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An amphiphilic, PK/PBAN analog is a selective pheromonotropic antagonist that penetrates the cuticle of a heliothine insect

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ABSTRACT

A linear pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) antagonist lead (RYF[dF]PRLa) was structurally modified to impart amphiphilic properties to enhance its ability to transmigrate the hydrophobic cuticle of noctuid moth species and yet retain aqueous solubility in the hemolymph to reach target PK/PBAN receptors within the internal insect environment. The resulting novel PK/PBAN analog, Hex-Suc-A[dF]PRLa (PPK-AA), was synthesized and evaluated as an antagonist in a pheromonotropic assay in *Heliothis peltigera* against 4 natural PK/PBAN peptide elicitors (PBAN; pheromonotropin, PT; myotropin, MT; leucopyrokinin, LPK) and in a melanotropic assay in *Spodoptera littoralis* against 3 natural PK/PBAN peptide elicitors (PBAN, PT, LPK). The analog proved to be a potent and efficacious inhibitor of sex pheromone biosynthesis elicited by PBAN (84% at 100 pmol) and PT (54% at 100 pmol), but not by MT and LPK. PPK-AA is a selective pure antagonist (i.e., does not exhibit any agonistic activity) as it failed to inhibit melanization elicited by any of the natural PK/PBAN peptides. The analog was shown to transmigrate isolated cuticle dissected from adult female *Heliothis virescens* moths to a high extent of 25–30% (130–150 pmol), representing physiologically significant quantities. PPK-AA represents a significant addition to the arsenal of tools available to arthropod endocrinologists studying the endogenous mechanisms of PK/PBAN regulated processes, and a prototype for the development of environmentally friendly pest management agents capable of disrupting the critical process of reproduction.

1. Introduction

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) peptides represent a multifunctional family that plays a significant role in the physiology of insects. The first member of the family, leucopyrokinin (LPK) was discovered in the cockroach *Leucophaea maderae* in 1986 [11] and since then over 30 peptides have been identified. They include

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PKs, myotropins (MTs), PBAN, melanization and reddish coloration hormone (MRCH), diapause hormone (DH), pheromonotropin (PT), and peptides derived from the PBAN gene or cDNA of various moths (termed pheromonotropic β and γ peptides), all of which share the common C-terminal pentapeptide FXPRL-amide (X = S, T, G or V) [4,25,26]. Functions of the PK/PBAN family include stimulation of sex pheromone biosynthesis in moths [4,14,25–27], and mediation of key functions associated with feeding (gut muscle contractions) [15,29], development (embryonic diapause, pupal diapause and pupariation) [12,16,18,19,33,34] and defense (melanin biosynthesis) [6,13] in a variety of insects (moths, cockroaches, locusts and flies). Studies have shown that all of the above functions can be stimulated by more than one peptide, and that the peptides do not exhibit species specificity [1,10,25,26]. The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides elicit their effects. Antagonists, particularly selective ones, can shed light on this issue.

As potent and specific as these PK/PBAN molecular messengers are, these neuropeptides in and of themselves are not suitable either as pest insect control agents or tools for insect neuroendocrinologists. Direct use of neuropeptides for insect control is impractical because the insect cuticle contains an apolar lipid matrix that, for the most part, inhibits penetration of polar compounds like peptides and because they are rapidly degraded by peptidases in the hemolymph and tissues within insects [17,21]. Therefore, the development of agonists and antagonists with enhanced cuticle/gut penetrability can overcome at least one of these limitations and represents a key step in the development of control techniques employing analogs of insect neuropeptides capable of disrupting critical life processes regulated by the PK/PBAN class.

