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Discovery of a linear lead antagonist to the insect pheromone biosynthesis activating neuropeptide (PBAN)☆

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Abstract

We report the discovery of a linear lead antagonist for the insect pheromone biosynthesis activating neuropeptide (PBAN) which inhibits sex pheromone biosynthesis in the female moth *Heliothis peltigera.* Two approaches have been used in attempting to convert PBAN agonists into antagonists. The first involved omission of the C-terminal amide and reduction of the sequence from the N-terminus in a linear library based on PBAN 1–33NH_2. The second involved replacement of L amino-acids by the D hydrophobic amino acid D-Phe in a linear library based on PBAN28–33NH_2. Screening of the two libraries for pheromonotropic antagonists resulted in the disclosure of one compound out of the D-Phe library (Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2) which inhibited sex pheromone production by 79 and 64% at 100 pmol in two moth colonies and exhibited low agonistic activity. Omission of the C-terminal amide in PBAN 1–33NH_2 and its shorter analogs did not lead to the discovery of an antagonistic compound.

Keywords: PBAN; Pheromone biosynthesis; Insect neuropeptides; *Heliothis peltigera*; Neuropeptide antagonists

1. Introduction

The pheromone biosynthesis activating neuropeptide (PBAN) is an important neuropeptide that mediates some of the key functions in insects. PBAN was first reported in 1984, as the neuropeptide that regulates sex pheromone production in female moths [51] and its amino acid sequence was revealed in 1989 from *Helicoverpa zea* (Hez-PBAN; PBAN 1–33NH_2) [52]. Since then, six other PBAN molecules have been isolated from five additional moth species, and their entire primary structures have been determined [9,16,29,32,33,42] and c-DNA and genes have been cloned [9,14,16,29–31,40]. PBAN molecules were found to be C-terminally amidated neuropeptides consisting of 33–34 amino acids, and comparison of their primary structures revealed that they share a high degree of homology and an identical pentapeptide C-terminal sequence (Phe-Ser-Pro-Arg-Leu-NH_2) which composes the active core required for its biologic activity [2,3,5,6,34,49,53,55]. Since 1984, the presence of PBAN-like activity has been demonstrated in a variety of moths as well as other non-Lepidopteran species, and its mode of action has been studied extensively (for review see [19,56]).

Further studies on the regulation of sex pheromone biosynthesis in moths have revealed that additional neuropeptides isolated from various insects, all of which share the common C-terminal pentapeptide of PBAN (Phe-Xxx-Pro-Arg-Leu-NH_2; Xxx = Ser, Gly, Thr, Val), have the ability to evoke sex pheromone biosynthesis upon injection into female moths [1,18,37,38,64]. Among these peptides are the pyrokinins (Lem-PK, Lom-PK-I and Lom-PK-II) and the locustamyotropins (Lom-MT-I to IV) (myotropic peptides isolated from the Madeira cockroach *Leucophaea maderae* (Fabricius) and the migratory locust, *Locusta migratoria* [47,63]; pheromonotropin (Pss-PT) an 18-amino acid peptide isolated from *Pseudaletia (Mythimna) separata* (Walker) [44] and diapause hormone (Bom-DH) isolated from the silkworm, *Bombyx mori* (L.) [28]. These peptides have recently been designated the pyrokinin/PBAN family. In addition to their ability to stimulate sex pheromone biosynthesis in moths, members of this family have been found to

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be responsible for a variety of other physiological and behavioral functions such as: melanization and reddish coloration in moth larvae [4,43], contraction of the locust oviduct [62], myotropic activity of the cockroach and locust hindgut [46,62], egg diapause in the silkworm [28] and acceleration of pupariation in the fleshfly *Sarcophaga bullata* (Parker) larvae [48].

Despite the intensive studies on the bio-activity of this family of peptides, very little is known about the endogenous mechanism and the structural, chemical and cellular basis of their activity. It is still not known which endogenous peptide(s) mediate(s) each of their functions in vivo, whether each function is mediated by a different peptide and whether each peptide mediates one or several functions. It is also not clear which receptors mediate these functions, what are the characteristics of these receptors, and whether the receptors share functional homologies. One way to get a better insight into the mode of action of this family of neuropeptides is by the use of antagonists.

