Ketomethylene and Methyleneamino Pseudopeptide Analogues of Insect Allatostatins Inhibit Juvenile Hormone and Vitellogenin Production in the Cockroach *Blattella germanica*

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Metabolic studies on insect allatostatins have suggested that the dipeptide Leu-Tyr may be a target for endopeptidases. In order to increase resistance to degradation, methyleneamino \(\Psi(\text{CH}_3\text{NH})\) and ketomethylene \(\Psi(\text{COCH}_3)\) peptide bond surrogates have been introduced at the position Leu\(^2\)-Tyr\(^4\) of the allatostatin Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-amide (BLAST-2), and Leu\(^2\)-Phe\(^4\) of [Phe]\(^5\)BLAST-2, respectively. Assays of inhibition of juvenile hormone (JH) synthesis *in vitro* by corpora allata from the cockroach *Blattella germanica* showed that both analogues were similarly active to the respective model peptides. The methyleneamino analogue was further tested *in vivo* as an inhibitor of JH synthesis, and *in vivo* as an inhibitor of vitellogenin production by the fat body of *B. germanica*. The analogue was less active than BLAST-2 when tested *in vitro*, but more active than it when tested *in vivo*. © 1997 Elsevier Science Ltd. All rights reserved

BLAST-2, allatostatin 2 of *B. germanica*, Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-amide CA, corpora allata JH, juvenile hormone PTMA, Asp-Arg-Leu\(^1\)\(\Psi(\text{CH}_3\text{NH})\)Tyr-Ser-Phe-Gly-Leu-amide PTMB, Asp-Arg-Leu\(^1\)\(\Psi(\text{COCH}_3)\)Phe-Ser-Phe-Gly-Leu-amide Vg, vitellogenin

INTRODUCTION

Cockroach allatostatins are neuropeptides that have a characteristic Tyr-Xaa-Phe-Gly-Leu-amide C-terminus. They were discovered as a result of their inhibitory activity on juvenile hormone (JH) synthesis by the corpora allata (CA) (see Stay et al., 1994 for review), but further studies have described other biological properties in these insects, such as antimitotic activity in gut tissues (Lange et al., 1993, 1995a; Duve et al., 1995), and inhibition of vitellogenin (Vg) production by the fat body (Martín et al., 1996).

Within the order Dictyoptera, several allatostatins have been isolated and identified in *Diploptera punctata* (Woodhead et al., 1989, 1994; Pratt et al., 1989, 1991), *Periplaneta americana* (Weaver et al., 1994) and *Blattella germanica* (Bellés et al., 1994). Furthermore, a total of 13 allatostatins have been deduced from a cloned cDNA sequence of *D. punctata* (Donly et al., 1993), and 14 have been inferred in *P. americana*, using the same approach (Ding et al., 1995). Other members of this family have also been discovered in the blowfly *Calliphora vomitoria* (Duve et al., 1993, 1994, 1996; Duve and Thorpe, 1994; East et al., 1996), in the cricket *Gryllus bimaculatus* (Lorenz et al., 1995), and in the desert locust *Schistocerca gregaria* (Vanden Broeck et al., 1996). However, in *C. vomitoria* and *S. gregaria* these allatostatins do not act upon JH biosynthesis but have myoinhibiting properties.
Studies on the degradative metabolism of allatostatins carried out on *D. punctata* (Garside et al., 1997) suggest that the dipeptide Leu-Tyr, which occurs in some native allatostatins of this species (Stay et al., 1994), may be one of the targets for endopeptidases. All allatostatins identified to date in *B. germanica* have this dipeptide (Bellés et al., 1994). However, we have focused our attention on BLAST-2 (Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-amide) because it also occurs in *D. punctata* (where it has been named allatostatin IV by Woodhead et al., 1989, dipstatin 5 by Stay et al., 1994 or dip-allatostatin 5 by Garside et al., 1997), and has been used as a model for metabolism and structure-activity studies in this species (Garside et al., 1997; Hayes et al., 1994, respectively). Preliminary studies in vivo using radiolabelled BLAST-2 and techniques of microdialysis on *B. germanica* (Vilaplana et al., unpublished), indicated that its half life in the internal milieu of adult females is between 5.4 and 5.7 min. In addition, Leu-Tyr-Ser-Phe-Gly-Leu-amide and Tyr-Ser-Phe-Gly-Leu-amide, were identified as putative metabolites by comparison with retention times of synthetic standards, thus suggesting that the bond Leu-Tyr may also be a target for endopeptidases in *B. germanica*.

