Peptides 30 (2009) 608–615

Contents lists available at ScienceDirect

Peptides

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

Biostable β-amino acid PK/PBAN analogs: Agonist and antagonist properties

Ronald J. Nachman [a,*], Orna Ben Aziz [b], Michael Davidovitch [b], Pawel Zubrzak [a], R. Elwyn Isaac [c], Allison Strey [a], Gloria Reyes-Rangel [d], Eusebio Juaristi [d], Howard J. Williams [e], Miriam Altstein [b,**]

[a] Areawide Pest Management Research, Southern Plains Agricultural Research Center, U.S. Department of Agriculture, College Station, TX 77845, USA
[b] Department of Entomology, The Volcani Center, ARO, Bet Dagan, 50250, Israel
[c] Faculty of Biological Sciences, University of Leeds, Clarendon Way, Leeds LS2 9JT, UK
[d] Department of Chemistry, Centro de Investigacion y de Estudios Avanzados del IPN, Mexico, D.F., Mexico
[e] Department of Chemistry, Texas A&M University, College Station, TX 77840, USA

ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form 11 November 2008
Accepted 12 November 2008
Available online 21 November 2008

Keywords:
β-Amino acids
PBAN
Sex pheromone biosynthesis
Cuticular melanization
Pupariation
Hindgut contraction
Insect neuropeptide agonist/antagonist

ABSTRACT

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family plays a significant role in a multifunctional array of important physiological processes in insects. PK/PBAN analogs incorporating β-amino acids were synthesized and evaluated in a pheromonotropic assay in *Heliothis peltigera*, a melanotropic assay in *Spodoptera littoralis*, a pupariation assay in *Neobellieria bullata*, and a hindgut contractile assay in *Leucophaea maderae*. Two analogs (PK-βA-1 and PK-βA-4) demonstrate greatly enhanced resistance to the peptidases neprilysin and angiotensin converting enzyme that are shown to degrade the natural peptides. Despite the changes to the PK core, analog PK-βA-4 represents a biostable, non-selective agonist in all four bioassays, essentially matching the potency of a natural PK in pupariation assay. Analog PK-βA-2 is a potent agonist in the melanotropic assay, demonstrating full efficacy at 1 pmol. In some cases, the structural changes imparted to the analogs modify the physiological responses. Analog PK-βA-3 is a non-selective agonist in all four bioassays. The analog PK-βA-1 shows greater selectivity than parent PK peptides; it is virtually inactive in the pupariation assay and represents a biostable antagonist in the pheromonotropic and melanotropic assays, without the significant agonism of the parent hexapeptide. These analogs provide new, and in some cases, biostable tools to endocrinologists studying similarities and differences in the mechanisms of the variety of PK/PBAN mediated physiological processes. They also may provide leads in the development of PK/PBAN-based, insect-specific pest management agents.

© 2008 Elsevier Inc. All rights reserved.

1. Introduction

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family of peptides plays a multifunctional role in the physiology of insects. In 1986 the first member of the family, leucopyrokinin (LPK), was isolated from the cockroach *Leucophaea maderae* [14] with over 30 members of this peptide class identified thereafter. All family members share the common C-terminal pentapeptide FXPRL-amide (X = S, T, G or V) and include subfamilies such as pyrokinins, myotropins (MTs), PBAN, diapause hormone (DH), melanization and reddish coloration hormone (MRCH), pheromonotropin (PT), as well as pheromonotropic β and γ peptides derived from the cDNA of moths [3,29,30]. The PK/PBAN family has been shown to stimulate sex pheromone biosynthesis in moths [3,29–31], and mediate critical functions associated with feeding (gut contractions) [21,33], development (egg diapause, pupal diapause and pupariation) [15,22,23,25,28,35,36] and defense (melanin biosynthesis) [5,18] in a variety of insects. The peptides do not exhibit species specificity and experiments have shown that all of the functions listed above can be stimulated by more than one peptide [1,11,29,30]. The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides operate and agonists and antagonists, particularly selective ones, can shed light on this issue.

While PK/PBAN molecular messengers are both potent and specific, they are not suitably designed to be effective either as pest insect control agents and/or tools for insect neuroendocrinologists. Neuropeptides are rapidly degraded by peptidases in the hemolymph and tissues within insects and generally exhibit poor bioavailability [2,23,26,27]. The development of potent agonists and antagonists with enhanced biostability can overcome at least one of these limitations and can represent a key step in the development of pest management techniques based on neuropeptide analogs capable of disrupting critical life processes regulated by the PK/PBAN family.

* Corresponding author. Tel.: +1 979 260 9315; fax: +1 979 260 9377.
** Corresponding author. Tel.: +972 3 968 3710; fax: +972 3 968 3835.
E-mail addresses: nachman@tamu.edu (R.J. Nachman), vinnie2@agri.gov.il (M. Altstein).

0196-9781/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2008.11.007

The PKs are hydrolyzed by tissue-bound peptidases at a primary susceptibility site between the P and R residues within the C-terminal pentapeptide sequence that defines members of this family of neuropeptides [27]. Incorporation of β-amino acids can enhance resistance to peptidase attack and modify biological activity [7,16]. Indeed, this strategy has been successfully applied to the insect kinin neuropeptide family [34,37], leading to the identification of several potent selective and non-selective agonists with enhanced biostability characteristics. In this article we describe the synthesis of a number of analogs of the PK/PBAN C-terminal hexapeptide core in which the important residues Tyr^1, Phe^2, and Pro^4 are replaced with β^3-amino acid and/or their β^2-homo-amino acid counterparts. All analogs were blocked at the N-terminus with an Ac group, which confers resistance to hydrolytic degradation by an additional class of peptidases, the aminopeptidases [13]. We have shown that the C-terminal PK hexapeptide analog is susceptible to degradation by the pure peptidases neprilysin (NEP) and angiotensin converting enzyme (ACE), whereas two of the β-amino acid analogs were found to demonstrate significantly enhanced resistance to these same enzymes.

