Biological Activities of Natural Sesquiterpene Lactones and the Effect of Synthetic Sesquiterpene Derivatives on Insect Juvenile Hormone Biosynthesis

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Three natural sesquiterpene lactones have been assayed for their biological activity on locust (*Locusta migratoria)* nymphs. Since results obtained in vivo pointed to disruptions on juvenile hormone (JH)-regulated physiology, we tested the sesquiterpene lactones and a family of synthetic sesquiterpene derivatives for their ability to disturb the rate of JH biosynthesis by locust corpora allata (CA) in vitro. All the active compounds in vitro share a basic sesquiterpene double-ring structure as well as substituents with different chemical complexities. Compounds that shared an eudesmane base with different oxidation states on C3 and C12 carbon atoms were tested as JH biosynthesis inhibitors. Supplementation of the locust corpora allata culture media with exogenous JH precursors together with data obtained with corpora allata from a moth (*Agrotis ipsilon*) suggested that epoxidase is a target of the inhibition process.

Keywords: *Insect; juvenile hormone biosynthesis; inhibitors; sesquiterpene lactones; sesquiterpene amides; biological activity; epoxidase inhibition*

INTRODUCTION

There is an increasing amount of evidence indicating that many of the secondary metabolites of plants provide defense against insect predators to the species that may accumulate them (Mann, 1987b; Guella et al., 1996; Marvier, 1996). In fact, many important insecticides incorporate either natural products with plant origins or structural analogues obtained by chemical synthesis (Camps, 1988; Sparks, 1990).

Among these compounds, natural terpenes display a range of activities suggesting that they should be examined as agents of pest control. For example, eudesmane sesquiterpenes, including both 6,12- and 8,12-olide moieties, make up a group of natural compounds widely present in the plant kingdom (Mann, 1987a; Sparks, 1990; Borkosky et al., 1997; Garcia et al., 1996). These natural products have already attracted much interest because of their wide spectrum of biological properties: antimicrobial (Bork et al., 1996), allelopathic (Seigler, 1996), and particularly the cytotoxic (Wang et al., 1996) and antitumor (Beekman et al., 1996) activities associated with the α-methylene γ-lactone group (Chen et al., 1996; Robles et al., 1995). Some reports also ascribe insect antifeedant and repellent effects to sesquiterpene lactones (Mullin et al., 1991; Felton, 1996) or to synergism with some insect phototoxynes (Bowers et al., 1995).

New approaches for alternative pesticides tend in part to target hormone-regulated functions, on account of the peculiarities of the insect endocrine system. In this way, it should be noted that reports about the effect of plant eudesmane derivatives on insect JH-related functions such as reproduction or development are scarce.

In this paper, we present the results obtained by testing three natural sesquiterpene lactones extracted from *Centaurea paui* (Compositae) on the development of 3rd and 5th instar larvae of *Locusta migratoria* (Orthoptera). These chemicals produced differential mortality rates depending upon the instar of the treatment and affected molting and metamorphosis to different extents. Natural sesquiterpene lactones can be chemically changed to sesquiterpene compounds by ring opening aminolysis (Blay et al., 1996). This allows the synthesis of related sesquiterpene compounds that can be obtained either as majority or as side products having a wide variety of substituents. This prompted us to test the natural lactones and some of the other synthetic derivatives for their effect on the rate of JH biosynthesis of adult locust corpora allata (CA) in vitro. Most of the synthetic compounds tested inhibited CA activity in vitro, and they were also tested for their ability to produce anti-JH effects on 3rd instar nynphs of *L. migratoria*.

Tests of the relevance of the molecule substituents for biological effects often require previous characterization of the activities of a series of structurally related synthetic compounds so an empirical base for establishing their structure−activity relationships can be set (Nishimura, 1990). In the first series of chemicals, we tested those that shared a sesquiterpene base but differed in chemical complexity of substituents on the C3 and C12 carbon atoms. We then tested a second

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batch of synthetic compounds that were chemically derived from an active member of the first series. Data concerning the two series of chemicals, as well as their structural formulas, are presented and discussed.

