available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/peptides

Backbone cyclic pheromone biosynthesis activating neuropeptide (PBAN) antagonists: Inhibition of melanization in the moth *Spodoptera littoralis* (Insecta, Lepidoptera)☆

Orna Ben-Aziz [a], *Irina Zeltser* [a], *Kalpana Bhargava* [b], *Michael Davidovitch* [a], *Miriam Altstein* [a,*]

[a] *Department of Entomology, The Volcani Center, Bet Dagan 50250, Israel*
[b] *Department of Molecular Biology and Biochemistry School of Biological Sciences, University of Missouri, Kansas City, MO 64110, USA*

ARTICLE INFO

Article history:
Received 7 March 2006
Received in revised form
2 April 2006
Accepted 4 April 2006
Published on line 26 May 2006

Keywords:
PBAN
MRCH
Cuticular melanization
Neuropeptide antagonists
Spodoptera littoralis
Insect neuropeptide

ABSTRACT

Antagonistic and agonistic activities of backbone cyclic (BBC) pheromone biosynthesis activating neuropeptide (PBAN) analogues were evaluated in an attempt to identify potent melanotropic antagonists, to gain an insight into their structure–activity relationship (SAR), and to discover molecules with selective and non-selective melanotropic and pheromonotropic properties. Eight potent melanotropic BBC antagonists and seven agonists were disclosed. SAR studies revealed that the structural requirements of the melanotropic and pheromonotropic agonists and antagonists are different. The cyclic structure of the BBC peptides was unimportant for antagonistic activity, and linearization retained their melanotropic and pheromonotropic antagonistic properties. Comparison of the antagonistic activities of the BBC and precyclic peptides with respect to both functions revealed eight selective antagonists (six that were selective melanotropic antagonists and two selective pheromonotropic antagonists) and four non-selective (melanotropic and pheromonotropic) antagonists. The selective melanotropic antagonists exhibited both, pure or mixed agonistic/antagonistic activities. The selective pheromonotropic compounds were pure antagonists. All non-selective compounds were pure antagonists. Comparison of the agonistic activities of the BBC peptides with respect to both functions revealed six selective melanotropic agonists and one non-selective agonistic compound. All compounds (whether selective or non-selective) exhibited pure agonistic activity. Discovery of the selective compounds hints at the possibility that the receptors that mediate the respective activities may have different properties.

1. Introduction

Insects display a wide variety of coloration and are capable of altering their pigmentation in response to external and internal factors by synthesizing pigments that may be located in the epidermal cells or the cuticle [31]. Pigmentation in insects (whether cuticular or epithelial) is controlled by endocrine and neuroendocrine factors. In locusts and many other grasshopper species (Acrididae), pigmentation and color polymorphism has been found to be controlled by a variety of neuroendocrine factors [39] such as the dark color-inducing neurohormone (DCIN, originally isolated from the CC of *Schistocerca gregaria* and *Locusta migratoria*) [40], and the adipokinetic hormone II of

☆ Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No. 500/06, 2006 series.

* *Corresponding author*. Tel.: +972 3 968 3710; fax: +972 3 968 3835.
E-mail address: vinnie2@agri.gov.il (M. Altstein).

0196-9781/$ – see front matter
doi:10.1016/j.peptides.2006.04.001

Schistocerca gregaria (Scg-AKH) and AKH of *Gryllus bimaculatus* (Grb-AKH) [41,42].

In noctuid moths cuticular pigmentation is controlled by the pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family. The first indication of the possible involvement of this family of neuropeptides in the control of larval cuticular melanization was demonstrated in the common army worm, *Leucania separata*, by Ogura and coworkers [29,30,38]. The hormone, which was termed melanization and reddish coloration hormone (MRCH), was later found to initiate the melanization of the integument of other moth larvae, such as *L. loreyi* [20], *Spodoptera litura* [24] and *Mamestra brassicae* [10]. Several MRCH peptides were partially purified from *Bombyx mori* [17–20,38], and the primary structure of one of them, MRCH-I, was elucidated in head extracts of adult insects [21]. MRCH-I was found to be an amidated peptide consisting of 33 amino acids and identical to PBAN of *B. mori* (Bom-PBAN-I) [14]. This neuropeptide was also found to share 80% homology with the primary structure of Hez-PBAN, isolated from *Helicoverpa zea* [33,35]. Both natural and synthetic Bom-PBAN-I/MRCH-I were found to induce cuticular melanization in larvae of *L. separata*, *S. litura*, and *S. littoralis* and to stimulate sex pheromone production in adults of *B. mori* and *S. litura* [21]. The c-DNA of *B. mori* PBAN/MRCH was cloned by Kawano et al. [13], as were many other peptides of this family of neuropeptides, all of which share a common C-terminal sequence of Phe-Xxx-Pro-Arg-Leu-NH_2 (X = Ser, Gly, Thr, Val) [2,32].

Cuticular melanization was also found to be induced by Hez-PBAN in *S. littoralis* larvae [6] and by other members of the PK/PBAN family including: Pss-pheromonotropin (Pss-PT, also termed Pss-MRCH), an 18-amino acid neuropeptide isolated from larval heads of *Pseudaletia* (=*Leucania*) *separate* [15,22], by a pheromonotropic melanizing peptide (PMP) isolated from *H. zea* (which bears 83% sequence homology with Pss-PT) [34], by *L. migratoria* myotropin-I and -II (Lom-MT-I and Lom-MT-II) and *Leucophaea maderae* PK (LPK) [11,16]. An unidentified factor, extracted from the nerve cord system, which differs from Bom-PBAN-I/MRCH-I or Hez-PBAN, was reported to be involved in cuticular melanization of *Manduca sexta* larvae [11]. Bursicon, a 40 kDa neurosecretory protein, has also been reported to induce melanization. The neuropeptide is produced by neurons in *M. sexta* and its presence has been detected in a variety of different insects. Bursicon triggers sclerotization and melanization of newly formed cuticles [23].

Studies performed in many laboratories including ours indicate that the PK/PBAN family (which currently comprises over 30 neuropeptides) is a multifunctional family of peptides, and that in addition to their ability to stimulate cuticular melanization in moths, these peptides mediate key functions associated with feeding (gut muscle contractions) [25,36], development (pupariation and diapause) [12,27,28] and mating behavior (sex pheromone biosynthesis) [2,35] in a variety of insects (moths, cockroaches, locusts and flies). These studies have shown that all of the above functions can be stimulated by more than one peptide, and that the peptides do not exhibit species specificity. For a detailed review see Refs. [2,9,32]. The functional diversity of the PK/PBAN family raises many questions with respect to the mechanisms of actions by which these neuropeptides elicit their effects, and to the nature of their receptors. Currently, our understanding of the modes of action of the various melanotropic peptides, the possible existence of multiple receptors for each function and/or for each neuropeptide, and their interactions with other components of the neuroendocrine or endocrine system is very limited, and requires further investigation. Antagonists, especially selective ones can shed light on some of these questions.

