INSECT JUVENILE HORMONE III IN THE SEDGE, CYPERUS IRIA L.: DISTRIBUTION, BIOSYNTHESIS AND POSSIBLE BIOLOGICAL FUNCTION(S).

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A thesis submitted in conformity with the requirement for the degree of Doctor of Philosophy
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Insect juvenile hormone III in the sedge *Cyperus iria* L.: Distribution, biosynthesis and possible biological function(s).

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Abstract

In insects, juvenile hormones regulate critical physiological processes such as metamorphosis and reproduction. To date, there has been only one report of the identification of an insect juvenile hormone in a plant; juvenile hormone III (JH III) has been isolated from the sedges *Cyperus iria* L. and *C. aromatics* (Ridley) Mattf and Kük. The presence of these compounds in plants is not understood.

The temporal and tissue distribution of JH III was determined in *C. iria* over an eight-month period, from the seedling to senescent plant. Levels increased in immature plants until flowering, at which time a transient decrease in JH III content of all plant tissues was observed. Juvenile hormone III subsequently increased in mature plants until senescence when, again, amounts fell in the aerial tissues. This pattern was not observed in the subterranean tissues, where JH III was predominantly localized.

The high levels of this unique compound throughout development suggests that JH III may have an important biological function in *C. iria*. The ability of this plant to interfere with insect development, presumably because of the presence of JH III, has been demonstrated. However, conclusive evidence of the protection of the plant from insect herbivory by this compound has not been established. Therefore, other potential biological activities were investigated. Juvenile hormone III delayed lettuce seed germination and demonstrated potent inhibitory activity against rice seedling growth.
This activity may confer an ecological advantage to *C. iria* by affecting the germination and growth of neighbouring, competing plants. The antimicrobial activity of JH III (13.3 μg) was tested on a taxonomically diverse range of fungi; no cytotoxic effect was observed.

At present, little is known about the biosynthesis of JH III in *C. iria*. To elucidate this pathway, a cell suspension culture of *C. iria* was established. Through enzyme inhibition studies, it has been conclusively demonstrated that the sesquiterpenoid skeleton of JH III is biosynthesized, at least partially, through the classical mevalonate pathway. Precursor feeding studies also suggest that the later steps of the biosynthetic pathway are similar to the insect pathway.
Hormone juvénile des insectes III dans *Cyperus iria* L.: Distribution, biosynthèse et fonction(s) biologique(s) éventuelle(s).

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Résumé

Dans les insectes, les horomones juvéniles règlent les processus physiologiques critiques tel la métamorphose et la réproduction, mai on ne comprend pas leur présence dans les plantes. À ce jour, il n’y a eu qu’un seul rapport de l’identification d’une hormone juvénile des insectes dans une plante; on a isolé l’hormone juvénile III (JH III) des *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf et Kük.

On a déterminé la distribution temporelle et de tissus de la JH III dans le *C. iria* sur une période de huit mois, de la plantule jusqu’à la plante sénescent. Les niveaux se sont accrus dans les plantes immatures jusqu’à la floraison, auquel moment on a observé une décroissance transitoire en contenu de JH III dans sous les tissus des plantes. Subséquemment, JH III s’est accrue dans les plantes mûres jusqu’à la sénescence quand, de nouveau, les montants se sont amoindris dans les tissus aériens. On n’a pas observé ce modèle dans les tissus souterrains, où la JH III était principalement localisée.

Les niveaux élevés de ce composé unique tout au long du développement suggèrent que la JH III peut avoir une fonction biologique importante dans le *C. iria*. On a démontré la capacité de cette plante de perturber le développement des insectes, probablement à cause de la présence de la JH III. Cependant, on n’a pas établi de témoignage probant de la protection de la plante par ce composé de l’herbivorisme des insectes. Donc, on a examiné d’autres activités biologiques potentielles. L’hormone
juvénile III a retardé la germination des semences de laitue et a démontré une activité inhibitrice contre la croissance des plantules de riz. Cette activité peut conférer un avantage écologique au C. iria en ayant un effet sur la germination et sur la croissance des plantes avoisinantes et concurrentielles. On a analysé l’activité antimicrobienne de la JH III (13.3 µg) sur une gamme de eumycètes divers taxonomiquement; aucun effet cytotoxique n’a été observé.

À présent, on ne sait pas grand-chose au sujet de la biosynthèse de la JH III dans le C. iria. Pour élucider cette voie, on a établi une culture de C. iria à cellules en suspension. Par des études d’inhibition d’enzymes, on a démontré de façon concluante que le squelette sesquiterpénoïde de la JH III est biosynthésisé, du moins en partie, par la voie mévalonate classique. Des études d’alimentation précurseurs suggèrent aussi que les étapes subséquentes de la voie biosynthétique sont similaires à la voie des insectes.
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As science research benefits from the synthesis of ideas from different disciplines, so my dissertation had depended on the encouragement, friendship and contribution of ideas from many key individuals whom I would like to thank.

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Thesis Organization

Thesis chapters are comprised of manuscripts that have been published or are presently submitted for publication. The chapters may have been slightly modified from the submitted manuscripts to avoid extensive redundancies. Dr. Stephen Tobe and I co-authored "Insect Juvenile Hormones in Plants" which was published in the series Studies in Natural Products Chemistry, Vol. 24. This review has been condensed into the first chapter of this thesis and some of the chemical data is reproduced in Appendix I. Chapter III has been accepted for publication in Phytochemical Analysis. This work is co-authored with Dr. Walt Goodman (Department of Entomology, University of Wisconsin, Madison, Wisconsin) and Dr. Tobe. Dr. Goodman kindly taught me the radioimmunoassay and supplied antiserum for the experiments. Chapter IV had been submitted to Phytochemistry. The chapter included here is a slightly more inclusive version of the manuscript submitted. Again, Dr. Goodman provided the antiserum for the experiments. Chapter V has been submitted to the Chemoecology and the antifungal assays were performed in collaboration with James Scott (Department of Botany, University of Toronto, Toronto, Ontario). Chapter VI has been accepted by Plant Cell Reports (Plant Cell Reports (1999) 19: 20-25) and was a collaborative study with Dr. Peter Teal (United States Department of Agriculture, Gainesville, Florida). In this work, I established suspension cultures of Cyperus iria and Dr. Teal performed gas chromatography-mass spectroscopy identification of the compounds. The work in chapter VII has not been written as a manuscript. Again, in these experiments, we are grateful to Dr. Goodman for JH III antiserum. All co-authors have contributed intellectual and editorial input. Copyright permission to reprint these manuscripts has been granted from all respective publishers.
As Gregor Samsa awoke one morning from uneasy dreams he found himself transformed in his bed into a gigantic insect.

What has happened to me? he thought.

- The Metamorphosis, Franz Kafka
  (reprinted with permission from Pantheon Books)
Chapter I. Literature review

Introduction

One defensive strategy of plants against insect herbivory is the production of secondary metabolites that interfere with the physiology of the insect (Rosenthal and Janzen, 1979; Rosenthal and Berenbaum, 1991). It is thought that targeting the insect endocrine system makes it difficult for the insect to evolve counteradaptive strategies (Bowers, 1991). Insect juvenile hormones (JHs) are sesquiterpenoids which are involved in the regulation of insect metamorphosis and reproduction (Wigglesworth, 1985; Gilbert et al., 1996). In some insect species, these hormones are also involved in the regulation of other processes associated with diapause and behaviour (Wigglesworth, 1985). Plants have been shown to contain compounds which can either mimic JH activity or act as antagonists by inhibiting JH biosynthesis. The purpose of this introduction is to highlight some of these phytochemicals which are known to interfere with the endocrine system of insects, specifically with the function of insect JH.