METHODS AND MATERIALS

Insects. African locusts (*L. migratoria*) were reared in gregarious conditions on young barley and corn seedlings supplemented with bran, in crowded cages at 30 ± 2 °C and a 16 h light:8 h dark photoperiod (16L:8D). Black cutworms (*Agrotis ipsilon*) were reared on an artificial diet and maintained in individual plastic cups until pupation under 16L:8D at 21 ± 1 °C. Adults were held in plastic boxes and had access to a 20% sucrose solution (Duportets et al., 1996).

In Vivo Assays. Topical application of the chemicals (100 μg doses) was performed in 2 μL of acetone with a Hamilton syringe; controls were treated with the same volume of acetone. Each experiment was repeated three times with 10 nymphs per iteration.

In Vitro Measurement of JH Biosynthesis. JH biosynthesis was analyzed using the radiochemical assay first described by Pratt and Tobe (1974). Corpora allata from 9-day-old *L. migratoria* adult females were incubated at 28 °C in TC 199 (Flow Laboratories) supplemented with Ficoll (20 g/L, Sigma) and [^{14}C]methylmethionine (final concentration of 0.3 mM, specific activity of 1.5 Bq/pmol). When necessary, farnesol or farnesoic acid was added to the culture medium (Couillaud et al., 1988). After a 3 h incubation, corpora allata and medium were extracted twice with hexane and submitted to either normal phase HPLC or TLC.

Corpora allata from 4-day-old *A. ipsilon* moths were incubated in TC 199 and sodium [2-^{14}C]acetate (final concentration of 943 mM, specific activity of 1.08 Bq/pmol). After incubation for 4 h, the medium was extracted twice with ethyl acetate and submitted to reverse phase HPLC.

Normal phase HPLC separation was performed on a silica column (250 × 4.6 mm, Chrompack, Middelburg, NL) and eluted with a hexane/2-propanol (93/7) mobile phase.

Reverse phase HPLC was performed on a polymer column (PLRP-S, 5 mm diameter, 5 cm length, 10 nm, Flow Zinser, Frankfurt, Germany) and eluted with a 4 to 80% acetonitrile linear gradient on 5 mM HEPES at pH 6.2 (Halarnkar and Schooley, 1990; Duportets et al., 1996).

Radioactivity monitoring was performed on line by solid scintillation counting (Ready Safe, Beckman). The mobile phase was also monitored by UV detection at 245 nm.

TLC separations were performed on precoated silica sheets (Merck, reference 5735) in a xylene/ethyl acetate (4/1) solvent system. The radioactivity of bands corresponding to JH III sliced from the sheet was determined by liquid scintillation counting with a Beckman LS 2800 spectrometer.

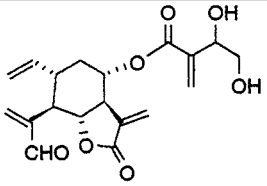
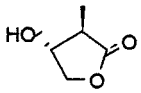
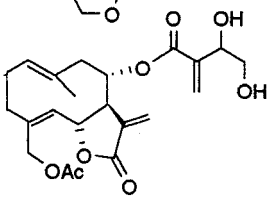
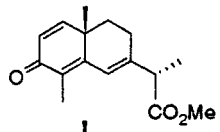
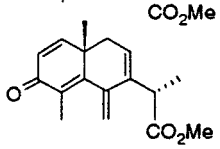
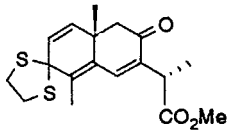
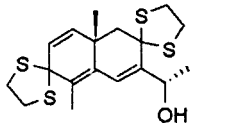
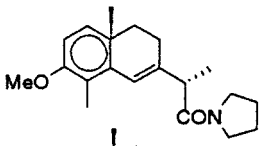
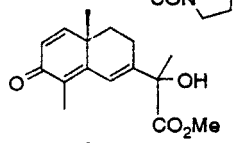
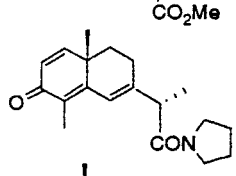
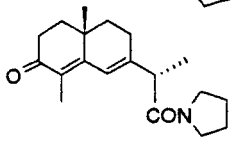
Osmotic Pressure Measurement. The osmotic pressure of the culture medium of CA-containing chemicals to be tested was determined at the freezing point using a Knauer osmometer (Berlin, Germany) by comparison with a NaCl standard curve.