In the past few years we have developed a strategy for generation of antagonists on the basis of an agonistic neuropeptide. The strategy (termed insect neuropeptide antagonist, INA) [2] was applied to the PK/PBAN family and resulted in the synthesis of two backbone cyclic (BBC) conformationally constrained libraries: BBC-Ser and BBC-D-Phe. The first sub-library was based on the C-terminal hexapeptide sequence (Tyr-Phe-Ser-Pro-Arg-Leu-NH_2) of PBAN1-33NH_2, which comprises the active core of the PK/PBAN molecules [2,3,6,26], and the second on a potent linear antagonist of PBAN that was found in our previous studies to have pheromonotropic antagonistic properties [44]. Examination of the antagonistic activities of both libraries led to the discovery of several highly potent pheromonotropic antagonists that were able to inhibit effectively sex pheromone biosynthesis in *Heliothis peltigera* [1,2,4,43].

In the present study we tested the agonistic and antagonistic melanotropic activities of the Ser and D-Phe BBC libraries, as well as those of a few of their derivatives, by means of a quantitative melanotropic bioassay that was previously optimized in our laboratory with *S. littoralis* larvae [7], for the detection of melanotropic antagonists, determination of the structure–activity relationship (SAR) of their requirements, and for the discovery of selective and non-selective melanotropic and pheromonotropic compounds.

2. Materials and methods

2.1. Insects

S. littoralis larvae were kept in groups of 100–200 insects in plastic containers (40 cm × 30 cm × 20 cm). Sawdust was placed at the bottom of each container and the top was covered with cheesecloth. Larvae were fed on castor bean leaves and kept in a thermostatically regulated room at 25 ± 2 °C with a light/dark regime of 14/10 h and 60% relative humidity.

H. peltigera moths were reared on an artificial diet as described previously [8]. Pupae were sexed and females and males were placed in separate rooms with a light/dark regime of 14/10 h, at 25 ± 2 °C and 60–70% relative humidity. Adult moths were kept in screen cages and supplied with a 10% sugar solution. Moth populations were refreshed every year with males caught from the wild by means of pheromone traps, as described previously [8]. All females used in this study were 3.5–4.5 days old.

2.2. Peptide synthesis

2.2.1. Chemicals

Protected amino acids, Rink amide methylbenzhydrylamine (MBHA) resin and coupling reagents were purchased from Novabiochem (Laufelfingen, Switzerland). Other chemicals were purchased from Sigma, St. Louis, MO, USA or Merck, Darmstadt, Germany. Solvents and reagents for peptide synthesis were purchased from Baker (Phillipsburg, NJ, USA).

2.2.2. Synthesis of PBAN 1-33NH_2

Hez-PBAN [33] was synthesized on an ABI 433A automatic peptide synthesizer on Rink amide MBHA resin by means of the FastMoc™ chemistry as described previously [44].

2.2.3. Synthesis of the BBC peptide library and the precyclic peptides

BBC peptides (Ser and D-Phe sub-libraries and the small BBC peptides, Figs. 1 and 2) and the precyclic peptides (Fig. 3) were synthesized by the simultaneous multiple peptide synthesis (SMPS) ''tea bags'' methodology on Rink amide MBHA resin by means of 9-fluorenylmethoxycarbonyl (Fmoc) chemistry as previously described [4,43].

2.2.4. Purification and characterization of peptides

The purity of all peptides (linear and cyclic) was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) on ''LichrosorbR'' C-18 (LICHROCARTE 250-10 cat. 16817) using acetonitrile: H_2O (+0.1% trifluoroacetic acid) as previously described [4,43,44] and was found to be in the range of 90–95%. Purified peptides were characterized by time-of-flight mass spectrometry (TOF-MS) and amino acid analysis of hydrolyzates. The analytical methods and data of the peptides were described by Refs. [4,43,44].

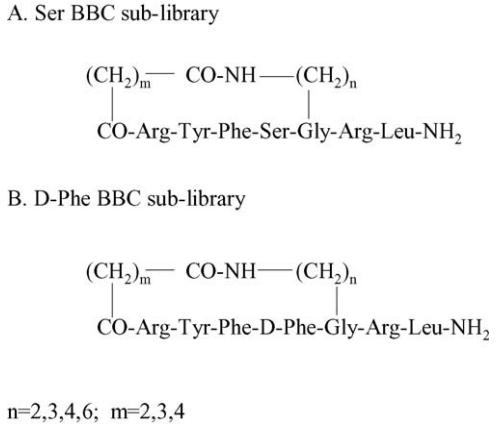


Fig. 1 – General structure of the BBC Ser (A) and the D-Phe (B) sub-libraries of peptides. Peptides of the Ser sub-library are referred to in the text by consecutive numbers BBC 4 to BBC 15 representing *n* = 2 and *m* = 2–4; *n* = 3 and *m* = 2–4; *n* = 4 and *m* = 2–4; *n* = 5 and *m* = 2–4, respectively. Peptides of the D-Phe sub-library are referred to in the text as BBC 19 to BBC 30 and represent the same combinations of n and m (see Tables 1 and 2).

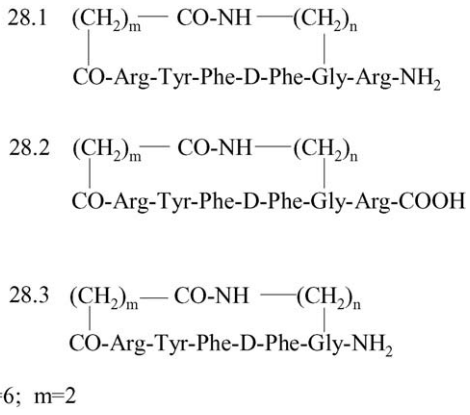
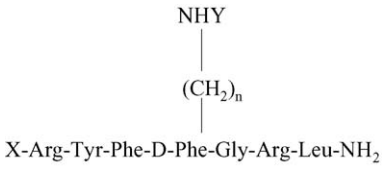


Fig. 2 – General structures of BBC 28 C-terminally modified analogs (small BBC peptides).



Peptide	X	Y	n	m
20-L-1	Ac	Ac	2	
28-L-1	Ac	Ac	6	
20-L-2	OC-$(CH_2)_m$-COOH	H	2	3
28-L-2	OC-$(CH_2)_m$-COOH	H	6	2

Fig. 3 – General structures of the precyclic linear peptides.

2.3. Melanotropic bioassay

The melanotropic bioassay was performed as previously described [7]. Melanotropic agonistic activity of the BBC or precyclic peptides was determined by an evaluation of the ability of the injected peptides (at 1 nmol) to induce cuticular melanization in larvae (in the absence of PBAN1-33NH_2). Larvae injected with 5 pmol of PBAN1-33NH_2 served as a reference for agonistic activity. Antagonistic activity was determined by monitoring the ability of the BBC or precyclic peptides (at 1 nmol), injected together with the elicitor PBAN1-33NH_2 (at 5 pmol), to inhibit the cuticular melanization. Larvae injected with 5 pmol of PBAN1-33NH_2 served as a reference for maximal stimulation and those injected with 50 mM HEPES, pH 7.6 served to determine the basal cuticular melanization of the ligated insects in both the agonistic and antagonistic activity experiments.

