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1,5-Disubstituted imidazoles inhibit juvenile hormone biosynthesis by the corpora allata of the mosquito *Aedes aegypti*

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Abstract

We investigated the effect of fifteen 1,5-disubstituted imidazoles (1,5-dis) on juvenile hormone III (JH III) and methyl farnesoate (MF) biosynthesis by the corpora allata (CA) of the mosquito *Aedes aegypti* in vitro. Four compounds (TH-35, TH-83, TH-62 and TH-28) significantly decreased JH biosynthesis in the CA dissected from 3-day old sugar-fed females. The decrease of JH synthesis was not always associated with increased MF. TH-30 and TH-83 increased MF levels, while TH-85 and TH-61 significantly decreased MF levels. Five compounds (TH-26, TH-60, TH-83, TH-35 and TH-30) significantly inhibited JH biosynthesis in the CA dissected from females 15 h after a blood meal. Four 1,5-dis (TH-30, TH-26, TH-28 and TH-66) caused MF increases in CA from blood-fed females. 1,5-Disubstituted imidazoles had higher inhibitory activity on JH synthesis when substituted at position 5 by a 3-benzyloxyphenyl group and at position 1 by a benzyl group (such as TH-35). Inhibition of JH and MF biosynthesis by TH-35 was age-dependent and influenced by nutritional status; inhibition differed when evaluated in the CA dissected from sugar-fed females at different days after emergence and in the CA dissected from females at different hours after a blood meal. Inhibition was always higher when the CA was more active. The addition of TH-35 significantly reduced the stimulatory effect of *Aedes*-allatotropin and farnesoic acid on JH synthesis. This is the first report of an inhibitory effect of 1,5-disubstituted imidazoles on JH synthesis in Diptera.

Keywords: Aedes aegypti; 1,5-Disubstituted imidazoles; Blood-feeding; Corpora allata; Juvenile hormone synthesis; Methyl farnesoate

1. Introduction

Juvenile hormones (JH) are a class of regulatory sesquiterpenoids that control metamorphosis in immature insects and reproduction in adult insects (Gilbert et al., 2000). Inhibition of the final steps of JH biosynthesis (such as farnesoic acid methylation and methyl farnesoate epoxidation) constitutes an excellent target for insect control, since the enzymes that catalyze these steps (*O*-methyl transferase and methyl farnesoate epoxidase) are specific for insect sesquiterpenoid biosynthesis (Feyereisen, 1985a; Schooley and Baker, 1985).

The corpora allata (CA), a pair of endocrine glands with nervous connections to the brain, synthesize and secrete JH. A range of disubstituted imidazoles has been investigated for their ability to decrease JH synthesis in vivo and in vitro. Application of 1,5-disubstituted imidazoles to *Bombyx mori* and *Neobellieria bullata* produced precocious metamorphosis (Darvas et al., 1990; Kuwano et al., 1988, 1991, 1992), and inhibited JH biosynthesis by the isolated CA of the cockroach *Diploptera punctata* (Pratt et al., 1990; Unnithan et al., 1995; Kuwano, 1997). The precocious metamorphosis of *B. mori* by 1,5-disubstituted imidazoles was prevented by the topical application of methoprene, a JH mimic (Roussel et al., 1987; Kuwano et al., 1994). Another 1,5-disubstituted imidazole (KK-42) when administered by feeding, delayed the growth and development of nondiapause-bound and diapause-bound *Ostrinia nubilalis* larvae, suggesting that KK-42 inhibits JH production (Gelman et al., 1995).

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The discovery of new molecules that could disrupt the mosquito endocrine system is relevant for vector control. In this study, we investigated the effect of fifteen 1,5-disubstituted imidazoles on JH and methyl farnesoate (MF) biosynthesis by the CA of the mosquito *Aedes aegypti* in vitro.

2. Materials and methods

2.1. Chemicals

Fig. 1 shows the chemical structures of the fifteen 1,5-disubstituted imidazoles that were tested. The synthesis of 1,5-disubstituted imidazoles has previously been described: TH-26, TH-28, TH-30 and TH-35 (Kuwano et al., 1991), KK-42 (Kuwano et al., 1985), TH-62, TH-66, TH-84 and TH-85 (Kuwano et al., 1992) and TH-60, TH-61, TH-80, TH-81, TH-82 and TH-83 as described for TH-35 (Kuwano et al., 1991). All compounds showed a single spot on thin-layer chromatography. The structures of the compounds were confirmed by 1H NMR spectra which were recorded on a JEOL EX-400 (400 MHz) spectrometer.