Chemicals. Chemicals that were assayed were a gift from Dr. Blay (Departamento Química Orgánica, Universitat de València). Synthesis was carried out according to the methods of Blay et al. (1996). All other chemicals were either reagent or HPLC grade. Natural compounds were extracted and identified according to the methods Cardona et al. (1994).

RESULTS AND DISCUSSION

In Vivo Activity of Natural Sesquiterpenie Lactones and Synthetic Eudesmane Derivatives. In a first batch of experiments, we assayed three sesquiterpene lactones (chemicals **1**−**3**; see Table 1) extracted from *Centaurea paui* for their ability to disturb development of 5th and 3rd instar *L. migratoria* larvae. Each compound, dissolved in acetone, was topically applied on the insect tegument and its effect evaluated by the observation of the animals during the following days up to the adult stage.

Table 1. Effect of Different Sesquiterpene Compounds at 1.5 g/L on the in Vitro Spontaneous and Farnesol-Stimulated Rate of JH Biosynthesis by Locust Corpora Allata[a]

	Inhibition of spontaneous activity (%)	Inhibition of farnesol stimulated activity (%)
 (1)	0	-
 (2)	0	-
 (3)	0	-
 (4)	97	99
 (5)	99	99
 (6)	98	99
 (7)	99	99
 (8)	98	75
 (9)	45	-
 (10)	100	100
 (11)	99	99

[a] Numbers, expressed as a percentage of the control activities, are means of five individual determinations.

Lactone **1**, when applied to 5th instar larvae at a dose of 100 μg per insect, had no effect during the first week after its application. However, after control animals had metamorphosed to adults, treated animals still remained in the larval instar. Most of them (80%) died 1 week later without molting. Survivors (20%) molted about 10 days after the controls and gave inperfect adults with minor abnormalities such as twisted wings. Aside from this, feeding deterrence was observed in treated animals. A dose of 100 mg per insect applied to 3rd instar larvae did not produce any morphogenetic changes during the next two larval instars. However, the metamorphosis was delayed for about 5 days in treated animals. During this extra period, most of the treated animals died (60%). Survivors molted to the adult stage without apparent abnormalities. No feeding deterrence was detected in this latter case.

Lactone **2**, when applied to 5th instar larvae at a dose of 100 μg per insect, induced mortality. About 60% of the treated insects died between 3 and 8 days following the topical application. Survivors molted about 5 days after the controls and became normal gregarious adults. When the same dose of lactone **2** was applied to 3rd instar larvae, the next two larval−larval molts were delayed for 5 and 7 days, respectively. The duration of the 5th instar was not delayed, and survivors (about 50%) gave normal adults (100%). No feeding deterrence was observed in the assay of lactone **2**.

Lactone **3** showed acute toxicity when topically applied to 5th instar larvae at a dose of 100 μg per animal. All treated insects died within 2 days after treatment. Surprisingly, such rapid mortality was not observed when 3rd instar larvae were treated with the same dose. In the latter case, deaths occurred at the end of the 3rd instar and during the 4th and 5th larval instars. The duration of the last larval instar was increased for 5 days. Survivors (30%) gave normal adults. No feeding deterrence was observed after application of lactone **3**.

