Review

Antibacterial Peptides Isolated from Insects

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Abstract: Insects are amazingly resistant to bacterial infections. To combat pathogens, insects rely on cellular and humoral mechanisms, innate immunity being dominant in the latter category. Upon detection of bacteria, a complex genetic cascade is activated, which ultimately results in the synthesis of a battery of antibacterial peptides and their release into the haemolymph. The peptides are usually basic in character and are composed of 20–40 amino acid residues, although some smaller proteins are also included in the antimicrobial repertoire. While the proline-rich peptides and the glycine-rich peptides are predominantly active against Gram-negative strains, the defensins selectively kill Gram-positive bacteria and the cecropins are active against both types. The insect antibacterial peptides are very potent: their IC_{50} (50% of the bacterial growth inhibition) hovers in the submicromolar or low micromolar range. The majority of the peptides act through disintegrating the bacterial membrane or interfering with membrane assembly, with the exception of drosocin, apidaecin and pyrrhocoricin which appear to deactivate a bacterial protein in a stereospecific manner. In accordance with their biological function, the membrane-active peptides form ordered structures, e.g. α-helices or β-pleated sheets and often cast permeable ion-pores. Their cytotoxic properties were exploited in *in vivo* studies targeting tumour progression. Although the native peptides degrade quickly in biological fluids other than insect haemolymph, structural modifications render the peptides resistant against proteases without sacrificing biological activity. Indeed, a pyrrhocoricin analogue shows lack of toxicity *in vitro* and *in vivo* and protects mice against experimental *Escherichia coli* infection. Careful selection of lead molecules based on the insect antibacterial peptides may extend their utility and produce viable alternatives to the conventional antimicrobial compounds for mammalian therapy. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antibacterial peptides; insect peptides; insects

INTRODUCTION

Insects are probably the first successful scholars of combinatorial chemistry. They demonstrate a remarkable evolutionary success that can be attributed to a variety of reasons [1], among which their potent antibacterial defense reactions play a major role [2]. These creatures are continuously exposed to potentially pathogenic microorganisms and eukaryotic parasites, but only a few encounters result in infection [3]. The great diversity of insects as we know it today was achieved not by high origination rates but rather by low extinction rates compared to other animal groups [1]. The class Insecta contains far more species than any other class of animals or the entire plant kingdom. Approximately 800 000 insect species, about 80% of all the animal species known to date, have been identified and named. In their defense mechanism, insects mainly rely on innate immunity [4]. Comparing the size of the DNA required to produce one immunoglobulin (Ig)G molecule with that of 20 peptides, including all the processing enzymes, it can be concluded that innate immunity appears to be 100 times more energy efficient than adaptive

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immunity [5]. This economical use of DNA results in considerably faster reaction time upon infection [6]. Antimicrobial peptides can be detected in insect haemolymph as early as 2–4 h after a septic injury [7]. Actually a prepro form of an antibacterial peptide is made almost 130 times faster than IgM, the first appearing immunoglobulin [8], about three times faster than the reproduction of the bacteria. A single insect produces approximately 10–15 peptide antibiotics [9], each peptide exhibiting a completely different activity spectrum [10].

These peptides are remarkably potent antibacterial compounds. In response to an experimental infection of *Drosophila melanogaster*, the overall haemolymph concentration of seven inducible antimicrobial peptides reaches the value of 200 μM, half of which is accounted for by the antifungal molecule drosomycin, and the other half by seven antibacterial compounds [11]. This means that the average antibacterial peptide concentration in the haemolymph is as low as approximately 15 μM and the active concentration should be lower than this figure. Moreover, if peptide stability in mammalian body fluids was comparable to that in haemolymph (actually it is not, as I will document later) the active dose of an antibacterial peptide drug based on these peptides can be expected to fall into the high μg/kg– low mg/kg range. Interestingly, while the seven antibacterial peptides made by *D. melanogaster* are not homologous at all, many other species produce peptides very similar to these seven peptides in size and amino acid composition. However, because the name of the peptide often reflects the insect origin rather than the structure, the relationship (and probably the genetic origin) or the 'sublibrary' category of these peptides are not obvious at first sight.

GENE EXPRESSION

The genes encoding the antibacterial peptides of *Drosophila*, *Lepidoptera*, *Hymenoptera* and other *Diptera* have largely been cloned [7]. In *Drosophila*, the intronless genes code for prepropeptides containing a signal sequence, a short prosequence and the mature peptide sequence. The prosequence can be located at either side of the mature peptide gene [12]. The promoter regions contain sequence motifs similar to *cis*-regulatory elements of mammalian acute-phase response genes [13], e.g. the NF-κB, the inducible transactivator involved in the expression of immune genes [14]. In mammals, NF-κB rapidly induces gene expression upon extracellular

stimulations that signal distress and pathogen invasion [15]. The gene cassettes of the mammalian immune response are generally similar to the dorsoventral patterning in *Drosophila*, which is initiated by ligand binding of the transmembrane protein Toll (equivalent of the IL-1 receptor in mammals) and which controls the fly's antifungal response [16]. While the Toll signalling pathway is sufficient for the synthesis of drosomycin, the genes encoding antibacterial peptides are dependent upon an additional complex regulatory cascade [17,18]. It is interesting to note that Toll's ligand, spätzle, the initiator of the whole cascade [7], is homologous to the cysteine-knot family of proteins that include, among others, a number of growth factors [19] and peptides involved in plant defense [20].