Although there are many examples of plant secondary compounds which interfere with insect physiology, there is debate as to the nature of the selective forces responsible for the evolution of these compounds (Rhoades, 1979; Futuyma and Keese, 1991). It has been argued that although these secondary metabolites serve to protect the plant against insect herbivory today, it is likely that they were initially selected as a response to vertebrate herbivory (Hay and Steinberg, 1991). However, phytochemicals which specifically interfere with the insect endocrine system possibly represent compounds which have evolved as plant adaptations to insect herbivory.
Insect juvenile hormones

To date, six JHs have been identified (Fig. 1). Structurally, they share a sesquiterpenoid skeleton, a methyl ester moiety on C-1 and an epoxide function. Juvenile hormone III (JH III), methyl-10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate, is the most common and has been isolated from Lepidoptera (moths and butterflies), Coleoptera (beetles), Hymenoptera (sawflies, wasps, ants and bees), Orthoptera (grasshoppers, locusts, katydids, crickets), Dictyoptera (cockroaches) and Isoptera (termites) (Schooley, 1977; Tobe and Feyereisen, 1983). In most of these insect Orders, JH III is the only JH present.

In the Lepidoptera, four other homologs have been isolated in addition to JH III. The ethyl branched homologs, juvenile hormone I (JH I), methyl-10R,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate, and juvenile hormone II, methyl-10R,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate, and their corresponding acids predominate in this order (Meyer et al., 1968, Judy et al., 1973; Sparagana et al., 1984). Two additional homologs, juvenile hormone 0 (JH 0), 10R,11-epoxy-3,7,-diethyl-11-methyl-2,6-tridecadienoate, and 4-methyl JH I have been isolated from embryos of the tobacco hornworm, Manduca sexta. Juvenile hormone 0 has also been isolated from males of the silkworm, Hylophora cecropia (Bergot et al., 1980; 1981; Schooley et al., 1984). In higher Diptera (flies), a unique JH bisepoxide, 6,7,10,11-bisepoxy-3,7,11-trimethyl 2E-dodecaneoate, has been isolated and appears to be the principle JH of some species in this Order (Lefevere et al., 1993).

Physiological role of insect juvenile hormones

Insect representatives of plants herbivores are widespread throughout the orders
Fig. 1. Structures of insect juvenile hormones (JHs).
JH 0

4-methyl JH I

JH I

JH II

JH III

JH III bisepoxide

4
Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, Phasmda and Thysanoptera. Even though cockroaches are not herbivorous, they can be used as a paradigm from which generalizations on the physiological action of JH in other insect species may be made. For reviews on the role of JH in other insects see Riddiford (1994), Wyatt and Davey (1996) and Gilbert et al. (1996).

Metamorphosis

Insect growth is discontinuous and comprised of discrete periods of metamorphosis, the change of form, which includes redifferentiation of body tissue and the shedding of the old cuticle. Pterygote (winged) insects undergo either complete (holometabolous, such as the Lepidoptera which show larval (caterpillar), pupal and adult stages) or incomplete (partial) metamorphosis (hemimetabolous, such as the cockroach in which the larval or juvenile form resembles the adult form). The regulation of this metamorphosis by insect JHs differs in holometabolous and hemimetabolous insects. This thesis will focus on the latter.

In the events leading up to metamorphosis, neurosecretory cells secrete prothoracicotropic hormone which acts on the prothoracic gland to stimulate the synthesis and release of the moulting hormone, 20-hydroxyecdysone (Gilbert et al., 1996). Ecdysone and its metabolites are responsible for the induction of the moulting process, whereas the titre of JH in the insect haemolymph in the interval prior to the moult determines the characteristics. In hemimetabolous insects such as the Pacific beetle cockroach, Diploptera punctata, the presence of JH III is necessary for the maintenance of juvenile characteristics: JH III biosynthesis by the corpora allata (CA) is relatively high in immature stages and there is a drop in the rate of production during the
Fig. 2. Rates of juvenile hormone III (JH III) biosynthesis by the corpora allata (CA) of the penultimate and final instars of the cockroach, *Diploptera punctata*. The rate of biosynthesis, which is closely correlated to haemolymph titers, was measured by an *in vitro* JH III radiochemical assay (Tobe and Pratt, 1974; Pratt and Tobe, 1974). Each point represents the mean of 3-12 determinations. Arrows indicate approximate times of ecdysis. (Adapted from Szibbo *et al.*, 1982. Reprinted with permission from Academic Press).
Penultimate instar

Last instar

JH III Biosynthesis (pmol h⁻¹ per CA gland) vs. Age (days)
penultimate and final nymphal stages prior to the moult to an adult (Fig. 2) (Szibbo et al., 1982).

*Gonadotrophic cycle*

In adult females of most insect species, JHs are involved in the regulation of reproduction and oocyte maturation. Juvenile hormone-dependent processes include the synthesis of vitellogenin, the precursor of the yolk protein, by the fat body and the release of vitellogenin and its subsequent uptake and incorporation into the oocyte (Engelmann, 1979; Tobe and Stay, 1985).

Most insects are oviparous, laying eggs externally which then undergo embryogenesis and development. However, there are also examples of viviparous insects in which the fertilized egg undergoes early development within the female and derives nourishment from her, and ovoviviparous insects in which the eggs are incubated within the reproductive tract of the female. In these last two situations, there is a period of gestation within the insect. These three categories are represented within the order Dictyoptera (cockroaches), allowing comparison of the relationship between reproductive strategies and JH III titres in these insects (Roth, 1970; Tobe, 1980).

In the oviparous brown-banded cockroach, *Supella longipalpa*, females produce successive oothecae (eggs and protective casing) at five to seven day intervals. Each cycle of maturing oocytes is closely associated with a cycle of JH III biosynthesis by the CA (Fig. 3) (Smith et al., 1989). Interestingly, mating is not necessary for the production and release of JH III as a similar trend is observed in virgin females. In another oviparous insect, the American cockroach, *Periplaneta americana*, mated adult females produce batches of eggs in rapid succession and the vitellogenic cycle encompasses two
Fig. 3. Juvenile hormone III (JH III) release from the corpora allata (CA) of the oviparous cockroach, *Supella longipalpa*. Juvenile hormone release was measured by the *in vitro* radiochemical assay. Values represent the means of 5-12 determinations. Approximate times of mating and oviposition are indicated by arrows. (Adapted from Smith *et al.*, 1989. Reprinted with permission from Elsevier Science).
asynchronous egg-laying periods. In this situation, there is continuous JH III biosynthesis (Weaver et al., 1975; Weaver and Pratt, 1977).

In both ovoviviparous and viviparous species of cockroaches, JH III biosynthesis is associated with vitellogenic growth of oocytes followed by periods of ovarian arrest and reduction of JH III biosynthesis during gestation (Tobe et al., 1985; Gadot et al., 1989). In mated females of the viviparous cockroach, D. punctata, JH III titre is closely correlated to oocyte development (Fig.4) (Tobe et al., 1985); high rates of biosynthesis correspond to the rapid growth of oocytes as compared with the low synthesis observed during the pre- and post-vitellogenic periods (Tobe, 1980). In adult virgin females, only basal JH III biosynthesis is observed. Similar results are observed in ovoviviparous cockroach, Nauphoeta cinerea (Lanzrein et al., 1985).

