-75 with the same buffer.

Membrane Permebilization Assays. The ability of peptides to permeabilize the bacterial inner membrane was determined by measuring the unmasking of the β-galactosidase activity in the *E. coli* ML-35 strain using the impermeant ONPG subtrate.[19] Mid-log phase bacteria were washed in 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and 5 mM D-glucose, and resuspended in 0.80 mL of the same buffer containing 1.5 mM ONPG. The production of *o*-nitrophenol over time was monitored spectrophotometrically at 405 nm in the absence or presence of peptide. The permeabilizing activity of the membrane active peptide SMAP-29[18] was also measured as a positive control.

The lytical effect of peptides on neutral and negatively charged liposomes, PC−CH and DPPC−DPPG, respectively, was tested by dye leakage experiments.[42] The lipid concentration was kept constant (60 μM), and increasing [peptide]/[lipid] molar ratios (R_i^{-1}) were obtained by adding aliquots of solutions of peptides. After they were rapidly and vigorously stirred, the time course of fluorescence change corresponding to CF escape was recorded at 520 nm (3 nm band-pass) with λ_{exc} 488 nm (3 nm band-pass). The percentage of released CF at time *t* was determined as $(F_t - F_0)/(F_T - F_0) \times 100$, with F_0 = fluorescence intensity of vesicles in the absence of peptide, F_t = fluorescence intensity at time *t* in the presence of peptide, and F_T = total fluorescence intensity determined by disrupting

the vesicles by addition of 50 μL of a 10% Triton X-100 solution in water. The kinetics of dye leakage was followed for 20 min. The activity of the pore-forming lipopeptaibol antibiotic trichogin GA IV[43] was also measured for comparison.

Acknowledgment. We thank Dr. D. Dal Zoppo for ESI mass spectra and Prof F. Formaggio for spectroscopic support. Funding was provided by the Italian National Research Council, C. N. R. (Progetto Finalizzato Biotecnologie), and the Italian Ministry of University and Research, MIUR.

