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A novel dihydroimidazoline, trans-Pro mimetic analog is a selective PK/PBAN agonist

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1. ABSTRACT

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family plays a significant role in the regulation of reproductive and developmental processes in a variety of insects. A *trans*Pro, type I beta-turn has been previously identified as important for the activity of PK/PBAN peptides. A PK/PBAN analog (**PPK-Jo**) incorporating a novel dihydroimidazole transPro mimetic motif was evaluated in four PK/PBAN bioassays (pheromonotropic, melanotropic, pupariation and hindgut myotropic). **PPK-Jo** proved to be a pure, selective melanotropic agonist in *S. littoralis*. The melanotropic receptor in *S. littoralis* demonstrates more tolerance to deviations from the ideal *trans*Pro structure than those of other PK/PBAN assays. The selective PK/PBAN agonist represents a new tool to better understand the endogenous mechanisms of these peptides and serves as a probe of the plasticity of PK/PBAN regulated systems and receptors. The dihydroimidazoline moiety is shown to function as a surrogate for a *trans*Pro in certain circumstances, and provides a novel scaffold with which to construct mimetic PK/PBAN analogs with enhanced selectivity and the potential to disrupt critical physiological processes in insect pests.

2. INTRODUCTION

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) peptides represent a multifunctional family that plays a significant role in the physiology of insects. Leucopyrokinin (LPK), isolated from the cockroach *Leucophaea maderae* in 1986 (1), was the first member of the family to be discovered. Since that time, over 30 peptides have been identified. They include PKs, myotropins (MTs), PBAN, melanization and reddish coloration hormone (MRCH), diapause hormone (DH), pheromonotropin (PT), all of which share the common C-terminal pentapeptide FXPRL-amide (X=S, T, G or V) (2-4). Functions of the PK/PBAN family include stimulation of sex pheromone biosynthesis in moths (1-6), and mediation of key aspects of feeding (gut muscle contractions) (7), development (embryonic diapause, pupal diapause and pupariation) (8-14) and defense (melanin biosynthesis) (15,16) in a variety of insects (cockroaches, flies, locusts and moths). All of the above functions can be stimulated by more than one peptide, and they demonstrate considerable cross-activity between various PK/PBAN assays, thereby lacking any species-specific behavior (3,4,17,18). The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by

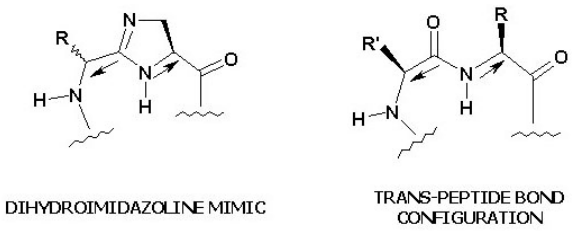

Figure 1. Comparison of the *trans*-peptide bond (right) and the dihydroimidazoline ('Jones') mimetic motif. A '*trans'* orientation is frozen within the five-membered ring structure.

which these neuropeptides elicit their effects. Selective agonists and/or antagonists of the PK/PBAN family can shed light on this issue.

In previous work, a highly rigid cyclic PK/PBAN analog *cyclo* (Asn[1])LPK (*cyclo* (NTSFTPTL)), featuring a *trans*Pro, type I beta-turn, was determined to retain significant bioactivity in several PK/PBAN bioassays, including hindgut contractile (cockroach *Leucophaea maderae*) (19), pheromonotropic (silk worm *Bombyx mori*) (20), oviduct contractile (cockroach *Leucophaea maderae*) (21), egg diapause induction (silk worm *Bombyx mori*) (21), and pupariation (flesh fly *Neobelieria bullata*) (22) assay systems. These results are consistent with the suggestion that a *trans* oriented Pro and the type I beta-turn structure holds broad significance for many physiological functions elicited by the PK/PBAN family of peptides.