2.4. Pheromonotropic bioassay

The pheromonotropic bioassay was performed with *H. peltigera* as described previously [5]. Agonistic activity of the BBC or precyclic peptides was determined by monitoring the ability of the injected peptides (at 1 nmol) to induce sex pheromone biosynthesis in females (in the absence of

PBAN1-33NH$_2$). Females injected with 1 pmol of PBAN1-33NH$_2$ served as a reference for agonistic activity. Antagonistic activity was determined by monitoring the ability of the BBC or precyclic peptides (at 1 nmol) to inhibit sex pheromone biosynthesis elicited by 0.5 pmol of exogenously injected PBAN1-33NH$_2$. Females injected with 0.5 pmol of PBAN1-33NH$_2$ served as a reference for maximal stimulation and those injected with 100 mM of phosphate buffer served to determine the basal pheromone biosynthesis at photophase in both the agonistic and antagonistic activity experiments. The pheromone content in buffer-injected moths did not exceed 20 ng/female.

2.5. *Statistical analysis*

The results were subjected to one-way ANOVA. The significance of differences among means was evaluated with the Tukey–Kramer HSD (honestly significant difference) test at $P < 0.05$.

3. Results

The first part of the study involved examination of the ability of BBC peptides from the Ser and D-Phe sub-libraries to inhibit the formation of cuticular melanin evoked by PBAN1-33NH$_2$ in *S. littoralis* larvae. The data in Tables 1 and 2 depict the presence of seven potent antagonistic peptides (BBC 15, 19, 20, 23, 27, 28 and 29) each of which exhibited over 60% inhibitory activity at 1 nmol. Most of the antagonistic peptides were among the compounds of the D-Phe library; only one peptide belonged to the BBC Ser library (BBC 15). The inhibitory potency of these peptides ranged from 61% (peptide no. 15) to a maximum of 83% (peptide no. 28).

Table 1 – Melanotropic agonistic and antagonistic activities of the Ser sub-library BBC peptides

Peptide #[a]	*n*	*m*	Activity[b] (%)	
			Agonistic	Antagonistic
4	2	2	42 ± 20 (*n* = 30)*	6 ± 8 (*n* = 4)
5	2	3	76 ± 9 (*n* = 30)	0 ± 1 (*n* = 10)
6	2	4	58 ± 19 (*n* = 30)*	14 ± 10 (*n* = 10)
7	3	2	72 ± 28 (*n* = 20)	4 ± 3 (*n* = 20)
8	3	3	99 ± 2 (*n* = 20)	1 ± 1 (*n* = 9)
9	3	4	68 ± 33 (*n* = 20)*	4 ± 2 (*n* = 19)
10	4	2	51 ± 8 (*n* = 10)*	1 ± 1 (*n* = 10)
11	4	3	64 ± 25 (*n* = 20)*	1 ± 1 (*n* = 9)
12	4	4	97 ± 9 (*n* = 10)	1 ± 1 (*n* = 10)
13	6	2	38 ± 11 (*n* = 10)*	18 ± 11 (*n* = 10)
14	6	3	76 ± 16 (*n* = 10)	35 ± 10 (*n* = 9)
15	6	4	41 ± 10 (*n* = 10)*	61 ± 9 (*n* = 18)*

[a] For peptide structure see Fig. 1.
[b] Agonistic activity is expressed as the ratio (as a percentage) between the extents of melanization elicited by the injection of each of the cyclic peptides and that elicited by PBAN1-33NH$_2$ (at 5 pmol). Antagonistic activity is expressed as 100 min the ratio (as a percentage) between the extents of melanization elicited by the injection of PBAN1-33NH$_2$ (5 pmol) in the presence and absence of each of the tested BBC peptides (at 1 nmol).
* An activity that differed significantly (at $P < 0.05$) from that of PBAN1-33NH$_2$ (at 5 pmol).

Table 2 – Melanotropic agonistic and antagonistic activities of the D-Phe sub-library BBC peptides

Peptide #[a]	*n*	*m*	Activity (%)	
			Agonistic	Antagonistic
19	2	2	58 ± 22 (*n* = 20)*	78 ± 8 (*n* = 60)*
20	2	3	17 ± 6 (*n* = 13)*	62 ± 14 (*n* = 50)*
21	2	4	5 ± 3 (*n* = 10)*	42 ± 24 (*n* = 40)
22	3	2	1 ± 1 (*n* = 10)*	18 ± 8 (*n* = 20)
23	3	3	23 ± 12 (*n* = 10)*	65 ± 7 (*n* = 30)*
24	3	4	49 ± 9 (*n* = 10)*	30 ± 1 (*n* = 20)
25	4	2	18 ± 7 (*n* = 10)*	55 ± 12 (*n* = 50)
26	4	3	31 ± 31 (*n* = 20)*	44 ± 17 (*n* = 40)
27	4	4	41 ± 13 (*n* = 9)*	76 ± 18 (*n* = 20)*
28	6	2	1 ± 1 (*n* = 12)*	83 ± 12 (*n* = 50)*
29	6	3	66 ± 1 (*n* = 20)*	65 ± 16 (*n* = 30)*
30	6	4	67 ± 40 (*n* = 20)	1 ± 1 (*n* = 30)

[a] For peptide structures see Fig. 1. All other details are as indicated in the legend to Table 1.
* An activity that differed significantly (at $P < 0.05$) from that of PBAN1-33NH$_2$ (at 5 pmol).

Since, backbone cyclization can, in principle, convert an antagonistic linear peptide into an agonist and vice versa, the antagonistic peptides of the D-Phe sub-library (which was designed on the basis of a potent D-Phe-substituted linear antagonist) [44] were tested for their melanotropic agonistic activity at the same concentration used to assess their antagonistic activity (1 nmol). Indeed, some of the D-Phe BBC peptides did show agonistic activity. Among the antagonists, BBC peptides nos. 19, 27 and 29 showed stimulatory activities that ranged from 41% to 66% (compared with that of PBAN1-33NH$_2$). Peptide no. 30, which was devoid of antagonistic activity, was also a potent agonist with an activity (67%) that did not differ from that of PBAN1-33NH$_2$ (Table 2). All other BBC peptides of the D-Phe sub-library, including the antagonistic peptides BBC 20, 23 and 28, showed low or no agonistic activity.

Since all of the BBC peptides in the Ser sub-library were derived from the putative active site of PBAN, they were tested for their melanotropic agonistic activity at the same concentration and under the same conditions as had been used to assess their antagonistic activity (1 nmol). The data in Table 1 depict five relatively potent agonists (nos. 5, 7, 8, 12 and 14), which exhibited high activities (ranging from 72% to 99%) that did not differ from that of PBAN1-33NH$_2$. All other BBC peptides of this sub-library also exhibited agonistic activity although lower than that of PBAN1-33NH$_2$. As indicated above, only one antagonist (BBC no. 15) was found among the peptides of this library.