INSECT ANTIBACTERIAL PEPTIDE FAMILIES

Experimental injection of bacteria into insects elicits the expression and synthesis of a number of antibacterial peptides and proteins, which are secreted into the haemolymph [3], the functional equivalent of blood. *D. melanogaster* has become the favourite model to investigate the molecular mechanisms of insect immunity. In *Drosophila*, the five distinct antibacterial peptides represent one of each of the major antibacterial peptide families [10]. These peptides are cecropin, defensin, drosocin, diptericin and attacin. Two additional antifungal peptides, drosomycin and metchnikowin, are also secreted. Figure 1 shows the sequences of one of each of the antibacterial peptide families and indicates the conserved and variable amino acid residues or sequence motifs.

Historically, cecropins were the first inducible antibacterial peptides to be isolated [21], characterized [22] and reviewed [23]. Insect cecropins are 35–39 amino acid residue-long peptides with amidated *C*-termini, expressed as preproproteins of 62–64 residues, and almost exclusively restricted to the Lepidoptera and Diptera orders of insects. In moths [21,24–26] the first residue, preceding the conserved Trp, is a Lys or an Arg, if any (Figure 1). In flies, the first amino acid is always a glycine [27–29]. The tryptophan is followed by Asn-Pro-Phe or Lys-Leu-Phe type tripeptide motifs in moth sequences or a Leu-Lys-Lys-Ile-Gly or similar pentapeptide motifs in fly sequences. Generally little sequence variations are found until Gly18. The *C*-terminal half of the peptide is highly conserved in the flies and less conserved in the moths, although

Cecropin A (*Hyalophora cecropia*):
  KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK

Defensin A (*Phormia terranovae*):

  ATCDLLSGTGINHSACAAHCLLRGNRGGYCNGKGVCVCRN

Drosocin (*Drosophila melanogaster*):
  GKPRPYSPRPTSHPRPIRV

Diptericin (*Phormia terranovae*):
  D[1]EKPKLILPTPAPPNLPQLV[20]
  G[21]GGGGNRKDGFGVSVDAHQKVWTSDNGRHSIGVTPGYSQHLGGPYGNSRPDYRIGAGYSYNF[82]

Thanatin (*Podisus maculiventris*):
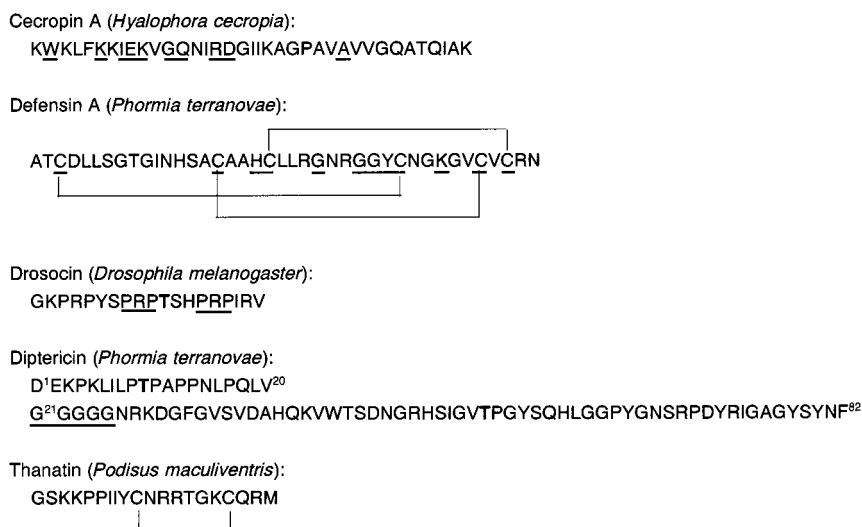 GSKKPPIIYCNRRTGKCQRM

Figure 1   Representative sequences from each of the insect antibacterial peptide families. The most conserved amino acid residues or sequence motifs are underlined. Glycosylated threonines are printed in bold.

the sequences are made up from the same residues, dominated by Ile, Val, Ala, interspersed with hydroxy- or turn-forming amino acids. The *C*-termini are Lys or Arg, except for the shorter *Bombyx mori* sequences in which the peptides end with a long-chain hydrophobic residue. Recently, a novel cecropin analogue was isolated from the mosquito *Aedes albopictus*, but this peptide shares only 37% homology with the closest relative from *D. melanogaster* [30] and, solely based on amino acid composition, can hardly be called a cecropin.

Even more confusion surrounds the insect defensins. These are 29–34 residue-long peptides, which contain six conserved cysteine residues involved in three disulfide bridges. Originally, insect defensins were suggested to be homologous with mammalian defensins based on the sequence similarity of residues 15–34 of the *Phormia* defensin (Figure 1) with the 4–24 fragment of rabbit defensin [31]. However, structural information collected in the beginning of the 1990s did not substantiate the original proposal [31]. Most importantly, the connectivities of the three intramolecular disulfide bridges are completely different in the two families of molecules [32,33]. It adds to the confusion that the names of the peptides within the order Insecta are inconsistent; these analogues are called 'sapecin', 'defensin' or 'royalisin'. The defensins appear to be the most wide-spread group of inducible antibacterial peptides and are present in insect orders as ancient as Odonata (dragonflies) [34]. Other analogues have been isolated from the flies, *Phormia*