In this manuscript, we first propose the dihydroimidazoline moiety (23,24) as a suitable mimic for a *trans* peptide bond (Figure 1), and specifically a *trans*Pro. This moiety has been reported by Jones *et al.* as a peptide bond isostere (23), but not specifically a mimic of a *trans* peptide bond. Second, we evaluate a PK/PBAN analog Ac-YF (Jo)RLa (PPK-Jo: PBAN Pyrokinin – Jones moiety) for its ability to elicit and/or inhibit pheromone biosynthesis in the moth *Heliothis peltigera* and melanization in the Egyptian cotton leaf worm *Spodoptera littoralis*. The ability of PPK-Jo to elicit other functions mediated by the PK/PBAN family: pupariation in the fleshfly, *Neobellieria bullata* and hindgut contraction in the cockroach *Leucophaea maderae* were examined as well. These evaluations were undertaken in an effort to determine whether differences could be detected between the PK/PBAN receptors associated with the four bioassay systems for tolerance to any variance from the natural *trans*Pro conformation; i.e., whether the analog could demonstrate selectivity between the related receptors. The development of selective analogs can aid in the study of the plasticity of PK/PBAN mediated systems and their associated receptors. The study further evaluates whether the dihydroimidazoline moiety could represent a novel scaffold with which to design pseudopeptide and/or nonpeptide PK/PBAN mimetic analogs that may disrupt critical PK/PBAN processes in pest insects.

3. MATERIALS AND METHODS

3.1 Insects

S. littoralis larvae were kept in groups of 100-200 insects in plastic containers (40 x 30 x 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 (25). 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 (25). All females used in this study were 3.5 days old.

Larvae of the fleshfly, *N. bullata* were reared in batches of 200-300 specimens on beef liver in small open disposable packets made from aluminum foil as described (26). Fully-grown larvae that left the food were allowed to wander in dry sawdust until the first puparia appeared 36 to 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.

Leucophaea 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* (1).

3.2. Peptide and pseudopeptide analog synthesis and purification

3.2.1. Synthesis of PBAN 1-33NH_2, PT, LPK, MT and YFTPRLa

Hez-PBAN (4) was synthesized on an ABI 433A automatic peptide synthesizer on Rink amide 4-methylbenzhydrylamine (MBHA) resin by means of the FastMoc™ chemistry as described previously (12). Syntheses of *Pseudaletia (Mythimna) separata* Pss-PT (11), *Leucophaea maderae* (Lem-LPK) (1), and *Locusta migratoria* (Lom-MT-II) (7) and the PK/PBAN fragment-analog YFTPRLa 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) (12,27) 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.

3.2.2. PPK-Jo analog synthesis and purification

Synthesis of the analog **PPK-Jo** (Ac-YF (**Jo**)RLa) was accomplished by adapting the previously

described solution-phase synthesis of Jones *et al.* (23) to a new solid phase strategy using RinkAmide resin (Kaczmarek *et al.*, unpublished data) 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 (28). Details of the solid phase strategy will be described elsewhere.

3.3. Pheromonotropic bioassay

The pheromonotropic bioassay was performed with *H. peltigera* as described previously (2). Briefly, adult females were injected in the abdomen with 10 ul double distilled water (DDW) or the test peptides. Stimulatory (e.g., agonistic) activity of the **PPK-Jo** and the LPK derived parent peptide 1323 was determined by monitoring their ability to induce sex pheromone biosynthesis at 1, 10, 100 pmol and 1 nmol. Females injected with 1 pmol PBAN served as a reference for stimulatory activity. Antagonistic activity was determined by monitoring the ability of the **PPK-Jo** at 100 pmol and 1 nmol to inhibit sex pheromone biosynthesis that was elicited by PBAN, PT, LPK and MT at 1, 1, 10 and 30 pmol, respectively. 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 (2). Briefly, samples were analyzed by an HP 6890 gas chromatograph using a 30 m x 0.25 mm RTX-5 column (Restek, Belle Fonte, PA). Samples were injected in the splitless mode and detected by an FID detector. The temperature of the injector and detector was 220°C. The column was kept at 60°C for 2 min and then programmed at a rate of 15°C/min to 155°C, and held at this temperature for 20 min, and subsequently programmed at a rate of 10°C/min to 200°C and held at this temperature for 10 min. Quantification of peaks was performed by an HP integrator and pheromone content was monitored with the most abundant component, (Z)-11-hexadecenal (Z11-16-Ald). All experiments were performed with 8-10 females per treatment.

