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Stimulating Action of Methyl 12,12,12-Trifluorofarnesoate on in Vitro Juvenile Hormone III Biosynthesis in *Blattella germanica*

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Methyl 12,12,12-trifluorofarnesoate (MTFF) at a dose of 10 μM, stimulated in vitro juvenile hormone (JH) release in corpora allata (CA) from 6-day-old, freshly ecdysed, and 8-day-old (period of ootheca transport) adult virgin females of *Blattella germanica*. In addition, MTFF also induced intraglandular accumulation of JH and MF in treated CA. Trifluorofarnesoic acid (TFFA) and trifluorofarnesol (TFF) exhibited the same properties, although to a lesser extent than MTFF. The detection of MTFF in TFFA-treated CA suggested that TFFA and TFF were biotransformed into MTFF by the CA enzymatic system and that this ester might be responsible for the activity observed. Equivalent experiments carried out with farnesoic acid (FA) resulted in a more significant stimulation of JH production. This is not surprising, because exogenous FA is readily epoxidized at C10-C11 double bond and methylated to afford JH. Conversely, analytical data have shown that the C6-C7 double bond of MTFF is epoxidized by the CA enzymatic system, whereas that at C10-C11 remains practically unaltered.

Key words: German cockroach, corpora allata, farnesoic acid, 12,12,12-trifluorofarnesoic acid, 12,12,12-trifluorofarnesol

INTRODUCTION

The peculiar properties of fluorine have been widely used to alter the behavior of bioactive compounds. In this context, the preparation of trifluoromethyl

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derivatives of methyl farnesoate and JH* III, in which the methyl group at C-3 or C-7 was replaced by CF_3, constituted the first examples of fluorinated analogs of these hormones [1,2]. The C-3-substituted derivative, in the acid form, inhibited the O-methyl transferase in CA from adult female *Locusta migratoria* [3]. On the other hand, the C-7 trifluoromethyl analog showed moderate juvenilizing responses when assayed in vivo on larvae of *Tribolium confusum* and *Blattella germanica* [4].

Nevertheless, we deemed that fluorine's disruptive effects could be more efficiently exerted in the vicinity of the terminal double bond, as this site is epoxidized in the last stage of JH biosynthesis. Thus, charge density and polarization changes induced by fluoro substituents should modify the reactivity of the olefin moiety toward the oxidative process, with a concomitant perturbation in the JH III production system. Accordingly, we recently prepared fluorinated analogs of MF and/or JH III in which fluorine replaces hydrogen atoms at C-10 [5] or at the C-12 methyl group (MTFF) (Fig. 1) [6].

This paper reports on the in vitro stimulatory activity of MTFF on JH production by CA from adult virgin females of *Blattella germanica*. This species produces JH III [7], and we have recently studied the in vitro spontaneous activity of CA during the first gonadotropic cycle [8]. For comparative purposes, a parallel study with FA has also been carried out. Addition of FA to the incubation medium containing isolated CA enhances JH biosynthesis (see [9] and references therein). In the order Dictyoptera, this stimulatory effect has been demonstrated in *Periplaneta americana* [10,11] and *Diploptera punctata* [12,13], both producing JH III as the only JH homolog. In these species, exogenous FA is readily used as substrate in the two last steps of JH III biosynthesis, i.e., methylation to afford MF followed by epoxidation of the terminal double bond [14].

The effects of MTFF and FA on JH production were investigated at three different stages in the first gonadotropic cycle: previtellogenic, vitellogenic, and postvitellogenic. In vitellogenic females, the study was extended to the corresponding trifluoromethylfarnesoic acid and alcohol; in addition, the intraglandular contents of JH III and MF were also measured. Furthermore, a rigorous identification of the biosynthesized compounds was carried out by chromatographic and spectral techniques.

MATERIALS AND METHODS

Insects

Adult *B. germanica* were reared at 26 (±1)°C as described elsewhere [15]. Freshly ecdysed virgin females isolated from the colony were used at the appropriate age, which was additionally assessed by measure of the basal oocyte length.

*Abbreviations used: BOL = basal oocyte length; BSA = bovine serum albumin; CA = corpora allata; CC = corpora cardiaca; CI = chemical ionization; 6,7-EMF = methyl 6,7-epoxyfarnesoate; 6,7-EMTFF = methyl 6,7-epoxy-12,12,12-trifluorofarnesoate; 10,11-EMTFF = methyl 10,11-epoxy-12, 12,12-trifluorofarnesoate; FA = farnesoic acid; FEAR = fractional endocrine activity ratio; GC/MS = gas chromatography mass spectrometry; JH = juvenile hormone; MF = methyl farnesoate; MTFF = methyl 12,12,12-trifluorofarnesoate; RT = retention time; SIM, selected ion monitoring; TFF = 12,12,12-trifluorofarnesol; TFFA = 12,12,12-trifluorofarnesoic acid.