These problems have been previously addressed with PK/PBAN agonists through the development of pseudopeptide analogs with amphiphilic character, which confers an ability to penetrate the hydrophobic insect cuticle and simultaneously maintains the water solubility necessary for them to re-emerge in the insect circulatory system and reach their target receptor sites [2,20,21,23,30,32]. Experiments involving topical application of aqueous solutions (without addition of an organic solvent component) of members of the PK/PBAN family did not transmigrate the isolated cuticle of the tobacco budworm moth *Heliothis virescens* in statistically significant quantities [2,23,32]. Structural modification to produce PK analogs that feature amphiphilic properties greatly enhances their ability to both penetrate the hydrophobic cuticle, and maintains the aqueous solubility required to reach their target receptor once they encounter the hemolymph [2,23]. The development of a series of pseudopeptide analogs of this neuropeptide family began with the addition of various hydrophobic groups to the N-terminus of the C-terminal pentapeptide active core, which in conjunction with the polar/charged Arg side chain, confer an amphiphilic property. Hydrophobic groups appended to the N-terminus included fatty acids of various chain lengths, aromatic acids, as well as hydrophobic amines attached via a succinnic acid linker [17,21–23,30,31]. Many of these amphiphilic analogs showed greater *in vivo* potency in a pheromonotropic assay than the native 33-membered PBAN when delivered via injection into female *H. virescens* moths. When applied to dissected pieces of *H. virescens* cuticle, 24 h recoveries of a series of amphiphilic pyrokinin analogs ranged from 5 to 70%, dependant on the hydrophobic nature of the individual analog. In addition, prolonged pheromone production exceeding 20 h following a single topical application of an amphiphilic pyrokinin analog to *H. virescens* moths was observed. The nature of the hydrophobic moiety could also influence the duration of the slow release of a given amphiphilic pyrokinin analog. The results demonstrated that the insect cuticle could serve as a reservoir for the time-release of a physiologically active, amphiphilic analog of an insect neuropeptide [22].

In a previous study we demonstrated that replacement of the amino acid Ser in a sequence derived from the C-terminus of PBAN (YFSPRLa, which contains the signature sequence of the PK/PBAN family) with dF and addition of an amino acid Arg at the N-terminus resulted in the disclosure of a potent antagonist which was able to inhibit PBAN evoked pheromone biosynthesis in *Heliothis peltigera* [35]. The peptide (RYF[dF]PRLa) was a selective, pure (i.e., did not exhibit any agonistic activity) pheromonotropic inhibitor and did not inhibit melanin formation in *Spodoptera littoralis* [8]. The modification of the PBAN antagonist analog to enable penetration of the cuticle of an insect would be a logical extension of the previous studies on this lead antagonist [35] and those focused on the development of amphiphilic PK/PBAN agonists [22]. In this manuscript, we describe the synthesis of an amphiphilic version of this dF PK/PBAN antagonist that features an aliphatic amine appended to the N-terminus via a di-acidic linker (succinnic acid). This analog, Hex-Suc-A[dF]PRLa, is labeled PPK-AA (PBAN Pyrokinin-Amphiphilic Antagonist) and was evaluated for both agonist and antagonist activity in a pheromonotropic assay in adult female *H. peltigera* and a melanotropic assay in larvae of *S. littoralis*. Following this, it was evaluated for its ability to penetrate dissected pieces of the cuticle of another heliothine, *H. virescens*.

2. Materials and methods

2.1. Insects

S. littoralis larvae were kept in groups of 100–200 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 [9]. 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 [9]. All females used in this study were 3.5 days old.

H. virescens moths were obtained as pupae from North Carolina State University. Pupae were sexed and males discarded. Females were held in 30 cm^3 plastic cages equipped

with mesh sleeves to allow for removal of adults. Newly enclosed adults were placed in new cages daily held in environmental chambers at 25 ± 2 °C, 65% relative humidity with a 14:10 h light:dark photoperiod and provided with a 5% sucrose solution as food.

2.2. Peptide and pseudopeptide analog synthesis and purification

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

Hez-PBAN [28] and *Pseudaletia* (*Mythimna*) *separata* Pss-PT [14] 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 [11,35]. Synthesis of *Leucophaea maderae* LPK (Lem-LPK) [11] and *Locusta migratoria* (Lom-MT-II) [29] was carried out via 9-fluorenylmethoxycarbonyl (Fmoc) methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Advanced Chemtech, Louisville, KY) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) as described previously [17]. The purity of all peptides was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) [3,34] and was found to be in the range of 90–95%. Purified peptides were characterized by time-of-flight mass spectrometry (TOF-MS) and amino acid analysis of hydrolyzates.