In order to further characterize the conformational requirements of the antagonists, the most potent and purely antagonistic (i.e., devoid of agonistic activity) peptide, BBC 28, was modified in a manner that involved truncation of amino acids from its C-terminus (28-1 and 28-3, Fig. 2) and replacement of the C-terminal amide with a free carboxyl residue (28-2, Fig. 2). The peptides were tested for their melanotropic inhibitory activity under the same conditions as

Table 3 – Melanotropic agonistic and antagonistic activities of the small BBC peptides

Peptide #[a]	Activity (%)	
	Agonistic	Antagonistic
28-1	38 ± 23 (n = 30)*	71 ± 1 (n = 20)*
28-2	8 ± 8 (n = 19)*	46 ± 20 (n = 19)
28-3	56 ± 18 (n = 20)	20 ± 20 (n = 20)

[a] For peptide structures see Fig. 2. All other details are as indicated in the legend to Table 1.
* An activity that differed significantly (at $P < 0.05$) from that of PBAN1-33NH$_2$ (at 5 pmol).

the parent molecule. The results (Table 3) showed that BBC 28-1 exhibited a high antagonistic activity, inhibiting PBAN1-33NH$_2$ induced melanization by 71%. The other two peptides 28-2 and 28-3 were devoid of melanotropic antagonistic activity. Peptides 28-1 and 28-2 exhibited low or no agonistic activity, peptide 28-3, however, exhibited high agonistic activity that did not differ significantly from that of PBAN1-33NH$_2$ (Table 3).

The importance of the cyclic structure in determining activity was tested by comparing the agonistic and antagonistic melanotropic activities of precyclic linear peptides with those of their cyclic parent molecules. Four precyclic linear peptides (for structural details see Fig. 3) were synthesized on the basis of the active D-Phe BBC peptides 20 and 28 (Fig. 1) that exhibited high antagonistic activity with very low or no agonistic activity, and their melanotropic agonistic and antagonistic activities were examined. The results (Table 4) revealed that peptides 20-L-1 and 28-L-1 were potent antagonists with very low agonistic activity, similar to the activities exhibited by the parent molecules BBC 20 and 28, whereas peptides 20-L-2 and 28-L-2 exhibited low agonistic or antagonistic activities.

Comparison of the antagonistic melanotropic activity of the BBC peptides in *S. littoralis* with their pheromonotropic activity in *H. peltigera* revealed four selective BBC antagonists, i.e., peptides whose pheromonotropic and melanotropic activities differed significantly (Fig. 4): BBC peptides 15, 19 and 27, whose melanotropic inhibitory activities were significantly higher than their pheromonotropic inhibitory activities; BBC peptide 22, which was a selective pheromonotropic antagonist. BBC peptides 23 and 29 were also selective melanotropic antagonists although their melanotropic and pheromonotropic activities did not differ statistically. A similar comparison of the small BBC peptides revealed one selective melanotropic antagonist (28-1) and a selective pheromonotropic antagonist (28-3) (Fig. 5). Unlike in the case of the melanotropic activity where peptide 23-8 exhibited agonistic activity that did not differ from that of PBAN1-33NH$_2$ (at 5 pmol), in the case of the pheromonotropic activity none of the small BBC peptides showed agonistic activity. No selective antagonists were found among the precyclic linear peptides, and the same compounds that inhibited the formation of cuticular melanin elicited by PBAN1-33NH$_2$ in *S. littoralis* larvae also inhibited PBAN1-33NH$_2$ elicited sex pheromone production in *H. peltigera* (Fig. 6). Most of the selective melanotropic antagonists, BBC 15, 19, 27 and 29, also exhibited agonistic activity, whereas the selective pheromonotropic antagonist, BBC 22 and 28-3, were pure antagonists [4,43]. All of the non-selective melanotropic/pheromonotropic compounds (BBC 20, 28, 20-L-1 and 28-L-1) were pure antagonists.

A comparison of the melanotropic and pheromonotropic agonistic activities of all of the above peptides revealed a much larger number of melanotropic agonists than of pheromonotropic ones. Only one pheromonotropic agonistic peptide (BBC 14) was found in the Ser BBC sub-library, and no other such agonists could be detected in the other sub-libraries (D-Phe BBC, small BBC peptides and precyclic linear peptides) [4,43], in contrast, highly potent melanotropic agonists were found in both BBC sub-libraries and among the small BBC peptides (BBC 5, 7, 8, 12, 14, 30 and 28-3). Most melanotropic agonists, other than BBC 14, were selective compounds (Tables 1–3). Interestingly, all of these compounds were pure agonists. A summary of the selective and non-selective agonists and antagonists among all tested peptides is presented in Table 5.

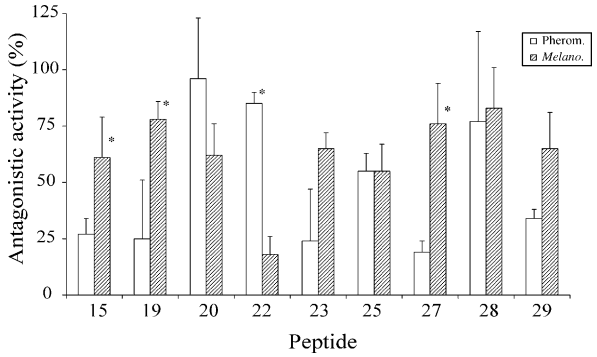


Fig. 4 – Comparison of the melanotropic and pheromonotropic antagonistic activities of D-Phe BBC peptides. Inhibitory melanotropic activity is expressed as 100 min the ratio (as a percentage) between the activity elicited by the injection of PBAN1-33NH$_2$ (0.5 and 5 for pheromonotropic and melanotropic activities, respectively) in the presence and absence of each of the tested BBC peptides (at 1 nmol). Pheromonotropic antagonistic data are taken from Ref. [4].

Table 4 – Melanotropic agonistic and antagonistic activities of the precyclic peptides

Peptide #[a]	Activity (%)	
	Agonistic	Antagonistic
20-L-1	13 ± 13 (n = 20)*	70 ± 1 (n = 20)*
20-L-2	23 ± 11 (n = 10)*	32 ± 10 (n = 20)
28-L-1	20 ± 21 (n = 20)*	73 ± 16 (n = 20)*
28-L-2	7 ± 2 (n = 20)*	46 ± 12 (n = 20)

[a] For peptide structures see Fig. 3. All other details are as indicated in the legend to Table 1.
* An activity that differed significantly (at $P < 0.05$) from that of PBAN1-33NH$_2$ (at 5 pmol).