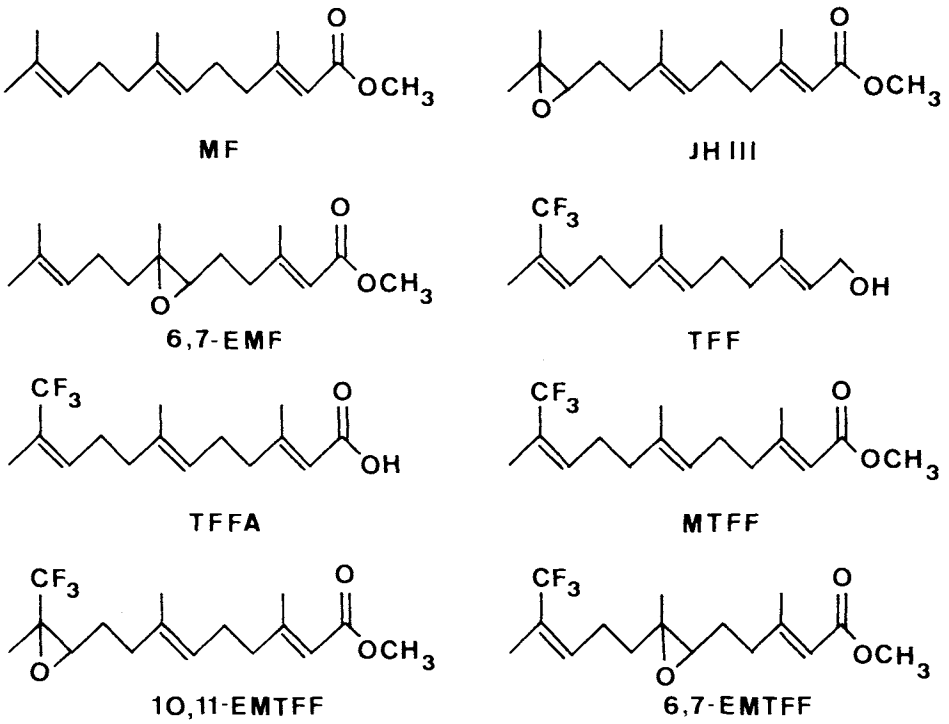

Fig. 1. Structures of juvenile hormone III (JH III); methyl farnesoate (MF); methyl 12,12,12-trifluorofarnesoate (MTTF); 12,12,12-trifluorofarnesoic acid (TFFA); 12,12,12-trifluorofarnesol (TFF); methyl 6,7-epoxyfarnesoate (6,7-EMF); methyl 6,7-epoxy-12,12,12-trifluorofarnesoate (6,7-EMTFF); and methyl 10,11-epoxy-12,12,12-trifluorofarnesoate (10,11-EMTFF).

Compounds and Analytical Techniques

All glassware was coated with 1% 201 silicone emulsion in water (Siliconas Hispania, Barcelona). JH III was from Sigma (Dorset, UK) MF was prepared in our laboratory by a procedure similar to that described for a bisdeuterated analog [7]. The trifluoromethyl compounds MTFF, TFFA, and TFF, as well as the epoxyderivative 6,7-EMTFF (Fig. 1), were synthesized and characterized in our department by Dr. FJ Sánchez (unreported data). Although the fluorinated compounds depicted in Figure 1 are shown in the Z configuration at C-12, they contained 10% of the corresponding (12*E*) isomer. [10-^{3}H(N)] JH III (11 Ci/mmol) was obtained from New England Nuclear (Dreieich, West Germany) and [*methyl*-^{3}H]methionine (80–85 Ci/mmol) from Amersham (Amersham, UK).

TLC analyses were performed on precoated silica gel plates (aluminium sheets, 0.2 mm thick with concentrating zone. Merck, Darmstadt, West Germany). HPLC analyses were carried out with a Waters (Milford, Mass.) modular system provided with two model 510 pumps, an automated gradient controller, a U-6K injector, and a 481 UV detector. Radioactivity was measured with a scintillation counter or with an Isomess TLC radioscanner (Münster, West Germany).