The β-amino acid PK/PBAN analogs PK-βA-1 to -4 (structures listed below) were also tested for their ability to elicit and/or inhibit pheromone biosynthesis in the moth *Heliothis peltigera* and melanization in the Egyptian cotton leaf worm *Spodoptera littoralis*. Their ability to elicit other functions mediated by the PK/PBAN family: pupariation in the flesh fly, *Neobellieria bullata* and hindgut contraction in the cockroach *L. maderae* were examined as well.

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 [10]. 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 [10]. All females used in this study were 3.5 days old.

Larvae of the flesh fly, *N. bullata* were reared in batches of 200–300 specimens on beef liver in small open disposable packets made from aluminum foil as described [36]. Fully grown larvae that left the food were allowed to wander in dry sawdust until the first puparia appeared 36–40 h later. The batch was ready for collecting when red spiracle (RS) stage larvae, distinguished by precocious tanning of the cuticle in the region of hind spiracles (peritreme), appeared. 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. β-Amino acid PK/PBAN analog synthesis and purification

Four β-amino acid PK/PBAN analogs were synthesized:

PK-βA-1: Ac-YFT[β^3P]RLa (1461)
PK-βA-2: Ac-Y[β^2homoF]TPRLa (1466)
PK-βA-3: Ac-Y[β^3F]TPRLa (1465)
PK-βA-4: Ac-[β^3F]FT[β^3P]RLa (1602)

Protected β-amino acids were purchased from Fluka (Buchs, Switzerland). PK/PBAN analogs were synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Applied Biosystems, Foster City, CA) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) under previously described conditions [37]. Crude products 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 and 100A pore size) on a Waters 600 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA) and Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: initial solvent consisting of 10% B was followed by the Waters linear program to 90% B over 25 min; flow rate, 2 ml/min. Delta Pak C18 retention times: PK-βA-1 (Ac-YFT[β^3P]RLa): 8.5 min; PK-βA-2 (Ac-Y[β^2homoF]TPRLa): 11.0 min; PK-βA-3 (Ac-Y[β^3F]TPRLa): 9.0 min; PK-βA-4 (Ac-[β^3F]FT[β^3P]RLa): 7.5 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; Solvent A = 95% acetonitrile made to 0.01% TFA; Solvent B = 50% aqueous acetonitrile made to 0.01% TFA; 100% A isocratic for 4 min, then a linear program to 100% B over 80 min. WatPro retention times: PK-βA-1 (Ac-YFT[β^3P]RLa): 6.25 min; PK-βA-2 (Ac-Y[β^2homoF]TPRLa): 6.0 min; PK-βA-3 (Ac-Y[β^3F]TPRLa): 6.25 min; PK-βA-4 (Ac-[β^3F]FT[β^3P]RLa): 6.0 min. Amino acid analysis was carried out under previously reported conditions [37] and used to quantify the peptide and to confirm identity, leading to the following analyses: PK-βA-1 (Ac-YFT[β^3P]RLa): F[1.0], L[1.0], R[1.0], T[0.9], Y[0.9]; PK-βA-2 (Ac-Y[β^2homoF]TPRLa): L[1.0], P[0.9], R[0.9], T[0.9], Y[0.9]; PK-βA-3 (Ac-Y[β^3F]TPRLa): L[1.0], P[0.9], R[0.9], T[0.9], Y[0.9]; PK-βA-4 (Ac-[β^3F]FT[β^3P]RLa): F[1.0], L[1.0], T[1.1], R[1.0]. The identity of the peptide analogs were confirmed via MALDI-TOF MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical Ltd., Manchester, UK) with the presence of the following molecular ions ($M+H^+$): PK-βA-1 (Ac-YFT[β^3P]RLa): 851.4 [calc MH^+ = 851.9]; PK-βA-2 (Ac-Y[β^2homoF]TPRLa): 865.9 [calc MH^+ = 866.0]; PK-βA-3 (Ac-Y[β^3F]TPRLa): 851.0 [calc MH^+ = 851.0]; PK-βA-4 (Ac-[β^3F]FT[β^3P]RLa): 849.5 [calc MH^+ = 849.1].

2.3. Synthesis of PBAN1-33NH_2, PT and LPK

Hez-PBAN [32] and *Pseudaletia* (*Mythimna*) *separata* PT (Pss-PT) [19] were synthesized on an ABI 433A automatic peptide synthesizer on Rink amide 4-methylbenzhydrylamine (MBHA) resin by means of the FastMoc™ chemistry as described previously [14]. Syntheses of *L. maderae* (Lem-LPK) [14] and PBAN fragment–analogs YFTPRLa and the acylated version (Ac-YFTPRLa, 1559) were 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 [27]. The purity of all peptides was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) [5] 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 hydrolysates.

2.4. Pheromonotropic bioassay

The pheromonotropic bioassay was performed with *H. peltigera* as described previously [4]. Stimulatory activity of PBAN, LPK, the β-amino acid PK/PBAN analogs and the LPK derived peptide1559

was determined by monitoring their ability to induce sex pheromone biosynthesis. Females injected with 1 pmol PBAN1-33NH_2 served as a reference for stimulatory activity. Antagonistic activity was determined by monitoring the ability of the tested peptides to inhibit sex pheromone biosynthesis that was elicited by PBAN1-33NH_2 at 1 pmol. Females injected with the elicitor 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 8–10 females per treatment.

2.5. Melanotropic bioassay

The melanotropic bioassay was performed as described previously [6]. Melanotropic stimulatory activity of PBAN, LPK, the β-analogs or the LPK derived peptide 1559 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 β-analogs (at 1 nmol), injected together with the elicitors PBAN1-33NH_2, PT or LPK (at 5, 5 and 15 pmol, respectively), to inhibit cuticular melanization. Larvae injected with the elicitors at the indicated doses served as 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. Each experiment also involved analysis of the intensity of the melanized area in untreated and ligated larvae. 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 8–10 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 PBAN1-33NH_2 (5 pmol).

