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Insect neuropeptides: Structures, chemical modifications and potential for insect control

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ABSTRACT

Insect neuropeptides are involved in almost all physiological processes in insects, such as diuresis, ecdysis, pheromone biosynthesis and control of muscle activity. Thus, these small peptide hormones and their receptors are promising targets for a novel generation of selective and non-neurotoxic insecticides. However, due to poor bioavailability, pharmacokinetics and short half-life the peptides themselves cannot be used as insect control agents. The past two decades have seen an increase in research into the discovery of non-peptide small molecules that function as mimics for neuropeptides. This review presents an overview on structure–activity studies, conformational analyses and peptidomimetic modifications of selected insect neuropeptides with a special potential for application in pest control.

1. Introduction

Insects form the largest class of the phylum Arthropoda. There are at least one million known species, more than 50% of all existing organisms on earth are insects. Besides multifaceted beneficial aspects numerous insect species cause massive crop damages and transfer serious diseases to humans and animals. One of the most characteristic features of many insects is their dramatic morphological change during development from a larva to an adult insect.

As early as 1922 the Polish scientist Stefan Kopeĉ proposed that substances in the brain control the moulting and metamorphosis processes.[1] Today we know that numerous physiological processes in insects are controlled by small, bioactive peptides. Because these regulatory peptides are synthesized in modified neurons, they are called neuropeptides. Neuropeptides are chemical messengers which are released from the neurons into the haemolymph of the insect to reach their distant target organs.

In the mid 1970s, about 50 years after Kopeĉ's publication on a brain hormone, the first two insect neuropeptide structures were determined: proctolin, a peptide from the cockroach *Periplaneta americana* with potent myotropic activities and one year later AKH, the adipokinetic hormone of locusts.[2,3] Only with the advent of genomics, protein mass spectrometry, and high-field NMR spectroscopy in the late 1980s and 1990s did knowledge on insect neuropeptides increase dramatically. Nowadays primary structures of several hundred neuropeptides are known.[4] Additionally, manifold structure–activity relationships, receptor-binding, and degradation studies have been established. In the *Drosophila melanogaster* genome there is evidence for at least 30 genes encoding neuropeptide precursors and more than 40 neuropeptide receptors.[5] Neuropeptides are ubiquitous in the nervous system of insects and they are by far the most diverse signalling substances, both structurally and functionally.[6] Neurohormone-regulated processes can however be grouped into four major functional categories: (1) growth and development, (2) behaviour and reproduction, (3) metabolism and homeostasis and (4) muscle movement.[7] (Fig. 1).

Insect neuropeptides and their receptors are promising targets for a novel generation of insecticidal agents offering levels of selectivity and environmental compatibility, absent from conventional, neurotoxic insecticides.[8] Additionally, the mechanisms responsible for the synthesis, maturation, transport, secretion, binding and inactivation provide further biochemical targets for neuropeptide derived insecticidal agents. However, insect neuropeptides have a number of characteristics that make them rather unsuitable candidates for insect control agents. Peptides are unstable in the environment, show poor solubility both in organic and aqueous solutions, and suffer rapid degradation in the digestive system of insects. Consequently, considerable effort has been directed into the discovery and development of effective delivery systems. In particular, the possible use of genetically modified baculoviruses as vectors for genes expressing neuropeptides and other insecticidal proteins has been studied intensively.[9]

From a chemist's point of view disruption of target binding or signal transduction by small organic molecules appear to be the most straightforward strategies regarding the practical application of a neuropeptide based insecticide. This review presents an over-

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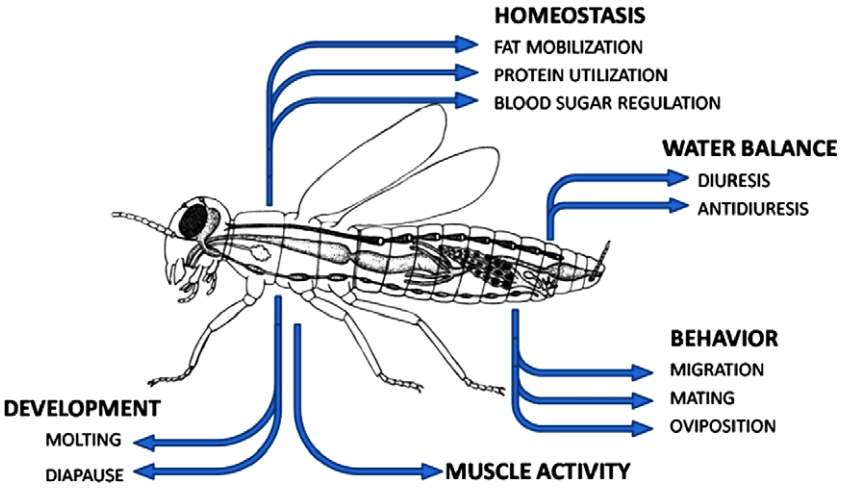


Figure 1. Overview on insect neuropeptides.

view on peptidomimetic approaches and chemical modifications of selected insect neuropeptides which have a special potential for the design of novel insecticidal agents. The majority of insect neuropeptides characterized thus far interfere with the contractile activity of visceral and skeletal muscles.[10] This mode of action is comparable to the neurotoxic action of most commercial insecticides and thus will probably find easier acceptance in the crop protection industry.

2. Neuropeptide families

2.1. Proctolin

Proctolin is present in extracts of the hindgut of the American cockroach *P. americana*. For its first isolation, purification and structure elucidation more than 125 kg cockroaches were needed.[11] So far proctolin has been identified in species from six orders of insect, as well as in other invertebrates, where it has been shown to exert myotropic effects (1–200 nM) on visceral and skeletal muscles. Proctolin has variously been assigned roles as a neurotransmitter, a neuromodulator, and a neurohormone.[12]

Since its primary structure became available in 1975 proctolin (**1**) was the subject of structure–activity studies by a number of research groups. In most cases biological testing was performed either with *Tenebrio molitor* (cardiotropic activity) or with *Schistocerca gregaria* (myotropic activity).[13] (Fig. 2)

Regarding the N-terminal position it was found that Arg[1] can only be substituted with other basic amino acids such as lysine or the arginine-mimics homoarginine and canavanine without reduction of myostimulatory activity. N-monomethylation of the guanidine group significantly reduced the myotropic activity. N,N-bismethylation, N-acetylation or nitration of the guanidine function as well as the exchange of L-arginine for D-arginine resulted in a complete loss of activity.[14] The significance of tyrosine in position 2 was examined in more detail. For instance, the hydroxy function could be replaced by several other *para*-substituents, some of which express 'superagonistic' activity on the cockroach hindgut (Table 1). Remarkably, the MeO-analogue showed 160% activity of proctolin while the structurally closely related EtO-derivative had only around 40% activity.[15]

Variation in the length of the tyrosine side chain appears to be critical in determining the level of biological activity. Elongation of the Tyr[2] side chain with only one additional methylene group regardless of the substitution in the *para*-position of the phenyl ring reduced the cardiotropic activity in *T. molitor* by 80% compared to proctolin. Truncation of the side chain by exchanging tyrosine for 4-hydroxy-phenylglycine and other *para*-substituted phenylglycines even caused a complete loss of myotropic activity at the physiological range of concentrations (10^{-9}–10^{-7} M). Obviously, the aromatic side chain has to keep the correct distance to the peptide backbone. Likewise, the presence of arginine in position 1, the L-configuration of tyrosine in position 2 and of leucine in position 3 are prerequisites for maintaining myotropic activity.

Furthermore, it is not possible to exchange Leu[3] with short unbranched amino acids like alanine or serine. However, up to 70% of

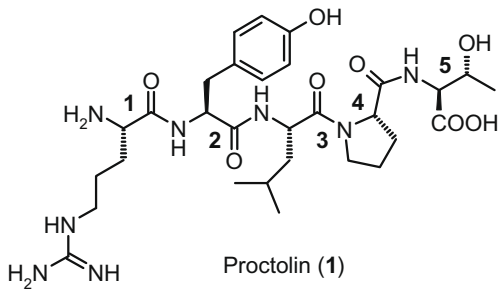


Figure 2. Structure of proctolin.

Table 1
Relative activity of phenyl-substituted analogues on cockroach hindgut relative to proctolin

Arg-Phe(p-X)-Leu-Pro-Thr	Relative activity (%)
X = OH (proctolin)	100
H	25
OMe	160
OEt	42
OPr	17
F	53
Cl	200
Br	228
I	136
NO_2	102
NH_2	46
N_3	177
Me	178

the cardiotropic activity in *T. molitor* is retained, when Leu is replaced by Ile, Lys, Asp or Arg, indicating some tolerance to structural modifications in this position.[16] As expected, D-proline in position 4 has no myotropic activity on the cockroach hindgut. The same was found for β-*homo*-Pro[4], Ala[4] and 4-methoxy-proline.[4] Very recently syntheses and conformational analyses of two diastereomeric 4-monofluoro-proline analogues were reported, unfortunately without any biological data.[17] According to structure–activity relationships and the narrow space for structural modifications it was speculated that L-Pro[4] is involved in the formation or stabilization of an essential secondary structure. *allo*-Threonin in position 5 gave full myotropic activity in a locust hindgut assay, which proves that the absolute configuration at the hydroxy group is uncritical. D-Thr[5], Thr[5]-$CONH_2$ as well as Ala[5] modifications did not produce any myotropic activity in the cockroach hindgut. Ser[5], Val[5], Arg[5] analogues and compounds containing amino alcohols in position 5 such as $H_2N–CH_2–CH(OH)–CH_3$ or $H_2N–CH_2–CH_2–CH_2–OH$ retained a cardiotropic activity in *T. molitor* of around 50–60%.[18] In contrast to the mono-D-amino acid analogues of proctolin, which were completely inactive, the bis-D-amino acid derivatives D-Arg-Tyr-D-Leu-Pro-Thr, Arg-D-Tyr-Leu-Pro-D-Thr and even more amazingly the all-D-proctolin demonstrated superagonistic cardiotropic activity in *T. molitor*.