In Vitro Techniques and JH Release Quantification

Glands from three different ages in the first gonadotropic cycle were used: a) from freshly ecdysed females; b) from 6-day-old specimens; and c) from females carrying the ootheca (8-day-old, 24 h after ootheca formation). CC-CA complexes were incubated in Millipore (0.22 μm) filtered TC-199 medium (0.2 ml) without glutamine and containing L-methionine (0.05 mM), Hank's salts, HEPES medium buffer (20 mM) plus Ficoll (20 mg/ml), to which [^{3}H]methionine (2 μCi) had been added to a final specific activity of 400 mCi/mmol. Conditions for dissection, measurement of basal oocyte length, and in vitro assay for rate of JH III production were as previously described [8]. After an initial 2-h incubation, each pair of glands was transferred to fresh medium containing the test compound in 0.1 μl ethanol at the appropriate concentration and incubated for 2 h. Finally, a 2-h posttreatment incubation was carried out after transferring the glands to fresh medium. In all cases, control incubations with medium containing 0.1 μl of ethanol were performed.

Quantification of Intraglandular JH III and MF

Glands from 6-day-old females were incubated in medium containing 10 μM of the test compound as described above. In the case of MTFF, a supplementary experiment without a posttreatment incubation was carried out. JH III and MF contents were quantified in the glands from these experiments [16]. The individual pairs of CC-CA were thoroughly rinsed, transferred to a tube containing methanol (200 μl), sonicated (Labsonic 1510 probe, Melsungen, West Germany), and centrifuged (2 min, 10,000 *g*). The supernatant was collected and the pellet washed with methanol and recentrifuged. The combined supernatants were concentrated under nitrogen and analyzed by HPLC (30 × 0.39 cm i.d. column packed with Spherisorb ODS-2, Tracer Analítica, Barcelona). Under our conditions, retention times for JH III, MTFF, and MF were 11.30, 18.70, and 20.24 min, respectively. Eluates corresponding to 1-min fractions were collected and radioactivity measured. The limit of detection for radioactive JH III standard was estimated at 0.03 pmol.

GC/MS Identification of JH III and MTFF Derivatives From In Vitro Incubations

CC-CA complexes (n = 10) from 6-day-old females were incubated for 9 h in TC 199 medium with 10-μM MTFF. When incubation was complete, the medium and glands were extracted separately using the procedure of Mauchamp et al. [17], although sonication (2 × 30 s at 60 W) was used for extraction of glands. Gas chromatographic separation of extracts was performed on a 25-m, 0.25-mm CP-Sil 5B capillary column (Chrompack, Les Ulis, France). Oven temperature was 200° C; carrier gas flow was 1 ml/min. The injector was an all-glass Ross Type (Girdel, Suresnes, France). The mass spectrometer (Nermag 10-10C, Rueil-Malmaison, France, interfaced to a data-acquisition system) has a 25-cm-long quadrupole and was used in the ammonia-positive CI mode.

Another set of experiments followed the approach of Baker et al. [18]: four CA were sonicated (2 × 30 s, 60 W) in 100 μl of 0.1 M phosphate buffer (pH: 7.4) containing 1% BSA. Then 40 μl of homogenate (1.6 CA equivalent) was mixed with 50 μl 1 mM NADPH and either 2 μl of 50 μM MF or 2 μl of 50 μM MTFF and incubated for 30 min at 30° C. After the addition of brine (50 μl), the medium

was extracted with ethyl acetate (2 × 0.8 ml), and organic extracts were evaporated and stored at −20° C until used. Quantification of the biosynthesized compounds was performed using the selected ion monitoring mode [17] after determination of the retention time and ions with highest relative intensity. MF or MTFF were used as internal standard to evaluate the yield of extraction and sample preparation.

RESULTS

Activity of MTFF

The effects of MTFF on in vitro JH release were first studied in incubations of CC-CA from 6-day-old females of *B. germanica*. These vitellogenic females show the highest spontaneous production of JH during the first gonadotropic cycle [8]. The MTFF concentrations used were 100 and 10 μM, and the results obtained in the period of treatment (Table 1) evidenced that stimulation of approximately 40% was induced in both cases. The FEAR [19] was also similar (ca. 0.72) for both MTFF concentrations. In addition, a persistence of the stimulating effect was observed in the posttreatment period of incubation (4–6 h), which was more apparent when the 100 μM dose was used.

In a supplementary set of in vitro incubations of CC-CA from 6-day-old females in the presence of MTFF (10 μM), the medium was analyzed by HPLC to ensure a rigorous identification of JH III. The analysis showed incorporation of tritium only at the RT corresponding to JH III. The rates measured with this method were (in pmol JH III/h × pair CA): 2.28 ± 0.60 (n = 5), 3.41 ± 0.97 (n = 4), and 3.62 ± 0.84 (n = 5) for the pretreatment, treatment, and posttreatment periods, respectively.