**Plant juvenile hormone mimics**

It is evident that JH III titers are precisely regulated in insects during development. Application of JH at specific times during development may result in the inappropriate retention of juvenile characteristics at the next moult. The morphological effects are dependent on the dose of hormone, the developmental stage of the insect and the sensitivity of the insect. In susceptible individuals, application of JH to final stadium larvae may produce "adultoids" at the next moult or supernumerary larvae. In the latter case, the insect moults into a giant immature stage retaining juvenile features, without reaching sexual maturity. Therefore, application of synthetic JH analogues to insects in their final larval stages could be useful in the control of insects which are pests in their adult stages such as mosquitos and fleas.
Fig. 4. Juvenile hormone III (JH III) haemolymph titres in mated adult females of the viviparous cockroach, *Diplopeta punctata*. Haemolymph titer was measured by derivitizing JH III and subjecting the resultant methoxyhydrin to gas chromatography-mass spectroscopy. Mating occurred on day 0. Arrows indicate approximate time of oviposition. (Adapted from Tobe *et al.*, 1985. Reprinted with permission from Birkhaeuser Publ.)
Application of JH or synthetic JH analogues to insect eggs disrupts embryonic development. This can range from immediate ovicidal effects to delayed developmental effects (Mundall et al., 1981). Application to eggs results in apparently normal larvae which ultimately moult into supernumerary instars instead of adults (Riddiford, 1972). It is interesting that the male linden bug, Pyrrhocoris apterus, treated with JH analogue, can transfer it to the female during mating, resulting in her sterility (Masner et al., 1968). In adult female insects, JH may be involved in the regulation of reproductive development. Therefore, application of JH or its synthetic analogues to preoviposition adult females or eggs may result in disruption of embryogenesis.

One defensive strategy of plants against insect herbivory is the production of secondary metabolites which mimic JH activity (Bowers, 1991). Many of these compounds have been extracted from diverse plant species and are termed “juvenoids” based on their activity in vitro. However, a number of criteria should be met before these compounds can be defined as JH mimics:

♦ Application of this compound should not block insect development but rather cause retention of juvenile characteristics in at least a single insect species, presumably a plant herbivore (Saxena et al., 1978).

♦ The compound should demonstrate the ability to mimic JH activity at the three principle stages of insect development: the eggs, the juvenile and the adult (female). Therefore, observation of ovicidal effects is suggestive of juvenoid activity but not definitive.

♦ In the assessment of efficacy, dose response curves are necessary and allow comparison of activity of different compounds.
These effects should represent the biological situation. For example, many of the studies described below have been performed using topical application of the putative juvenoid; this may not reflect the insect's natural contact with the chemical.

The plant must be considered; for example, the amount of compound in the tissues must be sufficient to produce a biological effect on the test insect. In the evaluation of this, it should be remembered that many plants have specialized tissues that contain high levels of secondary metabolites. For example, many conifer species contain specialized secretory structures, such as resin ducts, which contain high localized concentrations of terpenoids (Steele et al., 1995). There may also be dynamic variations in the amounts of these compounds in the plant, resulting from seasonal fluxes or synthesis induced upon stress. Also, there may be synergistic interactions with other compounds in the plant.

**Juvabione, the "paper factor"**

The best known example of a plant-derived JH mimic is juvabione or the "paper factor" which was identified after it was observed that fifth instars of the European linden bug, *Pyrrhocoris apterus*, metamorphosed into supernumary intermediates rather than mature adults when reared in the United States (Sláma and Williams, 1965). The apparent cause was traced to paper towels used in the rearing jars. Extracts from these paper towels were active against the Hemipteran, *P. apterus*, but inactive against two other Hemipterans, *Rhodnius prolixus* and *Oncopeltus fasciatus*, as well as pupae of the silkworms, *Hylophora cecropia*, *H. gloveri*, *Antheraea mylitta* and *Samia cynthia*. Therefore, it appeared that only insects in the family Pyrrhocoridae are sensitive to this JH analogue.
Extracts from several gymnosperms were assayed on *P. apterus* fifth instars (Table I) (Sláma and Williams, 1965, 1966a; Mansingh et al., 1970) and injected into pupae of the wax moth, *Galleria mellonella*. Some extracts caused a localized scaleless patch in the pupal cuticle at the site of injection. However, no abnormal effects on development were observed (Mansingh et al., 1970). The authors speculated that the absence of activity may be attributable to the dilute concentration of active ingredients in the extracts.

Juvabione, the compound responsible for this activity, was isolated from the balsam fir, *Abies balsamea* (L.) Miller, and identified as the methyl ester of todomatuic acid, (+)-4(R)-[1'(R)-5'-dimethyl-3'-oxohexyl]-1-cyclohexene-1-carboxylic acid (Bowers et al., 1966). This compound is a sesquiterpenoid (Fig. 6) with a cyclohexene group and an α,β-unsaturated methyl ester group.

Application of juvabione to fifth larval instars of *P. apterus* produced the expected supernumerary sixth instars at the subsequent moult (Bowers et al., 1966). At higher doses, juvabione was also active against the box elder bug, *Leptocoris trivittatus*, and the mealworm beetle, *Tenebrio molitor* (Table II). Similar results were obtained following treatment of these insects with JH III. These results suggest that the juvenoid activity of juvabione is not specific to insects from the family Pyrrhocoridae, as was previously thought, but these insects may be more sensitive to treatment. Pure juvabione was less active than the extract from balsam fir in the *P. apterus* assay (Sláma and Williams, 1966a; Bowers et al., 1966). Possible explanations for this are that there were differences in the strain of the insect tested or, more likely, that the extract contained other compounds which either functioned as juvenoids or which acted as synergists to juvabione. In subsequent studies, the juvenoid effects of juvabione have been confirmed
Table 1. Juvenoid activity of gymnosperm extracts.

<table>
<thead>
<tr>
<th>Plant:</th>
<th>Insect:</th>
<th>Stage:</th>
<th>Method of Application:</th>
<th>Response:</th>
<th>Ref:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balsam fir</td>
<td><em>Abies balsamea</em> (L.) Miller</td>
<td><em>Pyrrhocoris apterus</em></td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. apterus</em></td>
<td>eggs</td>
<td>Topical</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. apterus</em></td>
<td>adult females</td>
<td>Topical</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td><em>Galleria mellonella</em></td>
<td>pupae</td>
<td>Injection</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Fir</td>
<td><em>A. nordmanniana</em> (Steven) Spach</td>
<td><em>P. apterus</em></td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>+++</td>
</tr>
<tr>
<td>Douglas Fir</td>
<td><em>Pseudotsuga menziesii</em> (Mirb.) Franco</td>
<td><em>P. apterus</em></td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>+++</td>
</tr>
<tr>
<td>Canadian Hemlock</td>
<td><em>Tsuga canadensis</em> (L.) Carrière</td>
<td><em>P. apterus</em></td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>+++</td>
</tr>
<tr>
<td>Hemlock</td>
<td><em>T. heterophylla</em> Sarg.</td>
<td><em>G. mellonella</em></td>
<td>pupae</td>
<td>Injection</td>
<td>-</td>
</tr>
<tr>
<td>Yew</td>
<td><em>Taxus brevifolia</em> Nutt.</td>
<td><em>P. apterus</em></td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>+++</td>
</tr>
<tr>
<td>Species</td>
<td>Common Name</td>
<td>Extractant</td>
<td>Stage</td>
<td>Method</td>
<td>Potency</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------------</td>
<td>------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>American larch</td>
<td>Larix laricina (Du Roi) K.</td>
<td>P. apterus</td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Koch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European larch</td>
<td>L. decidua Mill.</td>
<td>P. apterus</td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>+</td>
</tr>
<tr>
<td>Southern pine</td>
<td>Pinus echinata Sarg.</td>
<td>P. apterus</td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>+</td>
</tr>
<tr>
<td>Pine</td>
<td>P. contorta Engelm.</td>
<td>G. mellonella</td>
<td>pupae</td>
<td>Injection</td>
<td>-</td>
</tr>
<tr>
<td>Red spruce</td>
<td>Picea rubra Sarg.</td>
<td>P. apterus</td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>-</td>
</tr>
<tr>
<td>Spruce</td>
<td>P. sitchensis (Bonn.) Carr.</td>
<td>G. mellonella</td>
<td>pupae</td>
<td>Injection</td>
<td>-</td>
</tr>
<tr>
<td>Juniper</td>
<td>Juniperus procumbens</td>
<td>P. apterus</td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>J. virginiana L.</td>
<td>P. apterus</td>
<td>5th instar larvae</td>
<td>Topical</td>
<td>-</td>
</tr>
<tr>
<td>Red Cedar</td>
<td>Thuja plicata Donn ex. D. Don</td>
<td>G. mellonella</td>
<td>pupae</td>
<td>Injection</td>
<td>-</td>
</tr>
</tbody>
</table>

