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Inhibition of PK/PBAN-mediated functions in insects: Discovery of selective and non-selective inhibitors☆

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ABSTRACT

The antagonistic properties of a few linear and backbone cyclic (BBC) conformationally constraint peptide libraries and their analogs, were tested for the ability to inhibit pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) mediated functions: sex pheromone biosynthesis in *Heliothis peltigera* female moths, cuticular melanization in *Spodoptera littoralis* larvae, pupariation in the fleshfly *Neobellieria bullata* and hindgut contraction in *Leucophaea maderae*, elicited by exogenously injected PBAN, pheromonotropin (PT), leucopyrokinin (LPK), myotropin (MT) or by the endogenous peptides. The data revealed differential inhibitory patterns within the same assay with different elicitors (in both the pheromonotropic and melanotropic assays) and among the different functions and disclosed selective antagonists, hinting at the possibility that the receptors that mediate those functions may differ from one another structurally.

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1. Introduction

The PK/PBAN family is a multifunctional family of peptides that plays a major role in the physiology of insects. The first member of the family, leucopyrokinin (LPK), was discovered in the cockroach *Leucophaea maderae* in 1986 [14] and since then over 30 peptides have been identified. They include pyrokinins (PKs), myotropins (MTs), pheromone biosynthesis activating neuropeptide (PBAN), melanization and reddish coloration hormone (MRCH), diapause hormone (DH), pheromonotropin (PT), and peptides derived from the PBAN gene or cDNA of various moths (termed pheromonotropic β and γ peptides), all of which share the common C-terminal pentapeptide FXPRL-amide (X = S, T, G or V) [2,25]. Functions of the PK/PBAN family include stimulation of sex pheromone biosynthesis in moths [2,25,27], and mediation of key functions associated with feeding (gut muscle contractions) [20,29], development (embryonic diapause, pupal diapause and pupariation) [16,21,24,30,31] and defense (melanin biosynthesis) [6,18] in a variety of insects (moths, cockroaches, locusts and flies).

Studies in several laboratories showed that all of the above functions can be stimulated by more than one peptide of the PK/PBAN family, and that the peptides do not exhibit species specificity (for detailed reviews, see [13,25]). The involvement of PK/PBAN neuropeptides in the above functions was demonstrated by means of a variety of *in vivo* and *in vitro*

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bioassays (pheromonotropic, melanotropic, egg and pupal diapause, pupariation and myotropic assays) that were developed and optimized in several laboratories (for review, see [2] and references therein, and [34]).

The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides elicit their effects. Currently, our understanding of the modes of action of this family at the cellular level, of the nature of the native (endogenous) peptides that mediate each function, and of the receptors that mediate them in moths and other insects is very limited and requires further investigation. Antagonists and, to a certain extent, agonists, 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 [1,2]) was applied to the PK/PBAN family and led to the design and synthesis of a D-Phe scan library of linear peptides [33] (which was based on a slight modification of the C-terminal hexapeptide sequence (YFSPRL-NH_2) of PBAN1-33NH_2 in which each amino acid was sequentially substituted with D-Phe), and two backbone cyclic (BBC) conformationally constrained libraries [3,32]: BBC-Ser (which was also based on a modification of the C-terminal hexapeptide sequence of PBAN1-33NH_2), and BBC-D-Phe (which was based on a potent linear antagonist of PBAN that was found in the D-Phe scan library [33]). Examination of the antagonistic activities of the above libraries led to the discovery of several highly potent selective and non-selective pheromonotropic and melanotropic agonists and antagonists that were able to effectively inhibit PBAN1-33NH_2 elicited sex pheromone biosynthesis in *Heliothis peltigera* [1–3,32,33] and cuticular melanization in the *Spodoptera littoralis* larvae [7,8].

In the present study we extended the examination of the agonistic and antagonistic properties of the above peptides and of some of their analogs (small BBC and precyclic peptides [8]), and tested their ability to inhibit pheromone biosynthesis and melanization evoked by other elicitors of the PK/PBAN family: PT, leucopyrokinin (LPK) and Lom-MT-II or by the endogenous mechanisms (e.g., native peptides). We also tested their ability to elicit and inhibit other functions mediated by the PK/PBAN family: pupariation in the fleshfly, *Neobellieria bullata* and hindgut contraction in the cockroach *L. maderae*.

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 the 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 [12]. Pupae were sexed and females and males were placed in separate rooms with a dark/light regime of 10:14 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 [12]. All females used in this study were 3.5 days old.

Larvae of the fleshfly, *N. bullata* were reared by 200–300 specimens per batch on beef liver in small open disposable packets made from aluminum foil as described [24,31]. Fully grown larvae that left the food were allowed to wander in dry sawdust until the first puparia appeared 36–40 h later. Then the batch was ready for collecting red spiracle (RS) stage larvae that can be distinguished by precocious tanning of the cuticle in the region of hind spiracles (peritreme). For the bioassay early RS larvae (2–3 h before pupariation) were used, unless indicated otherwise. *L. maderae* cockroaches were kept in plastic containers at 30 °C with a light:dark regimen of 12:12. Food and water were provided *ad libitum* [14].

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, PT, LPK and MT

Hez-PBAN [26] and *Pseudaletia* (*Mythimna*) *separata* PT (Pss-PT) [19], were synthesized on an ABI 433A automatic peptide synthesizer on Rink amide MBHA resin by means of the FastMoc™ chemistry as described previously [14,33]. Synthesis of *L. maderae* LPK (Lem-LPK) [14] and *Locusta migratoria* (Lom-MT-II) [28] was carried out via 9-fluorenylmethoxycarbonyl (Fmoc) methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Advanced Chemtech, Louisville, KY) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) as described previously [22].

2.2.3. Synthesis of the D-Phe scan peptide library

The peptides (Table 1) were synthesized by the simultaneous multiple peptide synthesis (SMPS) ''tea bags'' methodology on Rink amide MBHA resin according to Zeltser et al. [33].