2.6. Pupariation bioassay

The test was performed as described by Zd'arek et al. [36]. Briefly, the tested material was injected at doses of 0.5, 5, 50 and 500 pmol into flesh fly 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 dry 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 a 25% from the control group in R, C and T in each of the four trials.

2.7. 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 [8]. 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 β-amino acid analog at a concentration of 5×10^{-6} M (25 nmol) to determine if it could inhibit the initial response.

2.8. Enzyme hydrolysis trials

2.8.1. Angiotensin converting enzyme trials

Drosophila ACE (M_r, 67,000) was purified from a soluble extract of adults as described elsewhere [9,17] and yielded enzyme that appeared as a single band by SDS-PAGE. PK/PBAN β-amino acid analogs (100 μM) were incubated at 35 °C with 25 ng ACE in 0.1 M HEPES buffer, pH 7 (total volume, 20 μl) for 30 min. The reaction was stopped by the addition of 5 μl TFA to a final concentration of 2.7% (v/v) and the volume was made up to 260 μl with 0.1% (v/v) TFA before HPLC analysis.

2.8.2. Neprilysin degradation trials

PK/PBAN β-amino acid analogs (100 μM) were incubated at 35 °C with 20 ng human recombinant neprilysin (a gift from Dr. A.J. Kenny, School of Biochemistry and Molecular Biology, University of Leeds) in 0.1 M HEPES buffer, pH 7 (total volume, 20 μl) at 35 °C for 30 min. The reaction was stopped by the addition of 5 μl TFA to a final concentration of 2.7% (v/v) and the volume was made up to 260 μl with 0.1% (v/v) TFA before HPLC analysis.

HPLC analysis of the fragments after ACE and NEP degradation was performed using a Jupiter 5 μ, column (C18, 250 mm in length × 4.5 mm, internal diameter; Phenomenex, Macclesfield, UK) and UV detection at 214 nm, and a linear gradient (6–50%) of acetonitrile in 0.1% TFA over 25 min at flow rate of 1 ml/min. Rates of hydrolysis were calculated from the percentage decline of the substrate, as measured by changes in peak height, and in comparison with a substrate standard treated under the same conditions, but without enzyme. Hydrolysis never exceeded 20% of the starting substrate concentration to ensure that the reaction was linear with time. Assays were performed in triplicate. More experimental detail is presented in a previous manuscript [17].

2.9. Statistical analysis

The results of the pheromonotropic, melanotropic and hindgut contractile assays were subjected to one-way ANOVA. All data are presented as mean ± standard error mean. 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 T test software (JMP version 5.1.2, ©2004, SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Pheromonotropic bioassay

The results of a dose–response evaluation of the four β-amino acid PK/PBAN analogs as agonists in the *in vivo* pheromonotropic assay in *H. peltigera* indicate that the analogs demonstrated marked differences in their activity. Substitution of the Pro^4 with a β^3-amino acid or the Phe^2 with a β^2-homo-amino acid (as in PK-βA-1 (1461) and PK-βA-2 (1466), respectively) resulted in a marked loss in activity compared to the parent acylated C-terminal hexapeptide Ac-YFTPRLa (1559) (Fig. 1). However, substitution of the Phe^2 with a β^2-amino acid (as PK-βA-3, 1465) resulted in a highly potent agonist that exhibited activity even at 1 pmol, and substitution of both Phe^2 and Pro^4 with two β^3-amino acids (as in PK-βA-4, 1602) resulted in the most potent agonist which exhibited the highest activity at 1 pmol and at 1 nmol among all β substituted peptides.

Comparison of the activity at 1 pmol (Fig. 1) revealed that peptide PK-βA-4 is the most active one, likely due to enhanced biostability. The activity of this peptide (which stimulated pheromone biosynthesis to a level of 24 ± 10 ng, $n = 10$) was slightly higher than that of the parent peptide Ac-YFTPRLa (that stimulated pheromone biosynthesis to 11 ± 6 ng, $n = 10$) and equipotent with that of LPK at this dose (23 ± 12 ng pheromone, $n = 10$). At 10 pmol, the activity of PK-βA-4 was similar to that of the parent peptide Ac-YFTPRLa (61 ± 21 ng $n = 10$ and 60 ± 5 ng, $n = 10$, respectively) and to that of LPK (82 ± 14 ng, $n = 10$).

All peptides demonstrated high activities at 1 nmol (ranging from 68 to 126 ng) with PK-βA-4 being the highest amongst the β substituted peptides (68 ± 9 ng, 84 ± 6 ng, 80 ± 10 ng, and 118 ± 14 ng, $n = 10$ for PK-βA-1, PK-βA-2, PK-βA-3, and PK-βA-4, respectively, Fig. 1). Its activity was higher than that of all the other peptides and equipotent with that of the parent peptide Ac-YFTPRLa (118 ± 14 ng, $n = 10$ and 126 ± 15 ng, $n = 10$, respectively). Interestingly, the activities of both PK-βA-4 and the parent peptide 1559 at 1 nmol were higher than that of LPK (95 ± 15 ng, $n = 10$), although they did not differ significantly. The activity of all peptides, whether substituted or not, was significantly lower at 1 pmol than that of PBAN (Fig. 1).

The β-amino acid substituted peptides that were weak agonists (PK-βA-1 and PK-βA-2) were tested for pheromonotropic antagonistic activity at different doses. The data in Fig. 2 show that peptide PK-βA-1 exhibited a significant antagonistic activity at 100 pmol (a dose at which the peptide was devoid of agonistic activity). Peptide PK-βA-2 was inactive at all tested doses. The other peptides (PK-βA-3, PK-βA-4 and the control parent acylated peptide 1559) were tested for their antagonistic activity at 100 pmol and 1 nmol. The data revealed that all three peptides were devoid of any inhibitory activity at both doses.

3.2. Melanotropic bioassay

Unlike in the pheromonotropic assay substitution of Phe^2 and Pro^4 by one or two β^3 amino acids or a β^2-homo-amino acid did not result in marked differences between the peptides in the melanotropic activity. All peptides were active to some extent with three out of the four substituted peptides (PK-βA-2, PK-βA-3, and PK-βA-4) being highly potent even at 1 pmol (Fig. 3). No dose dependency was observed with any of the tested peptides and the response seemed to be of an ‘all or none’ nature. The parent acylated C-terminal hexapeptide Ac-YFTPRLa exhibited the highest activity matching the potency and efficacy of the 33-residue natural peptide PBAN (Fig. 3) at all tested doses.