TABLE 1. Activity of MTFF, TFFL, TFFA, and FA on the In Vitro JH Release by CA From Virgin Females of *B. germanica* in the First Gonadotrophic Cycle*

Treatment	(μM)	N	Age[a]	BOL[b]	pmols JH/h × pair CA		
					0–2 h	2–4 h	4–6 h
MTFF	100	9	6	1.84 ± 0.03	1.51 ± 0.10	**2.11 ± 0.09²**	2.77 ± 0.09
MTFF	10	9	6	1.85 ± 0.02	1.49 ± 0.12	**2.08 ± 0.11²**	2.05 ± 0.19
FA	10	6	6	1.85 ± 0.02	1.80 ± 0.12	**7.14 ± 0.75²**	3.42 ± 0.42
TFF	10	7	6	1.90 ± 0.02	2.07 ± 0.19	**2.38 ± 0.24²**	2.47 ± 0.25
TFFA	10	6	6	1.74 ± 0.01	1.64 ± 0.19	**1.91 ± 0.18²**	2.10 ± 0.25
Ethanol	(0.05%)	10	6	1.85 ± 0.02	1.46 ± 0.23	**1.63 ± 0.17⁰**	1.66 ± 0.17
MTFF	10	6	0	0.48 ± 0.01	0.21 ± 0.03	**0.47 ± 0.07²**	0.22 ± 0.03
FA	10	6	0	0.48 ± 0.01	0.31 ± 0.01	**1.38 ± 0.04²**	0.32 ± 0.07
Ethanol	(0.05%)	5	0	0.48 ± 0.01	0.48 ± 0.04	**0.53 ± 0.05⁰**	0.46 ± 0.05
MTFF	10	6	8	0.33 ± 0.01	0.10 ± 0.03	**0.69 ± 0.26¹**	0.09 ± 0.03
FA	10	5	8	0.33 ± 0.01	0.05 ± 0.04	**0.96 ± 0.04²**	0.06 ± 0.05
Ethanol	(0.05%)	5	8	0.34 ± 0.01	0.20 ± 0.05	**0.18 ± 0.08⁰**	0.17 ± 0.05

*JH release rates are given as $\bar{X}$ ± SEM, those values obtained from incubation in treated medium are indicated in boldface. Results of the *t*-test for paired data (comparison of the values from the treatment with those obtained in the pretreatment period of the same experiment) are summarized with the following superscripts: 0 (NS: $P < 0.05$), 1 ($P < 0.01$), 2 ($P < 0.001$).

[a]Chronological age in days; 8-day-old females are carrying the ootheca.

[b]Length (mm) of basal oocyte of the CA donors.

For comparative purposes, the stimulatory activity of FA at a dose of 10 μM was studied. In contrast with the results obtained with MTFF, the increase of JH release in FA-stimulated glands was clearly higher (approximately 300%), but the FEAR was lower (ca. 0.24). Moreover, the stimulatory effect also persisted in the posttreatment incubation period.

The stimulatory effect showed by MTFF led us to study the activity of this compound on incubated CC-CA from females in previtellogenic and postvitellogenic stages, which show the lowest spontaneous production of JH [8]. Results obtained at 10 μM are also depicted in Table 1. Again, MTFF stimulated JH release in both cases, although some differences were apparent. The absolute JH release values in these MTFF-stimulated glands were lower in comparison with those measured in the CC-CA from 6-day-old females. However, the percentage increase of JH release rates was higher: 124 and 590%, as average, in previtellogenic and postvitellogenic females, respectively. Moreover, FEAR was lower in the case of postvitellogenic specimens (ca. 0.15) and showed intermediate values (ca. 0.45) for the previtellogenic females. Conversely, stimulatory effects were not persistent in the posttreatment period of incubation.

Comparative experiments carried out with FA at the same concentration (10 μM) showed (Table 1) that the stimulatory activity was higher, in either absolute or in relative terms (average percentage increases of 300 and 1,200% in previtellogenic and postvitellogenic females, respectively). For previtellogenic specimens, the FEAR was lower (ca. 0.23) than that obtained in MTFF-stimulated glands, and it reached the lowest values (ca. 0.05) in postvitellogenic specimens.

Activity of TFF and TFFA

To determine if compounds related to MTFF could exhibit higher or lower stimulatory activity on JH production, the trifluoromethyl derivatives TFFA and TFF were assayed. As illustrated in Figure 1, these compounds are the respective trifluoro analogs of FA and farnesol. We anticipated that they could be substrates of the JH biosynthetic enzyme system, which converts farnesol into methyl farnesoate, as the transformation involved (oxidation to carboxylic acid and methylation) would occur at the other extreme end of the fluorine substitution in the sesquiterpene chain (see next heading).