These results prompted us to replace the Leu3-Tyr4 peptide bond of BLAST-2 with the methylethenamino \[\Psi(CH_2NH)\] and ketomethylene \[\Psi(COCH_2)\] surrogates, respectively, with the aim of increasing the resistance to degradation of the octapeptide. Both peptide bond surrogates have been widely used for the preparation of metabolically stable peptide analogues (see Spatola, 1983; Bladon, 1994, and references cited therein), and the methylethenamino surrogate has been used previously to increase the resistance of insect leucokinins to peptidases (Nachman et al., 1991). The opposite hydrogen bond properties of the CH2NH and COCH2 groups (donor or acceptor) were also considered for use as surrogates, because the pseudopeptides resulting from their incorporation into BLAST-2 could shed light on the influence of these properties on biological activity. Although studies on the systematic Ala scan of dip-allatostatin 5 (= BLAST-2) have revealed the relative importance of Tyr4 for the inhibition of JH biosynthesis (Hayes et al., 1994), we were interested in exploring the replacement of this residue with Phe. If this replacement were biologically acceptable, it would facilitate the synthetic pathway towards the ketomethylene BLAST-2 pseudopeptides. To test this possibility, [Phe3] BLAST-2 was also prepared.

Biological activity in vivo and in vitro, in terms of the inhibition of JH synthesis by the CA, and Vg release by the fat body, were carried out, comparing the pseudopeptide analogues with the prototype peptides.

**MATERIALS AND METHODS**

**Synthesis of \[\Psi(CH_2NH)\] and \[\Psi(COCH_2)\] pseudopeptides and pseudopeptide analogues**

For pseudodipeptide synthesis, amino acid derivatives were obtained from Bachem Feinchemikalien AG (Budendorf, CH). NMR spectra were recorded at 300 MHz. Reversed phase–high-pressure liquid chromatography (RP–HPLC) analyses were performed on a µ-Bondapak C18 (10 µm) stainless steel column (Waters; 3.0 × 300 mm) (Milford, MA, USA), with a 1 ml/min flow rate, using (40:60 or 50:50) CH3CN/0.05% TFA as the mobile phase, and a UV detector set at 214 nm. The methylethenamino pseudopeptide Boc-Leu\[\Psi(CH_2NH)\]Tyr(Bzl)-OH was synthesized with 70% yield, containing 10% of Boc-Leu\[\Psi(CH_2NH)\]Tyr-Oh, from N-Boc-leucinal and H-Tyr(Bzl)-OBzl,p-tosylate, following the method of Bravo et al. (1991), with some minor modifications. The ketomethylene dipeptide analogue Boc-Leu\[\Psi(COCH_2)\]-(R,S)-Phe-OH was prepared according to González-Muñiz et al. (1995), with 30% overall yield from Boc-Leu-OH.

Solid phase synthesis was used to complete the preparation of the pseudopeptide analogues and peptide prototypes. It was carried out in the manual mode on p-methylanhydrilamine resin using Boc chemistry protocols (Barany and Merrifield, 1980). The peptide-resins were cleaved and deprotected by treatment with HF/anisole (9:1) at 0°C for 1 h. The crude peptides were isolated after evaporation of HF, washed with ether, dissolved in 10% acetic acid and lyophilized. After preparative purification on a reverse phase column (Nucleosil: 2 × 25 cm, 20 µm) using CH3CN/water (both with 0.05% TFA added) gradients, the peptides were checked for purity by analytical HPLC on Nucleosil (0.4 × 25 m, 5 µm) using CH3CN gradients on water (see Results section). Their identification was confirmed by amino acid analysis and MALDI–TOF mass spectrometry.

**Insects**

Adults of *B. germanica* were reared in complete darkness at 30 ± 1°C and 60–70% relative humidity. Freshly ecdised virgin females were isolated and used at the appropriate physiological ages, which were assessed by measuring the basal oocyte length (Bellés et al., 1987). All dissections and tissue sampling were carried out on CO2 anesthetized specimens.