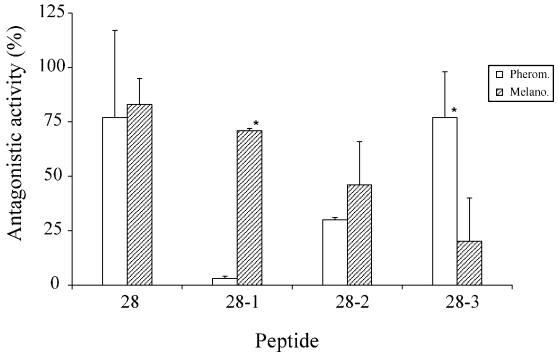


Fig. 5 – Comparison between the melanotropic and pheromonotropic antagonistic activities (inhibitory) of BBC peptide no. 28 and of the small BBC peptides derived from its structure. All experimental details are as described in the legend to Fig. 4. Inhibitory melanotropic activity is expressed as 100 min the ratio (as a percentage) between the extents of activity elicited by the injection of PBAN1-33NH$_2$ (0.5 and 5 for pheromonotropic and melanotropic activities, respectively) in the presence and absence of each of the tested BBC peptides (at 1 nmol).

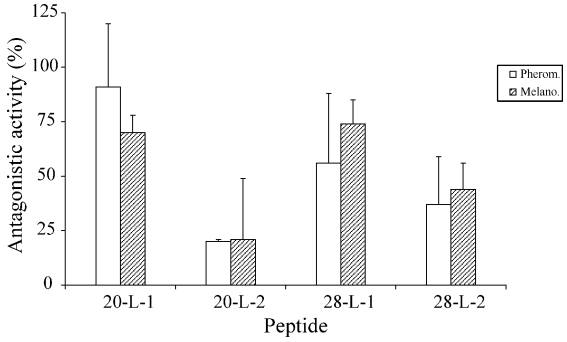


Fig. 6 – Comparison between the melanotropic and pheromonotropic antagonistic activities (inhibitory) of the precyclic peptides. All experimental details are as described in the legend to Fig. 4. Inhibitory melanotropic activity is expressed as 100 min the ratio (as a percentage) between the extents of activity elicited by the injection of PBAN1-33NH$_2$ (0.5 and 5 for pheromonotropic and melanotropic activities, respectively) in the presence and absence of each of the tested precyclic peptides (at 1 nmol). Pheromonotropic antagonistic data are taken from Ref. [43].

Table 5 – Summary of selective and non-selective agonistic and antagonistic activities of the BBC and the precyclic linear peptides

Peptide ($n + m$)[a]	Pure/mixed activity[b]	Melanotropic (M)/pheromonotropic (P) selectivity
Antagonistic peptides		
BBC 15 (6 + 4)	Mixed (M)	Selective M
BBC 19 (2 + 2)	Mixed (M)	Selective M
BBC 20 (2 + 3)	Pure (M + P)	Non-selective
BBC 22 (3 + 2)	Pure (P)	Selective P
BBC 23 (3 + 3)	Pure (M)	Selective M
BBC 27 (4 + 4)	Mixed (M)	Selective M
BBC 28 (6 + 2)	Pure (M + P)	Non-selective
BBC 29 (6 + 3)	Mixed (M)	Selective M
BBC 28-1 (6 + 2)	Pure (M)	Selective M
BBC 28-3 (6 + 2)	Pure (P)	Selective P
20-L-1	Pure (M + P)	Non-selective
28-L-1	Pure (M + P)	Non-selective
Agonistic peptides		
BBC 5 (2 + 3)	Pure (M)	Selective M
BBC 7 (3 + 2)	Pure (M)	Selective M
BBC 8 (3 + 3)	Pure (M)	Selective M
BBC 12 (4 + 4)	Pure (M)	Selective M
BBC 14 (6 + 3)	Pure (M + P)	Non-selective
BBC 30 (6 + 4)	Pure (M)	Selective M
BBC 28-3 (6 + 2)	Pure (M)	Selective M

[a] The letters $n + m$ represent the bridge size as indicated in Fig. 1 and Tables 1 and 2.

[b] The term mixed activity refers to a compound that exhibits both agonistic and antagonistic activities. Pure, refers to a compound that exhibits only one (agonistic or antagonistic) activity. Agonistic peptides are only those whose activity did not differ significantly from that of PBAN1-33NH$_2$. Results are based on Tables 1–4, Figs. 4–6 and previously published results [4,43]. For peptide structures see Figs. 1–3.

4. Discussion

The present study focused on evaluation of the antagonistic and agonistic activities of a variety of BBC peptides that were designed by means of the backbone cyclization and cycloscan concepts, in an attempt to discover potent melanotropic antagonists, to gain an insight into their agonistic and antagonistic SARs and to identify molecules with selective and non-selective melanotropic and pheromonotropic properties.

Two BBC sub-libraries were tested for melanotropic antagonistic and agonistic activities in *S. littoralis* larvae, by means of a quantitative bioassay that had previously been optimized in our laboratory [7]. One of these sub-libraries (the Ser sub-library)—was based on the C-terminal active sequence of the PK/PBAN family of insect neuropeptides (YFSPRL-amide) [5]. The other (the D-Phe sub-library)—was based on the sequence of a lead pheromonotropic antagonist (RYFdFPRL-amide), that had previously been discovered out of a D-Phe scan library [44]. All the cyclic peptides in each sub-library had the same primary sequence and the same location of the ring. The members of each library differed from each other in their bridge size and the position of the amide bond along the bridge [4].

Seven potent antagonistic peptides were discovered among the BBC peptides of the two sub-libraries, most of which were obtained from the D-Phe sub-library. Some peptides were found to be pure antagonists with little or no agonistic activity. Some, however, exhibited a mixed agonistic/antagonistic mode of action. All peptides of the Ser sub-library exhibited agonistic activity but only five peptides were as potent as PBAN1-33NH$_2$. Agonistic activity was also exhibited by the BBC peptides of the D-Phe sub-library, but only one peptide was as potent as PBAN1-33NH$_2$.

The distribution of the melanotropic agonistic and antagonistic activities between the two libraries was similar to that

obtained with the pheromonotropic activity, in the sense that most pheromonotropic antagonists emerged from the D-Phe sub-library, whereas the agonists were found among the BBC Ser peptides. However, in contrast to the pheromonotropic assay, which revealed no antagonists among the Ser sub-library, with no agonistic peptides emerging from among the BBC D-Phe peptides, in the case of melanotropic activity an antagonist was found among the Ser peptides and agonistic activity was displayed by most peptides in both sub-libraries, including those of the D-Phe BBC compounds.

Examination of the correlation between the bridge size and the melanotropic agonistic or antagonistic activity of the BBC peptides revealed quite a random pattern. Basically, no correlation was found between the bridge size and activity (whether purely agonistic or antagonistic, or mixed), and peptides having a wide range of bridge sizes expressed activity. This finding differs from that of the SAR analysis of both libraries with respect to the pheromonotropic activity of the BBC peptides, which showed that six of the eight active peptides (whether agonistic or antagonistic) had a bridge size of $m + n = 5$ or 6, and peptides with a bridge size of $m + n = 4$, 7 or 10 were inactive [4]. Another marked difference between the pheromonotropic and melanotropic activities was the large number of melanotropic peptides found to be agonistic, in contrast to only one pheromonotropic agonist (BBC 14). In general, it seems that pheromonotropic activity was much more dependent than the melanotropic activity on the peptides conformation [4].