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

The BBC peptides (Ser and D-Phe sub-libraries and the small BBC peptides, Figs. 1 and 2, respectively) and the precyclic peptides (Fig. 3) were synthesized by the SMPS ''tea bags'' methodology on Rink amide MBHA resin by means of Fmoc chemistry as described previously [3,32].

2.2.5. Purification and characterization of peptides

The purity of PBAN1-33NH_2, PT and the D-Phe scan library peptides as well as that of the BBC peptides was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) on ''LichrosorbR'' C_{18} (LICHROCARTE 250-10

Table 1 – Pheromonotropic antagonistic activity of the D-Phe scan-library of peptides on different elicitors

Tested peptide[a]	Elicitor			
	PBAN1-33NH_2 (0.3 pmol)	PT (1 pmol)	LPK (30 pmol)	MT (10 pmol)
Antagonistic activity (%)[b]				
dFYFSPRL-NH_2 (LA-1)	15 ± 10 (*n* = 10)	15 ± 4 (*n* = 40)	0 (*n* = 20)	0 (*n* = 30)
R**dF**FSPRL-NH_2 (LA-2)	1 ± 9 (*n* = 9)	12 ± 6 (*n* = 40)	2 ± 2 (*n* = 20)	0 (*n* = 30)
RY**dF**SPRL-NH_2 (LA-3)	18 ± 6 (*n* = 10)	20 ± 9 (*n* = 40)	10 ± 11 (*n* = 20)	0 (*n* = 30)
RYF**dF**PRL-NH_2 (LA-4)	63 ± 8 (*n* = 10)*	24 ± 10 (*n* = 40)	3 ± 3 (*n* = 20)	0 (*n* = 20)
RYFS**dF**RL-NH_2 (LA-5)	53 ± 10 (*n* = 10)*	16 ± 9 (*n* = 40)	15 ± 15 (*n* = 20)	0 (*n* = 20)
RYFSP**dF**L-NH_2 (LA-6)	65 ± 7 (*n* = 10)*	13 ± 6 (*n* = 40)	4 ± 3 (*n* = 20)	0 (*n* = 20)
RYFSPR**dF**-NH_2 (LA-7)	45 ± 8 (*n* = 10)*	10 ± 8 (*n* = 40)	20 ± 3 (*n* = 20)	3 ± 3 (*n* = 20)

An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. Values of the inhibitory effects of the peptides with PBAN as an elicitor are based on previously published results [33] and are presented here only for comparison purposes.
[a] The amount of the tested peptides was 100 pmol in experiments in which PBAN1-33NH_2 was used as an elicitor and 1 nmol in the presence of all other elicitors.
[b] Antagonistic activity is expressed as 100 minus the ratio (as percentage) between the sex pheromone production elicited by each of the elicitors in the presence and absence of the LA peptides.

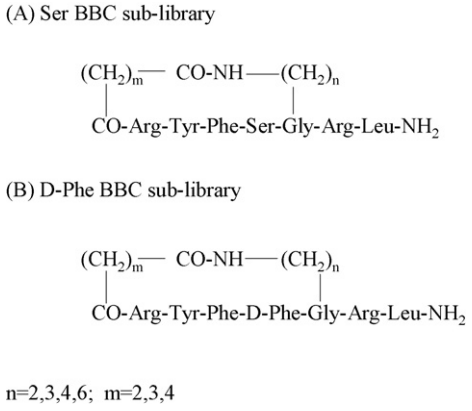


Fig. 1 – General structure of the BBC Ser (A) and the D-Phe (B) sub-libraries of peptides. Peptides of the Ser sub-library are referred to in the text by consecutive numbers BBC-4 to BBC-15 representing *n* = 2 and *m* = 2,3,4; *n* = 3 and *m* = 2, 3, 4; *n* = 4 and *m* = 2, 3, 4; *n* = 5 and *m* = 2, 3, 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 2, 5, 8 and 9).

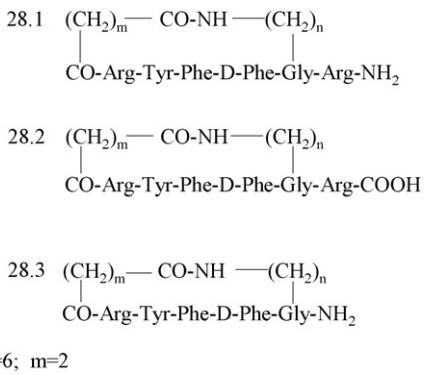
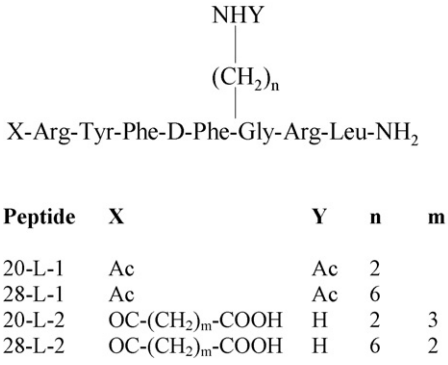


Fig. 2 – General structure of BBC-28 reduced size analogs (small BBC peptides).



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 structure of the precyclic linear peptides.

cat. 16817) with acetonitrile (ACN):H_2O + 0.1% trifluoroacetic acid (TFA) as described previously [3,32,33] and was found to be in the range of 90–95%. The purified peptides were characterized by time-of-flight mass spectrometry (TOF-MS) and amino acid analysis of hydrolyzates. The analytical methods and the properties of the peptides were described previously [3,32,33].

