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A β-Amino acid Pyrokinin Analog Induces Irregular Pupariation Behavior in Larvae of the Flesh Fly *Sarcophaga bullata*

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Abstract: The developmental process of pupariation is accelerated by members of the pyrokinin class of neuropeptides in larvae of the flesh fly *Sarcophaga bullata*. A pyrokinin analog (Ac-Y[β^{3}Phe]TPRLamide), in which a Phe residue is replaced with a β-amino acid, accelerates pupariation in this fly at a potency (0.2 pmol/larva) that matches that of the native pyrokinin factor. At higher concentrations, this β-amino acid pyrokinin analog induces irregular pupariation behavior patterns that are suggestive of neurotoxic properties. Biostable analogs based on this structure may in future provide analog leads with the potential to disrupt the important pupariation process in flies.

Keywords: pyrokinin, β-amino acid, pupariation, *Sarcophaga bullata*, neuropeptide

INTRODUCTION

Pupariation in cyclorrhaphous flies is a process of transformation of the soft integument of a wandering larva into a hard ovoid case called the puparium. It

involves both behavioral activities (immobilization, retraction of the anterior segments, longitudinal body contraction), and sclerotization of the cuticle (deplasticization and hardening by phenolic tanning) that fixes the achieved morphological changes [1]. Pupariation in the flesh fly *Sarcophaga bullata* can be accelerated by members of the pyrokinin (FXPRLamide) class of neuropeptides, either native or isolated from other arthropod species [2-6].

In the present paper, we describe the synthesis and biological evaluation of a pyrokinin analog, Ac-Y[β^3Phe]TPRLamide (labeled **1465**), which features a replacement of the Phe residue in the pyrokinin C-terminal pentapeptide region with a β^3Phe counterpart. The β^3Phe differs from the natural Phe residue by the presence of an additional methylene group (-CH_2-) between the α-carbon and the carboxyl group [7, 8]. Pyrokinin analogs incorporating β-amino acids have not been previously reported, though β-amino acid analogs of the insect kinin neuropeptide class have been shown to both retain significant biological activity and exhibit enhanced biostability [8]. The β-AA pyrokinin analog **1465** is evaluated in the classic pupariation bioassay supplemented with a tensiometric technique designed to record and analyze details of motor patterns of behavior and biomechanical changes in larval cuticle associated with formation of the barrel-shaped puparium.

MATERIAL AND METHODS

Experimental flies

Larvae of the fleshfly, *Sarcophaga* (=*Neobellieria*) *bullata* were reared by 200-300 specimens per batch on beef liver in small open disposable packets made from aluminium foil as described [1].

Classical bioassay for acceleration of pupariation

The test was performed as described by Zdarek [1]. Briefly, the tested material was injected into early-red-spiracle larvae previously immobilised by chilling on ice. Control larvae were injected with solvent only. After removal from ice the injected larvae were kept in petri dishes lined with filter paper at 25 °C, and the time of anterior retraction (*R*), contraction (*C*) and tanning (*T*) was recorded. The effects of a tested compound were expressed as a difference between the mean time after which *C* and *T* occurred in the control as compared with experimental larvae. Eight to 12 larvae were injected in each group and the test was twice or thrice repeated. Volume of injected solutions was 1 μL.

Contact tensiometric measurements

A non-invasive (contact) tensiometric technique of indirect detection of cuticular tension was used as previously described [6]. The tensiograms start immediately after the recovery of the larva from chilling, i.e. approximately 5 minutes after injection. Briefly, changes of cuticular strain caused by both the muscular activity (rapid strain fluctuations) and shrinkage of the cuticle (slow rise of the baseline) were recorded via mechanical transduction of a strain gauge. During normal pupariation four distinct patterns of motor activity can be distinguished [6]. Briefly, the muscles of a crawling or digging larva produce anteriorly directed body peristalsis that is recorded as a dense continuous train of rather regular complex pulses. This locomotory pattern of the wandering phase (W) is replaced with a pattern of an immobilization (I) phase, when the larva ceases locomotion. The muscular contractions continue but the direction of peristalsis is reversed. We identify this period as the retraction (R) phase. Then the pulsation dramatically changes. The pauses disappear and the pulsation becomes composed of regular sharp peaks interspaced with broader multiple spike peaks. At the same time the baseline slowly rises indicating a steady increase of hemocoelic pressure due to shrinkage of the cuticle. During this contraction (C) phase the larva shortens into a white puparium and its cuticle becomes smooth. After about 20 minutes the broad multiple spike peaks disappear and the first signs of tanning of the cuticle appear. Muscular activity gradually ceases after several more minutes. At least 3 tensiograms of each dose were made and a representative one chosen for illustration in Figure 1.

Peptide analog synthesis

The pyrokinin analog Ac-Y[β^3Phe]TPRLamide **1465** was synthesized via Fmoc methodology on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) and purified chromatographically on Delta Pak C_{18} reverse-phase and Waters Protein Pak I125 columns under previously described conditions [8, 9]. Delta-Pak C-18 retention time: 12.0 min; Waters Protein Pak I125 retention time: 6.25 min; Amino acid analysis [8, 9]: L[1.0], P[1.0], R[1.0], T[1.0], Y[1.1]; MALDI-TOF-MS analysis [8, 9]: 852.8 [$M+H^+$].