The symbols represent the potency of the extract: +++ (high activity); ++ (moderate activity); + (slight activity); - (inactive).

References: 1 = Sláma and Williams, 1965; 2 = Sláma and Williams, 1966a; 3 = Mansingh et al., 1970; 4 = Sláma, 1969.
Fig. 5. Structures of phytojuvenoids.
Juvabione

Juvocimene I

Juvocimene II

Bakuchiol

Echinolone
Table II. Juvenoid activity of juvabione.

<table>
<thead>
<tr>
<th>Insect:</th>
<th>Stage</th>
<th>Method of Application:</th>
<th>Dose:</th>
<th>Ref:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hemiptera</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrrhocoris apterus</td>
<td>5th larval instar</td>
<td>Topical</td>
<td>5 µg</td>
<td>1</td>
</tr>
<tr>
<td>P. apterus</td>
<td>5th larval instar</td>
<td>Topical</td>
<td>1.7 µg*</td>
<td>4</td>
</tr>
<tr>
<td>Leptocoris trivittatus</td>
<td>5th larval instar</td>
<td>Topical</td>
<td>100 µg</td>
<td>1</td>
</tr>
<tr>
<td>Dysdercus cingulatus</td>
<td>5th larval instar</td>
<td>Topical</td>
<td>2.3 µg*</td>
<td>4</td>
</tr>
<tr>
<td>Graphosoma italicum</td>
<td>5th larval instar</td>
<td>Topical</td>
<td>- (500 µg)</td>
<td>4</td>
</tr>
<tr>
<td><strong>Coleoptera</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tenebrio molitor</td>
<td>pupae</td>
<td>Injection</td>
<td>500 µg</td>
<td>1</td>
</tr>
<tr>
<td>T. molitor</td>
<td>pupae</td>
<td>Injection</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>T. molitor</td>
<td>pupae</td>
<td>Topical</td>
<td>- (500 µg)</td>
<td>4</td>
</tr>
<tr>
<td>T. molitor</td>
<td>adults</td>
<td>Topical</td>
<td>- (100 µg)</td>
<td>5</td>
</tr>
<tr>
<td><strong>Lepidoptera</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manduca sexta</td>
<td>pupae</td>
<td>Injection</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Antheraea polyphemus</td>
<td>pupae</td>
<td>Injection</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Choristoneura fumiferana</td>
<td>eggs</td>
<td>Topical</td>
<td>100 µg/egg mass</td>
<td>2</td>
</tr>
</tbody>
</table>

A plus sign (+) represents an observed response where the effective concentration was not reported. A minus sign (-) indicates that the compound was inactive at the highest concentration tested (in brackets). Starred values (*) represent the ED$_{50}$; the dose at which 50% of biological activity is observed.

**References:** 1 = Bowers et al., 1966; 2 = Retnakaran, 1970; 3 = Rogers et al., 1974; 4 = Manville, 1976; 5 = Ibaraki and Sahota, 1976.
on fifth instars of *P. apterus* (Manville, 1976) and pupae of *T. molitor* (Rogers et al., 1974) and observed on fifth instar nymphs of the red cotton stainer, *Dysdercus cingulatus* (Manville, 1976). The variability in sensitivity of these insects may reflect differences in the rate of penetration across the cuticle, metabolism and excretion etc. (Table II). In subsequent studies, topical application of juvabione to *T. molitor* pupae was inactive (Manville, 1976). This reflects differences in the mode of treatment; activity was observed if the pupae were injected with juvabione but absent if it was topically applied.

Juvabione was inactive against pupae of the tobacco hornworm, *Manduca sexta*, and the polyphemus moth, *Antheraea polyphemus* (Rogers et al., 1974) and the last instar larvae of the pentamid bug, *Graphosoma italicum* (Manville, 1976).

Bioactivities of structurally related juvabiones have also been investigated. The isomer of juvabione, epijuvabione, and dehydroepijuvabione inhibited normal development of fifth instars of *P. apterus* (Cerný et al., 1967); epijuvabione was approximately ten times more effective than dehydroepijuvabione in the assay with *P. apterus* (Sláma et al., 1968). Both these compounds are also active on fifth instar nymphs of five *Dysdercus* species, which are also in the family Pyrrhocoridae: *D. intermedius*, *D. superstitiosus*, *D. fluvoniger discolor*, *D. chaquensis* and *D. cingulatus*. (Sláma et al., 1968; Suchý et al., 1968). These compounds are inactive against *G. italicum*, *T. molitor*, the Colorado potato beetle, *Leptinotarsa decemlineata*, the crickets, *Gryllus domesticus* and *Acheta domesticus*, the locust, *Locusta migratoria*, and the wax moth, *Galleria mellonella* (Cerný et al., 1967; Sláma et al., 1968). The juvenoid activity of dihydrojuvabione is similar to juvabione (Rogers et al., 1974). Bioactivity was observed against *P. apterus* fifth instars and *T. molitor* pupae, but this compound was inactive following injection into pupae of the Lepidopterans, *M. sexta*, and *A. polyphemus*. As
expected, the proposed juvabione biosynthetic intermediates, dehydrojuvabione, juvabiol and its isomer, and dehydrojuvabiol and its isomer, were, also, active against fifth larval instars of _P. apterus_, and _D. cingulatus_, but inactive against last instars of _G. italicum_ and the pupae of _T. molitor_ (Manville, 1976).

In addition to their effects on metamorphosis, these compounds also disrupt embryonic development. The viability of _P. apterus_ eggs is severely reduced following treatment of adult females or freshly laid eggs with partially purified "paper factor" (Sláma and Williams, 1966b); this activity is not observed following application of the extract to eggs of _O. fasciatus_ or _R. prolixus_. Potent ovicidal activity was observed following application of juvabione to eggs of the spruce budworm, _Choristoneura fumiferana_, a lepidopteran forest pest (Retnakaran, 1970). Interestingly, balsam fir, _A. balsamea_, the conifer from which juvabione was first isolated, is a host plant of the spruce budworm. Treatment of _C. fumiferana_ eggs with dihydrojuvabione also reduced hatching (Rogers _et al._, 1974). However, application of this compound to the adult female of the Douglas-fir beetle, _Dendroctonus pseudotsugae_, resulted in an increase in fecundity. This is significant because dihydrojuvabione has been isolated from the host plant of this beetle, the Douglas fir, _Pseudotsuga menziesii_ (Beissn.) Franco, suggesting that coadaptation of the beetle and the host plant may have occurred such that dihydrojuvabione is not only non-toxic but increases the fecundity of the insect (Rogers _et al._, 1974).