The crude peptides MT and LPK were purified on a Waters C_{18} Sep Pak cartridge and a Delta Pak C_{18} reverse-phase column (8 mm × 100 mm, 15 μm particle size, 100 A pore size) on a Waters 510 HPLC controlled by a Millennium 2010 chromatography manager system (Waters, Milford, MA). Detection was at 214 nm at ambient temperature. Solvent A = 0.1% aqueous TFA; Solvent B = 80% aqueous ACN containing 0.1% TFA. Conditions: initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. The peptides were further purified on a Waters Protein Pak I125 column (7.8 mm × 300 mm) (Milligen Corp., Milford, MA). Conditions—flow rate: 2.0 ml/min; isocratic with solvent = 80% ACN made with 0.01% TFA. The identity of the peptide analogs was confirmed via MALDI-TOF-MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical, Ltd., Manchester, UK). Amino acid analysis was carried out

as described previously [22] and was used to quantify the peptide and to confirm its identity. The purity of MT and LPK was found to be >95%.

2.3. Pheromonotropic bioassay

The pheromonotropic bioassay was performed with *H. peltigera* as described previously [4]. Stimulatory activity of the PK/PBAN peptides was determined by monitoring their ability to induce sex pheromone biosynthesis in females. Females injected with 1 pmol PBAN1-33NH_2 served as a reference for stimulatory activity. Antagonistic activity was determined by monitoring the ability of D-Phe linear peptides, BBC or precyclic peptides (at 100 pmol or 1 nmol) to inhibit sex pheromone biosynthesis that was elicited by an injected exogenous stimulator (PBAN1-33NH_2, PT, MT, or LPK each at 0.3–0.5, 1, 10 and 30 pmol, respectively, see Section 3). Females injected with the elicitors at the indicated concentrations served as a reference for maximal stimulation and those injected with 100 mM phosphate buffer served to determine the basal pheromone biosynthesis at photophase. The pheromone content in buffer-injected moths did not exceed 10 ng/female. The pheromone glands were excised 2 h post-injection and sex pheromone was extracted and quantified by capillary gas chromatography as described previously [4]. All experiments were performed with a minimum of 9 females per treatment.

2.4. Melanotropic bioassay

The melanotropic bioassay was performed as described previously [7]. Each experiment involved analysis of the intensity of the melanized area in untreated and ligated larvae, and in larvae that were ligated and injected with buffer, PBAN or the tested peptide. The cuticular melanization was quantified as the ratio between the optical density and the scanned cuticular area (in millimeters) and was compared between control and experimental animals. All experiments were performed with a minimum of 8 larvae per treatment. The only experiments taken into account were those in which the extent of melanization in buffer-injected larvae did not differ significantly from that of ligated animals, and did differ significantly from that of those injected with the elicitors (PBAN1-33NH_2, 5 pmol; PT, 5 pmol or LPK 15 pmol).

The melanotropic stimulatory activity of the PK/PBAN peptides was determined by evaluating their ability to induce cuticular melanization in larvae. Larvae injected with 5 pmol PBAN1-33NH_2 served as a reference for stimulatory activity. Antagonistic activity was determined by monitoring the ability of the D-Phe linear peptides, of the BBC or of precyclic peptides (at 1 nmol), injected together with the elicitors PBAN1-33NH_2, PT or LPK (at 5, 5 and 15 pmol, respectively, see Section 3), to inhibit cuticular melanization. Larvae injected with the elicitors at the indicated concentrations served as references for maximal stimulation, and those injected with 50 mM HEPES, pH 7.6 served to determine the basal cuticular melanization of the ligated insects.

2.5. Pupariation bioassay

The test was performed as described by Zd'arek [31]. Briefly, the tested material was injected at doses of 0.5, 5, 50 and 500 pmol into fleshfly larvae (*N. bullata*) at the early RS stage that previously had been immobilized by chilling on ice. Control larvae were injected with water only. After removal from the ice the injected larvae were kept at 25 °C in Petri dishes lined with filter paper, and the time of retraction (R), contraction (C) and tanning (T) was recorded. At the end of the RS stage the larva stops crawling and irreversibly retracts the first three front segments with the cephalopharyngeal apparatus ('the mouth hooks') (retraction—R); it then contracts longitudinally to become the barrel-shaped puparium (contraction—C) and its surface becomes smooth by shrinking of the cuticle, until it attains the shape of the 'white puparium' (WP). Some 50–60 min after C the WP starts to change color by phenolic tanning of the cuticle (T) and turns to an 'orange puparium'. The effects of each compound tested in the study were expressed as a difference between the control and experimental larvae, in the mean time between the occurrences of C and T. Eight to 12 larvae in each group were injected, and the test was repeated four times. Larvae were injected by means of a disposable calibrated glass capillary with a pointed tip. The volumes of injected solution ranged from 0.5 to 1.0 μl. The threshold dose was the dose that demonstrated differences of at least 25% from the control group in R, C and T in each of the four trials.

2.6. Myotropic bioassay

Hindguts of adult *L. maderae* cockroaches were isolated from the central nervous system (CNS) and dissected [14], suspended in a 5 ml chamber, and prepared for recording as previously described [11]. Threshold concentrations were determined for each analog by adding a known quantity (dissolved in 0.5 ml of bioassay saline) [14] to the bioassay chamber containing the hindgut. The threshold concentration was defined as the minimum concentration of analog required to elicit an observable change in the frequency (50%) or amplitude (10%) of contractions within 1 min and sustained for 3 min. Threshold concentrations were obtained from measurements of three to five cockroach hindguts on consecutive days. A test for potential antagonist activity was conducted by introduction of LPK at a concentration of 3×10^{-9} M (15 pmol) in the quantity required to produce a half-maximal response on the hindgut, followed by a BBC analog at a concentration of 5×10^{-6} M (25 nmol) to determine if it could inhibit the initial response.

2.7. Statistical analysis

The results of the pheromonotropic and melanotropic assays were subjected to one-way ANOVA. The significance of differences among means was evaluated with the Tukey–Kramer HSD (honestly significant difference) test at $P < 0.05$. The threshold concentration data for each active analog in the cockroach hindgut contractile assay were presented as (mean ± standard deviation) and were calculated using the Student's *t*-test software.