Remarkably, analog PK-βA-2 and PK-βA-4 elicited melanotropic activity at a dose of 1 pmol, which did not differ significantly from that of PBAN at the 5 pmol dose and from that of the unmodified parent hexapeptide (Fig. 3). These analogs therefore matched the potency of the unmodified parent peptide and PBAN, despite the structural modification to the Phe^2 position and were significantly much more active than LPK at the same dose. As in the pheromonotropic assay, analogs PK-βA-2, PK-βA-3 and PK-βA-4

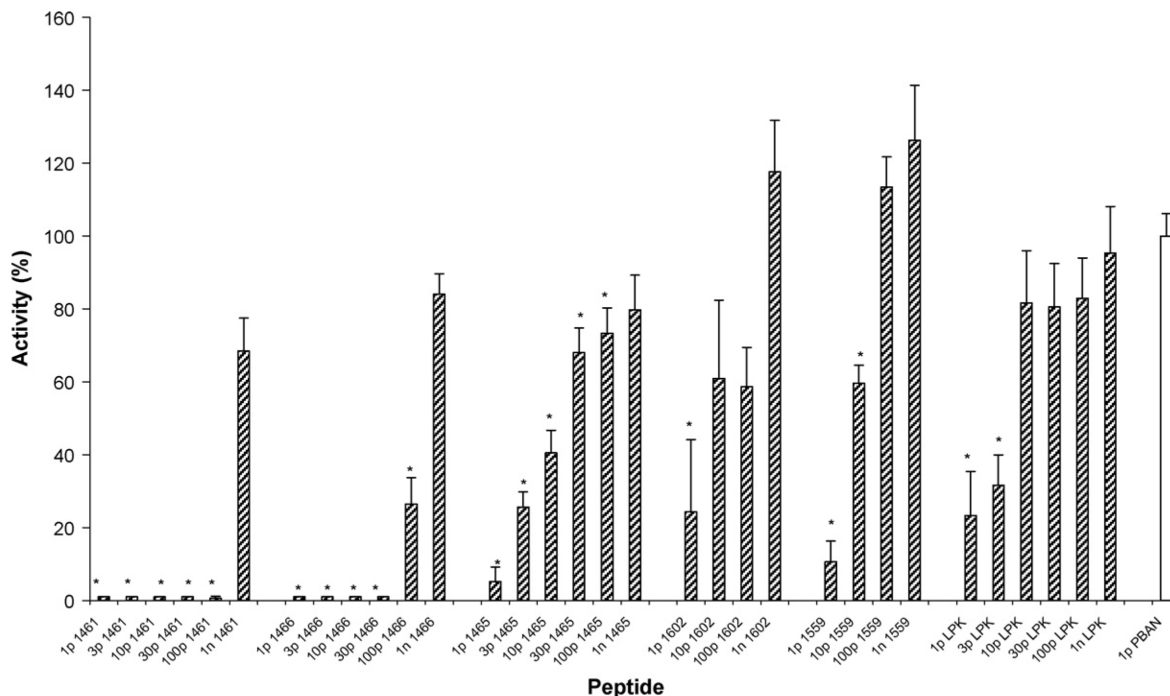

Fig. 1. *In vivo* dose–response agonist pheromonotropic activity of β-amino acid analogs: PK-βA-1 (1461), PK-βA-2 (1466), PK-βA-3 (1465) and PK-βA-4 (1602), the acetylated parent peptide 1559 and LPK in adult female *Heliothis peltigera*. Activity is expressed as the ratio (as a percentage) between the extents of pheromone biosynthesis elicited by the injection of each of the peptides at the listed doses and by PBAN1-33NH_2 (at 1 pmol) ± S.E.M. of 8–10 samples. Statistical analysis compared differences between the pheromonotropic agonistic activities obtained with a given peptide and PBAN1-33NH_2. An asterisk (*) indicates a significant difference in activity at $P < 0.05$, p: pmol; n: nmol.

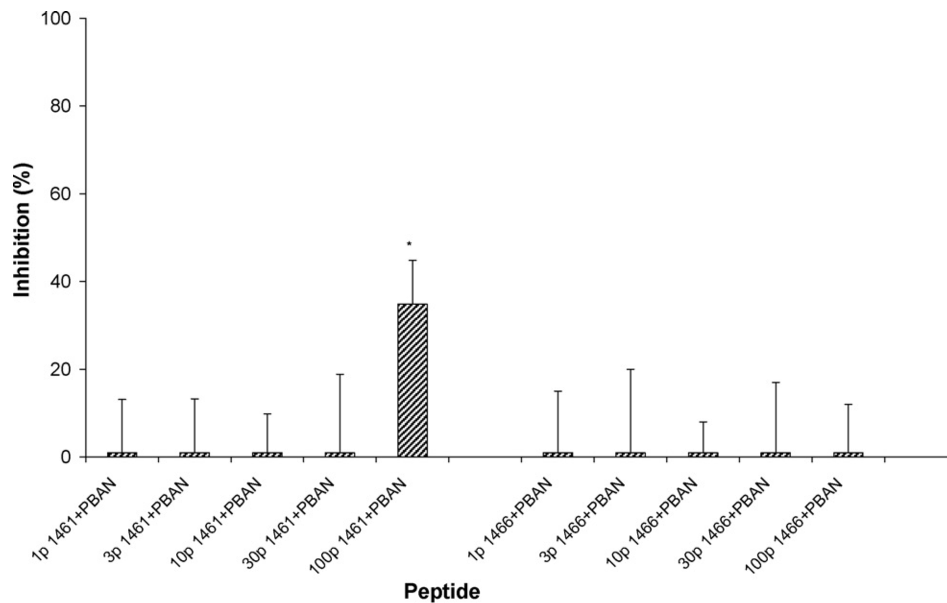

Fig. 2. *In vivo* inhibition of sex pheromone biosynthesis elicited by PBAN (at 1 pmol) by various doses of β-amino acid analogs (PK-βA-1, 1461 and PK-βA-2, 1466), in adult female *H. peltigera*. Antagonistic activity (i.e., inhibition) is expressed as 100 minus the ratio (as percentage) between the sex pheromone production elicited by the elicitor in the presence and absence of the tested peptides ± S.E.M. of 8–10 samples. Statistical analysis compared the amount of pheromone obtained with PBAN-1-33NH_2 in the presence and absence of the tested peptides. An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself. p: pmol.