2.2.2. Amphiphilic pseudopeptide analog synthesis and purification

Hexylamine (Hex) and succinic acid (Suc) were purchased from Aldrich Chemical (Milwaukee, WI). Synthesis of amphiphilic analog PPK-AA (Hex-Suc-A[dF]PRLa) was accomplished in a stepwise manner. A[dF]PR(PMC)L-Rink Amide resin complex was 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 [23]. The pseudopeptide analog was synthesized by condensation of succinic acid and hexylamine to the Rink Amide peptide complex above by stirring with 1 equiv. of 1,3-diisopropylcarbodiimide/i-hydroxy-2azabenzotriazole in dimethyl sulfoxide for 4 h at room temperature for each step. The crude pseudopeptide was cleaved from the resin and protecting groups removed by treatment with a mixture of trifluoroacetic acid (TFA) (90%), anisole (5%), thioanisole (4%) and 1,2-ethanedithiol (1%) for 1 h. The resin was removed via filtration and volatile reagents were removed with a Savant Speed Vac concentrator. 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. Retention time on C_{18} column: 12.0 min. Retention time on Protein Pak 125 column: 6.0 min. The pure peptides were analyzed and quantified via amino acid analysis. Each peptide sample was purged with N_2 and the peptide hydrolyzed with vapor phase HCl for 24 h at 105 °C. Precolumn derivatization and HPLC analysis was accomplished by the standard Pico Tag method supplied by Waters (Milford, MA). The observed amino acid ratios were as expected for PPK-AA: A[1.0], F[1.0], L[1.0], P[0.9], R[1.1]. Mass spectra were obtained on a Kratos Kompact Probe MALDI-TOF machine (Kratos Analytical, Ltd., Manchester, UK) using α-cyano-4-hydroxycinnamic acid as a matrix, recording the presence of the following molecular ions for PPK-AA: 785.5 [calc MH^+ = 784.0].

2.3. Pheromonotropic bioassay

The pheromonotropic bioassay was performed with *H. peltigera* as described previously [5]. Stimulatory activity of the PK/PBAN peptides or PPK-AA (at 1 nmol) 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 amphiphilic, D-Phe linear analog PPK-AA to inhibit sex pheromone biosynthesis that was elicited by an injected exogenous stimulator (PBAN1-33NH_2, PT, MT or LPK at 1, 1, 10 and 30 pmol, respectively, see results). Females injected with the elicitors at the indicated concentrations served as a reference for maximal stimulation and those injected with 100 mM phosphate buffer served to determine the basal pheromone biosynthesis during the 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 [5]. The experiment was performed with a minimum of 8 females per treatment.

2.4. Melanotropic bioassay

The melanotropic bioassay was performed as described previously [8]. The melanotropic stimulatory activity of the PK/PBAN peptides or PPK-AA 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 amphiphilic, D-Phe linear analog (at 1 nmol), injected together with the elicitors PBAN1-33NH_2, PT or LPK (at 5, 5 and 15 pmol, respectively, see results), to inhibit cuticular melanization. Larvae injected with the elicitors at the indicated concentrations served as a reference for maximal stimulation, and those injected with 50 mM HEPES, pH 7.6 served to determine the basal cuticular melanization of the ligated insects. Each experiment involved analysis of the intensity of the melanized area in untreated and ligated larvae, and in larvae that were ligated and injected with buffer, PBAN or the tested amphiphilic analog. The cuticular melanization was quantified as the ratio between the optical density and the scanned cuticular area (in millimeters) and was compared between control and experimental animals. All experiments were performed with a minimum of 9 or 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 (at 5 pmol).

2.5. Isolated cuticle penetration assay

Cuticle penetration assays were conducted using tissue obtained from the abdomen of female moths. Pieces of cuticle, ca. 0.4 cm^2, were prepared as described elsewhere [32] and floated cell-side down in wells of ELISA plates (Corning, 96-well Easy Wash) that had been previously blocked by filling with 1% gelatin in 10 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.25) (PBS) [32]. Analog PPK-AA (0.5 nmol) was applied to the center of the cuticle pieces in a 0.5 μl drop of H_2O using micromanipulator and observed for 5 min to insure that the drops did not slide off. Lids were applied to the plates and the plates were placed on an orbital shaker operated at 80 rpm. Cuticle was removed from wells at 1, 2, 4, 6, 8, 20 and 24 h after application of the analog. After incubation, 100 pmol of internal standard (*p*Glu-Arg-Phe-NH_2) were added and the contents of the wells were prepared for chemical analysis. Six replicates were made for each data point.