Synthetic sesquiterpene compounds (**4**−**11**, Table 1) were screened for anti-JH in vivo effects by applying them to 3rd instar nymphs of *L. migratoria* at a dose of 100 μg per insect. Most of them caused a strong mortality (about 70%), except compound **10** for which 40% of the survivors showed no apparent abnormalities and compound **9** which showed the weakest mortality (about 30% were survivors after metamorphosis), and this probably allowed the detection of 40% intermediate adults with respect to the survivors.

In vivo effects of the lactones used in this study are complex and quite difficult to interpret. Feeding deterrence of sesquiterpene lactones has been reported earlier (Streibl et al., 1983; Mullin et al., 1991), but in this work, feeding deterrence was only observed for lactone **1** when larvae were treated at the final larval stage. No feeding deterrence was found in the other cases. The biological effect of lactone **1** seems to be more or less specific to the last larval instar. The final larval instar differs from the previous ones mainly in the endocrine induction of the metamorphosis. Absence of JH plays a major role in the induction of metamorphosis, and disturbance of the JH endocrine system may be responsible for the observed effects. Since JH also exhibits ecdysiotropic effects (Williams, 1959; Riddiford, 1972), disturbance of JH metabolism could be responsible for the observed prolongation of the larval stage. We thus investigated the possible effect of the natural lactones and synthetic lactone derivatives on the rate of JH production by the juvenile hormone-producing glands in vitro.

Effect of Natural Lactones and Synthetic Lactone Compounds on the Spontaneous JH Biosynthesis Rate in Vitro. The three natural lactones (**1**−**3**) together with a series of related synthetic sesquiterpe compounds (**4**−**10**) (Table 1) were tested for their effect on the spontaneous JH biosynthesis rate by locust corpora allata in vitro. The chemicals were added to the incubation medium at a final concentration of 1.5 g/L. As shown in Table 1, some of the compounds exhibited a strong inhibition of the JH biosynthesis. Addition of the most active compounds (**4**−**8**, **10**, and **11**) to the CA culture medium resulted in an almost complete inhibition of hormone production, whereas compound **9** caused an inhibition of 45%.

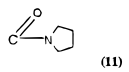
We verified that the chemicals tested did not vary the pH or the osmolarity of the CA culture medium, as this could be responsible for the observed inhibition (Couillaud, 1991). Compound **7** was highly insoluble in aqueous solution and thus was not further investigated.

It has been reported that some lactones inhibit early steps of the isoprenoid biosynthesis pathway in vitro (Schooley and Baker, 1985; Sparks, 1990), but surprisingly, the three natural lactones (**1**−**3**) that had been assayed in vivo did not modify the spontaneous rate of JH biosynthesis. It could be hypothesized that lactones would be hydrolyzed in the aqueous media of CA incubation. Otherwise, it could be possible that the effect observed in vivo did not result from a direct effect on JH biosynthesis. Finally, the natural lactones may undergo some activation in vivo that would not occur in our in vitro system, and their biological activity could be due to their sesquiterpene base instead of their lactone substituents. This latter hypothesis is supported by the strong inhibitory effect observed with compounds **4**−**11**, which share the sesquiterpene base but lack lactone function.

Effect of Sesquiterpene Compounds on the Farnesol-Stimulated JH Biosynthesis Rate in Vitro. The strong inhibitory effect of compounds **4**−**6**, **8**, **10**, and **11** motivated further characterization of their activity. To determine the target enzymes of the inhibitors on the JH pathway, we tested their inhibitory effect at a dose of 1.5 g/L on CA incubated in medium supplemented with trans−trans farnesol, a late intermediate of JH biosynthesis. Exogenous farnesol (200 μM) is believed to enter the biosynthetic pathway and to saturate enzymes beyond its formation. It has been shown that it stimulates JH production up to 10 times and is convenient for homogenizing the rate of hormone production in locust CA (Couillaud et al., 1988). As shown in Table 1, inhibition of JH biosynthesis was maintained on farnesol-stimulated corpora allata. The inhibition was above 90% for most compounds tested. As addition of exogenous farnesol did not restore hormone production, it can be concluded that the compounds tested act at least in part upon enzymes downstream from farnesol synthesis.