At present, however, there is no defined method to obtain antagonists on the basis of a known neuropeptide agonist and to determine which conformation will lead to a highly potent inhibitory activity. Until now most of the peptide and non-peptide antagonists were discovered by serendipity [67]. Two general approaches have emerged for the discovery of lead antagonists: the first is based on structure-activity relationship (SAR) studies of agonistic compounds; the second on random screening of libraries or mixtures of naturally occurring compounds. The first method has led to the discovery of most peptidic antagonists and the second to the discovery of non-peptidic antagonists (for reviews see [26,27,67]).

A few empiric practices have emerged from the studies that led to the conversion of agonists to antagonists: (*i*) systematic replacement of the naturally occurring L-amino acids by their non-natural D isomers or replacement of amino acid residues with D hydrophobic amino acid residues, such as D-Phe or D-Trp (for review see [26,27] and below); (*ii*) omission of amino acid residues from agonistic sequences, or omission or replacement of functional side chains (e.g. [Sar^1]-Angiotensin II (1–7)amide where Asp^1 in angiotensin was replaced with Sar and Phe^8 was omitted and replaced by an amide group, or [D-Phe^6, Des-Met^{14}]-Bombesin (6–14)ethyl amide where the N-terminal six amino acids and Met^{14} were omitted) [13]; (*iii*) replacement of a C-terminal amide with a free acid (as in bombesin and gastrin) [39,59]; (*iv*) reduction of peptide bonds, as in the case of bombesin [12]; (*v*) conformational and/or topographical alteration (for review see [24,25,27] and [8,11]). Implementation of the above empiric practices for given agonists necessitates detailed knowledge of the SAR and, if possible, any information regarding the bioactive conformation.

The discovery of the entire amino acid sequence of PBAN 1–33NH_2 led to a considerable effort aimed at the elucidation of the neuropeptide's SAR [3–6,34,36–38,49, 53,55]. Detailed studies performed by many laboratories including ours, which used synthetic Hez-PBAN and shorter peptides derived from its sequence, revealed that: (*i*) the C-terminal amide in PBAN 1–33NH_2 is essential for bioactivity [2,3,32,36,54]; (*ii*) the C-terminal pentapeptide that is common to all members of the pyrokinin/PBAN family, comprises the active core required for biologic activity [34,36–38,46,47,49,53,55]; and (*iii*) the C-terminal hexapeptide sequence derived from Hez-PBAN (PBAN 28–33NH_2 - Tyr-Phe-Ser-Pro-Arg-Leu-NH_2) is as active as the full-length PBAN [3–6]. These findings provide the basis for our present study.

In the present study we exploited our knowledge of the SAR of PBAN, and used an in vivo pheromonotropic bioassay [20] (suitable for screening libraries for agonistic and antagonistic activities) for the design, synthesis and screening of two libraries of linear peptides in which antagonists were sought. The first library, based on PBAN 1–33$NH_{2,}$ involved omission of the C-terminal amide and reduction of the sequence from the N-terminus; the second library, based on PBAN28–33$NH_{2,}$ involved replacement of L amino-acids by the D hydrophobic amino acid D-Phe.

We report the successful application of the D-Phe scan approach for the disclosure of a PBAN antagonist capable of inhibiting sex pheromone biosynthesis in the female moth, *Heliothis peltigera*.

2. Materials and methods

2.1. Chemicals

Protected amino acids, Rink amide methylbenzhydrylamine (MBHA) resin and coupling reagents were purchased from Nova Biochem (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. Peptide synthesis

2.2.1. Synthesis of PBAN 1–33NH_2

PBAN 1–33NH_2 was synthesized on an ABI 433A automatic peptide synthesizer, starting from 0.455 g of Rink amide MBHA resin (loading 0.55 mmol/g) by means of the FastMoc^{TM} chemistry and the following derivatives of amino acids: Fmoc-Leu-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Pro-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Phe-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Met-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH. A 1.52 g aliquot of peptidyl resin was treated with 16 ml of cleavage mixture (tri-fluoroacetic acid -TFA: H_2O: thioanisole: ethanedithiol 3.6: 0.1: 0.2: 0.1) for 15 min at 0°C and for 2h and 45 min at room temperature. After filtration, the resin was washed

Table 1
Amino acid sequence and analytical data of PBAN-derived N-terminally blocked and free peptides.