The effects of TFF and TFFA assayed at doses of 10 μM are summarized in Table 1. Both compounds elicited a slight and similar stimulation (approximately 15%), which persisted in the posttreatment period of incubation, and the FEAR was also similar (ca. 0.86) in both cases. This stimulatory activity was, however, clearly lower in comparison with that observed in the equivalent experiment performed with 10 μM of MTFF.

MF and JH Intraglandular Contents

To obtain more information about the possible mode of action of MTFF, intraglandular contents of MF and JH were measured in CA treated with MTFF and with TFF and TFFA (10 μM) as well. In addition, an assay with the same MTFF, but without the posttreatment period of incubation, was performed.

The results (Table 2) indicate that all treated CA have higher levels of MF and JH than do controls and that the influence of the posttreatment period of

TABLE 2. In Vitro JH Release by CA From 6-Day-Old Females of *B. germanica* Incubated with MTFF, TFF, TFFA, or FA (10 μM) for 2 h and Intraglandular Contents of JH and MF*

Exp.	Treatment	N	BOL[a]	JH release pmol JH/ h × pair CA 0–2 h	2–4 h	4–6 h	CA contents (pmol) JH	MF
1	MTFF	7	1.89 ± 0.02	1.85 ± 0.05	**2.58 ± 0.07**	2.77 ± 0.08	1.56 ± 0.11	0.31 ± 0.05
2	MTFF	7	1.84 ± 0.03	1.60 ± 0.12	**2.12 ± 0.18**	—	1.56 ± 0.20	0.24 ± 0.09
3	TFF	7	1.90 ± 0.02	2.07 ± 0.19	**2.38 ± 0.24**	2.47 ± 0.25	0.58 ± 0.09	0.41 ± 0.08
4	TFFA	6	1.74 ± 0.01	1.64 ± 0.19	**1.91 ± 0.18**	2.10 ± 0.25	1.04 ± 0.06	0.33 ± 0.03
5	FA	6	1.85 ± 0.02	1.80 ± 0.12	**7.14 ± 0.75**	3.42 ± 0.42	2.30 ± 0.10	0.61 ± 0.04
6	Ethanol (0.05%)	6	1.95 ± 0.04	1.47 ± 0.31	**1.55 ± 0.29**	1.58 ± 0.30	0.64 ± 0.20	0.06 ± 0.01
7	Ethanol (0.05%)	7	1.75 ± 0.03	2.60 ± 0.41	**2.59 ± 0.43**	—	0.54 ± 0.06	0.08 ± 0.02

*All values are expressed as $\bar{X}$ ± SEM and those obtained from incubation in treated medium are indicated in boldface.
[a]Length (mm) of basal oocyte of the CA donors.

incubation was negligible. CA incubated with TFF showed the highest relative accumulation of MF, followed by those treated with TFFA and MTFF (Table 2). Conversely, glands incubated with MTFF had the highest levels of JH, followed by those treated with TFFA and TFF. In addition, it is worth noting that HPLC analysis of TFFA-treated glands revealed the presence of MTFF (0.18 ± 0.04 pmol per pair CA; n = 6), indicating that an intraglandular methylation of this acid had occurred.

The relationship between JH release vs. JH synthesis (i.e., the intraglandular contents plus the released hormone, see [20]) was also studied. As shown in Figure 2, a linear correlation between both parameters can be obtained for each treatment. Thus, if the slope of the regression line is close to 1, then lower values of *b* coefficient (see the legend of Fig. 2) indicate major predominance of JH biosynthesis over JH release. Accordingly, MTFF-treated glands showed the highest relative accumulation of JH, followed by those treated with