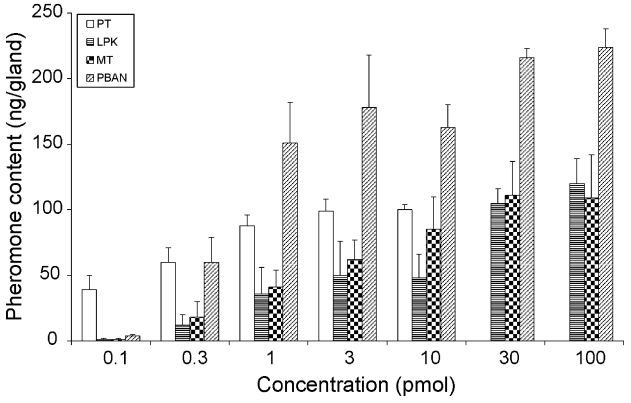


Fig. 4 – Dose response of the pheromonotropic activity of PT, LPK and MT in comparison with PBAN1-33NH$_2$. Note that PT was not tested at 30 and 100 pmol.

3. Results

3.1. *Stimulation of sex pheromone biosynthesis and cuticular melanization by PT, LPK and MT*

The first series of experiments was designed to determine the working concentration of each of the elicitors (i.e., PT, LPK and MT) in the pheromonotropic and melanotropic bioassays and to compare the kinetic properties of these peptides with those of PBAN1-33NH$_2$. The immediate objective of these experiments was to determine the lowest concentration that generated a perceptible response (i.e., stimulated *H. peltigera* females to produce pheromone at about 100 ng/gland or elicited melanization to a degree that differed significantly from that of ligated *S. littoralis* larvae).

As can be seen in Fig. 4, all peptides exhibited dose-dependent pheromonotropic activity, although their potency differed. PBAN1-33NH$_2$ was the most active peptide at almost all tested concentrations, other than at 0.1 pmol, where PT exhibited a higher activity. PT was equipotent with PBAN1-33NH$_2$ at 0.3 pmol and exhibited a lower activity than the latter at all other tested concentrations. LPK and MT exhibited similar activity profiles which were lower than those of PBAN1-33NH$_2$ or PT at all tested concentrations. The ability of all of the above peptides to activate sex-pheromone biosynthesis was demonstrated in several colonies reared in our laboratory. A dose-dependent activity was also exhibited by three of the four elicitors (PBAN1-33NH$_2$, PT and LPK) in the melanotropic bioassay (Fig. 5). MT did not show a dose-dependent response (data not shown). In light of the above experiments the concentrations that were chosen to stimulate sex pheromone biosynthesis by the above peptides were: 1, 30 and 10 pmol for PT, LPK and MT, respectively, and those chosen for melanin stimulation were 5 and 15 pmol for PT and LPK, respectively.

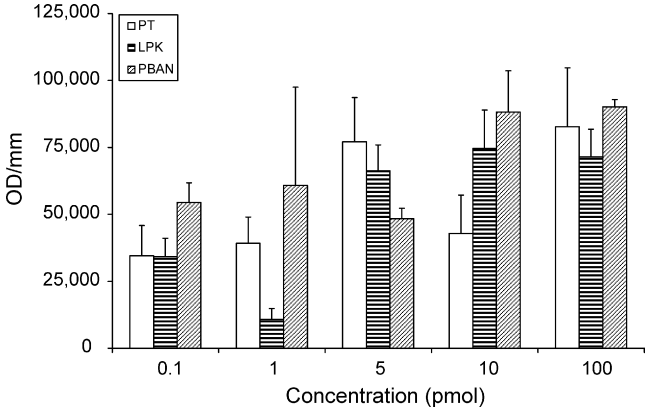


Fig. 5 – Dose response of the melanotropic activity of PT and LPK in comparison with PBAN1-33NH$_2$.

3.2. *Inhibitory effects of linear and BBC peptides on sex pheromone biosynthesis elicited by exogenously administered synthetic PK/PBAN peptides*

Next, the ability of the D-Phe scan linear peptides, the BBC and the precyclic peptides to inhibit sex pheromone biosynthesis elicited in *H. peltigera* females by PT, LPK and MT was monitored. As summarized in Tables 1–3 none of the peptides exhibited any significant inhibitory activity with any of the elicitors, other than with PBAN1-33NH$_2$. Four linear peptides of the D-Phe scan library (LA-4, -5, -6, and -7), six BBC peptides (BBC-20, -21, -22, -25 and -28 and the small BBC peptide 28-3) and two precyclic peptides (20-L-1 and 28-L-1) inhibited sex pheromone biosynthesis elicited by PBAN1-33NH$_2$ [3,8,32,33]. Peptides of the BBC-Ser sub-library did not exhibit any antagonistic activity with any of the tested elicitors (including PBAN1-33NH$_2$) (data not shown).

3.3. *Inhibitory effects of linear and BBC peptides on the cuticular melanization elicited by exogenously administered synthetic PK/PBAN peptides*

Examination of the effects of all of the above linear, BBC and precyclic peptides on PT- and LPK-elicited melanotropic activity revealed an entirely different pattern of inhibition from that of their pheromonotropic activities. Unlike the case of the pheromonotropic assay, melanization elicited by PT and LPK was inhibited by several of the tested peptides. Some peptides (i.e., LA-1, LA-3, LA-7, BBC-21, -25, -28, -29 and 20-L-2) inhibited only one elicitor, others (i.e., LA-4, LA-5, LA-6, BBC-15, 19, 23, 28-1, 28-3 and 28-L-1) inhibited two elicitors, and some (i.e., BBC-20 and -27) were able to inhibit melanization elicited by all three elicitors (Tables 4–6). Unlike in the case of the pheromonotropic activity, one peptide of the BBC-Ser sub-library (BBC-15) exhibited melanotropic antagonistic activity (elicited by PBAN1-33NH$_2$). All other peptides did not inhibit any of the tested elicitors (including PBAN1-33NH$_2$) (data not shown).