Peptide	Amino acid sequence	MS (calc)	MS (found)	aa analysis
LP-1	H-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	781	781	0.84: 0.86: 1.00: 1.02: 1.02: 1.00
LP-2	Ac-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	824	824	1.17: 1.47: 0.87: 0.89: 0.87: 1.00
LP-3	H-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	937	937.2	0.82: 0.95: 0.98: 1.01: 1.78: 1.00
LP-4	Ac-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	979	979.2	0.87: 1.02: 0.94: 1.11: 1.75: 1.00
LP-5	Bz-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	1041	1041.3	0.86: 0.87: 1.02: 1.08: 2.02: 1.00
LP-6	Ad-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	1099	1099.4	0.88: 0.90: 0.95: 1.00: 1.93: 1.00
LP-7	$AdCH_2CO$-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	1114	1113.5	0.64: 0.93: 0.99: 1.10: 1.73: 1.00

Ac: acetyl; Bz: benzoyl; Ad: adamantoyl. $AdCH_2CO$:adamantyl-acetyl. Peptide purity was above 95%.

with TFA (3 × 3 ml) and the peptide was precipitated by the addition of 200 ml of cold ether to the TFA filtrate. The peptide was isolated by centrifugation, washed with cold ether (2 × 200 ml) and dried in vacuo. The crude material (0.82 g) was purified by semi-preparative RP-HPLC. The yield was 377.2 mg of PBAN (purity according to HPLC: 95%). MW (determined by mass spectrometry–MS): calculated: 3901.5 found: 1301.3(M/3), 1950.8(M/2). Amino acid analysis: Asp (5.56), Ser (2.64), Glu (5.35), Thr (1.89), Arg (2.35), Ala (1.89), Tyr (1.87), Pro (3.54), Met (1.23), Phe (1.18), Ile (1.04), Leu (2), Lys (1.24).

2.2.2. Synthesis of PBAN analogs with free carboxy terminus

Peptides were synthesized by the Simultaneous Multiple Peptide Synthesis (SMPS) "tea bags" methodology [23] on p-benzyloxybenzyl Alcohol Resin (Wang resin). Syntheses were carried out in 5.5 × 5.5-cm polypropylene bags placed in polypropylene boxes. Each peptide was synthesized on 0.2 g of Wang resin in each bag. After washing with *N*,*N*-dimethylformamide (DMF), the resin was treated with 5 equivalents of symmetrical anhydride, obtained from 10 equivalents of Fmoc-Leu-OH and 5 equivalents of diisopropylcarbodiimide (DIC) in DMF for 20 min at 0°C and for 20 min at room temperature. The reaction was continued for 2 h at room temperature, after which the resin was washed with DMF, and unreacted hydroxyl groups were acetylated with Ac_2O. After washing, the Fmoc-piperidine test indicated loading of 0.6 mmol/g. The peptides were synthesized by successive additions of a three-fold excess of each of the amino acid derivatives: Fmoc-Arg(Pmc)-OH, Fmoc-Pro-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Phe-OH, Fmoc-Tyr(^{t}Bu)-OH, a three-fold excess of bromo-tris-pyrrolidino-phosphonuim hexafluorophosphate (PyBroP) or 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylethyluronium-tetrafluorborate (TBTU) as coupling reagents and a six-fold excess of diisopropylethylamine (DIEA). The Fmoc group was deprotected by 20% piperidine in DMF (2 × 30 min). The resulting peptidylresin was washed with DCM and dried in vacuo. The peptidylresin from each bag (0.25 g) was treated separately with 2.5 ml of cleavage mixture (TFA: H_2O: thioanisole: ethandithiol 3.6: 0.1: 0.2: 0.1) as described above for PBAN. The crude peptide was purified by semi-preparative HPLC and the yield was 40 mg. Purity according to HPLC was found to be 90–95%. Acetylation of PBAN 28–33COOH was carried out on the resin with a fifty-fold excess of Ac_2O in DMF for 30 min at room temperature.