3.4. Melanotropic bioassay

The melanotropic bioassay was performed as described previously (15). Briefly, larvae were injected with 10 ul DDW, or the test compounds dissolved in DDW. After injection larvae were kept at 25°C for 18-20 h, the cuticle was stripped off and scanned with an Astra 1220S scanner (Umax, Germany). Scanned images were evaluated by computerized image analysis using TINA (version 2.10g) software (Raytest, Germany). Melanotropic stimulatory activity of the **PPK-Jo** or the LPK derived parent peptide 1323 was determined by evaluating their ability to induce cuticular melanization in larvae at 1, 10, 100 pmol and 1 nmol. Larvae injected with 5 pmol PBAN served as a reference for stimulatory activity. Antagonistic activity was determined by monitoring the ability of the **PPK-Jo** and 1323 (at 100 pmol and 1 nmol), injected together with the elicitors PBAN, 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.

3.5. Pupariation bioassay

The test was performed as described by Žďárek (26). 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 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 to 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 LPK and/or **PPK-Jo** 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 ul. The definition of a 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.

3.6. Myotropic bioassay

Hindguts of adult *L. maderae* cockroaches were isolated from the central nervous system (CNS) and dissected (1), suspended in a 5 ml chamber, and prepared for recording as previously described (28). Threshold concentrations are determined by adding a known quantity of analog (dissolved in 0.5 ml of bioassay saline) (1) to the bioassay chamber containing the hindgut. The threshold concentration is 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 would be 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 **PPK-Jo** analog at a concentration

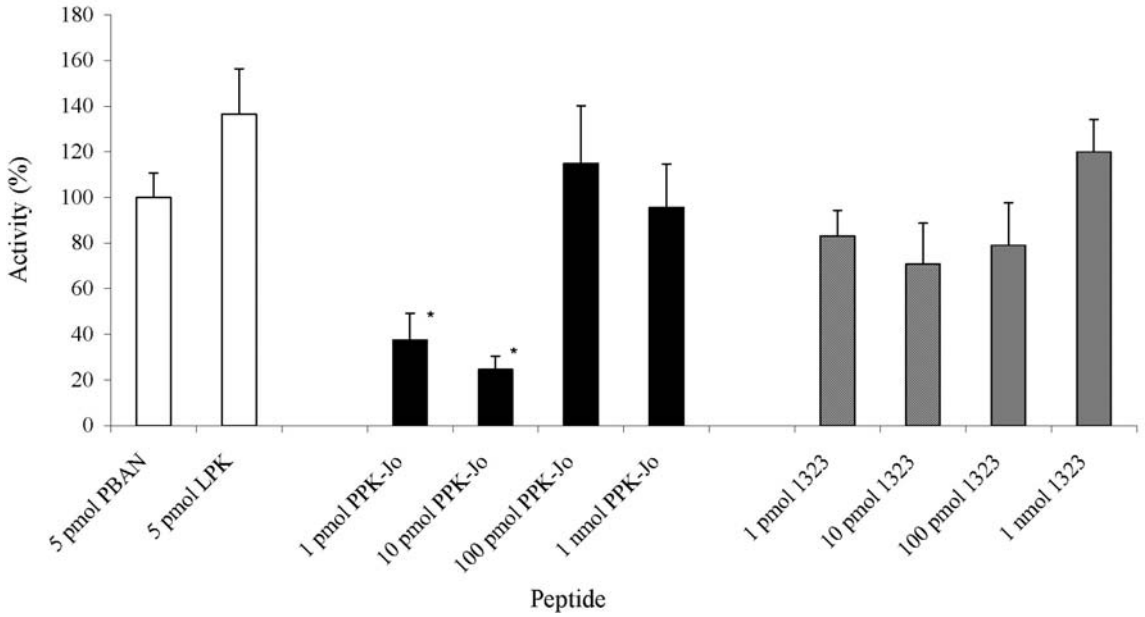

Figure 2. *In vivo* dose-response agonist melanotropic activity of **PPK-Jo**, the parent peptide 1323, PBAN and LPK in *S. littoralis* larvae. Activity is expressed as the ratio (as a percentage) between the extents of melanization elicited by the injection of each of the peptides at the listed doses and by PBAN at 5 pmol, (defined as 100%) ± SEM of 8 - 10 samples. Statistical analysis compared differences between the melanotropic agonistic activities obtained with a given peptide and PBAN. An asterisk (*) indicates a significant difference in activity at $P < 0.05$.

of 5 x 10^{-6} M (25 nmol) to determine if it could inhibit the initial response.