In balsam fir, juvabione is found predominantly in the wood; little activity is associated with the foliage or bark (Sláma, 1969). It is interesting that the free acids of juvabione and dehydrojuvabione, todomatumic acid and dehydrotodomatumic acid, were not detected in healthy balsam fir trees, _Abies grandis_ and _A. amabilis_ (Puritch and Nijholt,
1974). However, significant amounts of these compounds were present in the wood adjacent to sites infected by the balsam wooly aphid, Adelges piceae, suggesting that the synthesis of (+)-todomatuic acid and its derivatives or transport of these compounds to the site of infection may be induced upon insect infection.

There are minor discrepancies regarding the biological activity of these compounds. For example, in one assay, juvabione is effective against pupae of T. molitor (Bowers et al., 1966; Rogers et al., 1974) whereas in subsequent assays, it is inactive (Manville, 1976). This difference is probably due to the method of delivery (injection vs topical application) which may reflect the ability of juvabione to cross the pupal cuticle or to differences in the metabolism of this compound depending on the route of entry. There were also differences in potency of some compounds (Cerný et al., 1967; Sláma et al., 1968) depending on the sensitivity of the insect strain and the physiological state of the insect. For example, test compounds were applied to fifth larval instars of P. apterus but the age of the insects was not always specified. The activity of the juvenoid could be altered by the physiological stage of the insect and, in particular, the concentration of metabolic enzymes in the haemolymph which change dramatically during this period (Tobe et al., 1985; Hammock, 1985). This, along with contradictory reports of the structural stereoisomers of juvabione and its derivatives (Sláma and Williams, 1966b; Blount et al., 1969; Sakai and Hirose, 1973a, b), presents a very complicated story.

**Other phytojuvenoids**

There are many examples of putative JH mimics in plants (McDonough, 1984; Wakabayashi and Waters, 1985). In the following section, the activities of four of the better characterized JH mimics, juvocimene I and II, bakuchiol and echinolone, are
reviewed to illustrate the diversity of compounds which exhibit JH activity (Fig. 6) and the range of bioactivity (Table III).

Juvocimenes I and II are monoterpenoids isolated from the oil of sweet basil, Ocimum basilicum L. (Fig. 6) (Table III) (Bowers and Nishida, 1980). These compounds demonstrate potent JH activity following application to last instar nymphs of the milkweed bug, O. fasciatus. Juvocimene II is approximately 10 times more active than juvocimene I, with biological activities (ED₅₀) in the range of 5 pg and 50 pg, respectively (Bowers and Nishida, 1980). Therefore, these compounds are far more potent than juvabione when applied to Oncopeltus (Rogers et al., 1974).

Bakuchiol is a phenolic monoterpane (meroterpenoid) isolated from the seeds of Psoralea corylifolia L. (Fig. 6) (Mehta et al., 1973). Topical application of 10 µg of bakuchiol to fifth instar nymphs of D. koenigii results in their metamorphosis to nymph-adult intermediates (Bhan et al., 1980). This juvenoid activity is comparable to juvabione where application of 0.6 µg to fifth instars of P. apterus or 10 µg to last instar larvae of O. fasciatus produced morphological abnormalities in 50% of the adults (Rogers et al., 1974; Manville, 1976).

Root extracts of the American coneflower, Echinacea augustifolia DC., also showed juvenoid activity (Jacobson et al., 1975a); ether extracts showed high morphogenic activity on T. molitor pupae but none was observed on fifth instar nymphs of O. fasciatus (500 µg of extract). The active principle in the oil was identified as (E)-10-hydroxy-4,10-dimethyl-4,11-dodecadien-2-one or echinolone (Fig. 6) (Jacobson et al., 1975b). However, chemically synthesized echinolone was not active in the standard T. molitor pupal bioassay (Cooke, 1979). The author suggested this may be due to a failure
Table III. Putative juvenile hormone mimics isolated from plants.

<table>
<thead>
<tr>
<th>Compound:</th>
<th>Plant:</th>
<th>Plant part:</th>
<th>Plant species:</th>
<th>Insect:</th>
<th>Stage:</th>
<th>Method of application:</th>
<th>Dose:</th>
<th>Ref:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juvocimene I</td>
<td>Sweet basil</td>
<td>whole plant</td>
<td><em>Ocimum basilicum</em> L.</td>
<td><em>Oncopeltus fasciatus</em></td>
<td>5th instar larva</td>
<td>Topical</td>
<td>50 pg *</td>
<td>6</td>
</tr>
<tr>
<td>Juvocimene II</td>
<td>Sweet basil</td>
<td>whole plant</td>
<td><em>O. basilicum</em> L.</td>
<td><em>O. fasciatus</em></td>
<td>5th instar larva</td>
<td>Topical</td>
<td>5 pg *</td>
<td>6</td>
</tr>
<tr>
<td>Bakuchiol</td>
<td>Sweet basil</td>
<td>seeds</td>
<td><em>Psoralea cornifolia</em> L.</td>
<td><em>Dysdercus koenigii</em></td>
<td>5th instar larva</td>
<td>'Topical'</td>
<td>10 μg</td>
<td>4</td>
</tr>
<tr>
<td>Echinolone</td>
<td>American coneflower</td>
<td>roots</td>
<td><em>Echinacea augustifolia</em> DC.</td>
<td><em>Tenebrio molitor</em></td>
<td>pupae</td>
<td>Injection</td>
<td>0.97 μg</td>
<td>3</td>
</tr>
<tr>
<td>Thujic acid</td>
<td>Red cedar</td>
<td>wood</td>
<td><em>Thuja plicata</em> Donn</td>
<td><em>T. molitor</em></td>
<td>pupae</td>
<td>Injection</td>
<td>511 μg</td>
<td>1</td>
</tr>
<tr>
<td>Tagetone</td>
<td>Marigold</td>
<td>whole plants</td>
<td><em>Tagetes minuta</em> L.</td>
<td><em>D. koenigii</em></td>
<td>5th instar larva</td>
<td>Topical</td>
<td>NR</td>
<td>2</td>
</tr>
</tbody>
</table>

Starred values (*) represent the EC50; this is the dose where 50% of the biological response is observed. The effective dose of tagetone used was not reported (NR).

to synthesize the proper compound. However, the spectral data is consistent with that reported. Cooke was also unable to isolate echinolone from the roots of *E. augustifolia*.

Other phytochemicals which exhibit JH activity include juvadecene (1-(3,4-methylenedioxyphenyl)-trans-3-decene) isolated from roots of the pepper-tree, *Macropiper excelsum* Miq. (Nishida *et al.*, 1983), thujic acid (5,5-dimethyl-1,3,6-cycloheptatrien-1-carboxylic acid) extracted from the heartwood of western red cedar, *Thuja plicata* (Barton *et al.*, 1972), and tagetone ((E)-2,6-dimethyl-5,7-octadien-4-one) from marigold, *Tagetes minuta* L. (Saxena and Srivastava, 1973). There have been numerous other reports of juvenoid activity of plant extracts (Table IV); however, to our knowledge, the compound(s) responsible for this activity have not been isolated and characterized.