terranovae [35], *Eristalis tenax* [31], *Sarcophaga peregrina* [36], *D. melanogaster* [37], the mosquito, *Aedes aegypti* [38], the honey bee, *Apis mellifera* [39], the scorpion, *Leiurus quinquestriatus* [40] and the beetle, *Zophobas atratus* [41]. The peptide sequences are quite homologous within an order of insects, but considerably different across the orders. The first conserved Cys is preceded by two or three residues which are Ala-Thr or Leu-Thr (flies), Val-Thr (bee), Phe-Thr (beetle) or Gly-Phe-Gly (dragonfly and scorpion). The *C*-terminal domains are less conserved, although the Arg and Lys residues responsible for the net positive charge are characteristically located near the carboxy termini. A good representative of insect defensins is the most recently isolated analogue from the midge *Chironomus plumosus* [42].

Drosocin is the prototype of the medium-sized, proline-rich antibacterial peptides that have been isolated from Hymenoptera, Lepidoptera, Hemiptera and Diptera [43]. Other members of this family are pyrrhocoricin from the European sap-sucking bug *Pyrrhocoris apterus* [44], apidaecins from the honey bee [45] and formaecin from the ant *Myrmecia gulosa* [46]. More distant relatives are abaecin [47] and the lebocins [48], these last two from the silkmoth. As it is apparent from the list above, the names of these peptides provide no help in identifying their structural or functional relationships. Nevertheless, the ingenious combinatorial capabilities of insects are most obvious by examining the structural features of this category of peptides. The major

sequence motifs are the repeated Pro-Arg-Pro or Pro-His-Pro tripeptide segments. Perhaps these characteristic tripeptide building blocks are more important than single residue homologies. In addition to Pro and basic residues, the peptides are made of just a few different amino acids, such as Asn, Gly, Val, Ile and Tyr. Significantly, all family members, except abaecin and apidaecin, contain a glycosylated threonine in mid-chain position. Although a series of different glycoforms of drosocin can be detected in the haemolymph of *Drosophila*, the native peptide has never been observed to exist without the addition of any carbohydrate side-chain [11]. Still, as it was shown by using synthetic peptides and glycopeptides, the presence of the sugar is not necessary for the biological activity. While addition of the Gal-GalNAc disaccharide generally increases the antibacterial activity of drosocin [49], pyrrhocoricin-lacking sugar appears to be more potent [50]. The chemical nature of the peptide–sugar connection does not seem to play a role in the activity either. Drosocin variants, in which the native classical *O*-linked sugars in α-anomeric configuration are replaced with oxime-linked carbohydrates, work equally well [51,52].

Diptericin was once considered to belong to either the attacin-family or to the proline-rich peptide family [3,50]. This was based on the striking similarity of diptericin's carboxy-terminus to the glycine-rich proteins attacins and the amino-terminus to pyrrhocoricin. The attacin-sarcotoxin II family includes bacteria-inducible proteins of 20–28 kDa that have been identified in several lepidopteran and dipteran species [53,54]. Diptericin's size (82 residues) and the presence of the pentaglycine segment further supported the idea of structural similarity with the attacins. Diptericin-type peptides have been isolated from *P. terranovae* and *S. peregrina* [55] and a third diptericin sequence was deduced from the cDNA of *D. melanogaster* [56]. The *Phormia* diptericin carries two carbohydrate side-chains, one in the proline-rich domain (intriguingly on the drosocin and pyrrhocoricin analogue threonine residue) and one in the glycine-rich domain. Although a number of close homolog *Phormia* diptericins can be isolated with different carbohydrate lengths, at least one monosaccharide is attached to all of these molecules [57,58]. Treatment of a diptericin variant containing two disaccharides with *O*-glycosidase resulted in the loss of antibacterial activity [57], but this finding was not supported by experiments with a synthetic unglycosylated peptide [59] and synthetic glycosylated diptericin analogues [60,61]. The *C*-terminal region of hymenoptaecin isolated from honey bees [62] resembles that of diptericin, in addition to the similar size of these peptides. Hymenoptaecin, however, was not reported to carry sugar side-chains. Although the somewhat shorter beetle peptides coleoptericin (*Z. atratus*) [63] and holotricin-2 (*Holotrichia diomphalia*) [64] are dissimilar to the *Phormia* diptericin, their high Gly content justifies their discussion in the glycine-rich peptide family. The acaloleptins, also from a beetle, share significant sequence similarity with the three latter peptides [65].

Two additional antibacterial peptides, absent in *Drosophila* and without clear family classification, have been recently isolated from various insects. One of these peptides is the 21-mer thanatin from the spined stink bug, *Podisus maculiventris* [66]. Thanatin is important in the sense that it contains a disulfide bridge and displays sequence similarity to the brevinins, antimicrobial peptides isolated from frog skin [67]. The sequence of the second peptide, moricin, from *B. mori* [68], apparently does not resemble that of any other antibacterial peptide. Expression of lysozyme genes is strongly induced by bacteria, although this small protein is present in insects without bacterial insult [3], often serves as a digestive enzyme and should clearly be distinguished from the strictly antibacterial peptides.