(*E*, *E*) methyl farnesoate and (*E*, *E*) farnesoic acid (FA) were purchased from Echelon (Salt Lake City, UT, USA), and JH III from Sigma (St. Louis, MO, USA) or ICN (Irvine, CA, USA).

2.2. Insects

A. aegypti of the Rockefeller strain were reared at 28 °C and 80% relative humidity under a photoperiod of 16 h light: 8 h dark. Adults were offered a cotton wool pad soaked in a 3% sucrose solution until 16 h before blood feeding. In this paper, we will refer to the cotton wool pad sucrose-fed females as “sugar-fed”. The mosquitoes were fed pig blood equilibrated to 37 °C, and 1 mM ATP was added to the blood-meal immediately before use as previously described (Noriega et al., 1999).

2.3. In vitro radiochemical assay for CA activity and use of inhibitors

Preparation of isolated CA complexes from adult *A. aegypti* females and measurement of JH and MF biosynthesis were previously described (Li et al., 2003a,b). Briefly, the spontaneous biosynthetic rates for methyl (2*E*, 6*E*)-(10R)-10,11-epoxy-3,7,11-trimethyldodeca-2,6-dienoate (JH III) and its immediate precursor methyl farnesoate were determined in isolated pairs of CA by measuring the incorporation of a *methyl*-^{3}H group

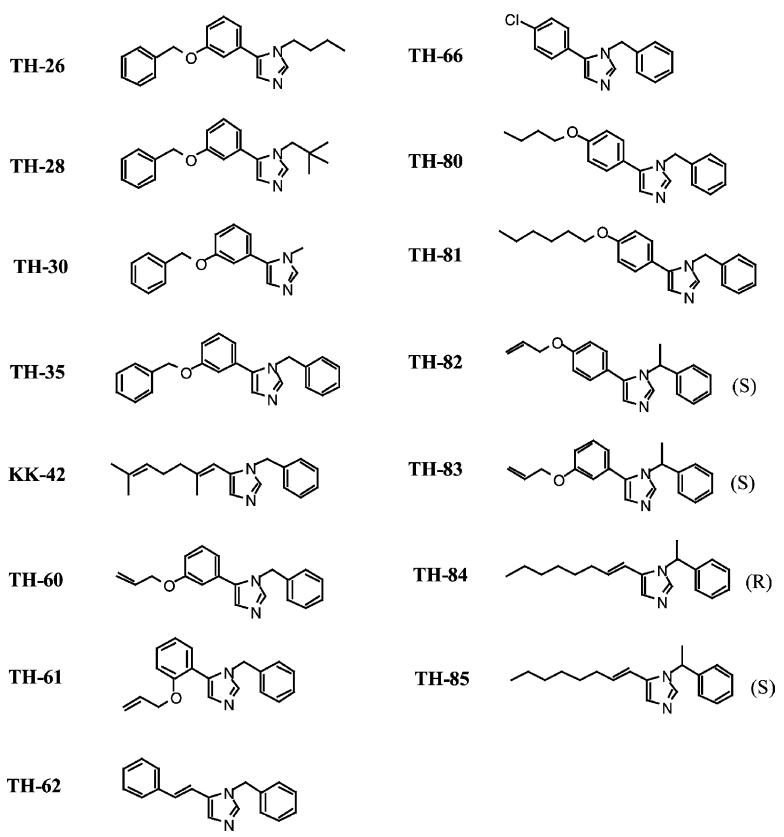

Fig. 1. Structures of the 1,5-disubstituted imidazoles tested in this study.

Table 1
Effect of 1,5-disubstituted imidazoles on biosynthesis of JH III and MF in vitro by CA complexes from 3-day old sugar-fed *Aedes aegypti* females. Individual CA complexes were dissected and incubated in the medium with 1 μM 1,5-disubstituted imidazoles. Each value is the mean (±SEM) of (*n*) measurements.