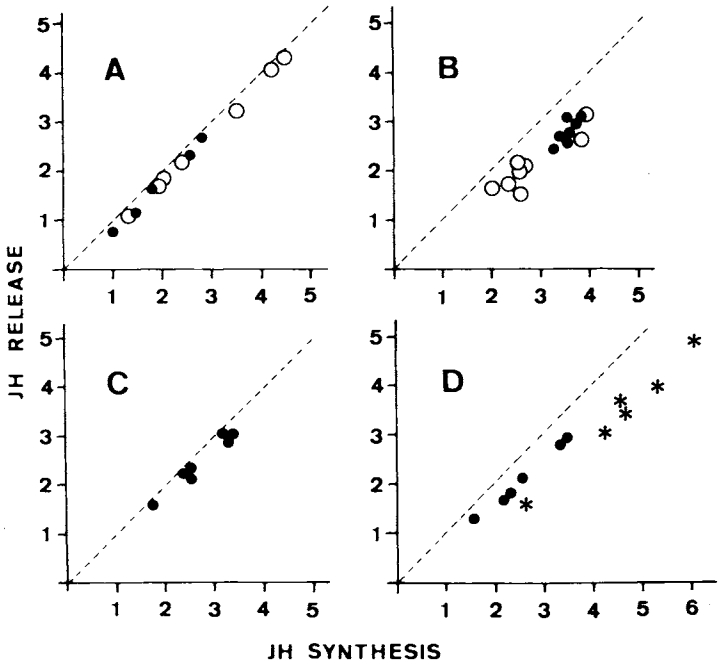

Fig. 2. Relationship between the rate of JH synthesis and the rate of JH release (pmol/h × pair CA) by individual pairs of CA subjected to different treatments. **A:** 2 h of incubation in a medium containing 0.05% of ethanol (control); **B:** 10 μM MTFF; **C:** 10 μM TFF; **D:** 10 μM TFFA (black points) or 10 μM FA (asterisks). Experiments carried out with each of two periods of pretreatment and posttreatment of 2 h are indicated by black points or asterisks, and those performed without posttreatment period of incubation are indicated by open circles. The coefficients of the linear regression (y = ax + b) calculated in each case are as follows. A (black points): a = 1.062, b = −0.339, r = 0.9948; A (circles): a = 0.979, b = −0.137, r = 0.9995; A (black points + circles): a = 1.000, b = −0.205, r = 0.9974; B (black points): a = 1.000, b = −0.790, r = 0.7496; B (circles): a = 0.665, b = 0.192, r = 0.9549; B (black points + circles): a = 0.781, b = −0.725, r = 0.9370; C: a = 0.946, b = −0.138, r = 0.9770; D (black points): a = 0.955, b = −0.405, r = 0.9937; D (asterisks): a = 0.955, b = −0.944, r = 0.9440.

TFFA and TFF. Certainly, the regression lines obtained for MTFF are not fully satisfactory, but they provide enough evidence to support the above ranking.

Results from experiments carried out with FA are also summarized in Table 2. In absolute terms, JH and MF contents of these FA-treated CA were the highest observed in this study. However, if contents are related to the JH release rates (i.e., MF or JH contents × JH release rates^{-1}), then they are equivalent to those obtained in MTFF-incubated glands (for JH) and those from TFF or TFFA treatments (for MF). Also, in this case, a linear correlation between JH release vs. JH synthesis (Fig. 2d) can be calculated with a satisfactory correlation coefficient. Moreover, the *b* coefficient was the lowest measured, which indicates that FA induces the highest intraglandular JH accumulation among the compounds herein investigated.

Mass Spectrometry Studies

Mass spectral studies were carried out to determine if MTFF could be epoxidized by the CA enzymatic system and to identify the possible epoxyderivatives. Analyses were performed after 9 h of CA incubation in a medium containing 10 μM of MTFF, and compounds were analyzed for in the medium were JH III, MTFF, 10,11-EMTFF and 6,7-EMTFF (Fig. 1).

A standard mixture was used to optimize GLC separation; 10,11-EMTFF was not included in the mixture because we were not able to obtain this compound by conventional methods of MTFF epoxidation. Good resolution was obtained for the standard compounds used; retention times were 2.07 min for MTFF, 2.45 min for 6,7-EMTFF, 3.18 min for JH III, and 3.25 min for 6,7-EMF. The clear separation between JH III and 6,7-EMF suggested that 6,7-EMTFF and 10,11-EMTFF, if present, would be well separated also.

Data on the identification of the ions obtained in the mass spectra (CI-NH_3) of JH III, MTFF, and 6,7-EMTFF are summarized in Table 3. As shown, it is not possible to localize the epoxy ring position in the chain since JH III and 6,7-EMF show identical ions, and by analogy we should expect the same fragmentation pattern to occur in both 6,7-EMTFF and 10,11-EFTFF isomers (Table 3). Thus, the presence of 10,11-EMTFF in the incubation medium should be detected as this derivative should show the same ions as 6,7-EMTFF, but with a different RT.

To increase the sensitivity of detection, SIM was used with the highest relative intensity ions. In the case of 6,7-EMTFF, ions 321 and 338 were selected and a peak was detected at RT 2.45. For MTFF, selected ions were 305 and 322, and detection occurred at RT 2.07. Because these ions also exist in the 6,7-EMTFF mass spectrum, they also appear at RT 2.45. The ions selected to identify JH III were 235, 252, and 267, and they all showed a peak at RT 3.18.

Under the experimental conditions used to quantify the hormone released by MTFF-stimulated CA into the medium, JH III was detected at RT 3.18 with ions 235, 252 and 267 (Fig. 3A), and the rate of release was estimated at 1.8 pmol/h × pair CA.