Supporting Information Available: HPLC profiles of the cyclization reactions of drosocin analogue **XII**[a] and apidaecin analogue **XIII**[a] and full data of amino acid analyses of peptides **I**−**XIV**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hoffmann, J. A.; Hetru, C.; Reichhart J. M. The humoral antibacterial response of *Drosophila*. *FEBS Lett.* **1993**, *335*, 63−66.
(2) Hultmark, D. Immune reactions in *Drosophila* and other insects: a model for innate immunity. *Trends Genet.* **1993**, *9*, 178−183.
(3) Cociancich, S.; Bulet, P.; Hetru, C.; Hoffmann, J. A. The inducible antibacterial peptides of insects. *Parasitol. Today* **1994**, *10*, 133−139.
(4) Otvos, L., Jr. Antibacterial Peptides isolated from insects. *J. Pept. Sci.* **2000**, *6*, 497−511.
(5) Hetru, C.; Bulet, P.; Cociancich, S.; Dimarcq, J.-L.; Hoffmann, D.; Hoffmann, J. A. Antibacterial peptides/polypeptides in the insect host defense: a comparison with vertebrate antibacterial peptides/polypeptides. In *Phylogenetic Perspectives in Immunity: The Insect Host Defense*; Hoffmann, J. A., Janeway, C. A., Natari, S., Eds.; R. G. Laudes Company: Texas, 1994; pp 43−65.
(6) Andreu, D.; Rivas, L. Animal antimicrobial peptides: an overview. *Biopolymers* **1999**, *47*, 415−434.
(7) Bulet, P.; Dimarcq, J.-L.; Hetru, C.; Lagueux, M.; Charlet, M.; Hegy, G.; Van Dorsselaer, A.; Hoffmann, J. A. A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. *J. Biol. Chem.* **1993**, *268*, 14893−14897.
(8) Cociancich, S.; Dupont, A.; Hegy, G.; Lanot, R.; Holder, F.; Hetru, C.; Hoffmann, J. A.; Bulet, P. Novel inducible antibacterial peptides from a hemipteran insect, the sap-sucking bug *Pyrrhocoris apterus*. *Biochem. J.* **1994**, *300*, 567−575.
(9) Mackintosh, J. A.; Veal, D. A.; Beattie, A. J.; Gooley, A. A. Isolation from an ant *Myrmecia gulosa* of two inducible O-glycosylated proline-rich antibacterial peptides. *J. Biol. Chem.* **1998**, *273*, 6139−6143.
(10) Bulet, P.; Hegy, G.; Van Dorsselear, A.; Hofmann, J. A.; Hetru, C. Insect immunity. The inducibile antibacterial peptide diptericin carries two O-glycans necessary for biological activity. *Biochemistry* **1995**, *34*, 7394−7410.
(11) Hara, S.; Yamakawa, M. A novel antibacterial peptide family isolated from silkworm, *Bombyx mori*. *Biochem. J.* **1995**, *310*, 651−656.
(12) Bulet, P.; Urge, L.; Ohresser, S.; Hetru, C.; Otvos, L., Jr. Enlarged scale chemical synthesis and range of activity of drosocin, an O-glycosylated antibacterial peptide of *Drosphila*. *Eur. J. Biochem.* **1996**, *238*, 64−69.
(13) Hoffmann, R.; Bulet, P.; Urge, L.; Otvos, L., Jr. Range of activity and metabolic stability of synthetic antibacterial glycopeptides from insects. *Biochem. Biophys. Acta* **1995**, *1426*, 459−467.
(14) McManus, A. M.; Otvos, L.; Hoffmann, R.; Craik, D. J. Conformational studies by NMR of the antimicrobial peptide, drosocin, and its nonglycosylated derivative: effect of glycosylation on solution conformation. *Biochemistry* **1999**, *38*, 705−714.
(15) Casteels, P.; Tempst, P. Apidaecin-type peptide antibiotics function through a nonporeforming mechanism involving stereospecificity. *Biochem. Biophys. Res. Commun.* **1994**, *199*, 339−345.
(16) Castle, M.; Nazarian, A.; Yi, S. S.; Tempst, P. Lethal effect of apidaecin on *Escherichia coli* involve sequential molecular interactions with diverse targets. *J. Biol. Chem.* **1999**, *274*, 33555−33564.
(17) Casteels, P.; Ampe, C.; Jacobs, F.; Vaeck, M.; Tempst, P. Apidaecins: antibacterial peptides from honeybees. *EMBO J.* **1989**, *8*, 2387−2391.
(18) Skerlavaj, B.; Benincasa, M.; Risso, A.; Zanetti, M.; Gennaro, R. SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes. *FEBS Lett.* **1999**, *463*, 58−62.
(19) Skerlavaj, B.; Romeo, D.; Gennaro, R. Rapid membrane permeabilization and inhibition of vital functions of gram-negative bacteria by bactenecins. *Infect. Immun.* **1990**, *58*, 3824−3730.
(20) Rothe, M.; Sander, A.; Fisher, W.; Mästle, W.; Nelson, B. Interchain reactions (cyclo-oligomerizations) during the cyclization of resin-bound peptides. In *Peptides—Proceedings of the 5th American Peptide Symposium*; Goodman, M., Meienhofer, J., Eds.; J. Wiley & Sons: New York, 1977; pp 506−509.
(21) McMurray, J. S.; Lewis, C. A.; Obeyesekere, N. U. Influence of solid support, solvent and coupling reagent on the head-to-tail cyclization of resin-bound peptides. *Pept. Research* **1994**, *7*, 195−206.
(22) Tam, J. P.; Wu, C. R.; Liu, W.; Zhang, J. W. Disulfide bond formation in peptides by dimethyl sulfoxide. Scope and limitations. *J. Am. Chem. Soc.* **1991**, *113*, 6657−6662.