were highly active at 1 nmol exhibiting an activity that did not differ significantly from that of PBAN but was significantly lower than that of the native peptide (1559) (Fig. 3). Weak melanotropic activity was detected for analog PK-βA-1 at all tested doses (Fig. 3).

An evaluation of the β-amino acid PK/PBAN analogs as antagonists in the *in vivo* melanotropic assay in *S. littoralis* against several natural elicitors is presented in Table 1. When evaluated against PBAN, PK-βA-3 is completely devoid of any inhibitory activity. Similar results were obtained with the two other natural elicitors PT and LPK (Table 1).

Each of the other analogs PK-βA-1, PK-βA-2, and PK-βA-4 at a 1 nmol dose inhibited the melanotropic activity of PBAN by 100%, 84%, and 65%, respectively, and all statistically significant. The acylated parent hexapeptide Ac-YFTPRLa inhibited PBAN melanotropic activity by a statistically significant 53%. PK-βA-4 showed a statistically significant inhibition of PBAN (73%) at a dose of 100 pmol as well (data not shown). The other two analogs PK-βA-1 and PK-βA-2 did not show any significant inhibition at doses of 100, 10 or 1 pmol (data not shown). Similar inhibitory activities were obtained with those peptides when Pss-PT was used as an elicitor where PK-βA-1, PK-βA-2, and PK-βA-4 inhibited PT elicited melanin formation by 99%, 99%, and 89%, respectively (Table 1). The peptides fail to inhibit LPK in the melanotropic assay (Table 1), with the exception of 10 pmol PK-βA-1 that inhibited LPK by 57% (data not shown).

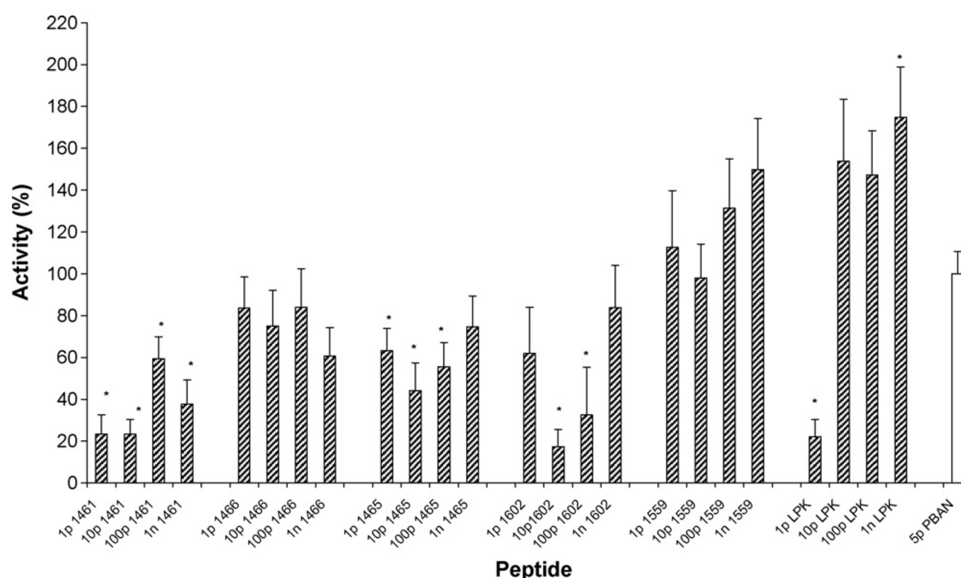

Fig. 3. *In vivo* dose–response agonist melanotropic activity of β-amino acid analogs PK-βA-1 (1461), PK-βA-2 (1466), PK-βA-3 (1465) and PK-βA-4 (1602), the acetylated parent peptide 1559 and LPK in *S. littoralis* larvae. Activity is expressed as the ratio (as a percentage) between the extents of melanization elicited by the injection of each of the peptides at the listed doses and by PBAN1-33NH_2 (at 5 pmol) ± S.E.M. of 8–10 samples. Statistical analysis compared differences between the melanotropic agonistic activities obtained with a given peptide and PBAN1-33NH_2. An asterisk (*) indicates a significant difference in activity at $P < 0.05$, p: pmol; n: nmol.

Table 1
Summary of antagonistic melanotropic activity of β-amino acid PK/PBAN analogs on different elicitors.

Peptide	Sequence	Antagonistic activity[a] (%)		
		Elicitors		
		PBAN1-33NH2 (5 pmol)	Pss-PT (5 pmol)	LPK (15 pmol)
β-AA PK/PBAN analogs (1 nmol)				
PK-βA-1 (1461)	Ac-YFT[β3P]RLa	100 ± 1 (n = 10)*	99 ± 1 (n = 10)*	38 ± 13 (n = 9)
PK-βA-2 (1466)	Ac-Y[β2homoF]TPRLa	84 ± 11 (n = 10)*	99 ± 3 (n = 10)*	33 ± 15 (n = 10)
PK-βA-3 (1465)	Ac-Y[β3F]TPRLa	1 ± 3 (n = 10)	1 ± 4 (n = 10)	1 ± 3 (n = 10)
PK-βA-4 (1602)	Ac-[β3F]FT [β3P]RLa	65 ± 17 (n = 8)*	89 ± 11 (n = 9)*	18 ± 15 (n = 10)
Control peptide (1 nmol)				
1559	Ac-YFTPRLa	53 ± 14 (n = 10)*	1 ± 9 (n = 8)	11 ± 21 (n = 10)

[a] Antagonistic activity (e.g. inhibition) is expressed as 100 minus the ratio (as percentage) between the melanin formation elicited by each elicitor in the presence and absence of the tested peptides.
* An activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself.