In addition, ions 321 and 338 were detected at RT 2.45 (Fig. 3B), which indicates that MTFF was epoxidized to 6,7-EMTFF, although at a very low (ca. 1%) ratio of conversion. Conversely, it is not possible to give a definitive answer as to the presence or the absence of 10,11-EMTFF, as a small peak observed for

TABLE 3. Identification of Ions of Several JH Homologues Obtained by Chemical Ionization Using NH_3 as Reagent Gas*

	$M+NH^+_4$	MH^+	$M+NH^+_4-CH_3OH$	MH^+-H_2O	MH^+-CH_3OH	$MH^+-(H_2O+CH_3OH)$
MF	268	251	236		219	—
MTFF	322	305	290		273	
JH III	284	267	252	249	235	217
6,7-EMF	284	267	252	249	235	217
6,7-EMTFF	338	321	306	303	289	271
(10,11-EMTFF)[a]	338	321	306	303	289	271

*This mode allowed the determination of theoretical ions corresponding to 10,11-EMTFF.
[a]Expected ions.

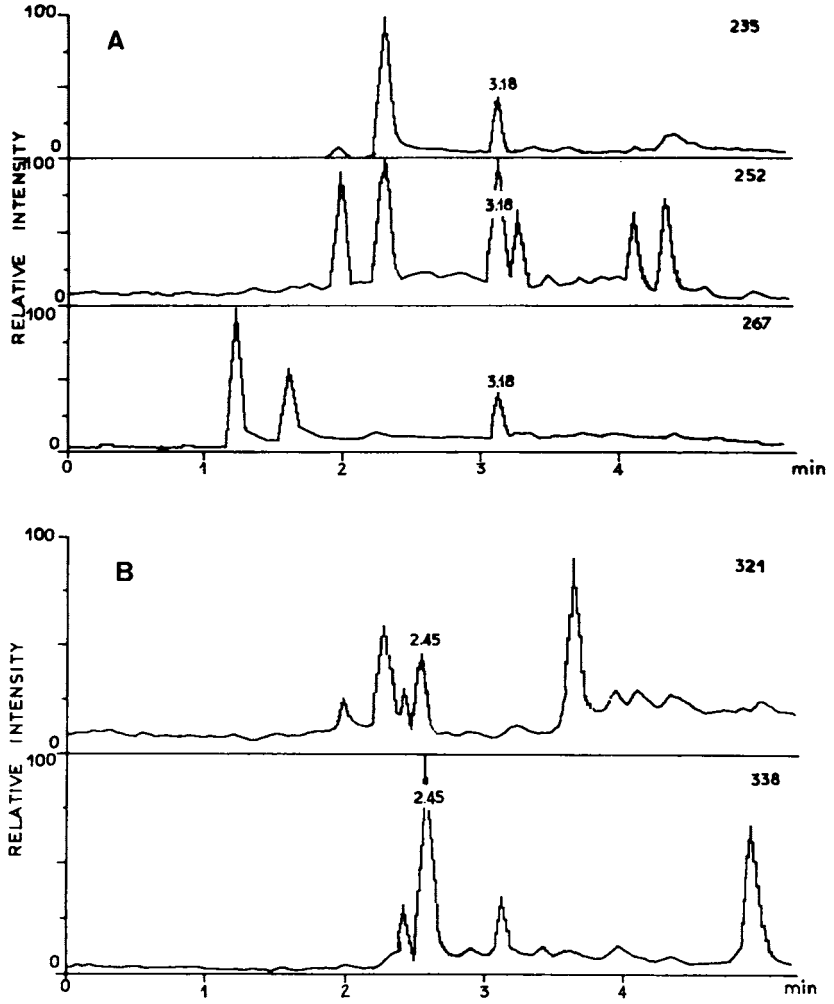

Fig. 3. **A:** Detection of JH III (RT 3.18) in incubation medium using selected ion-monitoring mode. Detection was performed with ions 235, 252 and 267. **B:** Detection of 6,7-EMTFF (RT 2.45) after CA incubation in the presence of MTFF. At m/z 321 and 338, a small peak is detected at RT 2.36 which could suggest the presence of small amounts of 10,11-EMTFF.

both ions 321 and 338 (Fig. 3B) at RT 2.36 could correspond to this compound. However, even assuming this tentative identification, 10,11-EMTFF would be present in small amounts in comparison with 6,7-EMTFF.

On the other hand, CA homogenates incubated in phosphate buffer with MF and NADPH showed the presence of JH III (approximately 300 pg). Conversely, in a parallel set of incubations carried out with MTFF, we did not detect the formation of any trifluoromethyl epoxy derivative, and JH III levels found in this case were below 100 pg.

DISCUSSION

The above results indicate that the trifluoroderivative MTFF enhances the in vitro JH production by CA from *B. germanica* females, and complementary analyses by HPLC have confirmed that the compound released by the incubated CA, and currently measured by TLC, is authentic de novo [^{3}H]JH III, whereas no other radiolabeled compounds were detected.