Compared to the numerous variations of amino acid side chains only a very limited number of backbone modifications have been published. The idea behind these backbone modified proctolin derivates is to stabilize the peptide against protease catalyzed degradation. In the depsipeptides **2** to **5** the amide function was replaced by an ester bond on varying positions in the chain (Fig. 3). While compound **3** (O between Tyr[2] and Leu[3]) was almost inactive at a concentration of 1 μmol/L (Table 2) in an isometric muscle-contraction bioassay employing hindguts from the locust *Locusta migratoria*, compound **2** (O between Arg[1] and Tyr[2]) retained significant activity and compound **4** (O between Pro[4] and Thr[5]) showed full activity.[19] The importance of the Tyr[2]-amide bond is underlined by the almost inactive compound **5** containing two ester groups and compound **9** with a hydroxymethylen-group instead of an amide bond.[20] More drastic structural modifications such as the incorporation of an oxazole-ring in the positions 3 and 5 caused a complete loss of activity.[21] Unfortunately, no biological data were given for the interesting tetrazole derivative **8**.[22]

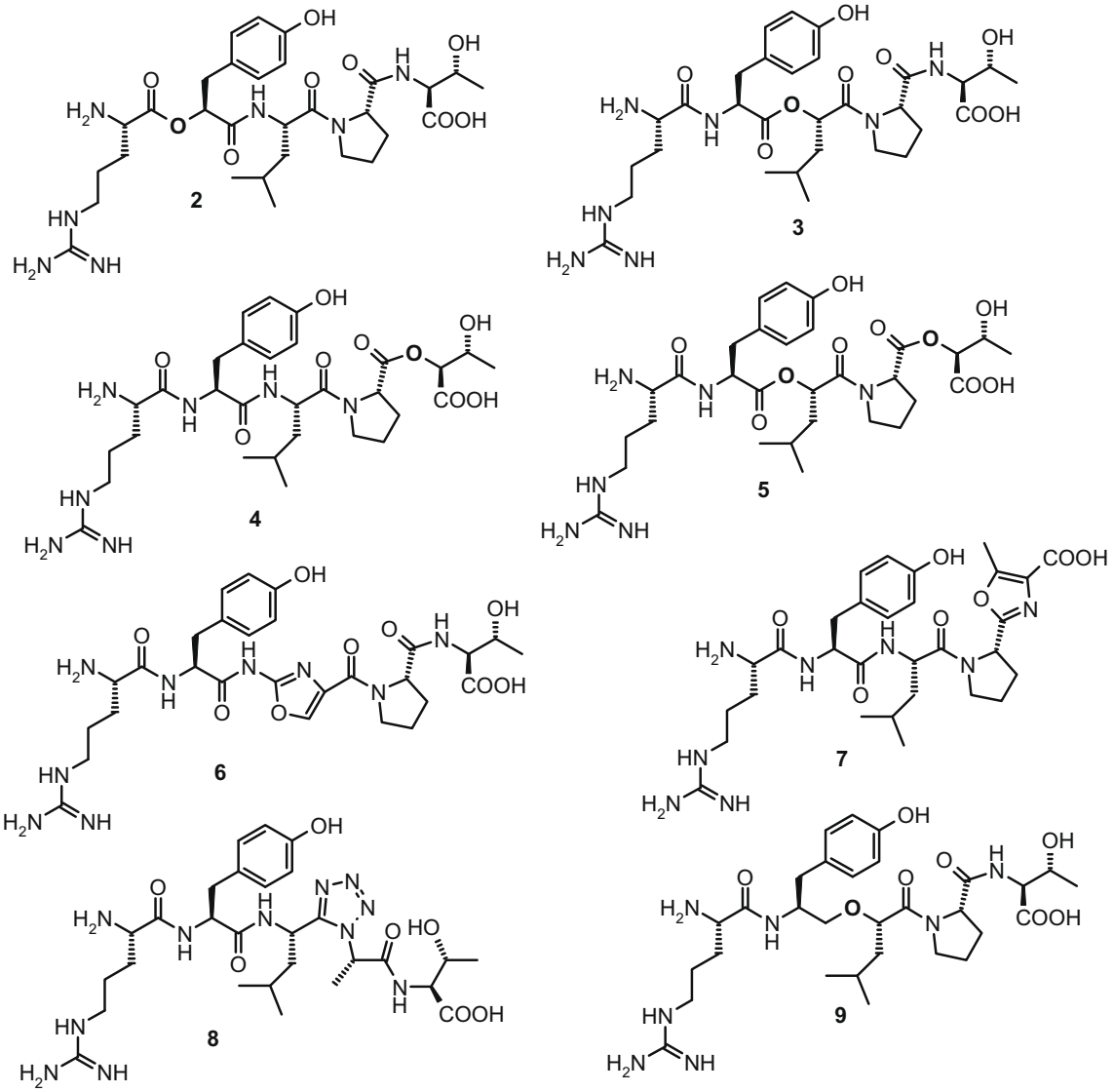


Figure 3. Proctolin analogues with backbone modifications.

Table 2
Relative myotropic activity on hindguts from *Locusta migratoria* at 1 μmol/L

Compound	Contraction (%)
1	100
2	66
3	30
4	106
5	27

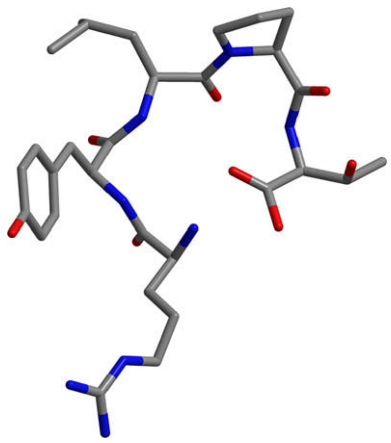


Figure 4. Preferred conformation of proctolin in DMSO-d_6.

A question which has so far remained unanswered, is what constitutes the biologically active conformation of proctolin? Several conformational studies performed by NMR- and CD-spectroscopy, as well as theoretical calculations have failed to yield a conclusive model. While in protic solvents no secondary structural elements exist, in DMSO-d_6 a preferred conformation with a *trans*-Pro and an inverse γ-turn between Thr-NH and Leu-CO was identified (Fig. 4).[23] However, the biological relevance of this secondary structure remains speculative because depsipeptide **4** still has full myotropic activity but can no longer form a γ-turn due to the ester bond in place of the Thr-NH.

Cyclic analogues of small and flexible peptides play an important role in the process of identifying the bioactive conformation because they restrict the conformational freedom of a linear peptide drastically and thus considerably simplify NMR spectra and theoretical calculations. Cycloproctolin **10** (Fig. 5) was inactive as a myotropic agonist, but was found to be a potent antagonist of proctolin-induced production of the second messengers $InsP_3$ and $InsP_4$. The same antagonistic effect was found for [α-Me-Tyr]-proctolin while [*N*-Me-Tyr]-proctolin was shown to be completely inactive.[24] NMR studies in DMSO-d_6 suggest a more compact structure for antagonists involving a loop rather than a turn between Tyr-NH and Thr-CO, possibly stabilized by a salt bridge between the N- and C-terminal groups. The inactivity of the N-Me-Tyr analogue is in full agreement with the postulated hydrogen bond between Tyr-NH and Thr-CO. Depsipeptide **2**, in which the Tyr-NH is replaced by a Tyr-O, shows a significantly reduced myotropic activity and thus emphasizes the importance of the Tyr-NH for receptor binding.

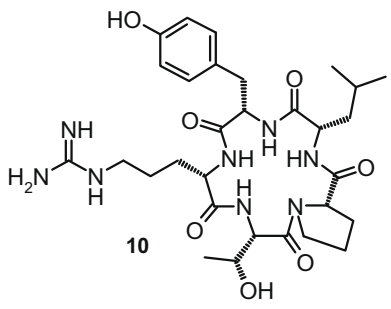


Figure 5. Cycloproctolin.