Table 2
Pupariation acceleration (*Neobellieria bullata*) and hindgut contractile (*Leucophaea maderae*) agonist activity of β-amino acid PK/PBAN analogs.

Peptide	Sequence	Threshold	
		Pupariation (pmol)	Hindgut contraction (10^{-9} M)[a]
LPK	pETSFTPRLa	0.3 [28]	1 [21]
PK-βA-3 (1465)	Ac-Y[β^3F]TPRLa	0.5	7 ± 4
PK-βA-2 (1466)	Ac-Y[β^2homoF]TPRLa	5	10 ± 8
PK-βA-1 (1461)	Ac-YFT[β^3P]RLa	500	15 ± 2
PK-βA-4 (1602)	Ac-[β^3F]FT [β^3P]RLa	<0.5	10 ± 3

[a] The threshold concentrations listed in the hindgut contraction column of the table are equivalent to 5, 35, 50, 75, and 50 pmol in the experimental chamber for LPK, PK-βA-3, PK-βA-2, PK-βA-1, and PK-βA-4, respectively.

3.3. Pupariation and myotropic bioassays

The results of an evaluation of the β-amino acid PK/PBAN analogs in the larval pupariation assay of *N. bullata* and isolated hindgut contraction assay of the cockroach *L. maderae* are summarized in Table 2. The PK Lem-PK is native to the *L. maderae* cockroach and also the first PK sequence used to establish the pupariation acceleration effect in larvae of the flesh fly *N. bullata* and is used as the control peptide. In the *in vitro* hindgut contractile assay, each of the four analogs PK-βA-1, PK-βA-2, PK-βA-3, and PK-βA-4 demonstrated stimulatory activity at threshold concentrations of 15, 10, 7, and 10×10^{-9} M (which correspond to 75, 50, 35, and 50 pmol, respectively, Table 2). These values are not statistically different from one another. Although the native LPK is more potent in the cockroach hindgut myotropic assay (1×10^{-9} M which corresponds to 5 pmol [21]) than the analogs, they remain relatively potent and within the broad physiological range of activity. In the *in vivo* larval pupariation assay of the flesh fly, analog PK-βA-1 shows only very weak to marginal activity, at a threshold dose of 500 pmol (Table 2). Analog PK-βA-2 demonstrates activity that is two orders of magnitude greater with a threshold dose of 5 pmol. Furthermore, analogs PK-βA-4 (<0.5 pmol) and PK-βA-3 (0.5 pmol) essentially match the potency of the control peptide LPK (Table 2).

3.4. Enzyme hydrolysis trials

Two of the analogs (PK-βA-1: Ac-YFT[β^3P]RLa and PK-βA-4: Ac-[β^3F]FT[β^3P]RLa) were selected for enzyme hydrolysis trials with pure NEP and ACE because they featured a β-amino acid replacement adjacent to the primary tissue-bound peptidase site located in the middle of the critical C-terminal PK/PBAN pentapeptide active-core sequence [27]. The other two analogs were not protected in this critical active-core region, and were therefore not considered likely to be 'biostable'. The results are summarized in Table 3. The placement of β-amino acids in PK-βA-1 and PK-βA-4 has stabilized them to both ACE and NEP peptidases. No hydrolysis of PK-βA-1 could be detected for either, whereas the hydrolysis of PK-βA-4 has been greatly reduced to 4% (ACE) and 9% (NEP) of the rates for the unmodified parent PK peptide YFTPRLa (Table 3).

4. Discussion

Despite the incorporation of an additional methylene group (–CH_2–) within the backbone of the C-terminal pentapeptide core region of PK/PBAN peptides, single and/or double replacement of amino acids with their β-amino acid counterparts led, in select cases, to significant retention of biological activity in several insect PK/PBAN bioassays. In some cases, the structural modification of the β-amino acid analogs led to a modification of the activity profile of the parent acylated, PK/PBAN C-terminal hexapeptide. Notably, the substitution of a β^3-Pro^4 for the Pro^4 in the middle of the core region, as in analogs PK-βA-1 and PK-βA-4, stabilized the resulting analogs to pure peptidases that degrade, and therefore inactivate, the natural PK/PBAN peptides. No hydrolysis of analog PK-βA-1 could be detected, even when extended incubation times were employed, when exposed to ACE and NEP peptidases, and the hydrolysis of PK-βA-4 was greatly reduced to 4% and 9%,

Table 3
Comparison of the rates of hydrolysis of β^3-Pro PK/PBAN analogs with the parent fragment.

Substrate	Rate[a] of hydrolysis by NEP (pmol/h)	Rate[a] of hydrolysis by ACE (pmol/h)
YFTPRLa	699	2357
PK-βA-1	0[b]	0[b]
PK-βA-4	30	209

[a] Rates are expressed as the mean of at least three assays with a standard error of less than 2%.
[b] No hydrolysis of PK-βA-1 was recorded, even when an extended incubation time was used.

Table 4
Comparison of pheromonotropic and melanotropic agonistic and antagonistic activities.