Compound	JH III (fmol/CA/h) (*n*)	MF (fmol/CA/h) (*n*)
Control	12.98 ± 1.90 (15)	7.69 ± 1.74 (15)
TH-35	2.84 ± 1.94* (10)	12.35 ± 4.64 (10)
TH-83	5.58 ± 0.34* (10)	13.50 ± 2.01** (10)
TH-62	5.60 ± 2.80** (10)	5.78 ± 2.46 (10)
TH-28	5.76 ± 0.91** (5)	8.77 ± 1.15 (5)
TH-26	8.14 ± 3.44 (10)	9.69 ± 1.89 (10)
TH-85	8.81 ± 3.05 (5)	0.71 ± 0.44** (5)
TH-60	8.87 ± 1.98 (10)	9.97 ± 2.73 (10)
TH-30	9.09 ± 1.66 (10)	47.98 ± 10.9*** (10)
TH-80	9.25 ± 5.49 (10)	6.47 ± 3.80 (10)
KK-42	10.35 ± 5.02 (25)	9.81 ± 2.93 (25)
TH-66	10.87 ± 6.37 (10)	8.15 ± 4.89 (10)
TH-84	11.75 ± 3.99 (10)	5.46 ± 1.33 (10)
TH-82	13.60 ± 1.83 (5)	4.62 ± 1.16 (5)
TH-61	16.59 ± 2.45 (15)	3.47 ± 0.39** (15)
TH-81	19.08 ± 6.46 (10)	5.86 ± 2.12 (10)

* Significantly different from the control value at $P < 0.01$.
** Significantly different from the control value at $P < 0.05$.
*** Significantly different from the control value at $P < 0.001$.

Table 2
Effect of 1,5-disubstituted imidazoles on biosynthesis of JH III and MF in vitro by CA complexes from *Aedes aegypti* females 15 h after a blood meal. The blood meal was given on the third day after emergence. Individual CA complexes were dissected and incubated in the medium with 1 μM 1,5-disubstituted imidazoles. Each value is the mean (±SEM) of (*n*) measurements

Compound	JH III (fmol/CA/h) (*n*)	MF (fmol/CA/h) (*n*)
Control	6.57 ± 1.11 (13)	2.23 ± 0.96 (13)
TH-26	1.32 ± 0.49* (5)	7.10 ± 1.90* (5)
TH-60	1.59 ± 0.36* (5)	2.74 ± 1.01 (5)
TH-83	1.69 ± 1.05* (5)	2.05 ± 0.60 (5)
TH-35	2.16 ± 0.81* (5)	1.21 ± 0.45 (5)
TH-30	2.38 ± 0.66* (5)	11.91 ± 4.26** (5)
TH-82	2.89 ± 0.78 (5)	2.44 ± 0.74 (5)
TH-61	3.30 ± 0.87 (10)	1.49 ± 0.28 (10)
TH-80	3.96 ± 2.42 (10)	3.08 ± 1.62 (10)
TH-28	4.35 ± 2.01 (10)	6.58 ± 2.03* (10)
TH-84	4.72 ± 1.50 (10)	2.13 ± 0.36 (10)
TH-62	4.78 ± 1.82 (10)	2.83 ± 1.90 (10)
TH-81	4.93 ± 2.25 (10)	3.94 ± 1.08 (10)
TH-85	5.53 ± 2.98 (10)	1.60 ± 0.18 (10)
KK-42	6.16 ± 0.77 (30)	4.56 ± 0.98 (30)
TH-66	9.95 ± 5.14 (10)	6.82 ± 2.06* (10)

* Significantly different from control at $P < 0.05$.
** Significantly different from control at $P < 0.01$.

from L-[*methyl*-^{3}H]methionine (specific activity 2.96–3.11 TBq/mmol; 80–84 Ci/mmol, Amersham Pharmacia, IL, USA), as described by Feyereisen and Tobe (1981) and Feyereisen (1985b) and modified by Li et al. (2003a,b). JH degradation during the experiment was checked by incubating ^{3}H-JH III in a medium in the presence or absence of CA complexes, and by analyzing the recovery of labeled JH. Between 95% and 99% of the hormone was recovered intact after 4 h of incubation (results not shown). Experiments with chemical inhibitors were conducted by the method of Brooks et al. (1985) which incorporates inhibitors into the medium without the inclusion of organic solvents.