**Quantification of JH synthesis**

Individual pairs of CA were incubated in 100 µl of TC 199 medium (Sigma, Madrid, Spain), containing L-methionine (0.1 mM), Hank’s salts, Hepes buffer (20 mM) plus Ficoll (20 mg/ml), to which L-[3H-methyl] methionine (Amersham, Buckinghamshire, UK) had been added to achieve a final specific activity of 7.4 Gbq/mmol. The experimental peptides or pseudopeptide analogues were added in aqueous solution to the incubation medium. Quantification of JH III produced was carried out in standard 3 h incubation periods. At the end of the incubation period, JH III was determined in the medium plus homogenized glands according to Piuachs and Couillaud (1992).
**Incubation of periovvaric fat body**

The incubation of periovvaric fat bodies was carried out in Grace’s medium, with l-glutamine and without insect hemolymph (Whitaker Bioproducts, Walkersville, MD, U.S.A.), as described by Martín et al. (1995b). The periovvaric fat bodies were dissected out of each female and washed for 10 min in Ringer’s saline, pre-incubated for 30 min in Grace’s medium, and then transferred to 300 μl of fresh medium, where the experimental incubation was carried out (in the dark, at 30°C and with gentle shaking). Because the periovvaric fat bodies from each specimen are symmetrical in terms of Vg production (Martín et al., 1995b), one was used as a control in each experiment. Vg released into the medium and its level in the incubated periovvaric fat body was measured by enzyme-linked immunosorbent assay (ELISA) (see below) at the end of the incubation period.

**Treatments in vivo**

Peptides or pseudopeptide analogues were administered by injection in Ringer’s saline at a dose of 50 μg in a volume of 2 μl to CO2 anesthetized 4-day-old virgin females. Controls received an equivalent treatment with solvent alone. The effects were measured after 24 h. Given the short half-life of BLAST-2, we expected that a period of 24 h would be appropriate to observe clear-cut differences between it and the pseudopeptide analogues, and to test the hypothesis that they would show improved resistance to endopeptidases.

**Preparation of samples for protein quantification and ELISA**

Fat body samples, either from incubation or from treatment in vivo, were thoroughly rinsed in Ringer’s saline and homogenized in Tris-buffer (0.4 M NaCl, 50 mM Tris–HCl, pH 7.3, 1 mM EDTA, 1 mM PMSF) with a plastic pestle. Homogenates were centrifuged at 10,000 rpm for 20 min, and the resulting supernatant was stored at −20°C. Ovaries were dissected in Ringer’s saline and homogenized in NaCl 0.4 M (1 mM PMSF); the cellular debris was pelleted by centrifugation (10,000 rpm, 20 min). The supernatant was collected and stored at −20°C. Hemolymph samples were obtained by cutting off one leg and applying gentle pressure to the abdomen. The hemolymph was collected in a micropipette, diluted in NaCl 0.4 M (1 mM PMSF) and stored at −20°C. Fat body, ovarian, and hemolymph-soluble proteins were quantified according to Bradford (1976), and Vg/vitellin titres were determined by ELISA.

**ELISA for Vg-vitellin**

For ELISA studies, an aliquot accounting for 400–500 ng of total soluble proteins from hemolymph, ovarian or fat body tissues was used. The sample was dissolved in carbonate buffer (0.05 M, pH 9.6) and absorbed to ELISA microplates by incubation overnight at room temperature, with gentle shaking. To analyse the incubation medium, aliquots of 5 μl were dissolved in fresh Grace’s medium and absorbed to microplate wells at 4°C overnight (Martín et al., 1995b). Incubation with antibodies and revealing procedures were as described by Martín et al. (1995a).

**Western blot analysis**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Enhanced Chemiluminescence (ECL) Western blotting of Vg was carried out as described (Martín et al., 1995b). Samples of 70–80 μg of total soluble fat body proteins were applied onto a 7.5% SDS–PAGE gel and protein bands were transferred to a nitrocellulose membrane. After the transfer, proteins were visualized by Ponceau S stain and the molecular weight standard proteins were located and marked. The polyclonal antibody against Vg was the same as that used in the ELISA, and was applied at a dilution of 1:18,000. ECL Western blotting (Amersham) was used following the manufacturer’s protocol. Chemiluminescent immunobLOTS were exposed to X-ray film for 30 s to 1 min. Bands corresponding to pre-pro-Vg, pro-Vg and Vg subunits were identified according to Martín et al. (1995a, b, 1996). At least five replicates were obtained for each Western blot analysis. Densitometry of the gels was carried out with a Molecular Dynamics (Sunnyvale, CA, U.S.A.) computing densitometer. Densitometry values were expressed as arbitrary absorbance units.