3.7. Statistical analysis

The results of the pheromonotropic and melanotropic 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 $P < 0.05$. All statistical analyses were calculated using JMP version 5.1.2, ©2004, SAS Institute Inc. Cary, NC, USA.

3.8. Molecular Modeling

Analogs were prepared for modeling on Sybyl Tripos 6.3 software, using the Biopolymers module (Tripos Associates Inc., MO, USA) by modification of an appropriate amino acid, addition of Geistiger-Huckel charges, and simulated annealing using Tripos Force Field on a Silicon Graphics O2 computer. For visual comparison, four corresponding backbone atoms in the turn region of the lowest energy form of each compound were superimposed, leaving side chain atoms and atoms from other amino acids free. In the circled portion of the computer graphics illustration is a superposition of a *trans*-peptide bond preceding Pro (green) with the dihydroimidazoline ('Jones') motif (red) that indicates it is closely aligned (Figure 4).

4. RESULTS

4.1. Pheromonotropic bioassay

The results of an evaluation of **PPK-Jo** in the *in vivo* pheromonotropic assay in *H. peltigera* indicate that it failed to demonstrate any statistically significant activity even up to a dose of 1 nmol. Under the experimental conditions, 1 pmol of PBAN demonstrated a pheromonotropic response of 118 ng (considered as 100%). Even the parent PK/PBAN hexapeptide YFTPRLa (1323) elicited significant pheromonotropic responses at 100 pmol and 1 nmol (95 and 108 ng, respectively), attaining a response that was not statistically different from that of PBAN. At 1 nmol, **PPK-Jo** failed to show any antagonism of the pheromonotropic activity elicited by 1 pmol of PBAN, 1 pmol PT, 10 pmol MT or by 30 pmol LPK.

4.2. Melanotropic bioassay

Unlike in the pheromonotropic assay, the analog **PPK-Jo** demonstrated a strong agonist response in the melanotropic assay in the Egyptian cotton leaf worm *S. littoralis*, eliciting a response of 115 and 96% of that of 5 pmol PBAN at doses of 100 pmol and 1 nmol, respectively. The activity of the peptide at those doses did not differ significantly from that of the 5 pmol PBAN or LPK (Figure 2). Almost no activity was observed at the smaller doses of 1 and 10 pmol (Figure 2). The parent PK/PBAN hexapeptide YFTPRLa (1323) elicited a high response at all tested doses (1, 10 and 100 pmol and 1 nmol) exhibiting an activity of 83, 71, 79 and 120%, respectively compared to that of 5 pmol PBAN. The activity at all tested doses did not differ from that of 5 pmol PBAN or that of of 5 pmol LPK. However, YFTPRLa inhibited the melanotropic activity of 5 pmol PBAN or 5 pmol PT by a statistically significant 82% and 47%, respectively at 100 pmol, and by 82% and 87%, respectively at 1 nmol (Figure 3). Whereas analog **PPK-Jo** did not antagonize PBAN or any other elicitors (PT or LPK) even up to a dose of 1 nmol. Thus,