Effect of C3- and C12-Modified Derivatives on JH Biosynthesis in Vitro. All of the active compounds show a common double-ring sesquiterpene base with either alcohol or carboxyl derivatives (methyl ester or amide moieties) on C12 and other substituents with different chemical complexities on C3 (Table 1). The solubility of these chemicals on aqueuous media depends

Table 2. Effect of Different C3 and C12 Sesquiterpene Derivatives at 1.5 g/L on the in Vitro Farnesol-Stimulated Rate of JH Biosynthesis by Locust Corpora Allata[a]

	R		Inhibition of JH biosynthesis (%)
C3 Ketones		(11)	99
		(12)	72
C3 Alcohols		(13)	100
		(14)	97
		(15)	91

[a] Numbers, expressed as a percentage of the control activities, are means of five individual determinations.

strongly on the nature of substituents at positions C3 and C12, and hence, they are not suitable for quantitative comparison of their in vitro activity. Thus, we tested the effect of a second batch of more related C12 and C3 sesquiterpene derivatives on the rate of JH biosynthesis by farnesol-stimulated locust CA. Amides such as **11** (Table 1) have been reported to be useful starting materials for the synthesis of such sesquiterpene derivatives. Ketone amide, alcohol amide, alcohol amine, ketone amine, and ketone alcohol were synthesized (Blay et al., 1996) and tested.

As shown in Table 2, at a dose of 1.5 g/L, the five molecules inhibited farnesol-stimulated JH biosynthesis. However, molecule **12** was less efficient than the others. Since all of the molecules were active, we focused on C3 alcohols (**14** and **15**) and checked the effect of their corresponding C12 derivatives at a 10-fold diluted dose on spontaneous activity. Amide **14** still maintained more than 50% inhibition of spontaneous CA activity at a dose of 0.15 g/L, whereas amine **15** was less effective. The effect of compounds (**14** and **15**) was also tested on CA incubated in the presence of 200 μM farnesol. Both compounds were assayed at doses of 5, 0.5, and 0.05 mM (2.3, 3.3, and 4.3, respectively, in log scale; Figure 1). Amide **14** and amine **15** blocked JH synthesis with a 5 mM concentration. At 0.5 mM, amide **14** produced a 75% reduction of JH synthesized by the controls, whereas CA incubated in amine **15** showed a 50% reduction of the control production. At 0.05 mM, no significant inhibition was observed with either compound.

To check the reversibility of the inhibition, we performed two successive incubations (2 h each) of the glands in medium containing 200 μM farnesol and 5 mM amide **14** and in fresh medium containing 200 μM farnesol. During the first 2 h of incubation, a 90% inhibiton of JH biosynthesis with respect to the controls was obtained, whereas during the second 2 h, the inhibition decreased to 50%.

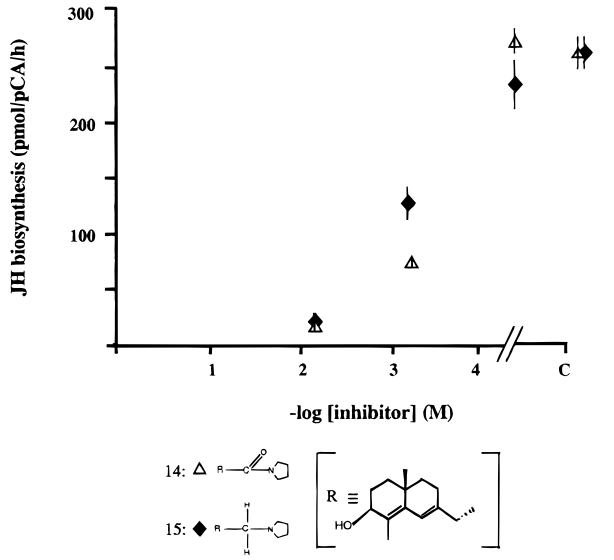


Figure 1. Farnesol-stimulated JH biosynthesis rates of locust CA in vitro in the presence of C3 alcohol amide (**14**) and C3 alcohol amine (**15**). Compounds were added to the incubation medium at 0.05, 0.5, and 5 mM concentrations. C represents control values. Values represent the mean of five experiments ± SE.