Attempts to further characterize the conformational requirements of the melanotropic antagonistic activity were carried out by examining the roles of three C-terminal moieties: Leu, Arg and amide group in the BBC peptides. Peptide BBC 28, which was found to be a pure antagonist and exhibited the highest activity, was chosen as the parent molecule from which the Leu, Leu plus Arg and Leu-amide were removed. Interestingly, the C-terminal part of the molecule was found to play a major role in determining both agonistic and antagonistic melanotropic properties, and removal of some of the residues modified the molecule's activity. Following removal of the Leu alone (BBC 28-1) the compound retained its antagonistic property. Removal of the Leu-NH_2 (BBC 28-2) resulted in a marked reduction of the antagonistic properties, with no change in the agonistic properties compared with those of BBC 28-1 and the parent peptide BBC 28. Removal of the Leu-Arg (28-3) resulted in total loss of the antagonistic activity and a marked increase in the agonistic activity. The findings indicate that Leu is not essential for antagonistic activity. Both the amide and the Arg acquire the molecule with antagonistic properties, as the absence of either moiety resulted in the loss of an inhibitory potency, but it seems that the two residues differ in their impacts on the agonistic activity. The amide, most likely, does not play a role in determining the agonistic activity, as the stimulatory potency in its presence or absence were found to be similar. Arg, on the other hand, has an important role in diminishing the agonistic activity, as its absence was found to result in an increase in the agonistic activity of the peptide. It is also possible that the overall structural change in peptide BBC 28-3 as compared with BBC peptides 28, 28-1 and 28-2, and not only the absence of Arg, imparted the high agonistic potency to this peptide.

Comparison of the pheromonotropic and melanotropic agonistic and antagonistic SARs of the small BBC peptides, revealed that the residues that determine agonistic and antagonistic activity in the two bioassays are different. Removal of Leu (in BBC 28-1) and Leu-amide (in BBC 28-2) eliminated antagonistic pheromonotropic activity, whereas removal of Arg-Leu (in 28-3) did not, which indicates that Arg and Leu had no effect (stimulatory or inhibitory) on the pheromonotropic activity. The amide may be essential but just its presence (as in BBC 28-1) was not sufficient for evoking antagonistic activity. None of the small BBC peptides exhibited any pheromonotropic agonistic activity.

The importance of the cyclic structure of the BBC peptides for their activities was tested by comparing the agonistic and antagonistic melanotropic activities of precyclic linear peptides with those of their cyclic counterparts. Four precyclic linear peptides (based on the active D-Phe BBC peptides 20 and 28) were synthesized. The building unit consisted of $(CH_2)_n$ side chains, in which $n = 2$ and 6, respectively, and a spacer consisted of a $(CH_2)_m$ reside in which $m = 3$ and 2, respectively. The peptides were tested for their melanotropic agonistic and antagonistic activities. The tests revealed that linearization of the peptides did not eliminate their activity, and both precyclic peptides, 20-L-1 and 28-L-1 retained their inhibitory properties. The activities of these peptides were similar to one another and to those of their respective BBC peptides 20 and 28 but differed from that of the linear lead antagonistic parent molecule (H-Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2, LA-4) [7], in both the level of antagonistic activity (which was higher for 20-L-1 and 28-L-1 than that of the LA-4) and in the diminution of agonistic activity, which was quite high in the LA-4 peptide and very low in the precyclic peptide, which indicates a possibility that the $(CH_2)_n$ side chain is important for the antagonistic activity and impairs the agonistic properties of the molecule.

Another interesting finding concerns the effects of the residues at the $\tilde{\alpha}$ and $\tilde{\varepsilon}$ amino groups of the molecule. Comparison of the activity of 20-L-1 with that of 20-L-2, and that of 28-L-1 with that of 28-L-2 revealed that the presence of the carboxylic group on the N-terminal end and of a free amino group on the side chain resulted in a marked decrease in antagonistic activity, i.e., the presence of a negatively charged residue at the N-terminus of the peptide eliminated the antagonistic activity. The above results were in accordance with previous findings on the effects of the introduction of acidic groups in the N-terminal part of the highly active and NK-1 selective analog Ac-Arg-Septide where the substitution of Asp for Arg resulted in an inactive peptide (Gilon et al., unpublished results). In contrast to the case of the BBC peptides, where marked differences were found between the melanotropic and pheromonotropic agonistic and antagonistic activities, there were no differences in the melanotropic and pheromonotropic SARs among the precyclic peptides, most likely because linear peptides have much a greater conformational flexibility and are thus much less receptor-selective.

Studies of the melanotropic activities of the PK/PBAN peptides have revealed that cuticular melanization can be

induced by several members of this family [15,16,22,34] and that C-terminal part of PBAN, which contains the signature sequence of the PK/PBAN family, constitutes the active site associated with the melanotropic activity (by analogy with what was already known for the pheromonotropic activity of this peptide family). It was, therefore, not surprising that in the present study the BBC peptides, which were designed on the basis of the C-terminal part of PBAN, and which have previously been shown to exhibit both agonistic and antagonistic pheromonotropic activities, also exhibited melanotropic agonistic and antagonistic activities. What is interesting, though, is the finding that the inhibitory and stimulatory patterns of the various BBC peptides differed with respect to the two activities.

Comparison of the pheromonotropic and melanotropic antagonistic activities of the BBC and precyclic peptides revealed eight selective compounds (six that were selective melanotropic antagonists and two pheromonotropic antagonist) and four non-selective (melanotropic and pheromonotropic) antagonists. The selective melanotropic antagonists exhibited both, pure or mixed agonistic/antagonistic activities. The selective pheromonotropic compounds were pure antagonists. All non-selective compounds were pure antagonists. Comparison of the agonistic activities of the BBC peptides with respect to both functions revealed six selective melanotropic agonists and one non-selective agonistic compound. All compounds (whether selective or non-selective) exhibited pure agonistic activity.

The finding that the pheromonotropic and the melanotropic inhibitory patterns of the BBC peptides were different hints at the possibility that sex pheromone biosynthesis in the pheromone gland of *Heliothis peltigera* and induction of cuticular melanization in *S. littoralis* may be mediated by different receptors (or receptor sub-classes), despite the fact that both functions are stimulated by the same peptide (PBAN1-33NH_2). Since the agonistic/antagonistic activity was determined in two different moth species and at different developmental stages (adult *H. peltigera* moths and *S. littoralis* larvae) it is possible that the different activities are caused by inter-species differences in receptor structure and/or properties (e.g., differing affinities toward the different compounds) or by the differing assay conditions. The possible presence of receptor sub-classes has previously been suggested in the light of similar results that were obtained with a D-Phe scan library which showed differing inhibitory and stimulatory patterns with respect to the two activities [7]. An alternative approach to prove the above is currently being studied in our laboratory: it involves the detailed examination of the antagonistic effects of the D-Phe scan library and the BBC peptides on various elicitors (Pss-PT, Lom-MT-I and LPK) in the same assay and the same moth species and developmental stage.