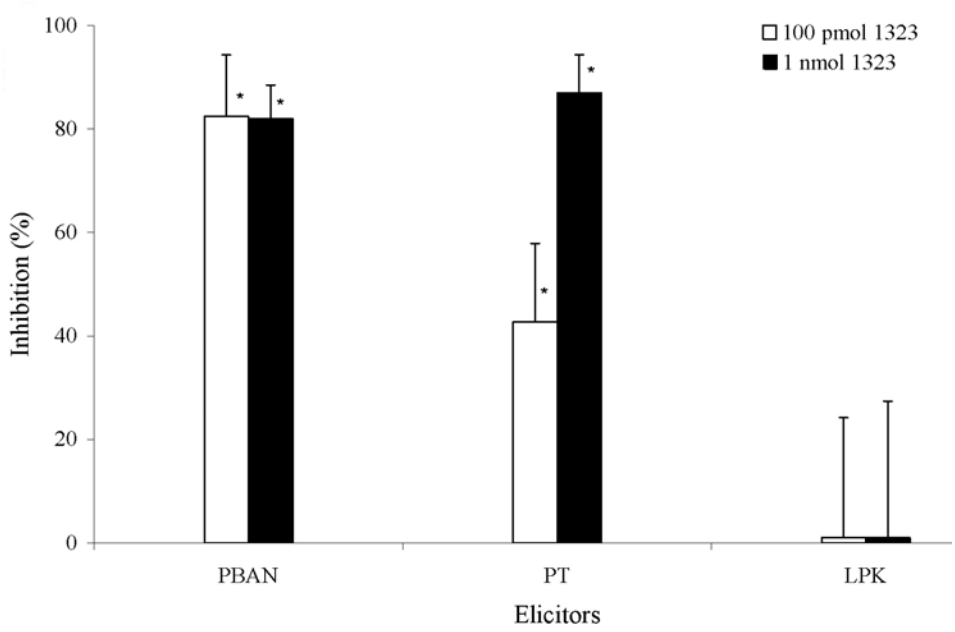

Figure 3. *In vivo* inhibition of melanin formation elicited by PBAN, PT and LPK (at 5, 5 and 15 pmol, respectively) by 100 and 1,000 pmol of the parent LPK analog 1323**,** in *S. littoralis* larvae. Antagonistic activity (i.e., inhibition) is expressed as 100 minus the ratio (as percentage) between the extent of melanization elicited in the presence and absence of the tested peptides ± SEM of 9-10 samples. Statistical analysis compared the degree of melanization obtained with PBAN 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.

unlike the parent PK/PBAN hexapeptide YFTPRLa, which is a non selective (e.g. capable of inhibiting both the pheromonotropic and melanotropic activities) mixed (e.g. having a mixed agonistic and antagonistic activity) compound, **PPK-Jo** is a pure selective agonist for the melanotropic assay, and is furthermore selective for this assay as it demonstrates no agonist activity in the other PK/PBAN assays as indicated below.

4.3. Pupariation and myotropic bioassays

Evaluation of **PPK-Jo** in the *in vivo* pupariation assay in the flesh fly *Neobellieria (Sarcophaga) bullata* indicated that it elicited no significant activity even up to a dose of 500 pmol. By comparison, both the natural PK/PBAN analog LPK (pQTSFTPRLa), and its C-terminal pentapeptide FTPRLa, demonstrated activity at a threshold dose of 0.3 pmol. In the *in vitro* cockroach (*Leucophaea maderae*) hindgut contractile assay, analog **PPK-Jo** was inactive even up to a tested concentration of 5×10^{-6} M (25 nmol). By comparison, the native PK/PBAN analog LPK (pQTSFTPRLa), and its C-terminal pentapeptide FTPRLa, demonstrated activity threshold concentrations of 1×10^{-9} (5 pmol) and 2×10^{-9} M (10 pmol), respectively. Analog **PPK-Jo** failed to exhibit any antagonist response in both the *in vivo* pupariation assay up to a dose of 500 pmol and in the *in vitro* hindgut contractile assays up to a concentration of 5×10^{-6} M.

4. DISCUSSION

The PK/PBAN family of neuropeptides elicits a broad array of physiological activities in insect bioassays, including pheromonotropic (1-5), cuticular melanization (15,16), pupariation (26), and hindgut myotropic (1,28) in a variety of insects. In each of these assays, the active core, the minimum sequence capable of eliciting significant activity, has been identified as the C-terminal pentapeptide FXPRLa (X = S,T,G or V) that defines the PK/PBAN family (29-34).

A conformational study of the rigid, cyclic PK/PBAN analog *cyclo* (NTSFTPRL) (*cyclo* (Asn^1)LPK) was previously carried out in aqueous solution containing no organic solvents using a combination of NMR spectroscopic and molecular dynamics (19,20). The specific conformation of this constrained, cyclic analog in aqueous solution was shown to be extremely rigid, featuring a *trans*-oriented Pro (specifically, a *trans* peptide bond preceding the Pro residue) in the second position of a type-I beta-turn over residues Thr-Pro-Arg-Leu within the active core region. The very large (for Thr-2, Thr-5, and Leu-8) and very small (for Ser-3 and Arg-7) coupling constants found indicated that the backbone of *cyclo* (Asn^1)LPK was rigidly held in a single or a few closely related conformations, since conformational averaging would have given averaged, intermediate values (19). This analog demonstrated significant activity in pheromonotropic (20), hindgut and oviduct contractile (19,21), egg diapause induction (21), and pupariation (26) PK/PBAN insect bioassays. Potent activity demonstrated by a PK/PBAN analog that contains an (E)-alkene, *trans*-Pro mimic in the four PK/PBAN bioassays included in this study (pheromonotropic, melanotropic, pupariation, and hindgut contractile) also provides strong evidence for a