Target Enzyme of Sesquiterpene Compounds. The persistence of the inhibition on farnesol-stimulated corpora allata suggested that the observed effects could be due to alterations on, at least, one of the enzymes catalyzing the last four steps of JH biosynthesis (alcohol dehydrogenase, aldehyde dehydrogenase, farnesoic acid methyl transferase, or methyl farnesoate epoxidase) (Schooley and Baker, 1985). To determine the precise target, we incubated locust corpora allata with 5 mM amide **14** and 200 μM farnesoic acid (FA). In addition to JH III, our chromatographic procedure (Figure 2A) allows the detection of methyl farnesoate (MF), the inmediate product of FA methyl transferase, and other more polar products, such as 12′-OH-JH III and 8′-OH-JH III, that have been proven to be derived from JH (Darrouzet et al., 1997). As shown in Figure 2B, the production profiles of the control CA (incubated in 200 μM FA) and the CA incubated in amide **14** and 200 μM FA both display quantitative and qualitative differences. Whereas JH III production is clearly inhibited by compound **14**, the levels of MF are comparable between the controls and the CA incubated in the presence of the inhibitor. Consistently, JH derivatives 12′-OH-JH III and 8′-OH-JH III are absent when the CA is incubated with compound **14** (Figure 2B). This inhibition in the presence of exogenous FA suggests that one of the two final enzymes, namely, methyl transferase or expoxidase, is affected. However, the level of MF produced by methyl transferase does not vary significantly between inhibitor and control assays, so it can be hypothesized that MF epoxidase is the enzyme affected. In this case, some increase of methyl farnesoate in the presence of a P45O-dependent epoxidases inhibitor could be expected (Unnithan et al., 1995). However, this was not the case probably due to complex factors that normally work to moderate the intraglandular level of this highly lipophilic intermediate (Price et al., 1987). In *Periplaneta americana*, for example,

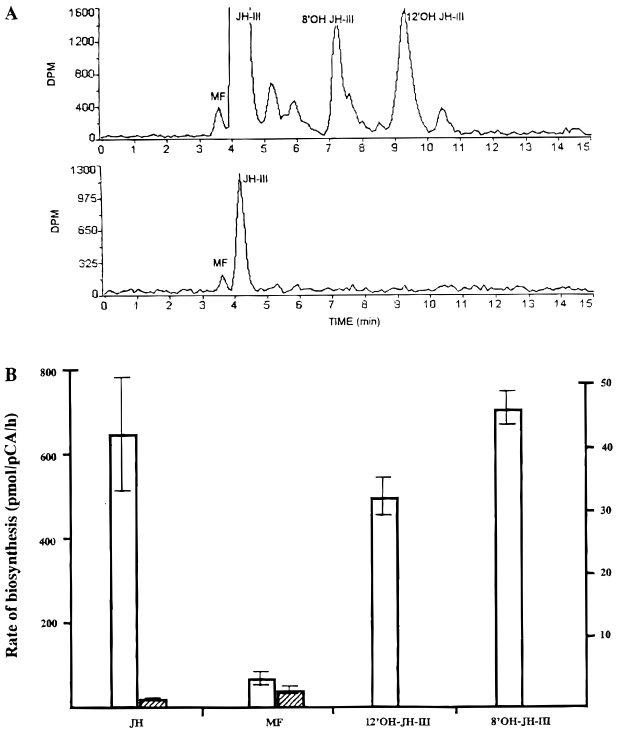


Figure 2. Inhibition of FA-stimulated JH III biosynthesis by C3 alcohol C12 amide (**14**) (5 mM). (A) Radiochromatogram obtained with normal phase HPLC of the hexane extract of incubation medium containing one pair of locust corpora allata without (upper) or with (lower) the compound. (B) Comparison of JH (left scale), MF, and JH catabolite (12′-OH-JH III and 8′-OH-JH III) levels (right scale) obtained with locust CA in control assays (open bars) and in the presence of inhibitor (shaded bars). Values represent the mean of five experiments ± SE.