In a previous study we found that most of the tested peptides exhibited mixed agonistic and antagonistic properties (especially with respect to the melanotropic activity); only a few compounds showed pure antagonistic activity [7,8]. Examination of the activities of only the pure antagonists (i.e., peptides with no agonistic activity) revealed one selective pheromonotropic antagonist (BBC-22, which inhibited pheromone production elicited by PBAN1-33NH$_2$), and three selective melanotropic antagonists (BBC-23 and BBC-28-1,

Table 2 – Pheromonotropic antagonistic activity of the D-Phe sub-library of BBC peptides on different elicitors

Tested peptide[a] (1 nmol)		Elicitor			
Peptide #	$n+m$	PBAN1-33NH$_2$ (0.5 pmol)	PT (1 pmol)	LPK (30 pmol)	MT (10 pmol)
Antagonistic activity (%)					
15	6 + 4	27 ± 10 (n = 10)	10 ± 7 (n = 30)	0 (n = 20)	0 (n = 20)
19	2 + 2	25 ± 14 (n = 18)	17 ± 9 (n = 50)	1 ± 1 (n = 20)	0 (n = 20)
20	2 + 3	96 ± 2 (n = 10)*	7 ± 5 (n = 50)	21 ± 21 (n = 20)	0 (n = 20)
21	2 + 4	81 ± 5 (n = 9)*	19 ± 6 (n = 50)	0 (n = 20)	3 ± 3 (n = 20)
22	3 + 2	85 ± 3 (n = 10)*	11 ± 5 (n = 50)	0 (n = 20)	0 (n = 20)
23	3 + 3	24 ± 21 (n = 9)	12 ± 8 (n = 50)	0 (n = 20)	0 (n = 20)
24	3 + 4	19 ± 7 (n = 10)	20 ± 9 (n = 40)	4 ± 4 (n = 20)	0 (n = 20)
25	4 + 2	55 ± 12 (n = 10)*	4 ± 2 (n = 40)	0 (n = 20)	0 (n = 20)
26	4 + 3	0 (n = 10)	17 ± 11 (n = 40)	5 ± 6 (n = 40)	0 (n = 20)
27	4 + 4	19 ± 19 (n = 9)	24 ± 11 (n = 40)	0 (n = 20)	0 (n = 20)
28	6 + 2	77 ± 5 (n = 19)*	32 ± 6 (n = 40)	11 ± 12 (n = 20)	0 (n = 20)
29	6 + 3	34 ± 16 (n = 9)	16 ± 14 (n = 30)	0 (n = 20)	0 (n = 20)
30	6 + 4	36 ± 14 (n = 10)	n.t.	n.t.	n.t.

Details are as indicated in the legend to Table 1. An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. Values of the inhibitory effects of the BBC peptides with PBAN as an elicitor are based on previously published results [3,32] and are presented here only for comparison purposes. n.t.: not tested.
[a] For peptide structures, see Fig. 1.

which inhibited melanin formation, elicited by PBAN1-33NH$_2$ and LPK, and the precyclic peptide 20-L-2, which inhibited only LPK elicited melanin formation) (Table 7). All other peptides inhibited both functions, although the inhibitory patterns differed between the respective functions: most melanotropic antagonists inhibited more than one elicitor, whereas the pheromonotropic antagonists inhibited only sex pheromone biosynthesis elicited by PBAN1-33NH$_2$.

3.4. *Inhibitory effects of BBC peptides on pupariation in flesh flies elicited by endogenous mechanisms*

The effects of the D-Phe sub-library of BBC peptides on pupariation are summarized in Table 8. The PK peptide LPK significantly accelerated the onset of the pupariation process in larvae of the fleshfly *N. bullata* at a potency threshold concentration of 0.25 pmol [23,31] when injected into larvae at the RS stage. Following injection, three of the D-Phe BBC peptides (20, 28 and 29) demonstrated an agonistic response, i.e., acceleration of the pupariation process. The threshold dose required for each of these three BBC peptides was 500 pmol, which indicates a very weak response. Only one of the BBC peptides, 25, was able to inhibit the pupariation process, leading to a statistically significant 30% delay in the onset of pupariation. The threshold dose inhibition by BBC-25 was determined to be 50 pmol. None of the BBC peptides, whether at 500 pmol or 1 nmol, could inhibit the acceleration of pupariation elicited by exogenously applied LPK (at a dose of 0.5 pmol).

3.5. *Inhibitory effects of BBC peptides on cockroach hindgut contraction elicited by LPK*

The effects of the D-Phe sub-library of BBC peptides on contraction of the hindgut of *L. maderae* are summarized in Table 9. Significant stimulation of hindgut contraction was

Table 3 – Pheromonotropic antagonistic activity of the small BBC and precyclic peptides on various elicitors

Tested peptide[a] (1 nmol)	Elicitor			
	PBAN1-33NH$_2$ (0.5 pmol)	PT (1 pmol)	LPK (30 pmol)	MT (10 pmol)
Antagonistic activity (%)				
28-1	3 ± 1 (n = 30)	16 ± 12 (n = 30)	0 (n = 20)	0 (n = 20)
28-2	32 ± 1 (n = 20)	15 ± 5 (n = 20)	17 ± 8 (n = 10)	0 (n = 10)
28-3	77 ± 4 (n = 40)*	23 ± 13 (n = 30)	1 ± 1 (n = 20)	0 (n = 20)
20-L-1	91 ± 3 (n = 20)*	21 ± 14 (n = 30)	19 ± 19 (n = 20)	n.t.
20-L-2	20 ± 3 (n = 20)	20 ± 11 (n = 30)	16 ± 7 (n = 10)	0 (n = 20)
28-L-1	56 ± 1 (n = 20)*	24 ± 9 (n = 30)	21 ± 10 (n = 20)	0 (n = 20)
28-L-2	37 ± 4 (n = 20)	19 ± 6 (n = 10)	n.t.	n.t.