Pigmentation in insects is quite a complex mechanism, which is controlled by endocrine and several neuroendocrine factors, some of which have not even been identified. The availability of the above antagonists as well as those that were reported by us in a previous paper [7] should shed light on the nature of the factors and on the similarity of their cellular mode of action in other Lepidopteran and non-Lepidopteran insects. One such application already emerged from this study. A close examination at the bioassay reveals that the post injection ligation of the larvae (namely disconnection of the anterior part of the larvae up to the third abdominal instar) does not result in a complete abolishment of the pigmentation, hinting at the possibility that other ganglia, posterior to those disconnected by the ligation, play a role in melanization. Interestingly, BBC 28 and two of its analogs (BBC 28-1 and 28-L-1), all of which are potent antagonists capable of inhibiting melanization induced by PBAN1-33NH_2, caused a decrease in the degree of melanization (20%, 62% and 73%, respectively) beyond that of the tied larvae to an extent that differed significantly from that of the ligated, buffer-injected larvae. The results demonstrate the ability of the above compounds to inhibit the endogenous melanization mechanisms, and hint at the possibility that the endogenous mechanism is mediated, at least in part, by PBAN1-33NH_2.

Availability of selective agonists or antagonists is of major importance in the study of neuropeptides in general, and of the PK/PBAN family of neuropeptides in particular. The PK/PBAN family of peptides is involved in the regulation of critical reproductive, developmental and digestive processes (e.g., sex pheromone biosynthesis, cuticular melanization, myotropic activity, oviposition, pupariation and diapause) in moths and other insects and it exhibits considerable functional and inter- and intra-specific diversity (for reviews, see [2,32]). Despite the intensive studies of the PK/PBAN peptides, which have yielded extremely interesting information on the chemical and molecular nature of the peptides (such as their origin, localization, target organ, route of transport, etc.), many aspects, especially those related to their modes of action, are still unresolved, and much remains to be learned about the structural, chemical and cellular bases of their activity, downstream cellular events, species specificity, receptor heterogeneity and functional diversity. The availability of antagonists for this family of neuropeptides, together with the availability of bioassays that have been developed for each of the above-mentioned functions [5,6,12,28,35,37] open the way to a better understanding of the endogenous mechanisms of this peptide family in moths and other insects as already indicated above.

The discovery of conformationally constrained selective and non-selective agonists and antagonists bears also a high potential for design of additional improved agonistic and antagonistic compounds (e.g., more potent, highly selective, metabolically stable and cost effective) for the further study of the above issues. Such novel, improved compounds, when available, may also be potential candidates for agrochemical applications that can serve, after formulation and preliminary field experiments, as prototypes for the development of a novel group of highly effective, insect-specific and environmentally friendly insecticides. Indeed, some of the above antagonists have already been used as a basis for the design and synthesis of additional improved compounds with higher inhibitory potency and bioavailability. These compounds are currently being screened for their ability to stimulate or inhibit cuticular melanization in *S. littoralis* larvae and sex pheromone biosynthesis in *H. peltigera*.

Acknowledgements

We thank Prof. Gilon of the Department of Organic Chemistry at the Hebrew University of Jerusalem, Israel for assistance in the design of the BBC and precyclic peptides. This research was supported in part by the Israel Ministry of Science and Technology, by the Israel Science Foundation, administered by the Israel Academy of Sciences and Humanities and by the US–Israel Binational Agricultural Research and Development Fund (BARD) (IS-3356-02).