IN VITRO ACTIVITY SPECTRUM, CONFORMATION, MODE OF ACTION

Both the range of activity (which is less precisely but more popularly called 'activity spectrum') and the conformation of the insect antibacterial peptides provide significant clues to their mode of action. This, in turn, defines the pharmaceutical potential and applicability of these natural products and their designed synthetic analogues. The incidence of serious bacterial infection is increasing despite remarkable advances in antibiotic chemotherapy [69]. It is not an overstatement to claim that one of the most serious and urgent topics of the health-care industry today is the rapid development of antibacterial compounds that kill bacteria in a manner completely different from those utilized by the currently marketed antimicrobial compounds, such as erythromycin, tetracyclines, penicillins, cephalosporins and even vancomycin. Currently, no drug resistance other than proteolytic cleavage could be attributed to the antibacterial peptides [4],

although some events during *in vitro* or *in vivo* assay conditions appear to inactivate the peptide antibiotics [70]. Antibacterial peptides may offer viable alternatives to current antimicrobials if the resistance of the pharmaceutical industry to peptides in general can be overcome.

All this justifies the discussion of the activity spectrum, conformation and the mode of action jointly. The current literature is just too large to include all data here; I attempt to summarize the relevant main features. For more detail, the reader is referred to the excellent original papers and recent peptide-centered reviews listed in Alessandro Tossi's web-site (www.bbcm.univ.trieste.it/ ∼ tossi). For the mechanistic features, I recommend the review by Andreu and Rivas [70] and for the structural explanation, that of Hwang and Vogel [71]. Table 1 contains *in vitro* activity data of representative antibacterial peptides of insect origin. This table is intended to demonstrate the selectivity of the families of the peptides to some bacterial strains and provide a rough estimate of the potency of the peptides. It needs to be mentioned that activity data

from different laboratories and even from the same laboratory, but acquired at different times, are often incoherent. One of the reasons is the use of isolated vs. synthetic material. When the first report on drosocin was published, researchers had to hand prick 30000 *Drosophila* and work up the abdomens to obtain material enough for the initial characterization of the peptide [43]. To more conveniently generate peptides in quantities suitable for detailed study of their various biological and biochemical properties, we chemically synthesize them, while others use bacterial expression systems [72]. The comparison of activity data based on peptides from different sources is not without controversy. While our synthetic glycosylated drosocin displayed antibacterial activities indistinguishable from the native material [49], markedly variable data were obtained for pyrrhocoricin, a very close family member [50]. Then, we explained the differences with the unsurpassed purity and accurate concentration-determination of the synthetic samples. However, additional uncertainties, such as assay temperature, composition and even the salt content of the

Table 1 *In vitro* Antibacterial Activity of Some Peptides of Insect Origin. The Minimal Inhibitory Concentration (MIC), also called Lethal Concentration (LC) or 50% of the Bacterial Growth Inhibition (IC_{50}) Data are Taken from the References Following the Peptide Names

Bacterial strain	MIC^a, LC^b or IC_{50}^c in μM				
	Cecropin A^b [74] *H. cecropia*	Defensin A^a [42] *C. plumosus*	Drosocin^c [49] *D. melanogaster* (glycosylated)	Diptericin^c [59] *P. terranovae* (unglycosylated)	Thanatin^a [66] *P. maculiventris*
Gram-negatives					
In general	Active	Inactive	Active	Active	Active
Escherichia coli D22	0.4^d	>50	0.2	<0.15	0.6
Pseudomonas aeruginosa	2.6		>40	>40	40
Salmonella typhimurium			0.5	0.3	1.2
Klebsiella pneumoniae			1.5	20	1.2
Enterobacter cloacae			2	20	2.5
Gram-positives					
In general	Active	Active	Inactive	Inactive	Active
Micrococcus luteus	1.4	0.6	0.35	>80	2.5
Bacillus megaterium	0.6	10	>40		5
Staphylococcus aureus		>50	>40		>40
Listeria monocytogenes		>50	>40		
Pediococcus acidilactici		2.5			40

^{d} Tested against *E. coli* D21.
The empty places indicate that the cited publications do not report these activities figures.

medium or strain-to-strain or culture-to-culture variations of the bacterial colonies influence the assay results [73].

Cecropins display broad spectrum activity against Gram-negative and Gram-positive bacteria [74]. Insect cecropins are without conformational preferences in water, but form amphipathic α-helices in organic solvents [75]. Actually, in cecropin A two separate helices are formed, with a kink around Gly23-Pro24, which is missing from an analogue mammalian cecropin variant [76]. If we accept the idea that water-halogenated alcohol mixtures efficiently mimic the dynamic hydrophobic–hydrophilic environment of the cell membrane [77], the helix-forming ability of the cecropins upon membrane contact indicates formation of membrane pores. Indeed, interaction of the cecropins with membrane lipids is thought to result in a general disintegration of membrane structure and lysis of bacterial cells. It was speculated that noncooperative binding of the peptides on the outer surface of the bacteria might help them to diffuse efficiently into the inner membrane, which is the likely target of cecropin-type antibacterial peptides [78]. This scenario is consistent with the broad spectrum antibiotic activity of the cecropins. Recently, the membrane insertion of cecropin A, with the long axis of the α-helix in the plane of the membrane, was proved experimentally by solid-state nuclear magnetic resonance (NMR) spectroscopy conducted in lipid bilayers [79]. Electron microscopy and immunocytochemistry further verified the binding of cecropin to *Escherichia coli* cell membranes [80]. In turn, this would suggest that the cecropins are also potentially toxic to eucaryotic cells. Interestingly enough, cecropin A and B demonstrate very little haemolytic activity, if any, against sheep erythrocytes [81] and, in our hands, cecropin A remained without toxicity up to the studied highest concentration of 50 μM against COS cells of primate origin as well.