In contrast to numerous in vitro studies with insect preparations investigations of in vivo activities of proctolin and analogous compounds are rare. It has been reported that injections of proctolin increase the amplitude of extracardiac haemocoelic pulsation in *T. molitor* pupae. In larva, pupa and adults of the fruitfly *D. melanogaster* proctolin decreases the heart rate. Due to insufficient penetration proctolin does not show any insecticidal activity by topical application to *Manduca sexta* larvae.[25]

By analysis of data from the *Drosophila* genome project preproproctolin, the biosynthetic precursor of proctolin, the proctolin receptor and a proctolin degrading peptidase have been identified.[12] In particular the *D. melanogaster* proctolin receptor opens the door for high-throughput screenings of small organic molecules as mimics for proctolin.[26]

2.2. Myokinins

The myokinins form a large group of insect neuropeptides which have been isolated from a number of insects, including species of Dictyoptera, Lepidoptera and Orthoptera. These peptides encompass 6–13 residues and have a highly conserved COOH-terminal pentapeptide sequence with the general formula FX_1X_2WGa-mide, where X_1 is S, H, N, Y and X_2 is S or P (Table 3). Myokinins are multifunctional neuropeptides expressing myotropic and potent diuretic activities.[27] Kinins stimulate Malphigian tubules, organs which are involved in the regulation of salt and water balance, with EC_{50} values in the range of 10^{-11}–10^{-12} M and act synergistically on the larger CRF-related (corticotrophin releasing factor) diuretic hormones in insects.[4,28] The helicokinins inhibit weight gain and increase mortality after injection into larvae of *Heliothis virescens*, a serious agricultural pest. Furthermore, the in vivo activity of helicokinin I increases significantly following addition of ACE inhibitors, such as captopril or enalapril, which suppress the proteolytic degradation of the kinins.[29] Their short sequences and interference with vital physiological processes render the myokinins prime candidates for the peptidomimetic design of metabolically stable neuropeptide based pest control agents.

Table 3
Selected myokinins from various insect species

Sequence	Name	Insect species	
SGAD**F**YP**WG**amide	Achetakinin I	*Acheta domesticus*	Cricket
AY**F**SP**WG**amide	Achetakinin II		
ALP**F**SS**WG**amide	Achetakinin III		
NSKYVSKQK**F**YS**WG**amide	Aedeskinin I	*Aedes aegypti*	Mosquito
NP**F**HA**WG**amide	Aedeskinin II		
NNPNV**F**YP**WG**amide	Aedeskinin III		
NP**F**HS**WG**amide	Culekinin I	*Culex salinarius*	Mosquito
NNANV**F**YP**WG**amide	Culekinin II		
WKYVSKQ**F**FS**WG**amide	Culekinin III		
Y**F**SP**WG**amide	Helicokinin I	*Heliothis zea*	Moth
VR**F**SP**WG**amide	Helicokinin II		
KVK**F**SA**WG**amide	Helicokinin III		
DPA**F**NS**WG**amide	Leucokinin I	*Leucophaea maderae*	Cockroach
DPG**F**SS**WG**amide	Leucokinin II		
DQG**F**NS**WG**amide	Leucokinin III		
A**F**SS**WG**amide	Locustakinin	*Locusta migratoria*	Locust

Table 4
Diuretic activity of glycine derivatives[31]

11	R	EC_{50} (nM)
a	OH	1000
b	NH_2	0.004
c	OMe	20
d	OEt	100
e	OCH_2Ph	50
f	SMe	5
g	NHMe	28
h	NMe_2	Inactive

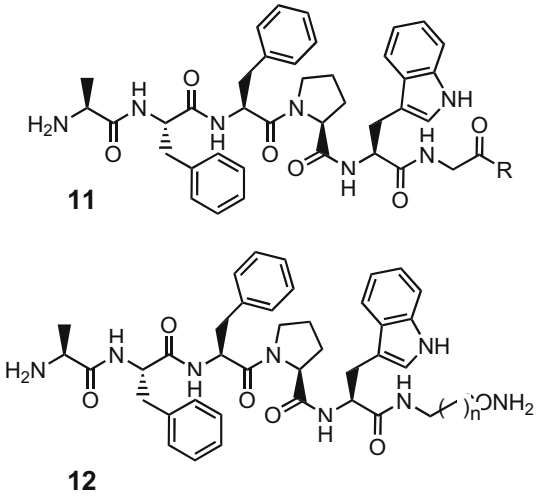


Figure 6. Myokinin analogues with variations at the C-terminus.

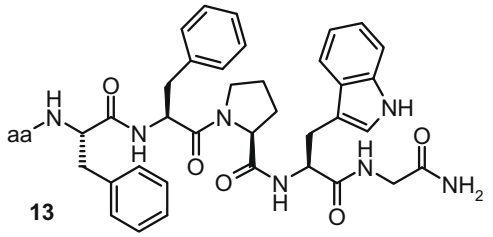


Figure 7. Myokinin analogues with variations in the N-terminus.

Table 5
Diuretic activity of chain-elongated glycine mimics[31]

12	*n*	EC_{50} (nM)
a	2–4	0.4–2.5
b	5	Inactive

Table 6
N-terminal amino acid variations of 13[35b]

13	aa	EC_{50} (nM)
a	Empty	0.1
b	Trp	0.042
c	Phe	0.029
d	Arg	0.020
e	Ala	0.005
f	Lys	0.005
g	Aib	0.005
h	Asp	0.002
i	Leu	0.002
j	Asn	0.001
k	Val	0.001

The C-terminal pentapeptide represents the active core and is sufficient for full myotropic and diuretic activity. Alanine-scans of several insect kinins revealed that the C-terminal Gly^1, Trp^2 and in particular Phe^5 are the most critical amino acids. Exchange of the variable amino acids in positions 2 and 3 with alanine does not cause a complete loss of myotropic and diuretic activity.[30] On a reduced level of activity the amide group can be replaced by an ester group (**11c**, AFFPWG-OMe) or a thioester (**11g**, AFFPWG-SMe) (Fig. 6). The latter was found to be four times more potent (EC_{50} = 5 nM) in a cricket diuretic assay than the methyl ester (EC_{50} = 20 nM). The more lipophilic benzyl ester AFFPWG-OCH_2Ph (**11e**) also retains some diuretic activity with an EC_{50} of 50 nM (Table 4). Increased lipophilicity as expressed by the thio- and benzyl esters are important for cuticle penetration and peptidase resistance. Kinin analogues with a C-terminal free carboxy group are usually devoid of any myotropic and diuretic activity. The monomethylated kinin analogue AFFPWG-NHMe (**11g**) stimulated Malpighian tubule secretion at an EC_{50} of 28 nM, whereas the bismethyl derivative AFFPWG-NMe_2 (**11h**) proved inactive. The carboxamide function can be separated from the glycine α-carbon by incorporation of one to four additional methylene spacers (**12a**) without loss of diuretic activity (cricket diuretic assay) while a fifth methylene group (**12b**) reduces the diuretic activity (Table 5).[31] C-terminal kinin aldehydes, such as Fmoc-RFFPW-CH_2CHO and Boc-VFFPW-CH_2CHO were four orders of magnitude less active in a cricket diuretic assay, but showed a significant in vivo weight gain inhibition in *Helicoverpa zea* larvae. However, it remains unclear whether the weight gain inhibition is caused by a specific diuretic activity of the kinin aldehyde or by an insecticidal effect of the toxic carbamate protecting group.[32] Amazingly, Fmoc-RFFPW-CH_2CHO expressed just the opposite effect in the housefly *Musca domestica*: in vitro and in vivo inhibition of diuresis[33].

A series of hexapeptide analogues XaaFFPWG-NH_2 (**13**) underlined the high variability of the N-terminus (Table 6). All derivatives showed high stimulation of fluid secretion in the cricket Malpighian tubule fluid secretion assay (Fig. 7). Analogues in which the Xaa position was occupied by Ala, Asn, Leu or Asp demonstrated superagonistic activity compared to the natural full-length achetakinins. Replacement of the critical N-terminal Phe residue in the pentapeptide core fragment FFPWG-NH_2 with a bulky carboranyl residue resulted in analogue **14** (Fig. 8) which retained around 60% of the maximal diuretic activity (EC_{50} = 0.2 nM).

This result is highly relevant because it is one of the few modifications in that position which does not delete biological activity. Furthermore the sterically demanding carboranyl moiety increases the lipophilicity and thus improves the ability of the achetakinin analogue **14** to penetrate the cuticle.[34]

A major challenge on the route towards a metabolically stable and cuticle penetrating neuropeptide analogue is the replacement of the natural amino acids with non-peptides.[35] Pseudopeptides derived from FFSWG-NH_2 and FFPWG-NH_2 in which the amide bonds between Phe^1 and Phe^2 have formally been reduced to –CH_2–NH– had only 1% of the myotropic activity on the cockroach hindgut compared with the parent peptides.[36]

Exchange of the N-terminal Phe by hydrocinnamic acid (Hca-Tyr-Pro-Trp-Gly-NH_2, **17**) afforded a myotropically highly active derivative (EC_{50} = 10 nM) whereas the structurally similar 6-phenylhexanoic acid containing pseudotripeptide (6Pha-Pro-Trp-Gly-NH_2, **18**) elicited myostimulation only at a significantly lower level (EC_{50} = 2 μM).[37] Analogues with reduced total length (**15**) of the peptide chain or lacking the conformation inducing proline residue (**16**) were in general weak myostimulants (Fig. 9). Taking all the structure–activity data together it becomes apparent that the C-terminal dipeptide Trp-Gly-NH_2 together with an N-terminal extension mimiking the phenyl ring of phenylalanine is the minimum requirement for efficient receptor-binding.