Analog	Sequence	Selectivity	Pheromonotropic activity		Melanotropic activity	
			Agonist	Antagonist	Agonist	Antagonist
β Analogs						
PK-βA-1 (1461)	Ac-YFT[β^3P]RLa	Pure antagonist (P + M) Non-selective	–[a]	√	–	√
PK-βA-2 (1466)	Ac-Y[β^2homoF]TPRLa	Mixed agonist/antagonist (M)	–[a]	–	√	√
PK-βA-3 (1465)	Ac-Y[β^3F]TPRLa	Pure agonist (P + M) Non-selective	√	–	√	–
PK-βA-4 (1602)	Ac-[β^3F]FT [β^3P]RLa	Pure agonist (P) Mixed agonist/antagonist (M) Non-selective agonist Selective antagonist (M)	√	–	√	√
Control peptide						
1559	Ac-YFTPRLa	Pure agonist (P) Mixed agonist/antagonist (M) Non-selective agonist Selective antagonist (M)	√	–	√	√

Results are based on Figs. 1–3 and Table 1.
[a] Evaluation was made based on activities up to 100 pmol. P: pheromonotropic; M: melanotropic.

respectively, of the rates observed for the unmodified parent PK/PBAN hexapeptide. Notably, the introduction of a second β^3-Phe as a replacement for the Phe^2 in the N-terminal region of the core sequence actually somewhat reduced, though did not reverse, the protection from peptidase hydrolysis afforded by the β^3-Pro^4 in the middle of the core. This result suggests that the active site of the enzymes are extended enough that substitutions some distance away from the susceptible region can influence hydrolysis rates, in this case a negative influence. The other two analogs did not involve incorporation of β-amino acids adjacent to the primary tissue-bound peptidase hydrolysis site [27] of the core sequence, and were therefore not evaluated against the two peptidases.

The double β-amino acid analog PK-βA-4 demonstrated non-selective agonism in all of the bioassays—that is pupariation, pheromonotropic, melanotropic, and hindgut contractile assays. Notably, PK-βA-4 virtually matched the activity of the natural PK Lem-PK in the flesh fly pupariation bioassay, and was equipotent or more active than the parent peptide and Lem-PK at most tested doses in the pheromonotropic assay. It therefore represents a potent, biostable pupariation and pheromonotropic agonist in flesh fly larvae and *H. peltigera* female moths. Several previous examples exist of biostable analogs of insect neuropeptides that feature less potent intrinsic biological activity over natural peptides, but nonetheless, demonstrate enhanced *in vivo* activity [12,20,24]. Like the parent acylated, PK hexapeptide, this double-β analog elicited a mixture of both agonist and antagonist responses in the melanotropic assay of *S. littoralis* and pure agonistic pheromonotropic activity in *H. peltigera* (Table 4).

The analog PK-βA-3 is a non-selective agonist in all four of the PK/PBAN bioassays. Indeed, it matches the potency of the natural PK Lem-PK in the flesh fly pupariation bioassay. Unlike the acylated parent hexapeptide, this analog is a pure pheromonotropic and melanotropic agonist, as it fails to demonstrate statistically significant inhibition of any of the natural elicitors used in the *H. peltigera* or *S. littoralis* assays (Table 4). The analog PK-βA-2 demonstrates a potent agonist response in the *S. littoralis* melanotropic bioassays, even demonstrating full efficacy at a dose of 1 pmol, as is observed for the unmodified acylated, parent PK hexapeptide (Fig. 3 and Table 4). The related analog PK-βA-2 features a β^2-homo-Phe as a surrogate for Phe rather than the β^3-Phe in PK-βA-3. In comparison with Phe, additional methylene groups are located between the alpha carbon and the amino group, as well as between the alpha carbon and the side chain group in β^2-homo-Phe. Only one additional methylene group is found in β^3-Phe, located between the alpha carbon and the carboxyl group. As a result of the more extensive structural change, analog PK-βA-2 features a complex activity profile in comparison with PK-βA-3 in the four bioassays. Like PK-βA-3, PK-βA-2 demonstrates an agonist response in the hindgut contractile, melanotopic and pupariation assays, although is an order of magnitude less potent than PK-βA-3 in the latter bioassay. However, PK-βA-2 fails to demonstrate an agonist response in the pheromonotropic assay. And whereas PK-βA-3 is a pure agonist in the melanotropic assay, PK-βA-2 demonstrates a significant antagonist response against the elicitors PBAN and Pss-PT.

The structural modification in analog PK-βA-1 that led to strong resistance to peptidase attack by ACE and NEP also led to modifications of its agonist profile in the various assay systems, increasing its selectivity over the acylated parent hexapeptide and/or natural PK/PBANs. This analog shows only very weak activity in the pupariation assay, placing it at the margin between activity and inactivity. Unlike the parent hexapeptide, analog PK-βA-1 demonstrates no statistically significant agonist activity in the melanotropic assay (Fig. 2) and at a wide range of doses in the pheromonotropic activity with the exception of 1 nmol where its activity reached 68% of that of PBAN (Fig. 1). It does, however, demonstrate antagonist activity against elicitors Pss-PT and PBAN in the melanotropic assay and against PBAN in the pheromonotropic assay (Fig. 2). It therefore represents a non-selective biostable antagonist (Table 4).

The β-amino acid analogs described in this study provide new, and in some cases, biostable tools to endocrinologists studying similarities and differences in the mechanisms of the spectrum of PK/PBAN-regulated physiological functions in insects. They also may provide leads in the development of novel insect-specific, environmentally favorable pest management agents capable of disrupting PK/PBAN-regulated systems.

Acknowledgments

This research was supported by the US–Israel Binational Agricultural Research and Development Fund (BARD) (IS-3356-02) (MA, RJN). We also acknowledge financial assistance from the Texas Advanced Technology/Research Grant (#000517-0103-

2001) (HJW, RJN), and a grant from the USDA/DOD DWFP Initiative (#0500-32000-001-01R) (RJN, PZ).

References

[1] Abernathy RL, Nachman RJ, Teal PEA, Yamashita O, Tumlinson JH. Pheromonotropic activity of naturally-occurring pyrokinin insect neuropeptides (FXPRLamide) in *Helicoverpa zea*. Peptides 1995;16:215–9.

[2] Abernathy RL, Teal PEA, Meredith JA, Nachman RJ. Induction of moth sex pheromone production by topical application of an amphiphilic pseudopeptide mimic of pheromonotropic neuropeptides. Proc Natl Acad Sci USA 1996;93:2621–5.

[3] Altstein M. Role of neuropeptides in sex pheromone production in moths. Peptides 2004;25:1491–501.

[4] 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;30:307–19.

[5] 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.

[6] 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.