Insect defensins selectively kill Gram-positive bacteria [7]. This feature is highly unusual as all other peptide families are more active against Gram-negative than Gram-positive strains (Table 1). Gram-positive bacteria have a simpler but thicker cell wall than Gram-negative bacteria. This cell wall consists primarily of multiple layers of peptidoglycan with teichoic acid polymers dispersed throughout. The acidic character of the peptidoglycan cell wall naturally binds the highly positively charged antibacterial peptides. In this regard, it is significant that defensin A loses its antimicrobial potency

in the presence of competing cations [82]. A comparison between the structure and the activity of fragments of tenecin 1, an antibacterial peptide belonging to the insect defensin family, indicated that a net positive charge was a prerequisite factor for activity [83]. Gram-negative strains have a cell wall that is thinner than that of Gram-positive bacteria. However, in Gram-negative strains an additional membrane, the outer membrane, composed of a lipid bilayer, some proteins and lipopolysaccharide (LPS), lies above the peptidoglycan layer. As predicted from their positive charge, many antibacterial peptides bind the negatively charged LPS [84]. Careful examination of the net positive charge/mass ratio of the various antibacterial peptide families indicates that this ratio is the smallest for the insect defensins, which, in turn, would explain defensin's less efficient permeability of the outer membrane of Gram-negative strains. When bound to the peptidoglycan layer of Gram-positive strains, defensin A disrupts the permeability barrier of the cytoplasmic membrane, resulting in the loss of cytoplasmic potassium, a partial depolarization of the inner membrane, a decrease of cytoplasmic adenosine triphosphate (ATP) and an inhibition of respiration [82]. It is proposed that these permeability changes reflect the formation of pores in the cytoplasmic membrane by defensin oligomers [82]. Sapecin also inhibits calcium-activated potassium pores [85], suggesting the existence of other functions involving host cells, perhaps unrelated antibacterial activity [3]. Defensins kill bacteria instantaneously; a 1-min contact with 0.5 μM concentration of peptide is sufficient to kill *M. luteus* in the growing or resting phase [35,40].

The three-dimensional structure of defensin A from *P. terranovae* consists of a flexible *N*-terminal loop, a central α-helix and a *C*-terminal twisted antiparallel β-pleated sheet [86]. This structure is very similar to other insect defensins, like sapecin [87], but somewhat different from that of mammalian defensins [71]. In mammalian defensins the α-helix is replaced with an additional β-sheet domain [88,89]. This structural difference may signal differences in the haemolytic activity. Mammalian defensins are moderately haemolytic [90], with the readily dimer-forming human defensins, being more cytotoxic than the rabbit defensins that are less prone to multimer formation [91,92]. Human NP-3 can dimerize via intermolecular β-sheet formation [89] and, if cytotoxicity is correlated with the presence of β-sheets [71], insect defensins with less sheet content are expected to be less cytotoxic than their mammalian counterparts.

The mode of action of the drosocin-pyrrhocoricin-apidaecin peptide family is markedly dissimilar to that of the cecropins and the defensins and makes these peptides suitable targets for drug development efforts. Drosocin is active in the low or sub-micromolar concentrations against approximately half of the Gram-negative strains studied, but, of the Gram-positives, kills only *M. luteus* [49]. Pyrrhocoricin is more active than drosocin [50] and some pyrrhocoricin analogues display a wide activity spectrum [93]. Analogues made of all-D amino acids remain inactive for all three peptides [49,93,94], suggesting that these peptides bind stereospecifically to a target bacterial protein rather than non-specifically disrupting the membrane integrity. In contrast, cecropin's antibacterial activity is not influenced by D-amino acid substitutions [95]. While amino- or carboxy-terminally truncated analogues of drosocin and pyrrhocoricin lose their ability to kill bacteria [49,50], defensin has an independently functioning active domain located at the *C*-terminus [83]. Another proof for the altered mode of action comes from the kinetics of killing. Antibacterial peptides with membrane lytic mode of action kill bacteria instantly. In contrast, drosocin needs 6–12 h to elicit its antibacterial activity *in vitro* [49,59]. This time-scale is consistent with the deactivation of a bacterial regulatory/housekeeping protein. This bacterial protein is unlikely to share sequence or structural homologies with appropriate mammalian proteins in the pyrrhocoricin-binding region because pyrrhocoricin is completely non-toxic to sheep erythrocytes and COS cells *in vitro* [93] and neither pyrrhocoricin nor drosocin show toxic effects in healthy mice up to the studied 50–100 mg/kg concentration [50,93].

As for apidaecin, recent results upheld the model of permease/transporter-mediated peptide uptake in bacterial cells [96]. The proposed mechanism involves an initial nonspecific encounter of the peptide with an outer membrane component, followed by invasion of the periplasmic space and by a specific and essentially irreversible engagement with a receptor/docking molecule that may be inner membrane-bound or otherwise associated. It is most likely a component of a permease-type transporter system. In the final step, the peptide is translocated into the interior of the cell where it meets its ultimate target, perhaps one or more components of the protein synthesis machinery [96]. When identifying the biopolymers involved in this process, we observed that pyrrhocoricin, drosocin and apidaecin bind bacterial LPS and the 70-kDa heat shock protein DnaK in a specific, and the 60-kDa bacterial chaperonin GroEL in a non-specific manner (Otvos L Jr. Interaction between heat shock proteins and antimicrobial peptides. *Biochemistry* 2000; submitted).