The kinin core sequence is inactivated by hydrolysis of the amide bond between Trp and the following amino acid, usually a serine or proline, by angiotensin-converting enzymes (ACE). The hydrolysis by *M. domestica* ACE can be completely prevented by incorporation of the unnatural, sterically hindered aminoisobutyric acid (Aib). The two kinin analogues Phe-Phe-Aib-Trp-Gly-NH_2 (**19**) and pGlu-Lys-Phe-Phe-Aib-Trp-Gly-NH_2 (**20**) both demonstrated subnanomolar stimulation of fluid secretion in isolated cricket Malphigian tubules with EC_{50} values of 5.6 pM and 2.8 pM, respectively (Fig. 10). Remarkably, **19** and **20** were not only stable against ACE degradation but also more potent by a factor of four to eight than most naturally occurring achetakinins. The high activity of these kinin analogues is explained by the turn-stabilizing effect of the sterically hindered aminoisobutyric acid, which facilitates efficient receptor-binding.[38] A comparable proteolytic stabilization was achieved by the incorporation of β^2- and β^3-amino acids in the peptide chain of Arg-Phe-Phe-Pro-Trp-Gly-NH_2. Acetylation of the N-terminal amino group affords an additional stabilization against aminoendopeptidases. With an EC_{50} value of 30 pM Ac-RFF[β^3Pro]WGa (**21**) proved the diuretically most potent compound in a series of eight single and double replacement (**22**) kinin analogues tested on isolated Malpighian tubules of the cricket *Acheta domesticus* (Fig. 10). Less active by a factor of around three was the kinin analogue Ac-R[β^3Phe]FPWGa. Exchange of Trp by β^2- or β^3-tryptophane resulted in an almost complete loss of diuretic activity.[39]

A detailed knowledge of the receptor-bound conformation or at least a preferred conformation in solution is a prerequisite for the rational design of peptidomimetic neuropeptides. Molecular dynamics simulations of leucokinin II (Asp^1-Pro^2-Gly^3-Phe^4-Ser^5-Ser^6-Trp^7-Gly^8-NH_2) suggest a β-turn preference for the residues Phe-Ser-Ser-Trp. Similar β-turns have been proposed for several other leucokinin (Phe^1-X^2-Ser^3-Trp^4-Gly^5-NH_2) and achetakinin (Phe^1-X^2-Pro^3-Trp^4-Gly^5-NH_2) core sequences. The exact nature of the β-turn (type I, type II or type VI) strongly depends on the amino acid sequence and the solvent. But in all cases MD simulations show the essential tryptophane and phenylalanine side chains to be located on the same side of the peptide backbone near each other. The three non-essential amino-terminal residues remain in an extended conformation. Noteworthy, the side chain of position 2 (Tyr, Ser), which is most variable with respect to structural modifications, turns away from Trp and Phe.

Based on these theoretical calculations and structure–activity data a qualitative binding model for the kinin core pentapeptide was developed by Nachman (Fig. 11).[30,54] The two critical residues Phe^1 and Trp^4 associate together and form a lipophilic aromatic surface that directly interacts with the receptor site. Gly^5 enforces receptor-binding by forming hydrogen-bonds with its amide function. According to this binding model the variable positions X^2 (Tyr) and X^3 (Pro, Ser) which form the central positions of the β-turn point away from the receptor surface and thus only show minimal interaction with the receptor.

There is a body of experimental evidence supporting the hypothesis of a β-turn in the biologically active kinin conformation. Extensive molecular modeling and nuclear magnetic resonance studies demonstrated that the myokinin analogue **19** adopts either a type II β-turn involving the residues 1–4 or a type I β-turn including residues 2–5. In particular, the 1–4 β-turn motive places the critical aromatic side chains Phe^1 and Trp^4 adjacent to each other and thus is in good accordance with Nachman's receptor-binding model.[40]

The postulated 1–4 β-turn motive can also be induced by *cis*-amide bond mimics, such as aminopyroglutamic acid (Apy) or a tetrazole residue. The insect kinin analogue Ac-Arg-Phe-(*2R,4S*)-APy-Trp-Gly-NH_2 (**23**) demonstrated high activity ($EC_{50} = 0.7 \times 10^{-8}$ M) and a 93% maximal response (Table 7) compared to the parent peptide in the cricket diuretic assay (Fig. 12). Three other stereoisomers tested expressed a significantly reduced diuretic activity and binding to the tick (*Boophilus microplus*) kinin receptor by at least a factor of 10. All members in a series of tetrazole ana-

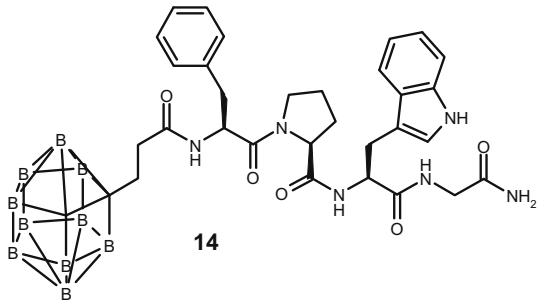


Figure 8. Carborane analogue **14**.

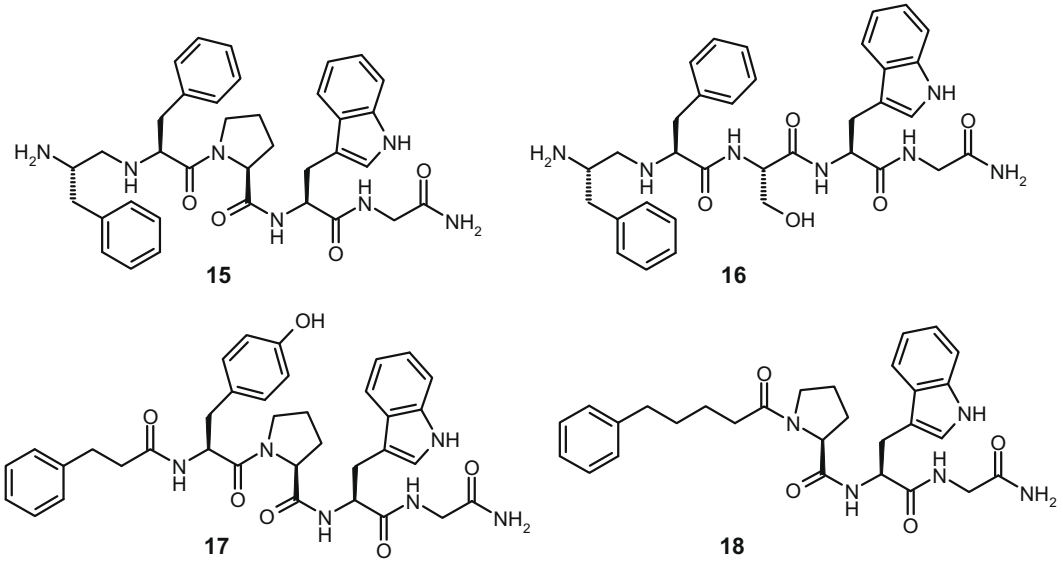


Figure 9. Myokinin pseudopeptides and truncated analogues.

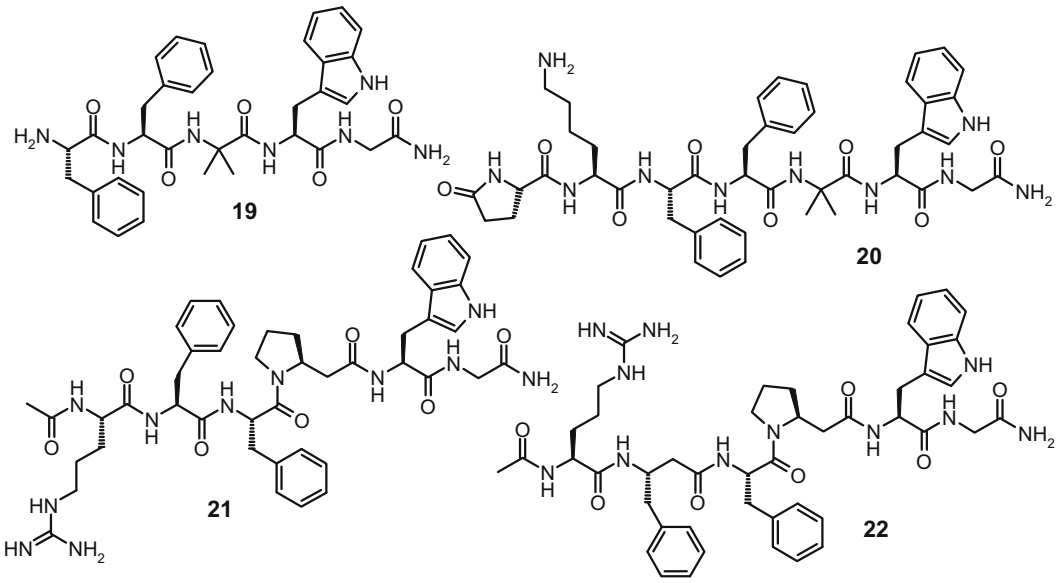


Figure 10. Metabolically stable myokinin analogues.