Plant secondary metabolites which mimic JH activity appear to be active on a narrow range of host species. What account(s) for this effect? The majority of bioassays use last larval instars of *P. apterus, O. fasciatus* and pupae of *T. molitor* to test for activity of the juvenoids. Are these the most sensitive insects? Six JHs have been identified to date; different homologs have been isolated from specific insect orders. Juvenile hormone III appears to be ubiquitous (Schooley, 1977; Tobe and Feyereisen, 1983) and, in most species, is the only JH present. Juvenile hormone I and II are important in the regulation of metamorphosis and ovarian maturation in Lepidoptera (Gilbert *et al.*, 1996) and the bis-epoxide appears to be the principle JH in higher Diptera (Lefevere *et al.*, 1993). Therefore, the nature of the JH in the test insect and the role that it plays in development must be considered in the selection of the bioassay; a compound which mimics the action of JH in *P. apterus* (Hemiptera) is unlikely to be active in a Lepidopteran insect.
<table>
<thead>
<tr>
<th>Plant species:</th>
<th>Common name:</th>
<th>Plant part:</th>
<th>Insect:</th>
<th>Stage:</th>
<th>Method of application:</th>
<th>Response:</th>
<th>Ref:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Iris endata</em> Thamb.</td>
<td>Iris</td>
<td>Roots</td>
<td><em>Dysdercus koenigii</em></td>
<td>5&lt;sup&gt;th&lt;/sup&gt; larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>1</td>
</tr>
<tr>
<td><em>Iris douglasiana</em> Herb.</td>
<td>Iris</td>
<td>Roots, stem, fruits</td>
<td><em>Oncopeltus fasciatus</em></td>
<td>5&lt;sup&gt;th&lt;/sup&gt; larval instars</td>
<td>Topical</td>
<td>++</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Tenebrio molitor</em></td>
<td>pupae</td>
<td>Topical</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Clethra alternifolia</em> L</td>
<td>Sweet pepperbush</td>
<td>Stem, leaves, fruits</td>
<td><em>O. fasciatus</em></td>
<td>5&lt;sup&gt;th&lt;/sup&gt; larval instars</td>
<td>Topical</td>
<td>++</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>T. molitor</em></td>
<td>pupae</td>
<td>Topical</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Sassafras albudum</em> (Nutt.) Nees</td>
<td>White sassafras</td>
<td>Root, root bark</td>
<td><em>O. fasciatus</em></td>
<td>5&lt;sup&gt;th&lt;/sup&gt; larval instars</td>
<td>Topical</td>
<td>++</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>T. molitor</em></td>
<td>pupae</td>
<td>Topical</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Eucalyptus camaldulensis</em> Dehn.</td>
<td>Murray redgum</td>
<td>Stem, bark</td>
<td><em>O. fasciatus</em></td>
<td>5&lt;sup&gt;th&lt;/sup&gt; larval instars</td>
<td>Topical</td>
<td>++</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>T. molitor</em></td>
<td>pupae</td>
<td>Topical</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Pinus rigida</em> Mill</td>
<td>Pitch pine</td>
<td>Twigs, leaves</td>
<td><em>O. fasciatus</em></td>
<td>5&lt;sup&gt;th&lt;/sup&gt; larval instars</td>
<td>Topical</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>T. molitor</em></td>
<td></td>
<td></td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Species</td>
<td>Part</td>
<td>Insect</td>
<td>Stage</td>
<td>Mode</td>
<td>Rating</td>
<td>ID</td>
<td></td>
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<td>---------------------------------</td>
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<tr>
<td><em>Chamaecyparis lawsoniana</em></td>
<td>Seeds</td>
<td><em>T. molitor</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>++</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(Andr. Murray) Parl.</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Anthocephalus cadamba</em></td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>++</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Lantana camara</em> L.</td>
<td>Yellow sage</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>++</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Calophyllum</em> sp. L.</td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>++</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Phyllanthus emblica</em></td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Erythrina indica</em> Lam.</td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Auracaria excelsa</em> R. Br</td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Annona reticulata</em> L.</td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Peltophorum inerme</em> Benth.</td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Manihot esculenta</em> Pohl.</td>
<td>Cassava</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Phyllanthus emblica</em> L.</td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Taberaemontana dichotoma</em></td>
<td>Stem</td>
<td><em>D. cingulatus</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Tagetes minuta</em> L.</td>
<td>Marigold</td>
<td>Whole plant</td>
<td><em>D. koenigii</em></td>
<td>5th larval instars</td>
<td>Topical</td>
<td>+++</td>
<td>2</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>Larva</td>
<td>Contact in water</td>
<td>-</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. aegypti</em></td>
<td>Pupae</td>
<td>Contact in water</td>
<td>-</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Musca domestica</td>
<td>pupae</td>
<td>Topical</td>
<td>-</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The symbols represent the potency of the extract: +++ (high activity); ++ (moderate activity); + (slight activity); - (inactive).

Originally, it was thought that these compounds could provide the structural basis for the design of pesticides because it is unlikely that insects would develop resistance to their own or closely related hormones (Williams, 1967). However, this has proven not to be the case. Insects that have developed resistance to insecticides may also show resistance to JH analogues (Dyte, 1972; Cerf and Georghiou, 1972; Vinson and Plapp, 1974). This cross-resistance appears to be related to an increase in metabolic enzymes, particularly mixed function oxidases, including cytochrome P₄₅₀ enzymes. Also, esterases are present at high levels in the insect haemolymph in the final instar and are thought to be partially responsible for the low JH III titers observed at this stage (Rotin et al., 1982; Tobe et al., 1985). Differences in such enzyme systems may account for the observed differences in the sensitivities of insect species to juvenoids.

Further difficulty with the use of these analogues as a means of pest control is that the window of sensitivity to these compounds is short, e.g. to eggs, during metamorphosis or reproduction. Treatment with these compounds may also result in an arrest of the insect in the feeding period. Also, these compounds have a relatively broad specificity and would not act exclusively on pest species. However, in certain circumstances, the synthetic JH analogues methoprene and hydroprene have been used successfully in insect control.

Much of the interest in phytojuvenoids has focused on the isolation of compounds to use as models for the development of stable, potent pesticides. As a result, there is little information regarding the biological nature of these compounds. In most bioassays, the juvenoid was applied topically, biasing the screen for lipophilic compounds that are able to penetrate the insect cuticle and underlying epidermis. In nature, these compounds can be absorbed, ingested or inhaled depending on the plant and the life history of the
insect on the plant. Little information is available regarding juvenoids that elicit these effects by oral administration. Similarly, there have been few reports regarding the amount and distribution of these compounds in the plant. To our knowledge, there is presently little or no information on whether the levels of these compounds in plant tissues are sufficiently high to affect insect herbivores or whether these compounds play another role(s) _in vivo_. Consequently, these compounds, which have been defined in the literature as insect JH mimics, do not meet our criteria. The present evidence suggests that they may function as phytojuvenoids but further studies must be performed.