On the other hand, we have verified (unpublished results) that epoxidation of MTFF with *m*-chloroperoxybenzoic acid, a reagent widely used as a biomimetic chemical probe for P-450 cytochrome mediated oxidations [21], affords only the corresponding 6,7-epoxyderivative, whereas the terminal C10-C11 double bond remains unaltered. MTFF may act as a stimulatory agent for JH production without being an effective substrate for the cytochrome P-450 mediated specific epoxidation on the terminal double bond. This hypothesis is supported by the results obtained in the GC/MS analyses of the media extracts after incubation of CC-CA with MTFF. With the analytical conditions given, which allow detection of the JH III released during the experiment, no significant peak attributable to a theoretical 10,11-epoxide of the MTFF was found. In addition, GC/MS analyses also revealed the presence of the corresponding 6,7-epoxyderivative (Fig. 1: 6,7-EMTFF) in the incubation medium, demonstrating the effective penetration of MTFF into the gland. This finding also indicated that the enzymatic system of these MTFF-treated CA was able to act on this less accessible C6-C7 position.

Complementary proof on the deactivation of the MTFF terminal double bond toward epoxidation came from an experiment with CA homogenates incubated in phosphate buffer, either with MF or MTFF, in the presence of NADPH. Results showed no evidence for trifluoromethyl epoxyderivative formation in the MTFF incubations, thus suggesting again that the CA epoxidase was unable to epoxidize the terminal double bond of MTFF, which is in agreement with our results from the peroxyacid oxidation.

The functional experiments showed that MTFF stimulates the CA from females in the three stages studied: freshly ecdysed (previtellogenesis), 6-day-old (vitellogenesis), and 8-day-old (postvitellogenesis), which show low, very high, and very low spontaneous production of JH, respectively. However, examination of the values measured in each case pointed out several apparent differences. The most conspicuous results were obtained in CA from postvitellogenic females, which showed the highest percentage of stimulation (590%) and the lowest FEAR values (ca. 0.15).

The study of the trifluoromethyl derivatives TFF and TFFA showed that they stimulate JH release, although to a lesser extent than MTFF. The detection of MTFF in TFFA-treated glands suggested that the above results could be due to a biotransformation of these two derivatives into MTFF mediated by the same CA enzymatic systems operating in the last steps of JH biosynthesis. Thus, MTFF may be the main, if not the only, compound responsible for the stimulatory activity observed.

Comparison of the stimulatory activity of FA with that induced by MTFF showed that in both cases the percentage of stimulation was inversely proportional to the activity of the gland in the pretreatment period. Such a relationship, in which CA producing JH at high rates are stimulated to a lesser degree

than low activity glands, seems quite general, because it has been observed in numerous other CA systems (see [22] and references therein). However, FA exerts a far higher stimulation, either in absolute or relative terms, although this is because this intermediate is a substrate in the last step of JH biosynthesis [9–13, 19].

In summary, the experimental results on MTFF led to three significant observations: the compound enhances JH III biosynthesis and induces MF intraglandular accumulation, its terminal olefin moiety is not a substrate for the epoxidase involved in the JH biosynthetic process, and this epoxidase is not substrate-specific as indicated by the formation of 6,7-EMTFF (see also [16,23]).

The occurrence of high levels of MF within the MTFF-treated CA could be explained by either an inhibition or a saturation of the terminal epoxidase. However, the parallel enhancement of JH release in these glands suggests the second possibility, which would lead us to postulate that MTFF stimulates de novo JH biosynthesis. In this sense, the levels of MF accumulation in the CA of *B. germanica* treated with FA (or MTFF) are similar to those described for *Periplaneta americana*, a species whose CA have limited epoxidative capacity [10].

Although it is not possible with these data to determine the mechanism of action accounting for the stimulatory activity of MTFF, the results described provide new information on the role of fluorine in the design of bioactive organic molecules. Recently we questioned the strategy of introducing a fluorosubstituent in a double bond to depress the reactivity of the olefin [5], and here we propose the potential use of a specifically located trifluoromethylvinyl moiety in a lipophilic structure for inducing perturbations in the cytochrome P-450 monooxygenase system.

In addition, MTFF appears to be one of the few synthetic JH analogs (see also [24]) that exhibits a stimulatory action on in vitro JH biosynthesis without being an intermediate of this process. The peculiar properties of MTFF should make it a useful experimental tool, not only in the insect field, but also in related areas of invertebrate endocrinology, particularly within crustaceans, as recent findings [25] suggest that MF may be a juvenile hormone in this arthropodan class.

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