Samples were analyzed by mass spectroscopy (MS) using a Thermo Finnigan LCQ DECA Max MS with electrospray ionization (positive ion). The MS was interfaced to a Thermo Finnigan liquid chromatography system consisting of a P4000 quaternary gradient pump, an AS 3000 autoinjector and a UV6000 diode array detector. Reversed phase liquid chromatographic separation (RPLC) was performed by injecting 20 μl of sample onto a Zorbax RX-C18 column (4.6 mm × 250 mm, 5 μm, Agilent Technologies) operated at 60 °C using the following solvents: A = methanol with 1.0% formic acid; B = water with 10 mM ammonium formate; C = 90% acetonitrile/10% H_2O with 10 mM ammonium formate. Conditions of chromatography were 4:90:6 (A:B:C) 1 min followed by a gradient to 4:58:38 (A:B:C) over 8 min followed by a second gradient to 4:0:96 (A:B:C) over 6 min and held at this for 2 min. Quantitation was accomplished by comparing the areas of base ions for PPK-AA (M/Z 785.23-786.23) and *p*Glu-Arg-Phe-NH_2 (M/Z 487–488) after calculating the ratio of intensities of these ions during analysis of equimolar amounts of the compounds analyzed without application to the cuticle.

2.6. Statistical analysis

The results of the pheromonotropic and melanotropic assays were subjected to one-way ANOVA. The significance of differences among means was evaluated with the Tukey–Kramer HSD (honestly significant difference) test at $P < 0.05$. Data from the cuticle penetration assay were analyzed using a one-way ANOVA and Fishers Least Significant Difference test using NCSS7 Statistical software and by regression analysis using GraphPad Prism® software.

3. Results

3.1. Pheromonotropic bioassay

As the focus of this study is on development of an amphiphilic antagonist analog, the PPK-AA (Hex-Suc-A[dF]PRLa) was evaluated for any residual agonist activity. This analog did not elicit any detectable level of sex pheromone production at 1 nmol (the activity obtained was 1% of that of 1 pmol of PBAN).

The linear D-Phe amphiphilic analog PPK-AA was tested for its antagonistic activity against different elicitors at 100 pmol and 1 nmol. As can be seen in Fig. 1, PPK-AA inhibited PBAN mediated activity, decreasing sex pheromone biosynthesis by 84 and 69% at 100 pmol and 1 nmol, respectively. PPK-AA also inhibited the elicitor PT by about 54% at 100 pmol, but proved less effective against MT, where the observed inhibition was not statistically significant from that obtained in the absence of PPK-AA. PPK-AA was completely ineffective in inhibiting LPK as a pheromonotropic elicitor. The combined agonistic and antagonistic examination of PPK-AA clearly reveals that it is a pure pheromonotropic antagonist, although it demonstrates a distinct pattern of selectivity with the natural elicitors, inhibiting two (PBAN, PT) out of four (PBAN, MT, PT, LPK).

3.2. Melanotropic bioassay

The amphiphilic analog PPK-AA was also evaluated for melanotropic agonistic activity at 100 pmol. The analog PPK-AA demonstrated very low or negligible stimulatory activity that was not statistically significant from buffer injected larvae (the activity obtained was 5% compared with that of 5 pmol PBAN).

The linear D-Phe amphiphilic analog PPK-AA was tested for antagonistic activity at 1 nmol with PBAN1-33NH_2, PT and LPK as stimulators. Inhibition activity less than 40% and was not considered as positive in this assay [8]. PPK-AA failed to meet the criteria as an inhibitor for any of the elicitors.

3.3. Isolated cuticle penetration assay

Following application of 500 pmol of the amphiphilic analog PPK-AA to the surface of isolated cuticle dissected from the abdomen of adult female *H. virescens* moths, the quantity transmigrating through was monitored at various time inter-

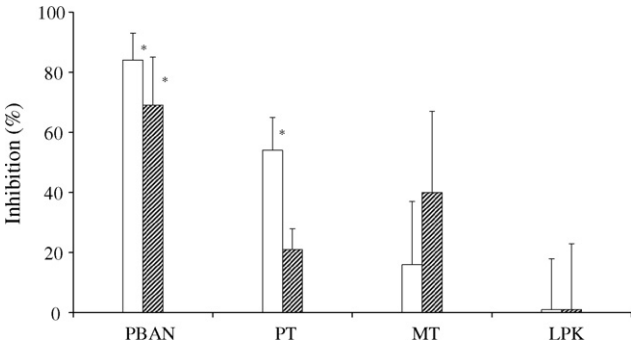

Fig. 1 – *In vivo* inhibition of sex pheromone biosynthesis elicited by PBAN, PT, MT and LPK by 100 pmol (open bars) and 1 nmol (dark bars) of the amphiphilic analog PPK-AA in adult female *Heliothis peltigera*. The data represent means ± S.E.M. (n = 8–10). An asterisk (*) indicates an activity that differs significantly (at $P < 0.05$) from that obtained by the elicitor itself.