**RESULTS**

**Characterization of peptides and pseudopeptide analogues**

The methyleneamino pseudodipeptide Boc-LeuΨ[CH2,NH]Tyr(Bzl)-OH was obtained as a white solid, tR(40:60 CH3CN/0.05% TFA) = 26.40 min, with 10% of the compound deprotected at the Tyr side chain Boc-LeuΨ[CH2,NH]Tyr-OH, tR = 6.80 min. 1H NMR (CDCl3, δ (ppm) 0.80 and 0.83 [2d, 6H, J = 6 Hz, 5-H (Leu)], 1.18 [m, 2H, 3-H (Leu)], 1.38 (s, 9H, Boc), 1.42 [m, 1H, 4-H (Leu)], 2.59 [m, 2H, 1-H (Leu)], 2.82 and 3.07 [2m, 2H, 3-H (Tyr)], 3.42 [m, 1H, 2-H (Tyr)], 3.53 [m, 1H, 2-H (Leu)], 5.02 (s, 2H, OCH2Ph), 6.55 (br s, 1H, NH2Boc), 6.89 and 7.14 [2d, 4H, J = 8 Hz, Ar(Tyr)], 7.38 (m, 5H, Ph). This mixture was used directly for the solid phase synthesis of the corresponding BLAST-2 analogue (herein abbreviated as PTMA). The ketomethylene pseudodipeptide Boc-LeuΨ[CO2H]-[R,S]Phe-OH was obtained as an epimeric mixture at the Phe residue, which could not be resolved and was used directly for the solid phase synthesis of the corresponding BLAST-2 analogue (herein abbreviated as PTMB). tR(50:50 CH3CN/0.05% TFA) = 11.15 min. 1H NMR (CDCl3, δ (ppm) 0.85–0.92 [2m, 6H, 5-H (Leu)], 1.23–1.36 [m, 2H, 3-H (Leu)], 1.40 (s, 9H, Boc), 1.63 [m, 1H, 4-H (Leu)], 2.30 and 2.46 [2m, 1H, 3'-H(Tyr)], 2.74 [m, 2H, 2-H and 3'-H (Tyr)], 3.17 and 3.13 [2d, 2H, J = 10 Hz, 3-H (Tyr)], 4.17 [m, 1H, 2-H (Leu)], 5.01
After preparative reverse-phase purification, the allato-
statin BLAST-2 (Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-
amide), the [Phe]BLAST-2 analogue (Asp-Arg-Leu-
Ser-Phe-Gly-Leu-amide), and the methyleneamino
peptide PTMA (Asp-Arg-Leu[CH₃NH]Tyr-Ser-
Phe-Gly-Leu-amide) were assessed for purity (ca. 95%)
by analytical HPLC, and their identities were confirmed
by amino acid analysis and MALDI-TOF mass spec-
trometry. The ketomethylene pseudopeptide PTMB
(Asp-Arg-Leu[COCH₃]Phe-Ser-Phe-Gly-Leu-amide)
synthesized from the epimeric Boc-Leu[COCH₃]-
(R,S)Phe-OH described above, gave four fractions by
preparative reverse-phase purification, each one with the
expected amino acid composition and mass spectrum.
This suggested that further epimerization at the α carbon
of the Leu moiety had occurred, probably during the activa-
tion of the pseudopeptide prior to its incorporation into
the full sequence. Three diastereomers (PTMB-1, -2
and -3) eluted at 17.2, 19.8 and 21.3 min, respectively,
on Nucleosil (0.4 x 25 cm, 5 μm), using a shallow linear
gradient of 25-35% CH₃CN (+0.036% TFA) into H₂O
(+0.045% TFA) over 30 min. The fourth diastereomer
(PTMB-4) eluted at 35.7 min in the same system and so-
vent, but using a 25% isocratic elution. Because the pre-
liminary results of the biological tests did not show sig-
nificant differences between the four PTMB diastereomers,
or between them and PTMA (see next section), further structural elucidation of the respective
diastereomers was not attempted.