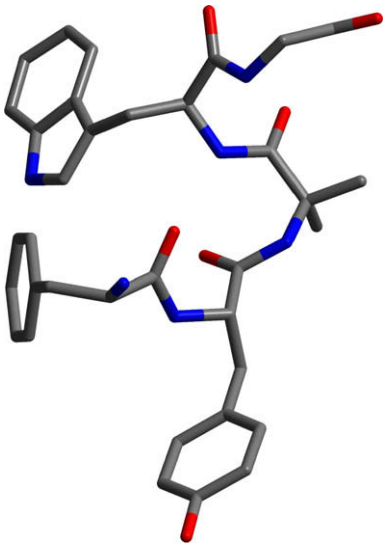


Figure 11. Qualitative receptor-binding model for myokinin neuropeptides.

Table 7
Diuretic activities of selected kinin analogues in a cricket Malpighian tubule fluid secretion assay

Compound	EC_{50} (nM)
19	0.0056
20	0.0028
21	0.03
23	7
24	3
25	340
Phe-Phe-Pro-Trp-Gly-Ala-NH_2	0.4
Phe-Phe-Pro-Trp-Gly-NH_2	0.1
Phe-Tyr-Pro-Trp-Gly-NH_2	0.055

logues (**25**) showed significantly lower diuretic activities, thus rendering the tetrazole scaffold a poorer mimic for the postulated *cis*-amide bond in the receptor bound conformation.[41]

A consequence of the presumed β-turn conformation is the spatial proximity of the C- and N-terminus. End to end cyclized kinin core sequences are expected to mimic the β-turn, to reduce the conformational freedom of the linear peptides, and to express enhanced receptor-binding due to reduced entropy loss. A major obstacle in verifying cyclic kinin analogues is the structural inflexibility of the Gly^5-NH_2. Structural modifications of the C-terminal primary amide function in general cause significant reductions of both the myotropic and diuretic activity. However, the C-terminal extended linear hexapeptide Phe-Phe-Pro-Trp-Gly-**Ala**-NH_2 and the corresponding cyclopeptide **24** (Fig. 12) demonstrated potent diuretic and myotropic activity albeit on a somewhat reduced level compared to the C-terminal unmodified peptide Phe-Phe-Pro-Trp-Gly-NH_2. Thorough computational and NMR analyses revealed that at least the *cis*Pro conformation of cyclo[Phe-Phe-Pro-Trp-Gly-Ala-NH_2] retains the β-turn of the linear active core pentapeptide. The essential Phe^1 and Trp^4 side chains are situated adjacent to each other whereas the highly replaceable position 2 side chain is located on the opposite surface.[30] Amino piperidinone **26** was designed to bridge the variable positions Phe-X_{aa}-X_{bb}-Trp-Gly-NH_2 in the kinine active core segment. According to Nachman's receptor interaction model a small library of simplified kinine analogues **26** was synthesized**.** NMR studies indicate that in the preferred conformation the indolyl side chain adopts an axial and the phenethyl residue an equatorial position.

In this conformation the two side chains come close together and mimic a β-turn conformation as it is found in the C-terminal kinine pentapeptide. Unfortunately, all compounds produced only very modest increases of fluid secretion in a Malpighian tubule assay.[42] It remains unclear whether the piperidinone is in principle an unsuited scaffold or **26** is oversimplified, since only the amino acid side chains were introduced and the critical glycine amide was omitted completely.

2.3. Pyrokinins and pheromone biosynthesis activating neuropeptides (PBANs)

Pyrokinins form a group of insect neuropeptides that share the common C-terminal pentapeptide sequence Phe-X-Pro-Arg-Leu-NH_2 (Table 8). The variable position X can be occupied by either Thr, Ser, Gly or Val.[43] Since the first isolation of a pyrokinin (leucopyrokinin) from the cockroach *Leucophaea maderae* in 1986 several

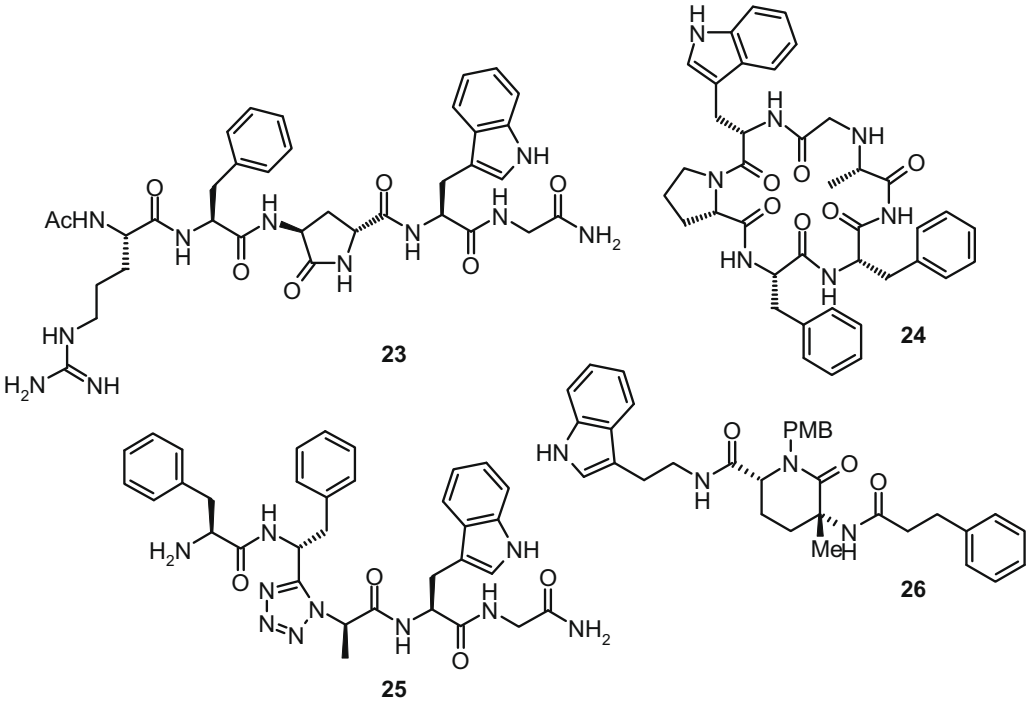


Figure 12. Conformational restricted myokinin-analogues.

Table 8
Selected pyrokinins and pheromone biosynthesis activating neuropeptides (PBANs)

Sequence	Name	Insect species
pQTSFT**PRL**amide	Lem-PK	*L. maderae*
pQ...QQP**FV**PRLamide	Lom-PK-I	*L. migratoria*
pQSVPT**FT**PRLamide	Lom-PK-II	
...VPAAQ**FS**PRLamide	Lom-MT-I	
EGD**FT**PRLamide	Lom-MT-II	
ME**FT**PRLamide	Hez-MT-I	*H. zea*
TMN**FS**PRLamide	Hez-MT-II	
II**FT**PRLamide	Bom-MT-I	*B. mori*
...THESLE**FI**PRLamide	Bom-MT-II	
...SRTKY**FS**PRLamide	Hez-PBAN	*H. zea*
...RTRY**FS**PRLamide	Bom-PBAN	*B. mori*
...SRNKY**FS**PRLamide	Lyd-PBAN	*L. dispar*

pyrokinins from different insect species have been isolated and characterized. Pyrokinins stimulate contractions of isolated hindgut preparations in nanomolar threshold concentrations. The pyrokinin active core pentapeptide sequence has also been found in the 33 residue pheromone biosynthesis activating neuropeptides (PBAN) from the corn earworm *Heliothis zea* and the silkmoth *Bombyx mori*.[44] In both PBANs the variable position 2 is occupied by a serine. The pyrokinins and PBANs show considerable cross-activity. For example, the PBANs of *H. zea* and *B. mori* demonstrate significant myostimulatory activity on the isolated cockroach hindgut. Conversely the myotropic pyrokinins from the cockroach and locust show considerable pheromonotropic activity. Recently, several neuropeptides sharing the FXPRLamide sequence with additional biological functions such as colour polymorphism and diapause induction were reported.[45] Due to their multifunctional role in insect physiology the pyrokinins and in particular the PBANs represent important leads for the development of insect neuropeptide based insecticides.