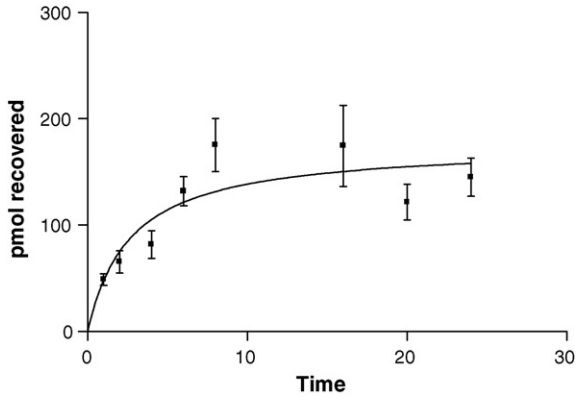

Fig. 2 – Penetration of the amphiphilic antagonist analog PPK-AA through the isolated cuticle dissected from the abdominal region of adult female *Heliothis virescens* at 1, 4, 6, 8, 16, 20, and 24 h intervals, as monitored via HPLC. The data represent means ± S.E.M. (n = 6).

vals via LC–MS measurements of the aqueous solution in the well. The results (Fig. 2) demonstrate that 130–150 pmol transmigrate the cuticular surface between 8 and 24 h; representing a high penetration rate of 25–30% of the applied sample. Previous experiments have demonstrated that neither PBAN nor its C-terminal pentapeptide, which do not feature amphiphilic properties, could transmigrate the isolated cuticle preparation of *H. virescens* [32].

4. Discussion

The linear dF PK/PBAN antagonist RYF[dF]PRLa [35] was modified to impart amphiphilic properties that would enhance penetration through the hydrophobic cuticle of pest heliothine insects and yet retain aqueous solubility in the hemolymph required to reach target PK/PBAN receptors within the internal environment of the insect. The modification was carried out by first appending a hydrophobic amine (hexylamine) via a di-acid linker (succinic acid) to the N-terminus of the C-terminal pentapeptide of the parent antagonist as had been previously achieved with a C-terminal pyrokinin pentapeptide agonist [22,31]. In the resulting analog Hex-Suc-A[dF]PRLa (PPK-AA), the F was replaced with an A in an effort to eliminate any residual agonist response and therefore maximize the antagonist profile. Amphiphilic analog PPK-AA demonstrated neither a detectable agonist response in the pheromonotropic assay of *H. peltigera* nor a statistically significant agonist response in the melanotropic assay in *S. littoralis*. In the pheromonotropic assay, PPK-AA demonstrated a selective ability to inhibit the elicitors PBAN and PT, but not the other PK elicitors MT or LPK (Fig. 1). PPK-AA proved to be a potent antagonist of the native pheromonotropin PBAN, inhibiting sex pheromone production by 84% at 100 pmol. In the melanotropic assay, PPK-AA at a dose of 1 nmol failed to meet the criteria of an inhibitor of any of the three PK/PBAN elicitors PBAN, PT or LPK. Therefore PPK-AA exhibited pure antagonism and was selective for the putative pheromonotropic receptor.

Testing on isolated cuticle dissected from the abdominal region of adult females of another heliothine moth, *H. virescens*, demonstrates that from an initial topical application of 500 pmol, a high percentage (25–30%, corresponding to130–150 pmol) transmigrates from the outer cuticular surface to the hemolymph side (Fig. 2). This experimental data indicates that the quantity that penetrates the abdominal heliothine cuticle is sufficient to reach and exceed the injected dose that led to 84% inhibition of sex pheromone biosynthesis in the *in vivo* pheromonotropic assay.

The results indicate that a peptide antagonist analog can be modified to impart amphiphilic character, and therefore an enhanced ability to transmigrate the cuticle of an adult female moth in physiologically significant quantities, without losing its intrinsic biological activity. The amphiphilic analog PPK-AA is a selective antagonist for the pheromonotropic response of the native hormone, and is therefore a significant addition to the neuroendocrinologist's arsenal of tools (in addition to the already existing linear and backbone cyclic (BBC) antagonists of the PK/PBAN family of peptides [3,7,35], and topically/orally active, biostable PK/PBAN agonists [2,20–24]) to study the endogenous mechanisms of this important aspect of reproduction in heliothine insects. The analog PPK-AA may serve as a prototype for the development of a novel group of highly effective, insect-specific and environmentally friendly pest management agents capable of disruption of the critical process of reproduction.

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