2.2.3. Synthesis of N-terminally blocked and free PBAN-derived peptides

Peptides (Tables 1 and 2) were synthesized by the SMPS "tea bags" methodology on Rink amide MBHA resin (loading 0.52 mmol/g) [23]. Each of the peptides was synthesized on 0.12 g of resin in every bag, using a six-fold excess of the amino acid derivatives: Fmoc-Leu-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-(D)Phe-OH, Fmoc-Tyr(^{t}Bu)-OH, and a six-fold ex-

Table 2
Amino acid sequence and analytical data of peptides of the D-Phe library.

Peptide	Amino acid sequence	MS (calc)	MS (found)	amino acid analysis
LA-1	H-D-Phe-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	911.2	911.3	0.45: 1.61: 1.07: 1.09: 0.84: 1.00
LA-2	H-Arg-D-Phe-Phe-Ser-Pro-Arg-Leu-NH_2	921.2	921.3	− : 1.87: 0.95: 1.34: 1.62: 1.00
LA-3	H-Arg-Tyr-D-Phe-Ser-Pro-Arg-Leu-NH_2	937.2	937.1	0.64: 0.93: 0.99: 1.10: 1.73: 1.00
LA-4	H-Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2	997.3	997.1	0.94: 1.30: − : 1.06: 2.08: 1.00
LA-5	H-Arg-Tyr-Phe-Ser-D-Phe-Arg-Leu-NH_2	987.2	987	0.72: 1.84: 0.91: − : 1.60: 1.00
LA-6	H-Arg-Tyr-Phe-Ser-Pro-D-Phe-Leu-NH_2	928.2	928.3	0.56: 1.78: 0.89: 0.75: 0.79: 1.00
LA-7	H-Arg-Tyr-Phe-Ser-Pro-Arg-D-Phe-NH_2	971.2	971.2	0.69: 1.99: 1.00: 0.97: 1.66: −

Peptide purity was above 95%.

cess of PyBroP or TBTU as coupling reagent, and a twelve-fold excess of DIEA in N-methylpyrrolidone (NMP). At the end of the peptide assembly the resin was dried in vacuo. For cleavage 150 mg of peptidylresin were treated with 1.5 ml of 90% TFA with scavengers as described above. The crude material (65–80% purity according to HPLC) was purified by semi-preparative HPLC, and yields of 20–40 mg were obtained. Purity according to HPLC was found to be 90–95%. Amino acid analysis and time of flight mass spectrometry (TOF-MS) data are shown in Tables 1 and 2.

Acetylation of peptides LP-2 and LP-4 was carried out on the resin with fifty-fold excess of Ac_2O in DMF for 30 min at room temperature. Acylation of peptides LP-5, LP-6 and LP-7 was carried out on the resin with 10 equivalents of benzoic acid (LP-5), 1-adamantanecarboxylic acid (LP-6) or 1-adamantaneacetic acid (LP-7), respectively and 10 equivalents of PyBroP and 20 eq. of DIEA in DMF for 2 h at room temperature.

2.3. Purification and characterization of peptides

2.3.1. Analytical HPLC

The purity of the peptides was assessed on a C18 (4.5 × 250 mm) column obtained from Vidac (USA) using the following gradient where A = acetonitrile (ACN) and B = H_2O (+0.1% TFA), 0–5 min 5%A + 95%B; 30 min 50%A + 50%B; 40–50 min 100%A.

2.3.2. Semi-preparative and preparative HPLC

Preparative and semi-preparative purification of crude peptides were performed by RP-HPLC on C18 (25 × 250 mm) and C8 (9 × 250 mm) columns, respectively, obtained from Merck (Darmstadt, Germany). Flow rates were 9 and 4.5 ml/min, respectively, for the preparative and semi-preparative purifications with the following gradient where A = ACN and B = H_2O (+0.1% TFA), 0–5 min 15%A + 85%B; 30 min 50%A + 50%B; 40–50 min 100%A.

The pure peptides (above 95%) were characterized by TOF-MS and electrospray mass spectrometry (ES-MS) and by amino acid analysis of hydrolysates.