Structure–activity studies have demonstrated that the C-terminal pentapeptide FTPRLamide represents the minimum active core for both myotropic and pheromonotropic activities in *Leucophaea* and *Bombyx*, respectively. Extension to the Hez-PBAN hexapeptide fragment increased the activity 2.5–3 times in both myotropic and pheromonotropic assays. The residues Phe and Arg proved most important for biological activity as well as the L-configuration of the amino acids.[46] Thus, replacement of L-Tyr or L-Arg with the corresponding D-amino acids resulted in an almost complete loss of activity.[47]

The carboranyl (Cbe) pseudotetrapeptide **27** (Fig. 13), consisting of only five residues was shown to be 10-fold more potent than the 33 residue containing natural PBAN in an in vivo pheromonotropic bioassay of the female tobacco budworm moth *H. virescens* (Table 9). Even more important, in contrast to natural HezPBAN the carboranyl-neuropeptide analogue **27** elicited pheromone production even after topical application to the lateral abdominal surface of *H. virescens*.[48] Topical activity and metabolic stability are major conditions for the development of an insecticidal agent. In a more recent publication it was shown that topical activity can be achieved in general with lipophilic residues coupled to the C-terminal active core of the insect pyrokinins. Pyrokinin tetrapeptides in which phenylalanine was replaced with hydrocinnamic acid (Hca, **29**), 1-pyrenebutyric acid (Pba, **31**) or 9-fluoreneacetic acid (Fla, **28**) induced pheromone production in *H. virescens* at doses of 50–500 pmol following topical application (Fig. 13). The fluorene- and pyrene-analogues stimulated a significant pheromone production for 18 h whereas the hydrocinnamic acid derivative **29** lost its activity already after 4 h.[49] The 7-bromofluorene derivative **30** induced pheromone production when applied topically to *H. virescens* in amounts of 1 nmol. After injection of 500 nmol **30** all moths died within 24 h. Both the bromofluorene and the active core Phe-Thr-Pro-Arg-Leu-NH_2 were critical for the toxic action, suggesting that the moths were poisoned by a specific receptor–ligand interaction.[50] An amphiphilic analogue of Locusta myotropin II (Lom-MT-II), in which a 6-phenylhexanoic acid-Ala building block was coupled to the N-terminus of Lom-MT-II, demonstrated pheromonotropic activity equivalent to the pheromone biosynthesis activating neuropeptide, when injected into females of *H. virescens*. Dissolved in water the PBAN analogue expressed significant

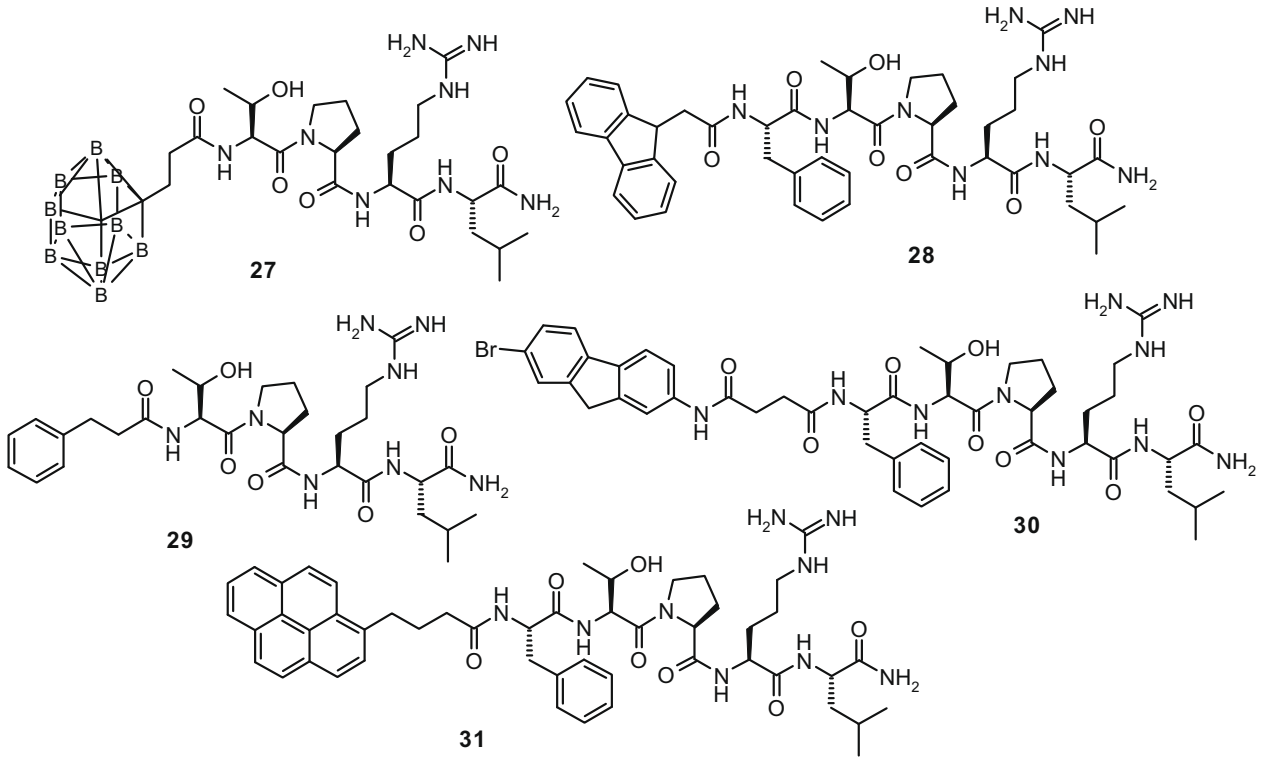


Figure 13. Lipophilic pyrokinin/PBAN analogues.

Table 9
Biological activities of carboranyl pyrokinin analogue **27** according to lit.[48]

Peptide	Myostimulation of the isolated cockroach hindgut (*L. maderae*) Threshold concentration (pmol)	Pheromone production in *H. virescens*	
		Via injection ED_{50} (pmol)	Via topical application ED_{50} (pmol)
Cbe-TPRLamide (**27**)	70	0.1	25
FTPRL-amide	2200	50	–
pQTSFTPRL-amide (leucopyrokinin)	650	5	–
HezPBAN (33 residues)	2600	1.0	Inactive

topical activity after administration to the abdomen of *H. virescens* females. Pheromone production was induced within less than 15 min. These results clearly prove that lipophilic neuropeptide analogues are able to penetrate the insect cuticle and induce endogeneous responses.[51]

Linear pyrokinin/PBAN pseudopeptide-analogues (**32**–**34**, **36**) with a suitable mimic for proline expressed significant myotropic activity in a cockroach hindgut assay with threshold concentrations in the lower nanomolar range (6 nM for **33**) which corresponds to 30% activity of the parent pentapeptide Phe-Thr-Pro-Arg-Leu-NH_2 (Fig. 14). In contrast, the straight-chain pseudopeptide **35** was significantly less active in the same bioassay (Table 10). Molecular modeling studies indicate that the adjacent carboxyl groups adopt a diequatorial orientation in the low-energy chair-conformation of the trans-cyclohexane system. The diequatorial conformation fits well with a β-turn found in the active pyrokinin analogue cyclo[Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu].[52]

Since receptor-bound conformations of an insect neuropeptide are not yet known, rigid cyclopeptides are used frequently as probes to sense the biologically active conformation.[53] The cyclic conformationally restricted pyrokinin analogue cyclo[Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu] retained a significant portion (threshold concentration 34 nM) of myotropic activity compared to its linear counterpart (threshold concentration 1.4 nM).[54] The same cyclooctapeptide retains 10% of the pheromonotropic activity of naturally occurring Bom-PBAN-I, a 33 amino acid peptide.[55] NMR and molecular dynamics analyses revealed a type I β-turn spanning the active core residues Thr-Pro-Arg-Leu in the cyclopeptide whereas the linear pentapeptide according to molecular dynamic simulations changes between an extended conformation and an open type I β-turn lacking a hydrogen bond between the first and forth residue.[56]

In an extensive molecular modeling study, using the X-ray structure of the G protein coupled receptor (GPCR) rhodopsin as a template a structure for the *Hez*PBAN receptor was predicted.[57] Based on this calculated structure, docking experiments with different types of β-turns all proposed for the C-terminus of HezPBAN allowed to build a binding-model for the HezPBAN C-terminal hexapeptide YFSPRLamide.[54,58] This model albeit not concise enough to provide clear evidence for the specific β-turn type, clearly supports the general hypothesis of a β-turn in the receptor bound conformation of HezPBAN.

While most efforts in insect neuropeptide research are focusing on metabolic stabilization and improved agonistic activities only a limited number of papers address neuropeptide antagonists. With respect to practical use antagonists which inhibit muscle activity or pheromone biosynthesis, should be at least of the same practical

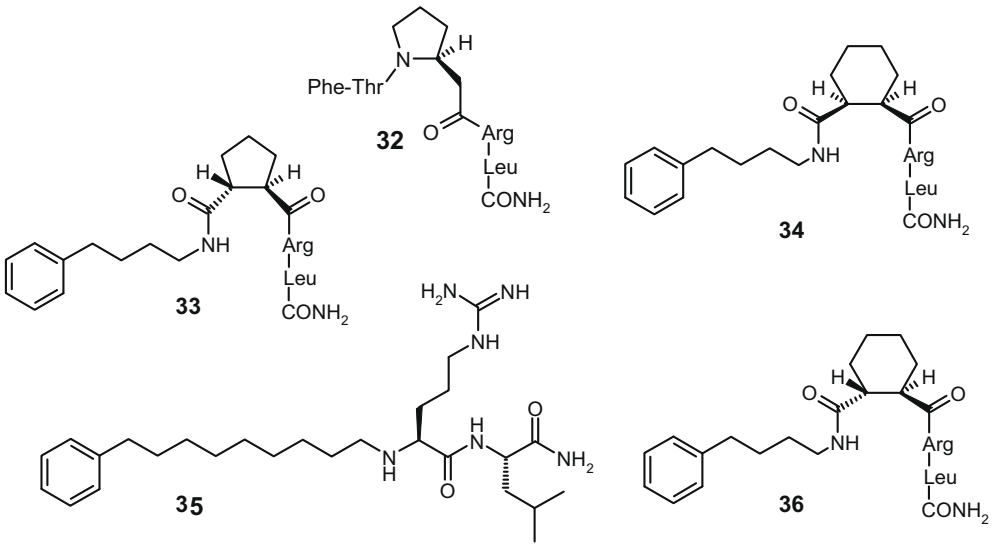


Figure 14. pseudopeptide analogues of pyrokinine/PBAN neuropeptides.