Details are as indicated in the legend to Table 1. An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. Values of the inhibitory effects of small BBC and precyclic peptides with PBAN as an elicitor are based on previously published results [8,32] and are presented here only for comparison purposes. n.t.: not tested.
[a] For peptide structures, see Figs. 2 and 3.

Table 4 – Melanotropic antagonistic activity of the D-Phe scan-library of peptides on different elicitors

Tested peptide (1 nmol)	Elicitor		
	PBAN1-33NH_2 (5 pmol)	PT (5 pmol)	LPK (15 pmol)
Antagonistic activity (%)[a]			
dFYFSPRL-NH_2 (LA-1)	50 ± 13 (*n* = 10)	71 ± 10 (*n* = 9)*	47 ± 15 (*n* = 10)
R**dF**FSPRL-NH_2 (LA-2)	1 ± 1 (*n* = 10)	16 ± 23 (*n* = 9)	29 ± 15 (*n* = 10)
RY**dF**SPRL-NH_2 (LA-3)	27 ± 15 (*n* = 9)	13 ± 21 (*n* = 9)	84 ± 10 (*n* = 9)*
RYF**dF**PRL-NH_2 (LA-4)	36 ± 9 (*n* = 19)	73 ± 10 (*n* = 10)*	94 ± 6 (*n* = 10)*
RYFS**dF**RL-NH_2 (LA-5)	51 ± 10 (*n* = 19)	65 ± 8 (*n* = 7)*	90 ± 5 (*n* = 10)*
RYFSP**dF**L-NH_2 (LA-6)	1 ± 1 (*n* = 10)	74 ± 14 (*n* = 10)*	76 ± 12 (*n* = 10)*
RYFSPR**dF**-NH_2 (LA-7)	1 ± 1 (*n* = 9)	0 (*n* = 10)	77 ± 9 (*n* = 10)*

An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. Values of the inhibitory effects of the peptides with PBAN as an elicitor are based on previously published results [7] and are presented here only for comparison purposes.
[a] Antagonistic activity is expressed as 100 minus the ratio (as percentage) between the melanin formation elicited by each elicitor in the presence and absence of the LA peptides.

elicited by exogenously applied LPK at a potency threshold concentration of $6.5 \pm 1.5 \times 10^{-10}$ M (3.25 pmol). Three of the D-Phe BBC peptides (27, 28 and 29) exhibited a very weak response, stimulating contractions of the isolated cockroach hindgut at a concentration range of $1.7–2.6 \times 10^{-6}$ M (8.5–13 nmol). The rest of the tested peptides showed no activity, either stimulatory or inhibitory. The D-Phe BBC peptides could not inhibit the spontaneous contractions of the cockroach hindgut, even at concentrations up to 5×10^{-6} M (25 nmol). In addition, the BBC peptides applied at a concentration of 5×10^{-6} M (25 nmol) failed to inhibit the response of exogenously applied LPK at a concentration (3×10^{-9} M, 15 pmol), which would elicit 50% of the maximal stimulatory response.

4. Discussion

The present study focused on examination of the stimulatory and inhibitory properties of a variety of linear and BBC peptides and of some of their analogs (small BBC and precyclic peptides), in an attempt to discover compounds that exhibited selective and non-selective activities (either agonistic or antagonistic) toward several functions (sex pheromone biosynthesis, cuticular melanization, pupariation and hindgut contraction) that are mediated by several elicitors of the PK/PBAN family (PBAN1-33NH_2, PT, MT, LPK).

Various chemical libraries were used in the study: the D-Phe scan library of linear peptides in which a few lead antagonists capable of inhibiting pheromonotropic and melanotropic activities elicited by PBAN1-33NH_2 were found [33], and two BBC sub-libraries: the Ser sub-library, which was synthesized based on a modification of the C-terminal active sequence of the PK/PBAN family (FXPRL-NH_2) [3], and the D-Phe sub-library, which was synthesized based on the sequence of a lead pheromonotropic antagonist (RYFdFPRL-NH_2), and in which a few potent selective melanotropic and pheromonotropic agonists and antagonists that inhibited PBAN1-33NH_2 were found [3,7,8,32,33].

BBC cyclic peptides, which exhibit greater conformational constraint than linear peptides, offer a major advantage for

Table 5 – Melanotropic antagonistic activity of the D-Phe sub-library of BBC peptides on various elicitors

Tested peptide[a] (1 nmol)		Elicitor		
Peptide #	*n* + *m*	PBAN1-33NH_2 (5 pmol)	PT (5 pmol)	LPK (15 pmol)
Antagonistic activity (%)				
15	6 + 4	61 ± 9 (*n* = 18)*	38 ± 12 (*n* = 9)*	1 ± 2 (*n* = 10)
19	2 + 2	78 ± 8 (*n* = 60)*	52 ± 9 (*n* = 10)*	24 ± 20 (*n* = 10)
20	2 + 3	62 ± 14 (*n* = 50)*	64 ± 11 (*n* = 10)*	63 ± 12 (*n* = 10)*
21	2 + 4	42 ± 24 (*n* = 40)	75 ± 7 (*n* = 10)*	32 ± 14 (*n* = 10)
22	3 + 2	18 ± 8 (*n* = 20)	19 ± 20 (*n* = 9)	43 ± 19 (*n* = 9)
23	3 + 3	65 ± 7 (*n* = 30)*	0 (*n* = 10)	45 ± 16 (*n* = 10)*
24	3 + 4	30 ± 1 (*n* = 20)	16 ± 12 (*n* = 10)	37 ± 25 (*n* = 10)
25	4 + 2	55 ± 12 (*n* = 50)	85 ± 8 (*n* = 10)*	30 ± 26 (*n* = 8)
26	4 + 3	44 ± 17 (*n* = 40)	0 (*n* = 8)	44 ± 18 (*n* = 10)
27	4 + 4	76 ± 18 (*n* = 20)*	87 ± 5 (*n* = 8)*	52 ± 12 (*n* = 10)*
28	6 + 2	83 ± 12 (*n* = 50)*	0 (*n* = 9)	20 ± 18 (*n* = 8)
29	6 + 3	65 ± 16 (*n* = 30)*	1 ± 1 (*n* = 9)	0 (*n* = 9)
30	6 + 4	1 ± 1 (*n* = 30)	n.t.	n.t.