2.3.3. Pheromonotropic bioassay

2.3.3.1. Insects *Heliothis peltigera* moths were reared on an artificial diet as described previously [15].

2.3.4. Determination of agonistic and antagonistic activities

Agonistic and antagonistic activities of the peptides were determined as described previously [7,21]. Agonistic activity was determined by injection of the tested peptide (at 10 or 100 pmol) into *H. peltigera* females and evaluation of the amount of sex pheromone generated in response to the injection. Females injected with 1 pmol PBAN1–33NH_2 served as a positive control and a reference for agonistic activity. Antagonistic activity was determined by monitoring the ability of the tested peptides (at 100 pmol) to inhibit sex pheromone biosynthesis elicited by 0.1, 0.5 or 1 pmol of synthetic PBAN1–33NH_2 (injected simultaneously with the tested peptide). Females injected with the same amounts of PBAN1–33NH_2 alone served as a reference for maximal stimulation and those injected with 0.1 M phosphate buffer served to determine the basal pheromone biosynthesis at photophase (which did not exceed 20 ng/female). Pheromone glands were excised 30 min or 2 h post injection, and sex pheromone was extracted and quantified by capillary gas chromatography as described previously [3,7]. All experiments were performed with 8 to 10 females per treatment.

2.4. Statistical analysis

The statistical analysis used was ANOVA. Differences among means were tested for significance by the Newman-Keuls test at $P < 0.01$.

3. Results

3.1. Activity of C-terminally free acid peptides

Studies in the first part involved examination of the ability of C-terminally free acid PBAN-derived peptides (truncated from the N-terminus) to inhibit the sex pheromone biosynthesis elicited by exogenously administered PBAN 1–33NH_2. The search for an antagonist requires first and foremost the delineation of agonistic activity. Therefore, the first set of experiments in this series involved analysis of the agonistic activity of these peptides. Peptides were synthesized and purified as described above, and tested by means of the pheromonotropic bioassay. The data revealed that the peptides were devoid of stimulatory biological activity (Table 3), strengthening the finding that the C-terminal amide is essential for agonistic bioactivity.

Examination of the ability of these peptides to inhibit sex pheromone biosynthesis elicited by exogenously administered PBAN 1–33NH_2 revealed that, despite the lack of agonistic activity, the peptides did not display a significant antagonistic activity (Table 3). The only peptide that exhibited a slight inhibitory effect was PBAN 9–18COOH (Table 3). Analysis of the inhibitory activity of this peptide on another colony of the same species and on another moth species (*H. armigera*) revealed that the peptide was unable to effectively block sex pheromone production.

3.2. Activity of N-terminally free and N-terminally blocked peptides

Since the above group of peptides was devoid of inhibitory activity, we decided to apply a different strategy for the discovery of antagonists; this strategy was based on D-Phe scan namely, generation of a linear library (termed D-Phe library) based on a parent sequence in which each

Table 3
Pheromonotropic agonistic and antagonistic activities of various C-terminally free acid PBAN-derived peptides.

Structure	Dose (pmol)	Agonistic activity (%)	Antagonistic activity (%)
PBAN 1-33COOH	10	1	0
	100	1	9
PBAN 9-33COOH	10	3	7
	100	12	37
PBAN 19-33COOH	10	2	27
	100	1	n.t.
PBAN 28-33COOH	10	n.t.	n.t.
	100	4	16
Ac-PBAN 28-33COOH	10	n.t.	n.t.
	100	3	0
PBAN 9-18COOH	10	0	0
	100	5	40

Agonistic activity was determined by injection of the individual peptides into *H. peltigera* female moths. Glands were excised 2 h post-injection and pheromone content was determined by capillary gas chromatography as described in Materials and Methods. Agonistic activity is expressed as the ratio (in percentage) between the sex pheromone content elicuted by a tested peptide, at the indicated dose, and PBAN 1-33NH_2 (at 1 pmol). The amount of pheromone evoked by PBAN 1-33NH_2 was 111 ± 8 (n = 11) ng/female and was defined as 100%. Antagonistic activity was determined by injection of each of the peptides (at the indicated doses) together with 0.1, 0.5 or 1 pmol PBAN 1-33NH_2 for 2 h. The antagonistic activity is expressed as 100 minus the ratio (as a percentage) between the pheromone content elicited in the gland by the injection of PBAN 1-33NH_2 in the presence and absence of each of the peptides. Pheromone content was monitored in 9-10 females for each of the tested peptides. Ac: acetyl.