2.4. Statistical analysis

Statistical analysis of the data was performed by *t*-test (unpaired with two-tailed), using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA, USA). The results were expressed as mean ± SEM, and considered significantly different at $P < 0.05$.

3. Results

3.1. Effect of 1,5-disubstituted imidazoles on JH III and MF biosynthesis by the CA in vitro

The effect of fifteen 1,5-disubstituted imidazoles on JH III and MF biosynthesis by the CA dissected from sugar-fed females 3 days after emergence is shown in Table 1. Four of the imidazoles significantly inhibited JH biosynthesis (TH-35, TH-83, TH-62 and TH-28). TH-30 and TH-83 increased MF levels, while TH-85 and TH-61 showed a significant decrease in MF levels (Table 1).

We also tested the effect of 1,5-disubstituted imidazoles on the CA dissected from females 15 h after blood feeding (Table 2). Five compounds significantly inhibited JH biosynthesis (TH-26, TH-60, TH-83, TH-35 and TH-30). Four compounds significantly increased MF levels (TH-30, TH-26, TH-28 and TH-66).

3.2. Changes in sensitivity of the CA to TH-35

The most potent JH III inhibitor (TH-35) was selected to study further changes of the CA sensitivity to 1,5-disubstituted imidazoles during adult life. Synthesis of JH III was very low (2.6 fmol/pair gland/h) immediately after adult emergence, irrespective of whether the CA were incubated with or without TH-35. Inhibition of JH biosynthesis by TH-35 was significant when the CA were dissected from 1-, 2- and 3-day old sugar-fed females (Fig. 2a). There was a significant linear relationship between the fmol of JH III synthesized by a pair of CA/h and the percentage of inhibition caused by the addition of TH-35 ($R^2 = 0.92$) (results not shown). Inhibition of JH biosynthesis by TH-35 was enhanced when the CA showed high JH biosynthetic activity.

Addition of TH-35 resulted in significant MF increases in glands dissected from sugar-fed females at 2, 3 and 5 days after emergence (Fig. 2b). Inhibition of JH biosynthesis by TH-35 was significant for CA dissected 1 and 12 h after blood feeding; there was no inhibition for CA dissected 24 and 48 h after blood feeding and inhibition was again significant for CA dissected 72 and 98 h after blood feeding (Fig. 3a). Addition of TH-35 did not result in significant increases of MF except in glands dissected 96 h after blood feeding (Fig. 3b).