Table 10
Myotropic activities of pyrokinin/PBAN pseudopeptide analogues[52]

Peptide	Threshold concn (nM)	EC_{50} (nM)
4Pbm-tCpd-Arg-Leu-NH_2, **33**	6	90
4Pbm-tChd-Arg-Leu-NH_2, **36**	64	900
4Pbm-cChd-Arg-Leu-NH_2, **34**	40	650
9Pna-Arg-Leu-NH_2, **35**	8000	—
Phe-Thr-hPro-Arg-Leu-NH_2, **32**	15	76
Phe-Thr-Pro-Arg-Leu-NH_2	2.2	32
Phe-Ser-Pro-Arg-Leu-NH_2	1.6	—
Phe-Gly-Pro-Arg-Leu-NH_2	2	—

Abbreviations: 4Pbm, 4-phenylbutylamino; tCpd, *trans*-DL-1,2-cyclopentanediacyl-; tChd, *trans*-DL-1,2-cyclohexane-diacyl-; cChd, *cis*-1,2-cyclohexane-diacyl-; 9Pna, 9-phenyl-nonyl; hPro, homoproline.

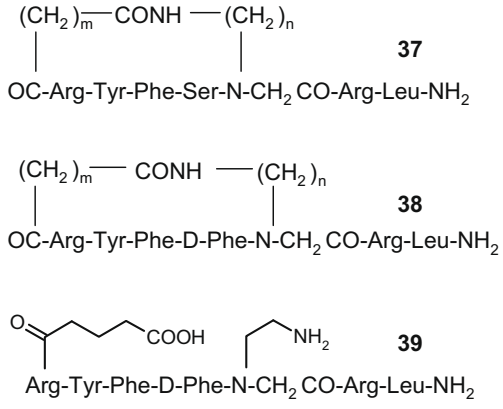


Figure 15. Pheromone biosynthesis inhibiting pyrokinine analogues.

value as neuropeptide agonists. In a D-Phe scan of the Hez-PBAN active core YFSPRLamide Altstein et al. identified an antagonistic peptide with the sequence Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2.[59] Based on this antagonist and the C-terminal hexapeptide sequence two cycloscan-libraries **37** and **38** with variable ring size and position of the bridging amide bond were synthesized (Fig. 15). Four highly active antagonistic backbone cyclized peptides ($m+n$ = 2+3, 3+3, 4+2, 6+2) which fully inhibited sex pheromone biosynthesis in *H. peltigera* at 1 nmol were found in the D-Phe library **38**.[60] In a more recent examination the antagonistic properties of several peptides and cyclic analogues were tested for their ability to inhibit or induce the additional physiological functions melanization (*Spodoptera littoralis*), hindgut contraction (*L. maderae*) and pupariation (fleshfly *Neobellieria bullata*). While no antagonist inhibited all biological functions three peptides were found with a selective pheromonotropic (n = 3, m = 2, library **38**) or melanotropic activity (n = 5, m = 2 and n = 3, m = 3, both library **38**). Additionally, a linear precyclic peptide **39** was found to selectively inhibit melanization in the fleshfly.[61]

Table 11
Selected tachykinins from insects, vertebrates and amphibians

Sequence	Name	Insect species
GPS**GF**Y**G**V**R**amide	Lom-TK-I	*L. migratoria*
APLS**GF**Y**G**V**R**amide	Lom-TK-II	
APQA**GF**Y**G**V**R**amide	Lom-TK-III	
APTA**F**Y**G**V**R**amide	Cav-TK-I	*C. vomitoria*
GLGNNA**F**V**G**V**R**amide	Cav-TK-II	
APS**GF**M**G**M**R**amide	Cus-TK-I	*C. salinarius*
APW**GF**T**G**M**R**amide	Cus-TK-II	
APT**GF**FAV**R**amide	Stom-TK	*S. calcitrans*
APS**GF**L**G**V**R**amide	Lem-TRP-I	*L. maderae*
…KRAPS**GF**L**G**V**R**amide	Lem-TRP-II	
…KKAPS**GF**L**G**T**R**amide	Lem-TRP-III	
RPKPQQ**FF**G**L**Mamide	Substance P	*Vertebrate*
pEADPNK**F**Y**G**LMamide	Physalaemin	*Amphibian*

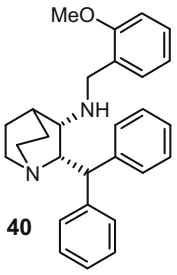
2.4. Tachykinins

In contrast to the insect neuropeptide families discussed so far tachykinins also have been identified in many vertebrate species (Table 11). Tachykinins are multifunctional brain/gut peptides, which are involved in the processing of sensory information, in the control of motor activities and in eliciting stimulatory responses in a variety of visceral muscles. The receptors for mammalian and insect tachykinins show a high degree of sequence conservation and their functional characteristics are similar. Vertebrate tachykinins and a few insect tachykinins share the common C-terminal sequence -FXGLMamide whereas the majority of insect tachykinins have a common –GFX_1GX_2Ramide C-terminus.[62]

Members of the vertebrate tachykinin (neurokinin) subfamilies also elicit myostimulatory activity in the cockroach hindgut bioassay. The most potent of these were substance P and physaelamin with threshold concentrations of 8 nM and 7 nM, respectively (Table 12). The potencies of the C-terminal pentapeptides of substance P (Phe-Phe-Gly-Leu-Met) and physealamin (Phe-Tyr-Gly-Leu-Met) proved to be 288 nM and 147 nM.[63] Noteworthy, the

Table 12
EC_{50} values of natural and synthetic peptides tested on different tachykinin receptors[64b]

Peptide	STKR EC_{50} (nM)	NK1 EC_{50} (nM)	NK2 EC_{50} (nM)
Lom-TK-I	181	N.D.	N.D.
Lom-TK-Ma	N.D.	110	N.D.
Lom-TK-LMa	N.D.	0.091	283
Substance P	N.D.	0.25	25
Neurokinin A	N.D.	2.0	1.1
Substance P-Ra	13	21	N.D.
Substance P-VRa	4.8	N.D.	N.D.



40

Figure 16. A non-peptidic tachykinin mimic.

Table 13
Selected myosuppressins and FLRFamides

Sequence	Name	Insect species
pQDV**DHVFLRF**amide	Lem-MS	*L. maderae*
PDV**DHVFLRF**amide	Scg-FLRF	*S. gregaria*
ADVG**HVFLRF**amide	Lom-MS	*L. migratoria*
GQERN**FLRF**amide	Lom-FaRP-I	
pQDVVHS**FLRF**amide	Mas-FLRF	*M. sexta*
TDV**DHVFLRF**amide	Neb-MS	*N. bullata*
TDV**DHVFLRF**amide	Ang-MS	*A. gambiae*

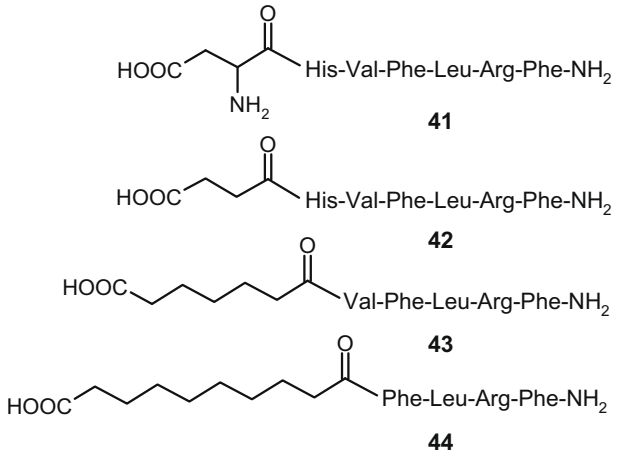


Figure 17. Myosuppressin pseudopeptide analogues.

substance P antagonist **40** (CP-96,345-1, Pfizer) demonstrated myostimulatory activity on a *L. maderae* cockroach hindgut preparation at a threshold concentration of 6.5 μM (Fig. 16). At concentrations higher than 10 μM CP-96,345-1 behaved as an antagonist, that completely blocked the myostimulatory response to substance P. The sequence homologies between the insect and vertebrate tachykinin receptors suggest that the non-peptidic compound **40** interacts with the insect tachykinin receptor on the *L. maderae* hindgut.[35c] A comparative study of a series of synthetic tachykinin-like peptides on recombinant neurokinins receptors (NK1, NK2) and on stomoxytachykinin receptors (STKR) revealed that the C-terminal pentapeptide FTGMRamide is sufficient for activation of the insect stomoxytachykinin receptor. Replacement of the highly conserved Phe and Met by Ala resulted in a loss of activity. The C-terminus also determines the selectivity for insect or mammalian receptors. Peptides with a C-terminal methionine amide show decreased insect but increased mammalian agonist activities and vice versa.[64]