Table 4
Agonistic activity of N-terminally blocked and free PBAN-derived peptides

Peptide	Activity (ng/female)	Activity (%)
PBAN 1-33NH_2	50 ± 5 (n = 19)[a]	100
H-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	43 ± 8 (n = 8)[a]	102
Ac-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	53 ± 5 (n = 10)[a]	126
H-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	35 ± 5 (n = 9)[a]	83
Ac-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	60 ± 7 (n = 10)[a]	105
Bz-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	73 ± 14 (n= 9)[a]	128
Ad-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	55 ± 5 (n = 10)[a]	96
AdCH_2CO-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2	52 ± 8 (n = 10)[a]	91

Activity was determined by injection of the individual peptides at a dose of 100 pmol into *H. peltigera* female moths. Glands were excised 30 min post-injection and pheromone content was determined by capillary gas chromatography as described in the Materials and Methods. Activity is expressed in ng/female ± SEM and as the ratio (in percentage) between the amount of pheromone that was elicited by a given peptide and the amount elicited by the injection of 1 pmol PBAN 1-33NH_2 (defined as 100%). Differences between means were tested for significance at $p < 0.01$. Means with the same letter do not differ significantly.

Ac: acetyl; Bz: benzoyl; Ad: adamantoyl; AdCH_2CO: adanantyl-acetyl.

amino acid is replaced with D-Phe (see Table 2). PBAN 28–33NH_2, which was found to constitute the active site of PBAN, and which exhibited the same activity as the full-length molecule [3–5], was chosen as a parent sequence. Two major considerations had to be taken into account prior to the synthesis of the D-Phe library and the evaluation of the inhibitory activity of the peptides: (*i*) their linear nature, which makes them susceptible to proteolytic enzymes that originate from the hemolymph or from tissues damaged in the course of the injection; and (*ii*) introduction of a D-Phe residue which may reduce their solubility. In order to test the effects of these two factors we performed a series of preliminary experiments. In the first set we examined whether the introduction of a basic amino acid such as Arg (which increases solubility) modifies the activity of PBAN 28–33 NH_2. In the second set we tested whether the introduction of an N-terminally blocking group (which increases metabolic stability) had a pronounced protective effect against possible degradation. Experiments in this part included blockage of the N-terminus with groups such as acetyl, benzoyl, adamantoyl and adamantyl-acetyl.

As indicated in Table 4, addition of Arg at the N-terminus of PBAN 28–33NH_2 did not affect the bioactivity, and the amount of sex pheromone produced following this modification did not differ significantly from that produced by unmodified PBAN 28–33NH_2. Introduction of the N-terminally blocking groups did not have any protective effect, and the bioactivity did not differ significantly from that of PBAN 28–33NH_2 or that of [Arg^{27}]PBAN 28–33NH_2. It is interesting to note that in a study performed by Kuniyoshi et al. [37] N-terminal blockage of PBAN 28–33NH_2 resulted in a marked potentiation in activity. Comparison of the bioactivities of the C-terminally derived hexa- and heptapeptides (in our study) with that of the PBAN 1–33NH_2 revealed that all peptides exhibited activities that did not differ significantly from that of the full length peptide (Table 4). These results confirm our previous observations of equipotency in the activities of PBAN 28–33NH_2 and PBAN 1–33NH_2 [3] and hint at the possibility that PBAN 28–33NH_2 and [Arg^{27}]PBAN 28–33NH_2 may not be very susceptible to proteolytic degradation (by aminopeptidases in this case). A similar conclusion has already been drawn by us on the basis of a previous study which compared the biologic activities of PBAN 28–33NH_2 in the presence and absence of protease inhibitors [3]. In the light of the above results we have decided that in all further studies we will include Arg at position 27 in the peptide to ensure higher solubility, without any modifications at the N-terminus.

3.3. Activity of the D-Phe scan peptides Library

The D-Phe library contained seven different peptides (Table 2). Peptides were synthesized, purified and characterized as described above, and their agonistic and antagonistic activities were tested with the in vivo pheromonotropic bioassay. As indicated in Table 5, three peptides (LA-4, LA-5 and LA-7, in Colony I) out of the seven showed profound antagonistic activity at 100 pmol, with LA-4 being the most effective peptide, inhibiting pheromonotropic activity by 79%.

Table 5
Pheromonotropic antagonistic activity of peptides derived from the D-Phe sean.