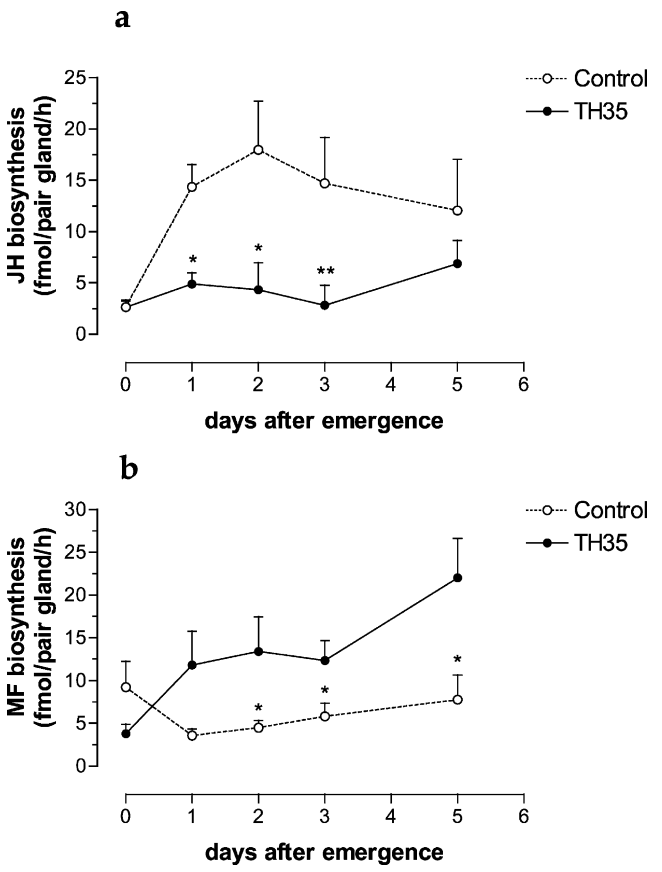

Fig. 2. Effect of TH-35 on the in vitro biosynthesis of JH III and MF by CA from 3-day old sugar-fed females. (a) biosynthesis of JH III and (b) biosynthesis of MF. Individual CA complexes were dissected and incubated in the medium with 1 μM TH-35. Control (○), TH-35 (●). Each point (±SEM) represents the mean of 10–25 independent determinations of individual CA complex. Asterisks denote significant differences from control values (unpaired *t*-test; ∗ $P \leq 0.05$ and ∗∗ $P \leq 0.01$).

3.3. Activity of 1,5-disubstituted imidazole on CA stimulated with farnesoic acid and Aedes-allatotropin

Previous studies in *A. aegypti* have demonstrated that the JH biosynthesis by the isolated CA can be stimulated by the addition of *Aedes*-allatotropin (*Aedes*-AT) or FA (Li et al., 2003a). We tested the effect of TH-35 on *Aedes*-AT- stimulated CA and FA-stimulated CA from 5-day old sugar-fed females. The addition of Aedes-AT (10^{-9} M) and FA (40 μM) resulted in a significant increase in JH III biosynthesis (Fig. 4a). The addition of TH-35 significantly reduced the stimulatory effect of *Aedes*-allatotropin and farnesoic acid on JH synthesis (Fig. 4a). Addition of TH-35 resulted in a significant increase in MF levels in AT-stimulated CA (Fig. 4b). In contrast, TH-35 had no significant effect on MF increases in FA-stimulated CA (Fig. 4b).

We tested the effect of TH-35 on FA-stimulated CA dissected from females at different hours after blood-feeding; the addition of TH-35 had no significant effect

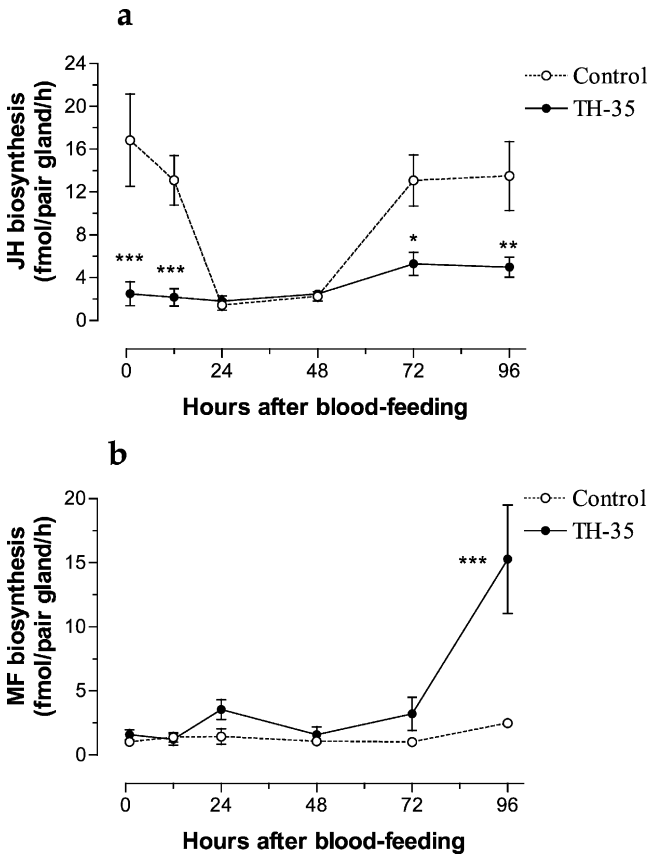

Fig. 3. Effect of TH-35 on the in vitro biosynthesis of JH III and MF by CA from blood-fed females. (a) biosynthesis of JH III and (b) biosynthesis of MF. Individual CA complexes were dissected and incubated in the medium with 1 μM TH-35. Control (○), TH-35 (●). Each data point (±SEM) represents the mean of 10–25 independent determinations of individual CA complex. Asterisks denote significant differences from control values (unpaired *t*-test; ∗ $P \leq 0.05$; ∗∗ $P \leq 0.01$ and ∗∗∗ $P \leq 0.001$).