**Plant juvenile hormone antagonists**

Phytochemicals, such as the precocenes, isolated from _Ageratum houstonianum_ Miller interfere with JH biosynthesis (Bowers _et al._, 1976). In sensitive insect species, application of these dichromenes to larval instars results in precocious metamorphosis to sterile adults or sterility in adult females following treatment. Pesticides based on these compounds would be useful in the control of insects that are primarily destructive in their immature stages. Other phytochemicals which potentially function as "antijuvenile hormones" include dimethyl sciadinonate, isolated from the leaves of avocado, _Persea americana_ Miller (Kaneko _et al._, 1963; Chang _et al._, 1975). Ingestion of this compound results in the direct pupation of fourth instar larvae of the silkworm, _Bombyx mori_, bypassing the fourth moult.

**Precocenes**

Precocene I, 7-methoxy-2,2-dimethyl chromene, and precocene II, 6,7-dimethoxy-2,2-dimethyl chromene, have been isolated from plants throughout the family
Asteraceae (Fig. 7) (Suga et al., 1975; Proksch et al., 1980; Castro et al., 1989; Castillo et al., 1989; Tamayo- Ferracini et al., 1989; Siebertz et al., 1990; González et al., 1991; Zdero et al., 1991). There is a wealth of information on the bioactivity of these compounds on various insect species (Wakabayashi and Waters, 1985; Staal, 1986). For simplicity, we will focus on the effects of precocene II on the susceptible Hemipteran bug, *Oncopestus fasciatus*.

Application of sublethal doses of precocene to the first, second and third instar nymphs of *O. fasciatus* results in premature metamorphosis to adultoids at the third, fourth and fifth stadium moult, respectively (Bowers et al., 1976). An "in-between" instar follows the stage treated with chromene and when the effects are observed. These effects can be reversed by the topical application of JH; a decrease in both mortality and the number of insects which undergo precocious metamorphosis is observed following application of JH I to second instars which have been pretreated with precocene II (Masner et al., 1979). Fourth instar larvae of *O. fasciatus* which were treated with precocene developed either into precocious adults or underwent apparently normal development through a fifth instar into an adult, with an increase in preoviposition period and reduced fecundity observed in females (Masner et al., 1979; Zamorano et al., 1981). This stage of *Oncopeltus* was less sensitive to precocene; a hundred fold increase in concentration was required to produce these effects (Masner et al., 1979). Treatment of these precocious adult females with JH resulted in mating, although oviposition did not ensue (Bowers et al., 1976). Application of precocene to last nymphal stages has no effect and these insects develop into normal, reproductive adults (Unnithan and Nair, 1979).
Fig. 7. Plant insect juvenile hormone antagonists, the ageratochromenes.
Precocene I

Precocene II
Treatment of adult female *O. fasciatus* with precocene resulted in sterilization (Bowers et al., 1976; Masner et al., 1979; Zamorano et al., 1981). Examination of the ovaries revealed a marked difference between control and treated insects (Bowers et al., 1976; Bowers and Martinez-Pardo, 1977). In normal insects, oocyte development begins two to three days after the adult moult and continues until day six, at which time oviposition normally begins. Following application of precocene to newly-emerged adult females, oocyte development is completely inhibited. Application of JH III to these insects resulted in a rapid increase in oocyte length, demonstrating that precocenes were not acting directly on the ovary. Treatment of gravid insects with precocene on day 5 after eclosion resulted in resorption of most oocytes. Laid eggs hatched and developed normally to third instar juveniles but then moulted into precocious adults (Bowers et al., 1976). Transplantation experiments in which precocene II was added to CA maintained *in vitro* from mated adult female *O. fasciatus*, followed by implantation into fifth instar juveniles, demonstrated that precocenes directly inactivate the CA and their effects are not the result of signals originating in the brain or other tissues (Müller et al., 1979). In complementary experiments on the cockroach, *Periplaneta americana*, Pratt and Bowers (1977) demonstrated that incubation of the CA with precocene II directly inhibits JH III biosynthesis and release, even though *in vivo* *P. americana* is relatively insensitive to these compounds.

The effects of precocenes on larval and adult *O. fasciatus* and the reversal of these effects by application of JH suggests that these compounds may be affecting JH biosynthesis by the CA. As with the ovary, profound differences are observed in CA volume between normal and precocene-treated animals (Bowers and Martinez-Pardo, 1977). After eclosion, the CA of mated adult females normally undergo a five-fold
increase in volume over the next nine days. Treatment of these animals with precocene on day 5 results in a reduction in size. If the insect is treated with precocene immediately following the adult moult, the CA size does not change; even if JH III is added on day 5 to these animals, no further development occurs. However, in this species, there is no definitive evidence to suggest that CA volume is correlated with the rescue of JH.

Morphological and ultrastructural studies of the CA of precocene-treated adult females of *O. fasciatus* demonstrate progressive necrotic degeneration, as compared to controls (Unnithan *et al.*, 1977; Liechty and Sedlak, 1978; Sedlak, 1985). These studies have shown that precocene II stimulates the atrophy of the CA. Its specific cytotoxicity is attributed to epoxidation of the precocenes by the epoxidase, which catalyzes the final step in JH III biosynthesis, generating extremely reactive 3,4-epoxy intermediates which alkylate cellular proteins (Ohta *et al.*, 1977; Soderlund *et al.*, 1980; 1981) causing atrophy of the CA and the resultant inhibition of JH biosynthesis (Bowers *et al.*, 1982). Therefore, destruction of the CA by precocene is responsible for its biological activity. This occurs through its epoxidation by an enzyme which is only active at times of JH biosynthesis. Therefore, precocenes have no effect on inactive CA such as those of last larval instars of *O. fasciatus* (Unnithan and Nair, 1979).

Generally, it is thought that these compounds are only effective *in vivo* against insects in the orders of Hemiptera, Homoptera, Dictyoptera and Orthoptera, although there are some exceptions (Wakabayashi and Waters, 1985; Staal, 1986). Holometamolous insects are believed to be insensitive; high doses of precocenes may affect development in these and other insect species but it is thought that this action does not occur through inhibition of JH biosynthesis. Rescue experiments with reversal of
precocene-induced effects counteracted with JH or JH analogues will allow
differentiation between endocrine specific and non-specific effects.

*In vitro* inhibition of JH biosynthesis in insect species that are insensitive to
precocenes *in vivo* has also been demonstrated (Feldlaufer and Bowers, 1982). It is
believed that the differences in susceptibility to precocenes may, in part, be related to
their metabolism by the different insect species. The metabolism of precocene II to the
diol was observed in nine insect species (Ohta *et al.*, 1977) and, presumably, occurs by
mixed function oxidases present in the gut and fat body. The wax moth, *G. mellonella,*
metabolized 77% of this compound as compared to 47% by *O. fasciatus* in the same time
period. This difference in metabolic rate may account, at least partially, for differences in
the sensitivity of these insect species to precocenes.

Precocene II has also shown antifeedant activity in a non-choice assay with the
Mexican bean beetle, *Epilachna varivestis* (Srivastava and Proksch, 1990). It also has
antifungal activity against *Pyricularia oryzae,* whereas precocene I was not (Satoh *et al.*, 1996). Neither compound demonstrated antibiotic activity against the yeasts,
*Saccharomyces cerevisiae* and *Candida albicans,* the gram-negative bacteria,
*Pseudomonas fluorescens* and *Escherichia coli,* and the gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus albus* (Proksch *et al.*, 1980).

The distribution of precocene I and II in flowering *A. houstonianum* has been
determined (Siebertz *et al.*, 1990). The highest amounts of precocene II is found in the
leaves (1.4 μmol/g fresh weight (FW) to 6.7 μmol/g FW in older leaves) and the
flowerheads (21.5 μmol/g FW). It is invalid to compare a dose that is topically applied
versus ingested. However, with this information on the amounts present in the plant, it
would be useful to determine if precocene II levels in the plant would be sufficient to
either deter feeding or interfere with development of a susceptible insect species.