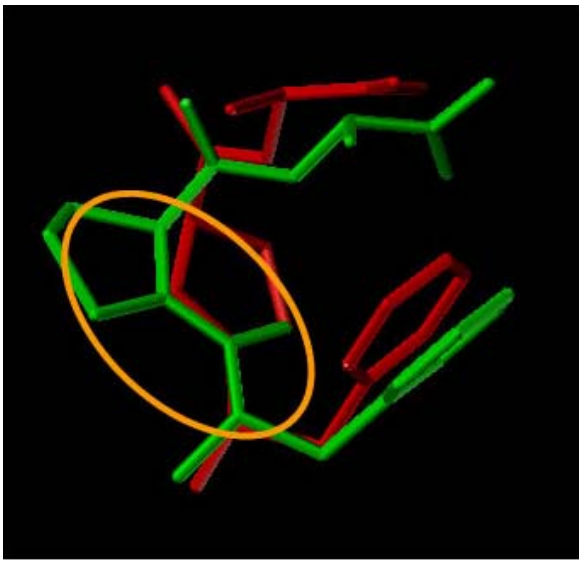

Figure 4. In the circled portion of the computer graphics illustration is a superposition of a *trans*-peptide bond preceding Pro (green) with the dihydroimidazoline ('Jones') motif (red) that indicates this motif can function as a surrogate for a *trans*Pro.

trans orientation of the peptide bond preceding Pro as an active PK/PBAN core conformation (35,Nachman and Altstein, unpublished data). In the (*E*)-alkene moiety of the aforementioned PK/PBAN analog, the peptide bond that binds the amino group of the Pro is locked into a *trans* orientation by replacement with a double bond, which lacks the ability to rotate between *trans* and *cis* orientations as does a normal peptide bond (36).

The dihydroimidazoline moiety (Figure 1) has been previously introduced by Jones and coworkers as a peptide bond isostere (with an amidine as an amide bond replacement) for the development of pseudopeptide analogs (23). However, it had not been previously proposed as a mimic of a *trans* peptide bond. As can be visualized in Figure 4, it is reasonable to assert that the dihydroimidazoline moiety can function as a mimic or surrogate of the *trans* peptide bond, and in particular, a *trans*Pro, locking a *trans* orientation within the constrained five-membered dihydroimidazole ring. Indeed, in Figure 4 we can see that, using a molecular modeling program, the dihydroimidazoline moiety can be readily superimposed upon a *trans*Pro. However, whereas the molecular modeling suggests that the dihydroimidazoline moiety can function as a mimic of a *trans*Pro, it is clear that it is not an exact mimic; and furthermore, is not as close a mimic as is the (*E*)-alkene moiety mentioned above (35). Therefore, analogs containing the dihydroimidazoline moiety provide an opportunity for selective interaction with closely related receptors, assuming that some receptors may display more tolerance to small deviations from the *trans*Pro structure of natural peptides.

Incorporation of the *trans*Pro surrogate, dihydroimidazoline moiety into a PK/PBAN C-terminal hexapeptide sequence led to analog **PPK-Jo** (Ac-YF (Jo)RLa). The analog is acetylated at the N-terminus to provide protection from aminopeptidase degradation. **PPK-Jo** demonstrated strong activity in the *in vivo S. littoralis* melanotropic assay, reaching a 115 and 96% activity at doses of 100 pmol and 1 nmol, respectively compared with that of 5 pmol PBAN (Figure 2) and matching the efficacy of 5 pmol LPK. The parent PK/PBAN hexapeptide analog YFTPRLa (1323) also elicited strong activity in this assay. However, YFTPRLa also demonstrated inhibition of the melanotropic activity of 5 pmol PBAN or 5 pmol PT at doses of 100 pmol and 1 nmol (Figure 3). Analog **PPK-Jo** shows no inhibition even up to a dose of 1 nmol. Unlike the parent PK/PBAN hexapeptide YFTPRLa, **PPK-Jo** is a pure melanotropic agonist in the *S. littoralis* assay.