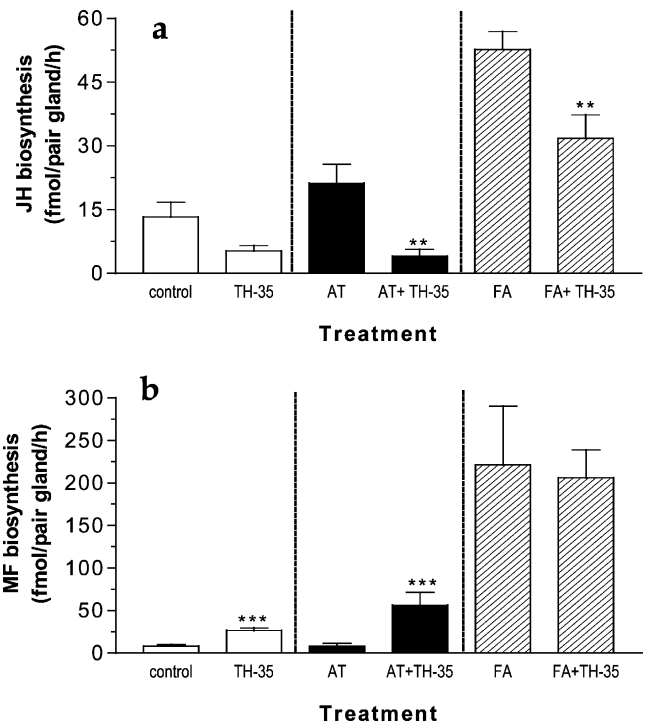

Fig. 4. Effect of TH-35 on the in vitro biosynthesis of JH III and MF by FA- and *Aedes*-AT-stimulated CA from 5-day old sugar-fed females. (a) biosynthesis of JH III and (b) biosynthesis of MF. Individual CA complexes were dissected and incubated in the medium at different additions. Control: medium, TH-35: 1 μM TH-35, AT: 10^{-9} M *Aedes*-allatotropin, TA + TH - 35: 10^{-9} M *Aedes*-AT plus 1 μM TH-35, FA: 40 μM farnesoic acid and FA + TH - 35: 40 μM FA plus 1 μM TH-35. Each data point (±SEM) represents the mean of 10–25 independent determinations of individual CA complex. Asterisks denote significant differences in each group (unpaired *t*-test; ∗∗ $P \leq 0.01$ and ∗∗∗ $P \leq 0.001$).

on JH biosynthesis (Fig. 5a). The addition of TH-35 resulted in significant increases of MF in FA- stimulated CA dissected at 1 and 12 after blood feeding (Fig. 5b).

4. Discussion

Fifteen 1,5-disubstituted imidazoles were tested in the short-term radiochemical assay for their effect on JH III and MF biosynthesis. It is interesting that most compounds showed different activity against CA from sugar-fed and blood-fed females. Inhibition of JH synthesis was significant for all the tested compounds characterized by the presence of a 3-benzyloxyphenyl group as the substituent at position 5 (TH-26, TH-28, TH-30 and TH-35). In addition, the presence of a 3-allyloxyphenyl group (TH-60 and TH-83) or a styryl group (TH-62) as substituents at position 5 significantly inhibited JH III biosynthesis. Since the activity of compounds is different against sugar-fed and blood-fed females, it is difficult to discuss the structure–activity relationships. Nevertheless, combined results from experiments with sugar and blood-fed females suggest that 1,5-disubstituted imidazoles have higher activity when substituted at position 5 by a *3-benzyloxyphenyl group* and at position 1 by a *benzyl group*. This 1,5-disubstituted imidazole is TH-35 and the prediction is consistent with our results, which showed TH-35 having the highest inhibitory activity on JH biosynthesis.

The activity of the most potent inhibitor (TH-35) was dependent on the age of the CA-donor sugar-fed female, and the time of dissection of the CA after a blood meal. Our studies suggest that when the spontaneous biosynthetic activity of CA is low, TH-35 had no significant effect on JH synthesis. However, inhibition of JH biosynthesis in vitro was enhanced when CA showed high JH biosynthetic activity.