Because native drosocin and pyrrhocoricin could not be shortened nor could the amino acid composition be changed without a loss of *in vitro* antibacterial activity [50,93], we hypothesized that the peptides have to assume a certain secondary structure to bind stereospecifically to the target protein. Clearly, this structure needs to be maintained in the design of potential drugs. Accordingly, we determined the bioactive conformation of native glycosylated drosocin and pyrrhocoricin as well as their non-glycosylated analogues by circular dichroism (CD) and two-dimensional NMR spectroscopy. For drosocin, no substantial difference between the predominantly random conformation of the glycosylated and non-glycosylated forms is observed, but there are subtle differences in the small populations of folded conformers [97]. In particular, the turn at residues 10–13 tends toward a more extended structure on glycosylation, while there is some tightening of the downstream turn at residues 17 and 18. Like drosocin, it appears that the structure of pyrrhocoricin is largely random coil and there is little change in the backbone conformation upon glycosylation [93]. For pyrrhocoricin, however, there is a subpopulation with organized structure at both the *N*- and *C*-terminus, indicating the presence of reverse-turns at the pharmacologically important terminal regions. The increase in the turn potential at the termini compared to drosocin may explain the increased *in vitro* antibacterial activity of pyrrhocoricin. Plate 1 shows representative models of glycosylated drosocin and pyrrhocoricin based on the NMR data (courtesy of David Craik and Ailsa McManus). Pyrrhocoricin's hypothetical structure is supported by the antibacterial activity of some of its cyclic peptide analogues. Cyclization stabilizes reverse-turns [98] and was expected to improve potency, but a head-to-tail cyclic pyrrhocoricin derivative without expanding the cycle lost activity compared to the analogue linear peptide, probably due to distortion of the extended domain in the middle of the peptide. Where the ring size was increased by repeating an internal octapeptide fragment, the resulting expanded cyclic analogue became highly active against both Gram-negative and Gram-positive bacterial strains [93].

Synthetic unglycosylated diptericin is inactive against the only Gram-positive strain tested, *M. luteus*, up to 80 μM concentration [59]. From the Gram-negative bacteria, diptericin exhibits sub-micromolar activity against two *E. coli* strains, D22 and 1106, and against *Salmonella typhimurium*. While some minor activity can be detected against *Klebsiella pneumoniae* and *Enterobacter cloacae*, the peptide has no effect on *Pseudomonas aeruginosa* and on *Agrobacterium tumefaciens*. This activity spectrum is similar to that of drosocin and pyrrhocoricin, with the exceptions of unglycosylated drosocin being less active on *E. coli* D22 and *S. typhimurium*, while *A. tumefaciens* appears to be very sensitive to pyrrhocoricin. However, the kinetics of killing *E. coli* suggests a completely different mode of action. Diptericin kills a major portion of the bacterial culture within 15 min and fully eliminates the bacteria after a 45 min incubation period in contrast to drosocin, which kills bacteria after 6 h of incubation [59], and the attacins, which also require a longer period to manifest their antimicrobial potency [99]. A single Asp→Glu mutation in the attacin-analogue *C*-terminal region of diptericin increases the IC_{50} value against *E. coli* D22 approximately ten-fold [60], although these activity differences may stem from different assay conditions. Introduction of a Cys residue (due to the synthesis *via* chemical ligation) results in another ten-fold activity decrease [61]. While the amino-terminal drosocin-analogue domain lacks any antibacterial activity, a minor residual activity could be detected for the *C*-terminal attacin-analogue region [60]. Significantly, glycosylation of Thr10 and Thr54 does not modify the activity figures at all [60,61]. The bactericidal properties of full-sized diptericin were studied on *E. coli* D22 and *S. typhimurium*. Full killing of either strain was detected at 40 μM [59]. Diptericin increases the permeability of the outer and inner membranes of *E. coli* D22 cells and it was suggested that the peptide acts by disrupting bacterial membrane integrity [60]. The antibacterial activity of the additional glycine-rich peptides, the attacins, sarcotoxins, coleoptericin and holotricin-2 is also restricted to a limited array of Gram-negative strains [3,100]. The attacins are thought to prevent bacterial cell division by inhibiting the biosynthesis of the outer membrane proteins [101].

However, the conformation of diptericin does not support the idea of membrane disintegration. According to CD evidences, diptericin lacks any periodic structure such as an α-helix or β-pleated sheet that would interfere with membrane assembly [59].

Inspection of the NMR spectra in water clearly suggests that the peptide adopts essentially random coil conformations. This is indicated by the small chemical shift dispersion of the amide resonances (~ 0.8 ppm), a lack of upfield-shifted methyl signals and a lack of downfield-shifted αH signals. In addition, there is almost a complete absence of NH-NH nuclear Overhauser effects (NOEs) in the 250 ms nuclear Overhauser enhanced spectroscopy (NOESY) spectrum, confirming the absence of helical populations. Indeed, the NOESY spectrum displayed relatively few NOEs overall for a peptide of this size [59]. Taken together, diptericin does not seem to belong to any known class of antibacterial peptides.