In the *in vivo H. peltigera* pheromonotropic assay, **PPK-Jo** failed to elicit significant agonist activity even up to a dose of 1 nmol, whereas the parent hexapeptide analog YFTPRLa elicited a significant activity at doses of 100 pmol and 1 nmol that was not statistically different from that of PBAN (Figure 2). **PPK-Jo** could not inhibit the pheromonotropic activity of 1 pmol of PBAN, 1 pmol PT, 30 pmol LPK or 10 pmol MT even up to a dose of 1 nmol.

In the *in vivo N. bullata* pupariation assay, **PPK-Jo** was unable to elicit significant agonistic activity even up to a dose of 500 pmol; whereas the PK/PBAN pentapeptide FTPRLa essentially matched the activity of the control PK/PBAN peptide LPK (pQTSFTPRLa; threshold: 0.3 pmol) (22,26). Neither the analog **PPK-Jo** nor FTPRLa was able to inhibit the activity of the control LPK even up to a dose of 500 pmol. In the *in vitro* cockroach *L. maderae* hindgut myotropic assay, **PPK-Jo** also failed to elicit a statistically significant response even up to a concentration of 5×10^{-6} M (25 nmol); whereas the native LPK and pentapeptide FTPRLa demonstrated strong myostimulatory activity at threshold concentrations of 1×10^{-9} M (5 pmol) and 2×10^{-9} M (10 pmol), respectively (37). **PPK-Jo** could not inhibit the myostimulatory response of LPK even up to a dose of 5×10^{-6} M (25 nmol).

Therefore, **PPK-Jo** is a pure, selective agonist for the melanotropic assay, showing no activity in the other three PK/PBAN assays (pheromonotropic, pupariation, and hindgut contractile). It is apparent that the receptor associated with the melanotropic assay in *S. littoralis* is more promiscuous than those of the other PK/PBAN assays, demonstrating more tolerance to deviations from the natural *trans*Pro structure. Differing inhibitory and stimulatory patterns among the PK/PBAN receptors were previously reported by us based on examination of the agonistic and antagonistic activities of backbone cyclic conformationally constrained peptides (38) and biostable beta-amino acid substituted PK/PBAN analogs (39) in the above mentioned four bioassays. In all of our previous studies the number of melanotropic selective compounds (whether agonistic or antagonistic) exceeded that of the other functions (i.e., other PK/PBAN receptors), hinting at the possibility that those functions may be mediated by structurally different receptors. Although the melanotropic

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. A more concrete proof for such differences has recently been demonstrated by us based on receptor cloning and comparison of the gene structures of *S. littoralis* larvae PK/PBAN receptors (mediating melanization) and pheromone gland receptors of *H. zea, B. mori* and *H. virescens* (40). The development of a selective PK/PBAN agonist can lead to a better understanding of the endogenous mechanisms of this important peptide class and can serve as a probe to study the plasticity of PK/PBAN regulated systems in insects and the receptors associated with them.

This work has demonstrated that the dihydroimidazoline motif can function as a mimic or surrogate of a *trans*Pro in certain circumstances, and has been identified as a novel scaffold with which to construct mimetic pseudopeptide and/or non-peptide analogs of the PK/PBAN, and other, peptide families that feature enhanced selectivity. The work further demonstrates that analogs containing the dihydroimidazoline moiety provide an opportunity for selective interaction with closely related receptors, as some receptors may display more tolerance to small deviations from the natural *trans*Pro structure of native peptides. Notably, the dihydroimidazoline motif in **PPK-Jo** introduces a major structural modification adjacent to the peptide bond connected to the amino group of Arg in the core pentapeptide, which has been identified as the primary tissue-bound peptidase susceptible site of the PK/PBAN core (27). Therefore, it seems plausible that incorporation of the motif could enhance the biostability of PK/PBAN analogs. It is envisioned that this moiety may also aid in the future development of mimetic analogs of insect neuropeptides with potential antagonist properties. Such selective mimetic agonist and/or antagonist analogs may provide leads in the development of novel insect-specific, environmentally favorable pest management agents capable of disrupting critical PK/PBAN-regulated systems.

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