The ability to inhibit JH biosynthesis was not always associated with MF increases. In sugar-fed females, TH-83 decreased JH levels and increased MF levels. In contrast, TH-30 significantly increased MF levels without affecting JH synthesis, while TH-61 showed only a significant decrease in MF levels. In blood-fed females, two

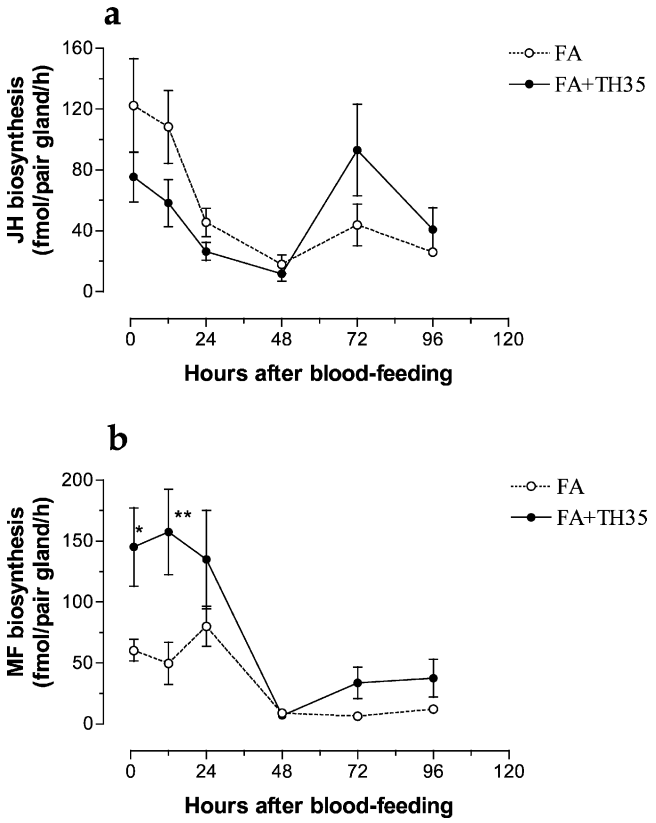

Fig. 5. Effect of TH-35 on the in vitro biosynthesis of JH III and MF by FA-stimulated CA from blood-fed females. (a) biosynthesis of JH III and (b) biosynthesis of MF. Individual CA complexes were dissected and incubated in the medium with 1 μM TH-35 plus 40 μM FA. FA (○), FA + TH - 35 (●). Each data point (±SEM) represents the mean of 10–25 independent determinations of individual CA complex. Asterisks denote significant differences from control values (unpaired *t*-test; ∗ $P \leq 0.05$ and ∗∗ $P \leq 0.01$).

imidazoles significantly decreased JH levels with concomitant increases of MF levels (TH-26 and TH-30), while the two compounds (TH-28 and TH-66) significantly increased MF levels without affecting the JH synthesis. It seems that TH-30 is a more selective inhibitor of MF epoxidase, because it inhibits JH III biosynthesis with increases of MF in both sugar-fed and blood-fed females. The ability of 1,5-disubstituted imidazoles to inhibit JH biosynthesis without concomitant MF increases was previously described for cockroaches; Brooks et al. (1985) found that in *Periplaneta americana*, some methyl farnesoate epoxidase inhibitors produced accumulation of MF while other did not. Price et al. (1987) also found that 1-citronellyl-5-phenyl disubstituted imidazole or piperonyl butoxide inhibited JH III biosynthesis by the CA of *Periplaneta americana* without causing a significant accumulation of MF.

Previous studies in *A. aegypti* have demonstrated that JH biosynthesis by the isolated CA can be stimulated by the addition of FA and *Aedes*-AT (Li et al., 2003a). The addition of TH-35 significantly reduced the stimulatory effect of *Aedes*-AT and FA on JH synthesis. The addition of TH-35 to AT-stimulated CA enhanced MF accumulation.

In summary, several 1,5-disubstituted imidazoles showed some degree of inhibition of JH biosynthesis. The inhibition of JH biosynthesis was not always associated with MF accumulation. The activity of the most potent inhibitor (TH-35) was dependent on the age of the CA-donor sugar-fed female, and the time of dissection of the CA after a blood meal. 1,5-Disubstituted imidazoles are suitable inhibitors to study the JH synthesis in mosquitoes and are promising tools for vector control.

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