From the rest of the antibacterial peptides, thanatin is active against both Gram-positive and Gram-negative bacteria [7], as is moricin [68]. Thanatin adopts a well defined anti-parallel *O*-sheet structure from residue 8 to the *C*-terminus, including the disulfide bridge [102]. Having said this, thanatin's structure appears to be quite different from the known structures of other insect antibacterial peptides with disulfide bridges, such as defensin or the antifungal drosomycin. Rather, the conformation of thanatin displays more similarities with those of various antibacterial peptides from different origins, such as the brevinins [103], protegrins [104] and tachyplesins, [105]. Similarly to these peptides, activity test experiments performed on severely truncated thanatin isoforms [66] stress the importance of the β-sheet structure and suggest that the activity against Gram-negative strains involves a site formed by the Arg20 side-chain embedded in a hydrophobic cluster [102]. For additional information on structure-activity relationships of insect antibacterial peptides, the reader is asked to consult an excellent recent review by Philippe Bulet and coworkers [106].

STABILITY AND *IN VIVO* PROPERTIES

The ultimate goal of all research that involves antibacterial peptides is the development of compounds suitable for human or veterinary therapy, although genes encoding antimicrobial peptides [107], including that of cecropin B [108], have been introduced to plants as well. The major concern of the use of peptides as therapeutics, of course, is their susceptibility to proteases [70]. Most insect antibacterial peptides are rich in Lys and Arg

residues, the targets of trypsin-like peptidases. In addition, cecropin is degraded by enzymes produced specifically for this purpose by *Bacillus larvae*, *Heterorhabditis bacteriophora* and *P. aeruginosa* [109–111]. Attacins are destroyed by inhibitor A, an exoprotease produced by *Bacillus thuringiensis* at the beginning of the stationary growth phase [112]. To be effective against bacterial infections, the peptides have to survive the actions of proteinases in the haemolymph of insects. This is less problematic for the defensins and the cecropins that exhibit their antibacterial effect rapidly, but can mount a bigger obstacle for the proline-rich peptides that act over an extended time period. However, as we showed by stability assays in a *Phormia* haemolymph, approximately half of the initial amounts of drosocin and pyrrhocoricin remained uncleaved after 15 h, indicating that the proteolytic activity of the haemolymph does not pose a major threat to the bioactivity of the native products [50]. Unfortunately, the peptides degrade considerably faster in plant leaf intercelluar fluid (IF) or mammalian serum. The half-life of cecropin B in potato IF is as low as 3 min and not much longer in tomato IF (7 min) [113]. Nevertheless, MB39, a close analogue of cecropin B, is significantly more resistant in all crops studied [113]. Because the bioactivity of cecropin A-derived peptides is variously affected by the presence of proteins extracted from leaves of tobacco and tomato plants, either total extracts or intercellular fluids, it is suggested that tobacco should not be used as a model for testing the possible protective effects of transgenically expressed, cecropin-based genes [114]. We observed a somewhat similar phenomenon in the degradation pathway of pyrrhocoricin analogues in human and mouse sera. While the degradation products, as determined by mass spectrometry, of native pyrrhocoricin differed in pooled human and mouse sera, they were largely identical when a modified pyrrhocoricin peptide, designed to resist serum peptidase cleavage, was studied [93]. *In vivo* stability of peptides in blood is currently modeled well by *in vitro* stability in serum or plasma (neglecting renal and hepatic clearance) [115]. Serum stability studies represent one of the most important secondary screening assays in peptide drug development, largely because they eliminate peptides that have short half-lives and are therefore unlikely to be therapeutically effective [115]. Indeed, glycosylated drosocin fails to protect mice against experimental *E. coli* infection when administered in 25–100 mg/kg doses due the complete degradation of the pep-

tide in mouse serum before it could exhibit its protective properties [50]. A relatively easy way to improve serum stability is the incorporation of D-amino acids [116]. This method, indeed, yielded a cecropin A variant resistant to trypsin and serum deactivation without sacrificing antibacterial potential [95]. Moreover, incorporation of D-amino acids into an antibacterial, cytolytic peptide resulted in a decrease of the undesired haemolytic activity [117]. However, this strategy is inappropriate for the proline-rich peptides that bind to their target protein in a stereospecific manner. Incorporation of an unnatural Lys analogue to the amino terminus renders a model, non-insect antibacterial peptide resistant to exopeptidase cleavage without a decrease in the antimicrobial activity [118] and may also block the actions of trypsin. Nevertheless, the design of new analogues has to take into account that both pig cecropin [119] and pyrrhocoricin [93] need a positive charge at the site of the original amino terminus to retain their bioactivity. Backbone modifications are similarly viable methods for improving protease resistance and perhaps modifying the activity spectrum [120]. This route is especially important because, for example, our broad spectrum cyclic pyrrhocoricin analogue undergoes extensive endopeptidase cleavage [93].