Peptide	Antagonistic activity (%)	
	Colony I	Colony II
LA-1	0	15
LA-2	0	0
LA-3	0	18
LA-4	79	64
LA-5	56	53
LA-6	0	65
LA-7	58	45

Experimental details were essentially as described in the legend to Table 3. Peptides were injected for 2 h. Antagonistic activity was determined by injection of each of the peptides (at 100 pmol) together with 0.1 (Colony II) or 0.3 (Colony I) pmol PBAN 1-33NH_2 for 2 h. The antagonistic activity is expressed as 100 minus the ratio (as a percentage) between the pheromone content elicited in the gland by the injection of PBAN1-33NH_2 in the presence and absence of each of the peptides. The amounts of sex pheromone elicited by 0.1 and 0.3 pmol PBAN1-33NH_2 were 86 $\pm$ 24 (n = 10) and 367 $\pm$ 22 (n = 9) ng/female, respectively, and were defined as 100%. Pheromone content was monitored in 9-10 females for each peptide.

Table 6
Agonistic activity of the D-Phe peptides.

Peptide	Pheromone (ng)	Activity (%) Compared with PBAN 1-33NH_2
PBAN 1-33NH_2	235 $\pm$ 27 (n = 9)[a]	100
Arg[27][PBAN 28-33NH_2]	49 $\pm$ 9 (n = 9)[b]	21
LA-1	99 $\pm$ 19 (n = 10)[b]	42
LA-2	291 $\pm$ 36 (n = 8)[a]	124
LA-3	226 $\pm$ 59 (n = 10)[a]	96
LA-4	61 $\pm$ 24 (n = 10)[b]	26
LA-5	142 $\pm$ 57 (n = 9)[a]	60
LA-6	13 $\pm$ 8 (n = 10)[b]	6
LA-7	57 $\pm$ 20 (n = 10)[b]	24

Activity was determined by injection of the individual peptides at a dose of 100 pmol into *H. peltigera* female moths of Colony I. Glands were excised 2 h post-injection. All other details are as described in the legend to Table 3. Agonistic activity is expressed as the ratio (in percentage) between the sex pheromone content elicited by the injected peptide and that elicited by 1 pmol PBAN 1-33NH_2 (defined as 100%). Differences between means were tested for significance at $p < 0.01$. Means with the same letter do not differ significantly.

In order to prove that the results were not specific to a particular moth colony which had been reared in the laboratory for several years (Colony I), the experiment was repeated with another colony (Colony II) of the same moth species, for which the moths had been collected from the wild a few month prior to analysis. The antagonistic activities of most of the peptides were similar in both colonies except for peptide LA-6, that exhibited antagonistic activity in Colony II but not in Colony I.

Antagonists may be devoid of agonistic activity, or they may exhibit full or partial agonistic activity. Since all of the peptides tested in the present study were derived from the putative active site of PBAN, the D-Phe peptides were tested for their agonistic activity at the same concentration and for the same time as had been used to assess their antagonistic activity (100 pmol and 2 h). As indicated in Table 6 most peptides exhibited agonistic activity; the only ones that exhibited low agonistic activity were LA-4 (26%), LA-6 (6%) and LA-7 (24%), which also exhibited high antagonistic activities (Table 5). A similar trend was found when agonistic activity was tested over a shorter time (30 min) during which most PBAN C-terminal derived peptides are active [3].

The fact that some peptides exhibited mixed activities (i.e. high antagonistic and low agonistic activities) indicates that they are partial agonists. A simple explanation by Hruby [27] is that the peptide binds to its receptor both in the agonistic conformation that partially activates the receptor and in the "non-activating" antagonistic conformation, which does not enable further activation of secondary messengers. Partial agonistic or antagonistic activity, especially among linear peptides in which the antagonist is based on the structure of an agonist, is a well known phenomenon and has been widely documented (for review see [27]).