Details are as indicated in the legend to Table 4. An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. Values of the inhibitory effects of the BBC peptides with PBAN as an elicitor are based on previously published results [8] and are presented here only for comparison purposes. n.t.: not tested.
[a] For peptide structures, see Fig. 1.

Table 6 – Melanotropic antagonistic activity of the small BBC and precyclic peptides on various elicitors

Tested peptide[a] (1 nmol)	Elicitor		
	PBAN1-33NH$_2$ (5 pmol)	PT (5 pmol)	LPK (15 pmol)
Antagonistic activity (%)			
28–1	71 ± 1 (n = 20)*	42 ± 24 (n = 10)	94 ± 4 (n = 10)*
28–2	46 ± 20 (n = 19)	n.t.	n.t.
28–3	20 ± 20 (n = 20)	54 ± 12 (n = 10)*	57 ± 15 (n = 10)*
20-L-1	70 ± 1 (n = 20)*	n.t.	n.t.
20-L-2	32 ± 10 (n = 20)	11 ± 21 (n = 10)	43 ± 12 (n = 10)*
28-L-1	73 ± 16 (n = 20)*	39 ± 16 (n = 9)*	0 (n = 10)
28-L-2	46 ± 12 (n = 20)	n.t.	n.t.

Details are as indicated in the legend to Table 4. An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. Values of the inhibitory effects of small BBC and precyclic peptides with PBAN as an elicitor are based on previously published results [8] and are presented here only for comparison purposes. n.t.: not tested.

[a] For peptide structure, see Figs. 2 and 3.

studying multifunctional peptide families. The conformational constraint confers on the peptides: (i) high selectivity, which results from restriction of the conformational space to a conformation that mediates one function of the peptide and excludes those that mediate other functions; (ii) enhanced metabolic stability which results from their exclusion of conformations that are recognized by degrading enzymes and consequent prevention of enzymatic degradation; (iii) increased biological activity, which is facilitated by the much slower equilibrium between the conformations; (iv) improved bioavailability, which results from their reduced polarity. These attributes, conferred by their conformational restraint, makes BBC peptides very powerful tools for exploring the modes of action of a family of peptides which exhibits a multifunctional pattern of activity.

Indeed, the employment of the above peptides exhibited differing inhibition patterns within the same assay evoked by different elicitors (in both the pheromonotropic and melanotropic assays) and so was the case among different functions (Tables 7–9). In the case of the pheromonotropic assay, peptides that inhibited pheromone biosynthesis elicited by PBAN1-33NH$_2$, showed no activity against any other tested elicitor, whereas they were able to inhibit a variety of melanotropic elicitors (Table 7). Although most peptides inhibited more than one elicitor, in most cases individual compounds differed in their power to inhibit different elicitors, and only rarely (as in the case of BBC-20) did a given compound inhibit all elicitors. No elicitor was inhibited by all peptides in any of the tested functions, despite the fact that all the D-Phe BBCs had the same primary sequence, the same location and the same ring chemistry, and they differed from each other only in their bridge size and the position of the amide bond along the bridge [3].

Differing inhibitory patterns were also revealed between assays: there were marked differences between the BBC inhibitory patterns of the pheromonotropic assay and those of the melanotropic, pupariation and myotropic assays. No BBC peptide inhibited the myotropic activity – although this activity was stimulated by LPK – which was effectively inhibited by two BBC peptides (BBC-22 and BBC-23) in the melanotropic assay. LPK-elicited pheromone biosynthesis was also not inhibited by any of the BBC peptides. Major differences were also found when the pheromonotropic and melanotropic antagonistic activities of the D-Phe scan, BBC,

Table 7 – Summary of selective and non-selective antagonistic BBC and precyclic peptides

Peptide	Selectivity	Pheromonotropic (P)				Melanotropic (M)		
		PBAN	PT	LPK	MT	PBAN	PT	LPK
BBC-20	Non-selective	√	–	–	–	√	√	√
BBC-22	Selective (P)	√	–	–	–	–	–	–
BBC-23	Selective (M)	–	–	–	–	√	–	√
BBC-25	Non-selective	√	–	–	–	-	√	–
BBC-28	Non-selective	√	–	–	–	√	–	–
BBC-28-1	Selective (M)	–	–	–	–	√	–	√
20-L-1	Non-selective	√	–	–	–	√	n.t.	n.t.
20-L-2	Selective (M)	–	–	–	–	–	–	√
28-L-1	Non-selective	√	–	–	–	√	√	–

Results are based on Tables 1–6 and previously published data [3,7,8,32,33]. Most peptides exhibit pure pheromonotropic and/or melanotropic antagonistic activity except for peptides BBC-20 and BBC-28, which showed weak agonistic responses in the pupariation assay (Table 8). For peptide structures, see Figs. 1–3. n.t.: not tested.