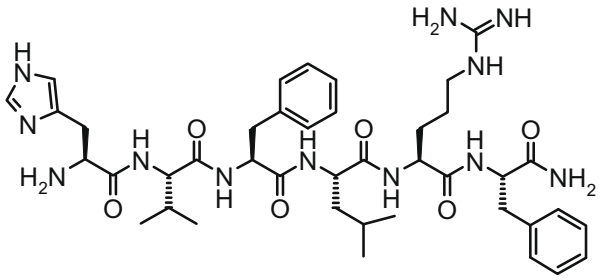
Comparable to the kinins the replacement of a glycine residue in the cockroach *L. maderae* tachykinin Ala-Pro-Ser-Gly-Phe-Leu-**Gly**-Val-Arg-NH_2 by aminoisobutyric acid (Aib) and additional blockage of the N-terminus with pyroglutamate resulted in a tachykinin analogue pGlu-Ala-Pro-Ser-Gly-Phe-Leu-Aib-Val-Arg-NH_2 with about the same potency as the parent peptide, but with complete resistance to hydrolysis by the angiotensin-converting enzyme (ACE) from *D. melanogaster*.[65] Conformational analyses of a sterically even more hindered locustatachykinin analogue (Gly-Phe-NMeTyr-Aib-Arg-NH_2), containing Aib instead of Gly and NMeTyr instead of Tyr confirmed a preferred cis-amide bond about the Phe-NMeTyr residues. According to molecular dynamics calculations the cis-geometry induces a type VI β-turn spanning residues 1–4. A very similar conformation found for the analogous substance P analogue (pGlu-Phe-NMePhe-Aib-Leu-Met-NH_2) may explain the physiological cross-activity.[66]

2.5. Myosuppressins

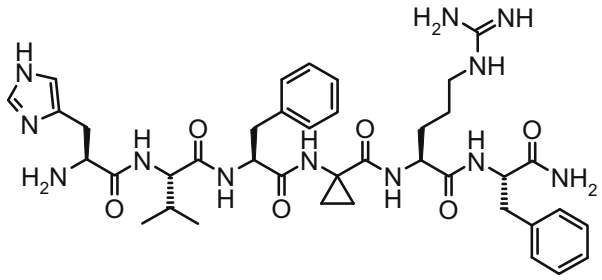
Myosuppressins have been isolated from various insect species, including the cockroach *L. maderae*, the locusts *S. gregaria* and *L. migratoria*, and the flies *N. bullata* and *D. melanogaster* (Table 13).[67] The myosuppressins (FLRFamide neuropeptide family) show potent inhibition of cardiac and visceral muscle contraction and thus express contrary activities compared to the neuropeptide families introduced so far in this review.[68] Myosuppressins share the C-terminal heptapeptide sequence Asp-His-Val-Phe-Leu-Arg-Phe-NH_2 which also comprises the smallest fragment capable of retaining the full myoinhibitory potency of the parent peptide.[35c,56] Despite eliciting contrasting biological responses the myosuppressins share Asp, Arg, and Phe residues in analogous positions with the sulfakinins.[69]

The active core heptapeptide sequence **41** can be further reduced by replacing up to three amino acids by dicarboxylic acid moieties, which mimic the carboxylic acid side chain of the critical Asp residue. The pseudohexapeptide analogue **42** with Asp exchanged for Suc (succinoyl) was found to have the same myoinhibitory activity with a threshold concentration (TC) of 0.15 nM compared to the C-terminal heptapeptide fragment (TC: 0.21 nM), suggesting that the N-terminal amino group has only minor significance for receptor binding. On the other side, pseudotetrapeptide **44** consisting of the C-terminal tetrapeptide and a Seb residue (sebacoyl) retained only weak myoinhibitory activity[35c] (Fig. 17).

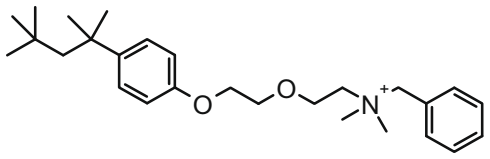
In contrast to almost all other insect neuropeptides a real non-peptidic mimic for the critical C-terminus is known (Fig. 18). Benzethonium chloride (Bztc, **47**) demonstrates significant inhibitory activity on the isolated cockroach hindgut, mealworm neuromuscular junction, and locust oviduct. In the *Leucophaea* cockroach hindgut assay **47** reversibly inhibits spontaneous contractions with an EC_{50} of 6.4×10^{-7} M which is in the range of the C-terminal pentapeptide but significantly less than the full length *Leucophaea* myosuppressin (EC_{50} = 2 nM). Receptor binding data provide additional evidence that the physiological effects of **47** are attributable to a direct interaction with the myosuppressin receptor. Compound **47** competetively displaces radiolabelled Scg-FLRFamide with a K_i of 6.3×10^{-8} M compared to 6.9×10^{-10} M for Scg-FLRFamide. Due to the flexible structure of **47** it is difficult to identify



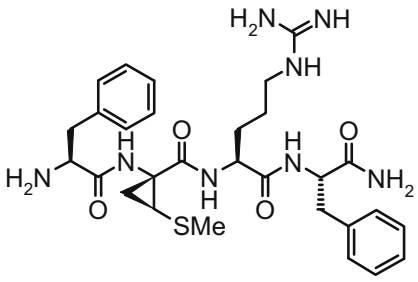
HVFLRFamide **45**



Cpa-myosuppressin fragment **46**



benzethonium chloride (Bztc) **47**



Cpm-myosuppressin fragment **48**

Figure 18. Myosuppressin mimics.

preferred conformations in solution by NMR. However, molecular modeling studies indicate several structural correspondences, for example between the two phenyl residues of **47** and the Phe side chains of **45**, between the quaternary ammonium group of **47** and the positively charged Arg[4] side chain of **45** and between the hydrophobic tail of **47** and either Leu[3] or Val[1] residues of **45**.[70]

Remarkably, the pseudohexapeptide His-Val-Phe-Cpa-Arg-Phe-NH_2 **46** and the pseudotetrapeptide Phe-Cpm-Arg-Phe-NH_2 **48** containing the conformationally restricted amino acid analogues cyclopropylalanine (Cpa) and cyclopropylmethionine (Cpm) completely antagonize the locust oviduct myoinhibitory activity of 100 nM HVFLRFamide at μM concentrations. Additionally, the pseudotetrapeptide analogue **48** was shown to bind to myosuppressin receptors of the locust oviduct with a better K_i (42 nM) compared to Scg-FLRFamide (K_i = 0.9 nM). NMR data of the pseudotetrapeptide Phe-Cpm-Arg-Phe-NH_2 **48** reveal a γ-turn including the residues Phe-Cpm-Arg, whereas a reverse γ-turn is postulated for VFLRFamide. The opposite γ-turn types may explain why the cyclopropyl analogues are still recognised but fail to activate their target.[56,71]

3. Outlook and conclusions

Neuropeptides are involved in the control of almost all key functions in insects and thus are prime targets for the development of a novel generation of selective, non-neurotoxic insecticides. During the past two decades a wealth of information on function, sequences, and structure–activity relationships of insect neuropeptides has become available, in particular by the tremendous efforts of Nachman and co-workers. Nuclear magnetic resonance studies together with molecular modeling calculations provided evidence for 'biologically active' conformations of relevant neuropeptides. Diverse peptidomimetic strategies were applied in order to improve the metabolic stability and pharmacological profile of the natural neuropeptides.

In the recent past the focus in insect neuropeptide research has shifted from the peptides with their specific chemistry and biology to the molecular biology of the neuropeptide receptors. Since the first report of a successful cloning of an insect neuropeptide receptor by Reagan in 1994,[72] numerous receptors have been characterized, in particular by the group of Grimmelikhuijzen.[73] Among those were the receptors for proctolin, PBAN, myosuppressin, leucokinin, oxytocin, neuropeptide F, adipokinetic hormone, and several others.[74] From genomic data of *D. melanogaster* it was concluded that the total number of neuropeptide receptors is in the range of 44.[5]

However, with respect to practical use insect neuropeptide research is not yet a real success story. Despite of all the information available on structures and conformations not one complete peptidomimetic analogue of an insect neuropeptide has been published or patented, perhaps with the exception of benzethonium chloride. So far, receptor assays have only been used for testing of single compounds or very small compound libraries. Not a single high-throughput screening of several thousand or even ten thousand compounds has been published to date neither in the scientific nor in the patent literature. To turn the insect neuropeptide story to a real success it needs even more efforts from both academia and industry. Scientists from academia should improve their peptidomimetic approaches for instance by adopting methodologies developed by medicinal chemists in the search for human neuropeptide analogues, integrin and somatostatin antagonists or protease inhibitors. The crop protection industry in general needs to get more interested in this highly promising field. Only large companies with their enormous compound collections will be able to accomplish successfully high-throughput receptor-screening campaigns and to develop a neuropeptide based commercial insecticide from an initial screening hit.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.061.

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