(23) Woody, R. W. Studies of theoretical circular dicroism of polypeptides. In *Peptides, Polypeptides and Proteins*; Blout, E. R., Bovey, F. A., Goodman, M., Lotan, M., Eds.; Wiley & Sons: New York, NY, 1994; pp 338−360.
(24) Marcaurelle, L. A.; Rodriguez, E. C.; Bertozzi, C. R. Synthesis of an oxime-linked neoglycopeptide with glycosylation-dependent activity similar to its native counterpart. *Tetrahedron Lett.* **1998**, *39*, 8417−8420.
(25) Casteels, P.; Romagnolo, J.; Castele, M.; Casteels-Josson, K.; Erdjument-Bromage, H.; Tempst, P. Biodiversity of apidaecin-type peptides antibiotics. *J. Biol. Chem.* **1994**, *269*, 26107−26115.
(26) Otvos, L., Jr.; O, I.; Rogers, M. E.; Consolvo, P. J.; Condie, B. A.; Lovas, S.; Bulet, P.; Blaszczyk-Thurin, M. Interaction between heat shock proteins and antimicrobial peptides. *Biochemistry* **2000**, *39*, 14150−14159.
(27) Kragol, G.; Lovas, S.; Varadi, G.; Condie, B. A.; Hoffmann, R.; Otvos, L., Jr. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. *Biochemistry* **2001**, *40*, 3016−3026.
(28) Kessler, H. Conformational and biological activity of cyclic peptides. *Angew. Chem, Int. Ed. Engl.* **1982**, *21*, 512−523.
(29) Otvos, L., Jr.; Bokonyi, K.; Varga, I.; Otvos, B. I.; Hoffman, T.; Ertl, H. C. J.; Wade, J. D.; McManus, A. M.; Craik, D. J.; Bulet, P. Insect peptides with improved protease-resistance protect mice against bacterial infection. *Protein Sci.* **2000**, *9*, 742−749.
(30) Lehrer, R. I.; Barton, A.; Ganz, T. Concurrent assessment of inner and outer membrane permeabilization and bacteriolysis in *E. coli* by multiple wavelength spectrophotometry. *J. Immunol. Methods* **1988**, *108*, 153−158.
(31) Filira, F.; Biondi, L.; Cavaggion, F.; Scolaro, B.; Rocchi, R. Synthesis of O-glycosylated tuftsins by utilizing threonine derivatives containing an unprotected monosaccharide moiety. *Int. J. Pept. Protein Res.* **1990**, *36*, 86−96.
(32) Biondi, L.; Filira, F.; Gobbo, M.; Scolaro, B.; Rocchi, R. Synthesis of glycosylated tuftsins and tuftsin-containing IgC fragment undecapeptide. *Int. J. Pept. Protein Res.* **1991**, *37*, 112−121.
(33) Kessler, H.; Wittmann, V.; Kock, M.; Kottenhahn, M. Synthesis of C-glycopeptides via free radical addition of glycosyl bromides to dehydroalanine derivatives. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 902−904.
(34) Marqusee, S.; Robbins, V. H.; Baldwin, R. L. Unusually stable helix formation in short alanine-based peptides. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5286−5290.
(35) Sarin, V. K.; Kent, S. B.; Tam, J. P.; Merrifield, R. B. Quantitative monitoring of solid-phase synthesis by the ninhydrin reaction. In *Peptide Structure and Biological Function*; Rich, D. M., Gross, E., Eds.; Pierce Chemical Co.: Rockford, IL, 1981; pp 221−224.
(36) Zimmer, S.; Hoffmann, E.; Jung, G.; Kessler, H. Head-to-tail cyclization of hexapeptide using different coupling reagents. *Liebigs Ann. Chem.* **1993**, 497−501.
(37) Coste, J.; Le-Nguyen, D.; Castro, B. PyBOP: a new coupling reagent devoid of toxic byproducts. *Tetrahedron Lett.* **1990**, *31*, 205−208.
(38) Brady, S. F.; Palaveda, W. J.; Arison, B. H.; Freidinger, R. M.; Nutt, R. F.; Veber, D. F. An improved procedure for peptide cyclization. In *Peptides: Structure and Functions*; Hruby, V. J., Rich, D. H., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; pp 127−130.
(39) Ellman, G. L. Quantitative monitoring of sufhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.
(40) Gennaro, R.; Scocchi, M.; Merluzzi, M; Zanetti, M. Biological characterization of a novel mammalian antimicrobial peptide. *Biochim. Biophys. Acta* **1998**, *1425*, 361−368.
(41) Osorio e Castro, V. R.; Ashwood, E. R.; Wood, S. G.; Vernon, L. P. Hemolysis of erythrocytes and fluorescence polarization

changes elicited by peptide toxins, aliphatic alcohols, related glycols and benzylidene derivatives. *Biochim. Biophys. Acta* **1990**, *1029*, 252−258.

(42) Sakamoto, S.; Mihara, H.; Matsuo, E.; Niidome, T.; Anzai, K.; Kirino, Y.; Aoyagi, H. Enhanced membrane-perturbing activities of bundled amphiphilic α-helical polypeptides on interaction with phospholipid bilayer. *Bull. Chem. Soc. Jpn.* **1995**, *68*, 2931−2939.

(43) Toniolo, C.; Crisma, M.; Formaggio, F.; Peggion, C.; Moanco, V.; Goulard, C.; Rebuffat, S.; Bodo, B. Effect of N^{α}-acyl chain length on the membrane-modifying properties of synthetic analogues of trichogin GA IV. *J. Am. Chem. Soc.* **1996**, *118*, 4952−4954.

JM020861D