[7] Cheng RP, Gellman SH, DeGrado WF. Beta-peptides: from structure to function. Chem Rev 2001;101(10):3219–32.

[8] Cook BJ, Holman GM. Comparative pharmacological properties of muscle function in foregut and hindgut of cockroach *Leucophaea-maderae*. Comp Biochem Physiol C-Pharmacol Toxicol Endocrinol 1978;61:291–5.

[9] Cornell MJ, Williams TA, Lamango NS, Coates D, Corvol P, Soubier F, et al. Cloning and expression of an evolutionary conserved single-domain angiotensin converting enzyme from *Drosophila melanogaster*. J Biol Chem 1995;270:13613–9.

[10] 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.

[11] Gade G, Hoffmann KH, Spring JH. Hormonal regulation in insects: facts, gaps, and future directions. Physiol Rev 1997;77:963–1032.

[12] Garside CS, Nachman RJ, Tobe SS. Injection of dip-allatostatins or dip-allatostatin pseudopeptides into mated female *Diploptera punctata* inhibits endogenous rates of JH biosynthesis and basal oocyte growth. Insect Biochem Mol Biol 2000;30:703–10.

[13] Gregory H, Hardy PM, Jones PM, Kenner DS, Sheppard RC. Antral hormone gastrin. Structure of gastrin. Nature 1964;204:931–3.

[14] Holman GM, Cook BJ, Nachman RJ. Primary structure and synthesis of a blocked myotropic neuropeptide isolated from the cockroach, *Leucophaea-maderae*. Comp Biochem Physiol C-Pharmacol Toxicol Endocrinol 1986;85: 219–24.

[15] 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.

[16] Juaristi E, Soloshonok VA, editors. Enantioselective synthesis of β-amino acids. 2nd ed., New York: Wiley; 2005.

[17] Lamango NS, Sajid M, Isaac RE. The endopeptidase activity and the activation by Cl^- of angiotensin-converting enzyme is evolutionarily conserved: purification and properties of an angiotensin-converting enzyme from the housefly, *Musca domestica*. Biochem J 1996;314:639–46.

[18] Matsumoto S. 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;36:427–32.

[19] 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.

[20] Nachman RJ, Garside CS, Tobe SS. Hemolymph and tissue-bound peptidase-resistant analogs of the insect allatostatins. Peptides 1999;20:23–9.

[21] 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.

[22] Nachman RJ, Holman GM, Schoofs L, Yamashita O. Silkworm diapause induction activity of myotropic pyrokinin (Fxprlamide) insect neuropeptides. Peptides 1993;14:1043–8.

[23] Nachman RJ, Radel PA, Abernathy RL, Teal PEA, Holman GM. Mimetic analog development of the insect pyrokinin/PBAN/diapause induction (FXPRLamide) neuropeptide family. In: Suzuki A, Kataoka H, Matsumoto S, editors. Molecular mechanisms of insect metamorphosis and diapause. Tokyo: Industrial Publishing and Consulting, Inc.; 1995. p. 97–106.

[24] Nachman RJ, Strey A, Isaac E, Pryor N, Lopez JD, Deng JG, et al. Enhanced *in vivo* activity of peptidase-resistant analogs of the insect kinin neuropeptide family. Peptides 2002;23:735–45.

[25] Nachman RJ, Strey A, Zubrzak P, Zdarek J. A comparison of the pupariation acceleration activity of pyrokinin-like peptides native to the flesh fly: which peptide represents the primary pupariation factor? Peptides 2006;27:527–33.

[26] Nachman RJ, Teal PEA, Ujvary I. Comparative topical pheromonotropic activity of insect pyrokinin/PBAN amphiphilic analogs incorporating different fatty and/or cholic acid components. Peptides 2001;22:279–85.

[27] Nachman RJ, Teal PEA, Strey A. Enhanced oral availability/pheromonotropic activity of peptidase-resistant topical amphiphilic analogs of pyrokinin/PBAN insect neuropeptides. Peptides 2002;23:2035–43.

[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] Predel R, Nachman RJ. The FXPRLamide (pyrokinin/PBAN) peptide family. In: Kastin A, editor. Handbook of biologically active peptides. Amsterdam, The Netherlands: Elsevier; 2006. p. 207–13.

[30] Rafaeli A, Jurenka R. PBAN regulation of pheromone biosynthesis in female moths. In: Insect pheromone biochemistry and molecular biology. New York: Academic Press; 2003. pp. 107–36.

[31] Raina AK, Klun JA. Brain factor control of sex-pheromone production in the female corn-earworm moth. Science 1984;225:531–3.

[32] 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;244:796–8.

[33] Schoofs L, Holman GM, Hayes TK, Nachman RJ, Deloof A. Isolation, primary structure, and synthesis of locustapyrokinin—a myotropic peptide of *Locusta-migratoria*. Gen Comp Endocrinol 1991;81:97–104.

[34] Taneja-Bageshwar S, Strey A, Zubrzak P, Williams H, Reyes-Rangel G, Juaristi J, et al. Identification of selective and non-selective, biostable β-amino acid agonists of recombinant insect kinin receptors from the Southern cattle tick *Boophilus microplus* and mosquito *Aedes aegypti*. Peptides 2008;29:302–9.

[35] Xu WH, Denlinger DL. Molecular characterization of prothoracicotropic hormone and diapause hormone in *Heliothis virescens* during diapause, and a new role for diapause hormone. Insect Mol Biol 2003;12:509–16.

[36] Zd'arek J, Myska P, Zemek R, Nachman RJ. Mode of action of an insect neuropeptide leucopyrokinin (LPK) on pupariation in fleshfly (*Sarcophaga bullata*) larvae (Diptera: Sarcophagidae). J Insect Physiol 2002;48:951–9.

[37] Zubrzak P, Williams HJ, Coast GM, Isaac RE, Reyes-Rangel G, Juaristi E, et al. β-Amino acid analogs of an insect neuropeptide feature potent bioactivity and resistance to peptidase hydrolysis. Biopolymers (Pept Sci) 2007;88:76–82.