Inhibition of JH in vitro

In order to study the inhibitory activity of the pseudo-
peptide analogues on JH synthesis, we first investigated
the sensitivity of CA from females of different ages to
10⁻⁶ M of BLAST-2. The results indicated that the highest
inhibitory activities were elicited in CA from 5- and 6-
day-old females, the first being the most sensitive to al-
lastatin activity, giving an average of 55% inhibition (Fig.
1). Therefore, all the following tests were carried out on
CA from 5-day-old females.

The methyleneamino pseudopeptide PTMA was tested
at the range of concentrations usual for BLAST-2: 10⁻⁵,
10⁻⁶ and 10⁻⁷ M (see Bellés et al., 1994). Results showed
that the pseudopeptide PTMA is apparently less active
that BLAST-2, although the differences are not statisti-
cally significant at any of the concentrations tested (Fig.
2). The four diastereomers of the ketomethylene pseudo-
peptide PTMB were tested at 10⁻⁶ M, in comparison with
BLAST-2 and the analogue [Phe]BLAST-2. Results
indicated that the inhibitory activities of BLAST-2 and
the analogue [Phe]BLAST-2 were similar, and that those
of the four diastereomers of PTMB were also similar to
one another (Fig. 3). Comparison of the activities elicited
by the PTMB diastereomers with that of [Phe]BLAST-
2, suggested that the introduction of the ketomethylene

pseudo peptide bond slightly decreased activity, although
the differences between these models were not statisti-
cally significant.

Another conclusion emerging from these results is that
both types of pseudopeptide were similarly efficient in
mimicking the biological properties of the corresponding
model peptide. Given this similar performance, the sim-
pler and more easily synthesizable PTMA was chosen
for further biological studies.

Inhibition of Vg in vitro

Periovaric fat bodies from 4-day-old females were
incubated with 10⁻⁶ M of BLAST-2 or PTMA according
to Martín et al. (1996). After 7 h of incubation, Vg was
measured in the incubated tissue and in the medium. Vg

\[ \text{Vg} \]

\[ \text{Control} \]

\[ \text{BLAST-2} \]

\[ \text{PTMA} \]

\[ \text{BLAST-2} \]

\[ \text{PTMA} \]

\[ \text{Control} \]

\[ \text{BLAST-2} \]

\[ \text{PTMA} \]

\[ \text{Control} \]

\[ \text{BLAST-2} \]

\[ \text{PTMA} \]

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\[ \text{PTMA} \]
content in the fat body was similar in both treatments and in controls (control: 36.20 ± 2.53; BLAST-2-treated: 32.66 ± 3.07; PTMA-treated: 40.44 ± 2.60; results expressed as μg of Vg/μg of fat body proteins, n = 4–18). Conversely, results on Vg contents in the medium (Fig. 4) indicated that BLAST-2 clearly inhibited Vg release. The average Vg contents in the medium treated with PTMA were lower than controls, but the differences were not statistically significant.

**Inhibition of JH in vivo**

Compounds (BLAST-2 or PTMA) were injected at a dose of 50 μg on 4-day-old females, and the CA were explanted 24 h later in order to measure the rates of JH synthesis. Results (Fig. 5(A)) indicated that differences between control and treated (either with BLAST-2 or PTMA) glands were not statistically significant. However, the average rates of synthesis in PTMA-treated glands were lower with respect to controls, whereas those of BLAST-2 were higher.

**Inhibition of Vg in vivo**

Fat body, hemolymph and ovary samples were obtained 24 h after the in vivo treatment (see previous section), in order to measure Vg or vitellin levels. Vg content in the fat body (Fig. 5(B)) showed that PTMA is a more efficient inhibitor of Vg production than BLAST-2 when tested in vivo. The hemolymph levels of Vg in PTMA-treated specimens were lower with respect to controls, whereas those in treated with BLAST-2 were higher (Fig. 5(C)), although the differences between control and treated (either with BLAST-2 or PTMA) samples were not statistically significant. Similarly, vitellin levels in the ovary showed no statistical differences when comparing control and treated samples (PTMA or BLAST-2) (Fig. 5(D)), although the average levels in PTMA-treated were the lowest.

In addition, SDS–PAGE studies indicated that the protein pattern (not shown) was qualitatively similar in control and treated (either with BLAST-2 or PTMA) insects. The corresponding ECL Western blot analysis (Fig. 6) showed that bands corresponding to precursor (pro-Vg), glycosylated (pro-Vg) and processed forms (Vg subunits) of Vg were less intense in BLAST-2 and PTMA-treated samples than in the controls. Results obtained from the integration of all bands (Fig. 6) were consistent with those from ELISA measurements (Fig. 5(B)).