Reports on *in vivo* activity of antibacterial peptides of insect origin are few and far between. The scarce existing studies investigated not only the antimicrobial properties, but also, due to cytotoxic effects, the antitumour activity. Cecropin B significantly increases the median survival time of mice bearing colon adenocarcinorna when administered intraperitonally at an 80 mg/kg dose [121]. Unfortunately, the peptide is lethal within 24 h at a dose of 100 mg/kg [121]. Expression constructs carrying cecropin were introduced into a human bladder carcinoma-derived cell line and the resultant cell clones were analysed for tumourigenicity in nude mice. Expression of cecropin resulted in reduction or even a complete loss of tumour induction [122]. In a recent study, a cecropin–melittin hybrid peptide [123] was delivered continuously using miniosmotic pump placed in the peritoneal cavity into juvenile coho salmon infected with *Vibrio anguillarum*, the causative agent of vibriosis [124]. Fish receiving 200 ng of the tandem peptide per day survived longer and had significantly lower accumulated mortalities than the control groups [124]. Drosocin is without toxicity up to a dose of 100 mg/kg when injected into mice, but becomes toxic to

compromised animals [50]. The *in vivo* toxicity of pyrrhocoricin and Chex-pyrrhocoricin-Dap(Ac) (an analogue in which Val1 is replaced with 1-amino-cyclohexane-carboxylic acid and Asn20 is replaced with β-acetyl-diamino-propionic acid) was studied in mice [93]. The peptides showed no toxicity up to the applied 50 mg/kg dose. The native peptide protected mice against *E. coli* infection at single intravenous doses of 10 mg/kg or 25 mg/kg applied 1 h after infection (with a boost after 5 h of infection), but at a dose of 50 mg/kg was toxic to compromised animals [93]. The Chex-pyrrhocoricin-Dap(Ac) derivative protected all 15 mice in the entire 10–50 mg/kg dose range. We are in the process of identifying the minimally active dose and the activity/toxicity ratio of native pyrrhocoricin and its designed analogues.

Pharmaceutical companies generally tend to be skeptical about considering peptides as therapeutically viable molecules. For antibiotics, this situation is even more serious because a number of factors deactivate otherwise potent antibacterial peptides *in vitro* [70]. For reproducible and meaningful results, many peptide families need to be tested in poor broth or 'minimal' media for which the standardized automatic assay conditions used by large companies are either incompatible, or have to be revalidated. Nevertheless, as the *in vivo* data with the pyrrhocoricin peptides indicate, some peptides are promising drug lead compounds. In a recent discussion with industry leaders we came to the conclusion that *in vitro* activity data on antibacterial peptides are useful, but largely do not predict *in vivo* results. Nevertheless, we have to take advantage of the insects' wise selection of the compounds that protect them from bacterial infections and the combinatorial library these animals have provided us. Careful modifications of the best native products coupled with prudent selection of the *in vitro* and/or *in vivo* assay conditions will enable us to fight a number of bacterial strains in this time of the emergence of bacterial resistance. Astonishingly, according to a recent study, high levels of antibacterial resistance have been found in gut bacteria from wild rodents that probably have never been exposed to classic antibiotics, prompting that more careful use of conventional antibiotics may not be enough to reduce antibiotic resistance [125]. In a decade-old review about antibacterial peptides, Boman gave the following title of a paragraph: 'In Defense of Peptides' [8]. It is my hope that we no longer need to defend the insect peptides. Rather, they will eventually defend us humans, too.

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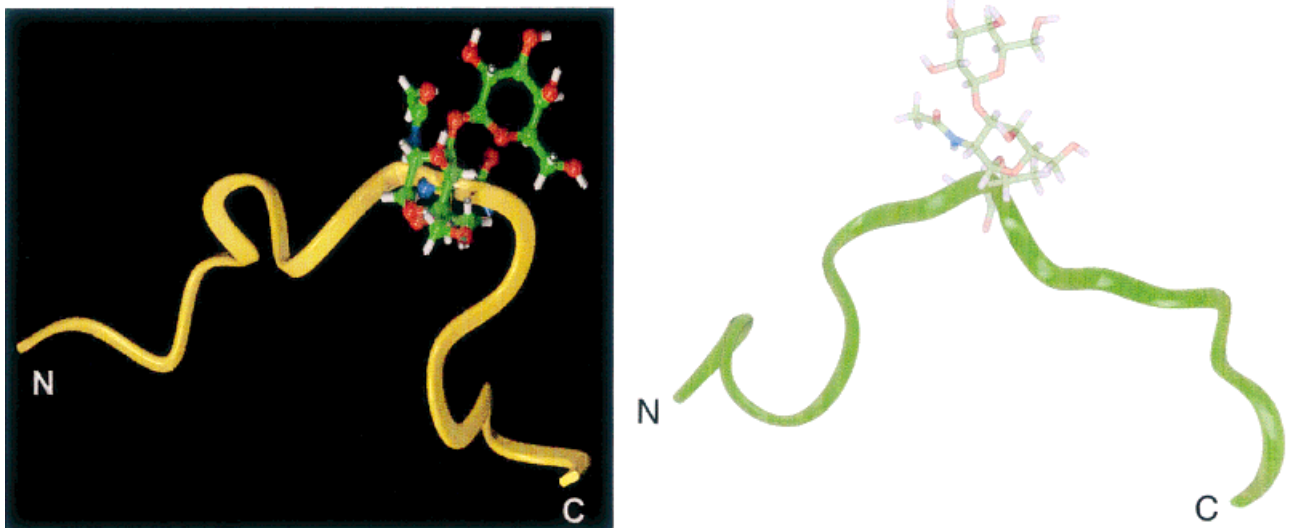
Plate 1   Representative models from simulated annealing and energy minimization calculations of glycosylated drosocin (left panel) and pyrrhocoricin (right panel). The initial parameters were set based on NMR NOE restraints. The coordinates are from [97] (drosocin) and [93] (pyrrhocoricin).