REFERENCES

[1] Altstein M. Novel insect control agents based on neuropeptide antagonists—the PK/PBAN family as a case study. J Mol Neurosci 2003;22:147–57.
[2] Altstein M. Role of neuropeptides in sex pheromone production in moths. Peptides 2004;25(9):1491–501.
[3] Altstein M, Ben Aziz O, Gabay T, Vogel Z, Barg J. Structure–function relationship in the melanotropic and pheromonotropic insect neuropeptide PBAN/MRCH. J Neurochem 1994;63:S62.
[4] Altstein M, Ben-Aziz O, Daniel S, Schefler I, Zeltser I, Gilon C. Backbone cyclic peptide antagonists, derived from the insect pheromone biosynthesis activating neuropeptide, inhibit sex pheromone biosynthesis in moths. J Biol Chem 1999;274:17573–9.
[5] Altstein M, Dunkelblum E, Gabay T, Ben Aziz O, Schafler I, Gazit Y. PBAN-induced sex-pheromone biosynthesis in *Heliothis peltigera*: structure, dose, and time-dependent analysis. Arch Insect Biochem Physiol 1995;30(4):307–19.
[6] Altstein M, Gazit Y, Ben Aziz O, Gabay T, Marcus R, Vogel Z, et al. Induction of cuticular melanization in *Spodoptera littoralis* larvae by PBAN/MRCH: development of a quantitative bioassay and structure function analysis. Arch Insect Biochem Physiol 1996;31:355–70.
[7] Ben Aziz O, Zeltser I, Altstein M. PBAN selective antagonists: inhibition of PBAN induced cuticular melanization and sex pheromone biosynthesis in moths. J Insect Physiol 2005;51:305–14.
[8] Dunkelblum E, Kehat M. Female sex-pheromone components of *Heliothis peltigera* (Lepidoptera: Noctuidae) chemical-identification from gland extracts and male-response. J Chem Ecol 1989;15:2233–45.
[9] Gade G. The explosion of structural information on insect neuropeptides. Prog Chem Org Nat Prod 1997;71:1–128.
[10] Hiruma K, Matsumoto S, Isogai A, Suzuki A. Control of ommochrome synthesis by both juvenile-hormone and melanization hormone in the cabbage armyworm, *Mamestra brassicae*. J Comp Physiol 1984;154(1):13–21.
[11] Hiruma K, Norman A, Riddiford LM. A neuroendocrine factor essential for cuticular melanization in the tobacco hornworm, *Manduca sexta*. J Insect Physiol 1993;39(4):353–60.
[12] Imai K, Konno T, Nakazawa Y, Komiya T, Isobe M, Koga K, et al. Isolation and structure of diapause hormone of the silkworm, *Bombyx mori*. Proc Jpn Acad Ser B Phys Biol Sci 1991;67:98–101.
[13] Kawano T, Kataoka H, Nagasawa H, Isogai A, Suzuki A. cDNA cloning and sequence determination of the pheromone biosynthesis activating neuropeptide of the silkworm, *Bombyx mori*. Biochem Biophys Res Commun 1992;189(1):221–6.
[14] Kitamura A, Nagasawa H, Kataoka H, Inoue T, Matsumoto S, Ando T, et al. Amino-acid sequence of pheromone-biosynthesis-activating neuropeptide (PBAn) of the silkworm, *Bombyx mori*. Biochem Biophys Res Commun 1989;163(1):520–6.
[15] Matsumoto S, Fonagy A, Kurihara M, Uchiumi K, Nagamine T, Chijimatsu M, et al. Isolation and primary structure of a novel pheromonotropic neuropeptide structurally related to leucopyrokinin from the armyworm larvae, *Pseudaletia separata*.. Biochem Biophys Res Commun 1992;182:534–9.
[16] Matsumoto S, Fonagy A, Schoofs L, Deloof A, Kurihara M, Nagamine T, et al. Induction of cuticular melanization in the armyworm larvae, *Pseudaletia separata*, by insect myotropic neuropeptides possessing FXPRLamide at the C-terminus. J Pestic Sci 1993;18:127–9.
[17] Matsumoto S, Isogai A, Suzuki A. Isolation of the melanization and reddish coloration hormone (Mrch) of the armyworm, *Leucania separata*, from the silkworm, *Bombyx mori*. Agric Biol Chem 1984;48(9):2401–3.
[18] Matsumoto S, Isogai A, Suzuki A. Isolation and amino terminal sequence of melanization and reddish coloration hormone (MRCH) from the silkworm, *Bombyx mori*. Insect Biochem 1986;16(5):775–9.
[19] Matsumoto S, Isogai A, Suzuki A. Purification and characterization of melanization and reddish coloration hormone (MRCH) in Lepidopteran insects. Prog Clin Biol Res 1988;256:437–51.
[20] Matsumoto S, Isogai A, Suzuki A, Ogura N, Sonobe H. Purification and properties of the melanization and reddish colouration hormone (MRCH) in the armyworm, *Leucania Separata* (Lepidoptera). Insect Biochem 1981;11(6):725–33.
[21] Matsumoto S, Kitamura A, Nagasawa H, Kataoka H, Orikasa C, Mitsui T, et al. Functional diversity of a neurohormone produced by the subesophageal ganglion: molecular identity of melanization and reddish coloration hormone and pheromone biosynthesis activating neuropeptide. J Insect Physiol 1990;36(6):427–32.
[22] Matsumoto S, Yamashita O, Fonagy A, Kurihara M, Uchiumi K, Nagamine T, et al. Functional diversity of a pheromonotropic neuropeptide: induction of cuticular melanization and embryonic diapause in Lepidopteran insects by *Pseudaletia* pheromonotropin. J Insect Physiol 1992;38:847–51.
[23] Mendive FM, Van Loy T, Claeysen S, Poels J, Williamson M, Hauser F, et al. *Drosophila* molting neurohormone bursicon is a heterodimer and the natural agonist of orphan receptor DLGR2. FEBS Lett 2005;579:2171–6.
[24] Morita M, Hatakoshi M, Tojo S. Hormonal-control of cuticular melanization in the common cutworm, *Spodoptera litura*. J Insect Physiol 1988;34(8):751–8.
[25] Nachman RJ, Holman GM, Cook BJ. Active fragments and analogs of the insect neuropeptide leucopyrokinin: structure–function studies. Biochem Biophys Res Commun 1986;137:936–42.
[26] Nachman RJ, Holman GM, Haddon WF. Leads for insect neuropeptide mimetic development. Arch Insect Biochem Physiol 1993;22(1–2):181–97.
[27] Nachman RJ, Holman GM, Schoofs L, Yamashita O. Silkworm diapause induction activity of myotropic pyrokinin (FXPRLamide) insect neuropeptides. Peptides 1993;14:1043–8.
[28] Nachman RJ, Zdarek J, Holman GM, Hayes TK. Pupariation acceleration in fleshfly (*Sarcophaga bullata*) larvae by the pyrokinin/PBAN neuropeptide family—structure–activity relationships. Ann NY Acad Sci 1997;814:73–9.
[29] Ogura N. Induction of cuticular melanization in larvae of armyworm, *Leucania separata* Walker (Lepidoptera: Noctuidae), by implantation of ganglia of the silkworm, *Bombyx mori* l. (Lepidoptera: Bombycidae). Appl Entomol Zool 1975;3:216–9.

[30] Ogura N, Saito T. Hormonal function controlling pigmentation of the integument in the common armyworm larvae, *Leucania separata* Walker. Appl Entomol Zool 1972;7:239–42.
[31] Raabe M. Pigment synthesis and breakdown—color change. Recent developments is insect neurohormones NY: Plenum Press; 1989. p. 289–312.
[32] Rafaeli A, Jurenka R. PBAN regulation of pheromone biosynthesis in female moths. Insect pheromone biochemistry and molecular biology NY: Academic Press; 2003. p. 107–36.
[33] Raina AK, Jaffe H, Kempe TG, Keim P, Blacher RW, Fales HM, et al. Identification of a neuropeptide hormone that regulates sex-pheromone production in female moths. Science 1989;244(4906):796–8.
[34] Raina AK, Kingan TG, Kochansky JP. A pheromonotropic peptide of *Helicoverpa zea*, with melanizing activity, interaction with PBAN, and distribution of immunoreactivity. Arch Insect Biochem Physiol 2003;53:147–57.
[35] Raina AK, Klun JA. Brain factor control of sex-pheromone production in the female corn-earworm moth. Science 1984;225(4661):531–3.
[36] Schoofs L, Holman GM, Hayes TK, Nachman RJ, Deloof A. Isolation, identification and synthesis of locustamyo-inhibiting peptide (Lom-Mip): a novel biologically active neuropeptide from *Locusta migratoria*. Regul Peptides 1991;36:111–9.
[37] Schoofs L, Vandenbroeck J, Deloof A. The myotropic peptides of *Locusta migratoria*: structures, distribution, functions and receptors. Insect Biochem Mol Biol 1993;23:859–81.
[38] Suzuki A, Matsumoto S, Ogura N, Isogai A, Tanaka S. Extraction and partial purification of the hormone inducing cuticular melanization in armyworm larvae. Agric Biol Chem 1976;40:2307–9.
[39] Tanaka S. Endocrine mechanisms controlling body-color polymorphism in locusts. Arch Insect Biochem Physiol 2001;47(3):139–49.
[40] Tawfik AI, Tanaka S, De Loof A, Schoofs L, Baggerman G, Waelkens E, et al. Identification of the gregarization-associated dark-pigmentotropin in locusts through an albino mutant. Proc Natl Acad Sci USA 1999;96(12):7083–7.
[41] Yerushalmi Y, Bhargava K, Gilon C, Pener MP. Structure–activity relations of the dark-colour-inducing neurohormone of locusts. Insect Biochem Mol Biol 2002;32(8):909–17.
[42] Yerushalmi Y, Pener MP. The response of a homochrome grasshopper, *Oedipoda miniata*, to the dark-colour-inducing neurohormone (DCIN) of locusts. J Insect Physiol 2001;47:593–7.
[43] Zeltser I, Ben-Aziz O, Schefler I, Bhargava K, Altstein M, Gilon C. Insect neuropeptide antagonist. Part II. Synthesis and biological activity of backbone cyclic and precyclic PBAN antagonists. J Pept Res 2001;58: 275–84.
[44] Zeltser I, Gilon C, Ben-Aziz O, Schefler I, Altstein M. Discovery of a linear lead antagonist to the insect pheromone biosynthesis activating neuropeptide (PBAN). Peptides 2000;21:1457–65.