feedback mechanisms have been demonstrated to prevent accumulation of this intermediate (Pratt et al., 1984).

Lepidopteran JH biosynthesis differs from the Orthopteran pathway in the sequence of the two last enzymatic steps. FA is first epoxidated to JH epoxy acid which is then methylated into JH (Feyereisen, 1985; Schooley and Baker, 1985). Furthermore, corpora allata of adult male moths are known for their lack of methyl transferase activity (Baskaran et al., 1988; Duportets et al., 1996). This is indeed the case for the glands of the male *A. ipsilon* that produced labeled JH epoxy acid (JH II acid; JH III acid and their respective conjugates B and A) when incubated in vitro with sodium [2-^{14}C]-acetate (Duportets et al., 1996). Addition of molecule **14** (5 mM) to the incubation medium resulted in a strong inhibition of spontaneous JH acid biosynthesis (Figure 3). This suggests that epoxidase could be the target of this C12 amide, assuming that the inhibitory mechanism in the moth corpora allata is the same as that in locust glands.

Taken together, our data clearly illustrate that the sesquiterpene compounds tested are good candidates for insect developmental regulators. The natural compounds induced severe modifications of the locust development but had no effect on the rate of JH production by the corpora allata in vitro. However, most of the related synthetic molecules exhibited a strong inhibitory effect on the hormone biosynthesis in vitro.

The compounds tested did not modify either pH or osmolarity of the CA incubation medium, and the effect

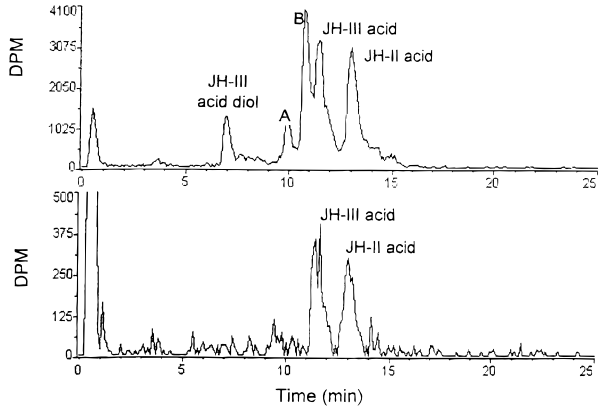


Figure 3. Inhibition of spontaneous JH acid biosynthesis by C3 alcohol C12 amide (**14**) (5 mM). Radiochromatogram obtained with reverse phase HPLC of the ethyl acetate extract of incubation medium containing three pairs of male moth CA without (upper) or with (lower) the compound.

of amide **14** could be eliminated by washing the CA in fresh culture medium. Therefore, we suggest some kind of reversible action rather than a general cytotoxic effect on the CA. In addition, the MF levels obtained by incubating the locust CA in the presence of exogenous FA suggest that enzymes upstream of the methyl farnesoate synthesis are not affected. The inhibition of JH expoxy acid synthesis in the CA of males of *A. ipsilon* indicates that the epoxidase activity could be the target enzyme. Thus, using a panel of in vitro assays, including both locust and moth JH-producing glands, and by stimulating the rate of JH biosynthesis by exogenous substrates, we could provide good information on the probable enzymatic target of these inhibitors as illustrated for amide **14**.

ABBREVIATIONS USED

CA, corpora allata; FA, farnesoic acid; JH, juvenile hormone; MF, methyl farnesoate.

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