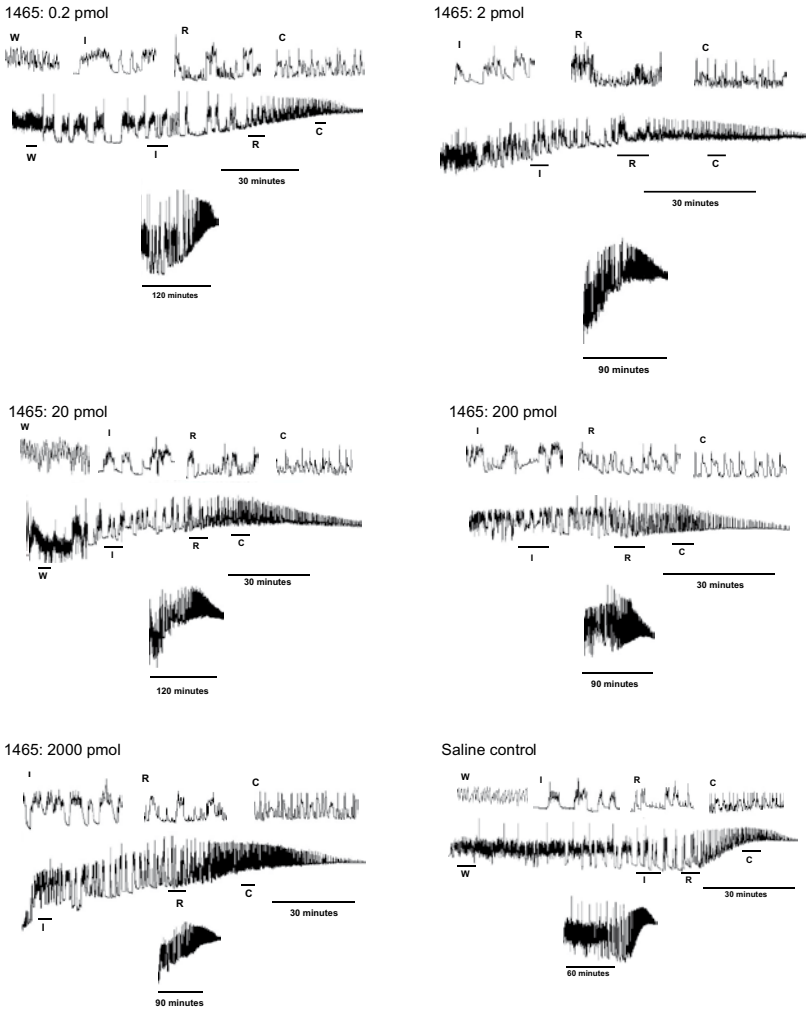

Figure 1. Tensiometric records of haemocoelic pulsations of *S. bullata* larvae injected with 0.2, 2, 20, 200 and 2000 pmol of **1465** analog and 1 µL of Ringer saline. In addition to an overall record of the entire event (middle), selected sections illustrating different motor patterns (crawling - W, immobilisation – I, retraction – R, contraction - C) expanded in time are shown above the record. The bottom record was made at a slow chart speed to graphically dramatize the rise of baseline that reflects elevation of hemocoelic pressure produced by shrinkage of the cuticle during formation of the barrel-shaped white puparium.

RESULTS AND DISCUSSION

The pupariation bioassay showed that the β-AA pyrokinin analog **1465** at a dose of 0.2 pmol accelerated puparial contraction slightly while tanning remained unaffected. A tensiometric analysis revealed that the larvae were also precociously immobilized as shown by an extended period of well-expressed *I* motor patterns (Figure 1). The *R* and *C* motor patterns were normal and occurred at a regular time. Tension in the cuticle began to rise in the middle of the immobilization phase, i.e. well before the *R* patterns occurred, which explains the slight acceleration of puparium contraction recorded by the bioassay. A dose of 0.02 pmol had no effect on timing of puparium formation or on patterns of pupariation behavior (record not shown). With the threshold dose being approximately 0.2 pmol per larva the **1465** analog is equivalent in potency to the native pyrokinin factor [4]. The doses of 2 and 20 pmol had a significant accelerating effect on timing of puparium formation, which was due to acceleration of shrinkage of the cuticle and tanning. The onset of *I* patterns of muscular activity was also accelerated and the *I* program was performed for an extended time. Higher doses (200 and 2000 pmol [0.2 and 2 nmol]) caused more irregularities in pupariation behavior: the *I* phase began almost immediately after recovery from chilling (there was no wandering) and violent convulsive contractions of overall musculature were observed during the *I* phase. This suggests that analog **1465** may demonstrate a neurotoxic action at the higher doses. The rise of the baseline pressure, which indicates shrinkage of the cuticle, was not as well pronounced as it was at lower doses. The ultimate levels of cuticle tension were achieved only during the *C* phase. Again, this suggests that the process of cuticular shrinkage was partially paralyzed by the higher doses of analog **1465**.

In conclusion, the β-AA analog **1465** demonstrates pupariation acceleration activity in the flesh fly *S. bullata* at a potency that matches the native pyrokinin factor. At higher concentrations, **1465** induces irregular pupariation behavior patterns that are suggestive of neurotoxic properties. Development of more biostable analogs based on **1465** that protect the primary peptidase hydrolysis site between the P and R residues in the core pyrokinin region [10] may provide analog leads with the potential to disrupt the critical pupariation behavior in these and other flies.

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