It is interesting to note that some of the peptides (LA-2, LA-3 and LA-5) exhibited high agonistic activities which did not differ significantly from that of PBAN 1–33NH_2 under conditions where C-terminal derived peptides usually have a very low activity (e.g. 2 h post injection) [3]. These activities were also significantly higher than that of the parent peptide [Arg[27]]PBAN 28–33NH_2. It is possible that replacement of Tyr[28], Phe[29] and Pro[31] with D-Phe resulted in a stereo-conformational change that confers to the molecule a higher affinity to the receptor. Similar results were obtained by Raina and Kempe [55] with PBAN 28–33NH_2, where replacement of Phe[29] with D-Phe increased the activity, and with several mammalian neuropeptides (e.g. luteinizing hormone-releasing hormone-LHRH, enkephalin and somatostatin), where replacement of L-amino acid residues with D-amino-acid residues at appropriate sites caused a considerable increase in potency (for review see [45]). Other substitutions with D-amino acids in PBAN 28–33NH_2 (e.g. D-Tyr[28], D-Tyr[29], D-Arg[32] and D-Leu[33]) [55] and D-Ala[31] or D-Pro[31] [36] resulted in a complete loss of agonistic activity.

In the light of the high antagonistic and low agonistic activities of LA-4 (H-Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2) we have chosen this peptide as a potential lead antagonist for further optimization of PBAN antagonists (see below and [7,21]). Peptide LA-6 was not chosen for this purpose (although its agonistic activity was slightly lower than that of LA-4) because of the variability it exhibited between the two moth colonies.

4. Discussion

The use of D-amino acids to convert agonists to antagonists has previously been applied to mammalian peptides such as LHRH [57,65], substance P (SP) and other neurokinins [17,50,60] vasopressin [61], bombesin [22], bradykinin [58,66], endothelin [10], enkephalin [11] and parathyroid hormone [41]. Recently, this approach was applied to the insect neuropeptide proctolin and resulted in the discovery of a few peptides with antagonistic activity [35].

Modifications at the C-terminus have also been used for the generation of antagonists, e.g. gastrin and bombesin [39,59], although this approach is less common than the use of D-amino acid scans. To the best of our knowledge, this approach has not been applied to insect neuropeptides for the discovery of antagonists.

Availability of agonists or antagonists is of major importance in the study of neuropeptides in general, and of the pyrokinin/PBAN family of neuropeptides in particular. The pyrokinin/PBAN family of peptides is involved in the regulation of critical reproductive, developmental and digestive processes (e.g. sex pheromone biosynthesis, cuticular melanization, myotropic activity, oviposition, pupariation and diapause) in moths and other insects (for review, see [19]) and exhibits considerable functional, and inter- and intraspecific cross reactivity [1,2,18,19,37,38,52,64]. The availability of antagonists for this family of neuropeptides, together with the availability of bioassays that were developed for each of the above-mentioned functions [3,4,28,48,63] open the way for a better understanding of the endogenous mechanisms of this group of peptides and provide tools for the discovery of additional functions mediated by the pyrokinin/PBAN neuropeptides in moths and other insects.

The fact that the D-Phe library, through which the lead antagonist, LA-4 was discovered, is based on the amino acid sequence that is common to all members of the pyrokinin/PBAN family suggests that, in addition to sex pheromone biosynthesis, this peptide (or other members of this library) may antagonize other functions mediated by this family of neuropeptides, and thus help in resolving the above issues.

The discovery of a lead antagonist is also important from another point of view, because this compound may be able to serve as an excellent lead compound for the design of further improved antagonists (e.g. conformationally constrained, highly selective and metabolically stable). Such improved and selective antagonists might provide further information on the endogenous peptides and their in vivo activities, shed light on the receptor(s) that mediate these functions, and correlate the various pyrokinin/PBAN peptides with their physiological functions in moths and other insects. Indeed, the LA-4 linear lead antagonist was used as a basis for the design and generation of conformationally constrained libraries out of which selective and metabolically stable backbone cyclic (BBC) antagonists, which inhibit cuticular melanization in *Spodoptera littoralis* and sex pheromone biosynthesis in *H. peltigera* female moths evoked by exogenously administered PBAN 1–33NH_2 or by the endogenous mechanism, were discovered [7,21]. Beyond the immediate benefits stemming from the use of such selective BBC peptides as antagonists, these compounds may serve as a basis for the design of further improved non-peptide mimetic compounds for agrochemical applications. Such compounds could serve, after formulation and preliminary field experiments, as prototypes for the development of a group of novel highly effective, insect-specific and environmentally friendly insecticides.

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