**DISCUSSION**

Methylecaminio \(\Psi[\text{CH}_2\text{NH}]\) and ketomethylene \(\Psi[\text{COCH}_2]\) peptide bond surrogates were introduced into the Leu\(^3\)-Tyr\(^4\) position of the cockroach allatostatin BLAST-2, and Leu\(^3\)-Phe\(^4\) of [Phe\(^a\)]BLAST-2, respectively, and the resulting pseudopeptide analogues were chemically characterized. The ketomethylene pseudopeptide analogue of [Phe\(^a\)]BLAST-2 was obtained as a mixture of the four diastereomers, which were isolated and studied. Inhibition of JH synthesis and Vg release by these pseudopeptide analogues were investigated in vitro and in vivo in the cockroach *B. germanica*.

The study on influence of age upon the inhibitory activity of BLAST-2 indicated that 5-day-old females were the most sensitive to allatostatic action, therefore all studies on JH synthesis were carried out at this age. The inhibitory activity was approximately proportional to the activity of the glands, as suggested by the parallel profiles of synthesis rates in the absence or presence of BLAST-2 (Fig. 1). This is in contrast with findings reported in *D. punctata* (Pratt et al., 1990), where the inhibitory activity of allatostatins during the gonado-
trophic cycle of mated females seems inversely related to the rates of JH synthesis (see also Stay et al., 1994). However, Pratt et al. (1990) used mated females (of D. punctata), whereas we have used virgin females (of B. germanica), which could account for the differences observed in the results of both studies. Conversely, our observations are closer to those reported by Weaver (1991) on virgin females of P. americana, where the ages showing highly active CA were most sensitive to allatostatin action.

With regard to the inhibition of JH synthesis in vitro, the substitution of Tyr⁴ by Phe⁴ in BLAST-2 did not significantly affect biological activity. This contradicts the results reported by Hayes et al. (1994), where Ala replacement studies indicated that Tyr⁴ plays an important role in biological activity. This apparent contradiction, however, may be explained by the fact that the substitution carried out here is clearly more conservative. The activity of ketomethylene and methyleneamino pseudopeptides showed that both analogues had similar efficiencies compared with the corresponding model peptides. This suggests that the Leu⁵-Tyr⁴ peptide bond of BLAST-2 is not critical for the interaction with the allatostatin receptor and for biological activity.

In vitro tests were also used to study the comparative effect of BLAST-2 and the methyleneamino (PTMA) pseudopeptide on Vg release by the fat body. They showed that both compounds inhibited Vg release, although the average inhibition was clearly lower in the case of the pseudopeptide.

In vivo activity was studied comparing BLAST-2 and PTMA at a dose of 50 μg. Previous reports on the in vivo activity of allatostatins indicated that doses required to elicit some effect at the level of JH inhibition were...
either repeated (ca. 3 μg twice a day for 3 days in *D. punctata*; Woodhead et al., 1993) or rather high (a single dose of 100 μg in *P. americana*; Weaver et al., 1994). As expected, a single dose of 50 μg was close to the threshold of effectivity of BLAST-2, and only in the case of Vg contents in the fat body, were values from specimens treated with this peptide significantly lower (*t*-test, \( P = 0.05 \)) than controls. Concerning JH synthesis, rates from BLAST-2-treated CA tended to be even higher than controls, which may be due to a rebound effect. Given the lower performance in *vitro* of the methyleneamino (PTMA) pseudopeptide when compared with BLAST-2, we would expect parallel results in *vivo*. However, the results suggested that the biological activity in *vivo* of the pseudopeptide analogue had been enhanced with respect to the native peptide. Vg contents in the fat body of PTMA-treated specimens were clearly lower (*t*-test, \( P = 0.01 \)) than controls, and vitellin contents in the ovary, hemolymph Vg, and rates of JH synthesis tended to be lower than BLAST-2-treated and control specimens.

Although the biological performance in *vivo* of the studied pseudopeptide analogues is still close to the native prototypes, the present results can be regarded as a first step towards designing more potent pseudopeptide analogues or true non-peptide agonists, in line with those described for other insect neuropeptides (Lange et al., 1995b; Albernathy et al., 1996), that may be useful tools for academic as well as practical uses.

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