Table 8 – Activity of D-Phe sub-library of BBC peptides on pupariation in flesh fly *Neobellieria bullata* larvae

Peptide #	$n + m$	Agonist response (threshold)	Endogenous inhibitory response (threshold)
19	2 + 2	None	None
20	2 + 3	+	None
21	2 + 4	None	None
22	3 + 2	None	None
23	3 + 3	None	None
24	3 + 4	None	None
25	4 + 2	None	++
27	4 + 4	None	None
28	6 + 2	+	None
29	6 + 3	+	None
30	6 + 4	None	None

(+) 500 pmol (weak); (++) 50 pmol.

and precyclic peptides were examined. Four selective antagonists were found, three of which were melanotropic (BBC-23, BBC-28-1 and 20-L-1) and one pheromonotropic (BBC-22) (Table 7). Selective agonists were also found amongst the BBC peptides in a previous study, in which six selective pure melanotropic agonists and one non-selective pure agonistic compound were discovered [8]. Five non-selective melanotropic and pheromonotropic antagonists were found (BBC-20, -25, -28 20-L-1 and 28-L-1), only one of which (BBC-25) also inhibited pupariation. However, it is interesting to note that this peptide inhibited pupariation that was elicited by the endogenous mechanism, whereas it did not affect pupariation that was elicited by exogenously injected LPK, which indicates that the endogenous mechanism may not be mediated by LPK.

The differing inhibitory and stimulatory patterns that were found in different assays indicate that those functions may be mediated by structurally different receptors. Although the selectivity may result from differences between the assays themselves (e.g., different insects, developmental stages, assay conditions, etc.) consideration should be given to the possibility that it may indicate diversity in the binding pockets or the ligand docking regions on the receptors—which in turn might indicate structural variations between the PK/PBAN receptors. The differences obtained within the same assay hint that the PK/PBAN peptides might mediate their activity *via* more than one receptor, or that different ligands exhibit differing binding patterns, which would result in differing binding affinities toward a given receptor.

The hypothesis that there are structural differences between the PK/PBAN receptors that mediate the different elicitor functions, was further strengthened by examination of the homology between a cloned PK/PBAN receptor (PK/PBAN-R) of *S. littoralis* that mediates cuticular melanization and has recently been cloned by us [35], with various pheromone gland PK/PBAN receptors that have been cloned from various moth species including a few Heliothinae species [9,10,15,35]. We found that the *S. littoralis* larval PBAN-R shared significant amino acid identity with other PBAN-Rs that were isolated from the pheromone glands of adult female moths (92, 81, 92 and 72% amino acid identity with the PBAN-R of *Helicoverpa zea*, *Bombyx mori*, *Helicoverpa armigera* and *Plutella xylostella*, respectively), with major variations occurring in the extracellular N-terminus and the C-terminal tail [35]. Currently, the structural mechanisms of the interactions between ligand and G-protein coupled receptors (GPCR) are still not clear, but the data that have been accumulated so far suggest that the extracellular N-terminus/loops are responsible for the ligand docking/interaction [17]. It is thus possible that whereas the FXPRL-NH_2 common PK/PBAN motif (which is an essential minimal part of the ligand) activates the receptor, the remaining amino acids of the ligand play different roles such as facilitating the docking of the ligand to the receptor. The major difference observed on the N-terminal region of the

Table 9 – Activity of D-Phe sub-library of BBC peptides on hindgut contraction in cockroach *Leucophaea maderae* adults

Peptide #	$n + m$	Stimulatory response (threshold—10^{-6} M)	Inhibitory response [elicitor: LPK] (threshold)
19	2 + 2	None	None
20	2 + 3	None	None
21	2 + 4	None	None
22	3 + 2	None	None
23	3 + 3	None	None
24	3 + 4	None	None
25	4 + 2	None	None
27	4 + 4	2.0 ± 0.01[a]	None
28	6 + 2	1.7 ± 0.1[a]	None
29	6 + 3	2.6 ± 0.7[a]	None
30	6 + 4	None	None

[a]Weak activity (threshold response corresponds to 10, 8.5 and 13 nmol for BBC-27, 28 and 29, respectively).

PBAN-R may, therefore, account for optimal conspecific ligand/receptor docking and interaction, resulting in different binding patterns of different ligands (e.g., elicitors) to the pheromone gland and larval receptors. The highly conserved TM helices between different PBAN-R might form a precise ligand interaction pocket which is required for the $FXPRLNH_2$ motif-induced receptor conformational change and receptor activation.

The proposed model may explain the differences between the inhibitory patterns of the BBC and those of the other antagonistic peptides, both within and between assays, and may provide an insight into the nature (competitive or non-competitive) of the inhibitors. It is most likely that the common sequence of the PK/PBAN elicitors ($FXPRLNH_2$) docks in the highly conserved region of the receptors, whereas the other sequences of the elicitor molecule dock at different sites, which may differ in between the different assays. In light of the above suggestions we may further hypothesize that the various BBC and other antagonists bind outside the $FXPRLNH_2$ docking pocket, and may, thus, be considered as non-competitive inhibitors; their inhibitory activity results, most likely, from interference with the docking of the other parts of the elicitor ligand with its receptor. Since different ligand molecules may dock in different regions within the same assay, and in between the different assays (due to the structural differences in the receptors) the degree of inhibition of a given elicitor (in different assays) or different elicitors (in the same assay) by a given BBC compound results in different inhibitory potencies and patterns. The non-competitive nature of the BBC inhibitors has been previously suggested by us in light of the results of an *in vitro* radioisotope-receptor binding study carried out with the native *H. peltigera* receptor [5].

In summary, the availability of conformationally constrained selective and non-selective agonists and antagonists together with the availability of bioassays that have been developed for each of the above-mentioned functions has opened the way to a better understanding of the endogenous mechanisms of this peptide family in moths and other insects. The information gained in the course of this study on the selective and non-selective antagonists for the various activities, as well as the data on the differing activity patterns of the PK/PBAN elicitors and on the structural changes in the receptors that mediate their activities, are of major importance for the design of additional, improved agonistic and antagonistic compounds (e.g., more potent, highly selective, metabolically stable and cost effective) for further study of the above issues. Such novel, improved compounds, when available, may also be potential candidates for agrochemical applications that could 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 novel 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*.

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