Considering that application of 0.5 mg of precocene II is ovicidal to *O. fasciatus* eggs treated by fumigation (Bowers *et al.*, 1976), 10 μg of precocene II resulted in precocious metamorphosis in *O. fasciatus* (Zamorano *et al.*, 1981) and that 0.05 μmol/leaf disc detered the feeding of *E. verivestis* (Srivastava and Proksch, 1990), it is possible that these compounds are present in sufficient levels to play a role in protecting the plant against insect herbivory.

**Insect juvenile hormone III in plants.**

To date, there has been only one report of the identification of an insect JH in a plant; in 1988, JH III and MF were isolated from the sedges *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf and Kük (Toong *et al.*, 1988). Little is known about the presence of this compound in the plant. The objectives of this thesis are to:

1. Determine the developmental distribution of JH III in the sedge, *C. iria*, and speculate as to its biological function in the plant.
2. Compare the biosynthetic pathway of JH III in the sedge, *C. iria*, with the pathway elucidated for the cockroach, *D. punctata*.

**References**


Rogers, I.H., Manville, J.F. and Sahota, T. (1974) Juvenile hormone analogs in conifers. II. Isolation, identification, and biological activity of cis-4-[1'(R)-5'-dimethyl-3'-oxohexyl]-cyclohexane-1-carboxylic acid and (+)-4(R)-[1'(R)-5'-dimethyl-3'-


Chapter II. Introduction

Juvenile hormones regulated critical physiological functions such as development and reproduction in most insect species. Although there are many examples of juvenile hormone mimics in plants (Bede and Tobe, 1999), there has only been one report of the isolation and identification of an actual insect JH in a plant; juvenile hormone III (JH III) and methyl farnesoate (MF) were both isolated from the sedges *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf and Kük (Toong *et al.*, 1988). Other insect hormones, such as phytoecdysteroids, have been identified in a diverse range of plant families. For example, 20-hydroxyecdysone, the insect moulting hormone, is found in *Polypodium vulgare* L. (Polypodiaceae (ferns)), *Ajuga* sp., *Zea mays* L., *Serratula tinctoria* L. and *Spinacia oleracea* L (Lamiaceae, Poaceae, Compositae, Chenopodiaceae, respectively) (angiosperms)) and has been implicated in plant-insect and pollination interactions (Grebenok *et al.*, 1991; Dinan, 1992; Tomás *et al.*, 1992; Corio-Costet *et al.*, 1993; Adler and Grebenok, 1995; Devarenne *et al.*, 1995; Reixach *et al.*, 1996). One objective of the present study is to determine the physiological role of JH III in the sedge, *C. iria*.

Juvenile hormone III and MF belong to a diverse class of compounds, the terpenoids, characterized by the common biosynthetic intermediate isopentenyl diphosphate (IPP). In higher plants, the activities of terpenoids range from photosynthesis and respiration (carotenoids, chlorophylls, ubiquinone), to membrane architecture (sterols) and to development (abscisic acid, gibberellins). They also may mediate a number of ecological interactions (review: Langenheim, 1994). For example, these compounds may be involved in plant defense against pathogens (fungal, bacterial), plant protection against herbivory (vertebrate, invertebrate) or in allelopathic interactions with
surrounding plants. In some cases, it has been demonstrated that individual compounds may have multiple ecological effects, thus adding further confusion to the question of which role(s) it plays in the plant.

Recently a novel role of terpenoids in plant communication has been proposed (Bruin et al., 1995; Penuelas et al., 1995). Terpenoids, such as (E)-β-ocimene, linalool, (3E)-4,8-dimethyl-1,3-7-nonatriene (DMNT) and (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene are released by some plants (e.g. corn, cotton, Lima bean) in response to insect herbivory. The volatile mixture may have one or any number of the following effects: A direct toxic action on the insect herbivore, a signal to induce defense responses systemically or in neighbouring plants or a signal attracting predators or parasitoids of the insect herbivore. Quantitative and qualitative changes in the plant's composition of these volatiles are specific to the plant species (e.g. corn vs cotton) and to the insect herbivore (Turlings et al, 1990; Dicke, 1994). For example, corn plants infested with larvae of the armyworm *Spodoptera frugiperda* or *S. exigua* emit subtle differences in the composition of the released volatiles which enable predators/parasitoids to distinguish between them (Turlings et al., 1993). Application of the respective caterpillar regurgitant to the plant is not sufficient to account for these differences. Therefore, it is believed that caterpillar feeding behaviour is also responsible for changes in volatile composition.

The physiological function of JH III in the sedge, *C. iria*, is unknown. As discussed in Chapter I, the JH titre in insect haemolymph titre is precisely regulated during development (Gilbert et al., 1996). The presence of JH at a critical period prior to the moult is necessary for the maintenance of juvenile characteristics during metamorphosis. In adult females of most insect species, JH is also involved in the regulation of ovarian maturation and reproductive function. Therefore, application of JH
or synthetic JH analogues to susceptible insects at specific stages or to insect eggs may result in the disruption of development. (Sehnal, 1983).

The dramatic effects of JH III on insect development suggests that it might be involved in protection of the plant against insect herbivory. In the grasshopper, Melanoplus sanguinipes, no effect in insect growth was observed when third stadium nymphs were fed on either wheat seedlings or C. iria (Toong, et al., 1988). There was no detected difference in insect growth. However, when these insects moulted to adults, those reared on C. iria displayed deformed wings and colour changes indicative of development under conditions of excess JH. The resulting adult females were also infertile; their ovaries contained only immature oocytes. In another laboratory study, C. iria leaves were shown to be larvicidal when added to water containing the mosquito, Aedes aegypti (Schwartz et al., 1998). In field studies, eggs of the Dipteran leafminer, Hydrellia sp., did not hatch when laid on C. iria leaves (Meneses and García de la Osa, 1988). These studies show that exposure to C. iria plants can interfere with insect development, presumably because of the presence of JH III.

However, in other reports, association of insect herbivores with C. iria had no apparent effect on development. For example, nymphs of the planthoppers Nisia strovenosa and N. nervosa were reared successfully on C. iria and C. rotundus L., although more insects reached maturity on C. rotundus (Dela Cruz, 1986). In a feeding preference study, the rice stink bug, Oebalus pugnax, fed on C. iria, although the mean number of feedings was fewer than that observed on other plants such as the Vasey grass, Paspalum urvillei Steud. (Naresh and Smith, 1984). Whether these insects are able to avoid the detrimental effects of JH III through metabolism (Dowd et al., 1983) or sequestration of this compound or by avoiding plant tissues rich in JH III is not known.
It is also possible that JH III reduces plant competition by inhibiting the germination and growth of plants in the immediate vicinity. In the search for allelopathic agents in invasive weeds, MF and farnesol (two biosynthetic intermediates of JH III in insects (Cusson et al., 1991)), were isolated from tubers of the related sedge, C. serotinus Rottb. (Komai et al., 1981). Both of these compounds demonstrated potent inhibitory activity on shoot growth of lettuce and rice seedlings. Cyperus iria is also an extremely aggressive weed and the structural similarity between these compounds and JH III suggests that JH III may have a similar biological activity (Holm et al., 1977; Catling, 1992)

Plant allelochemicals may also be released into the environment as a defence against nematodes (Hasan, 1992). Juvenile hormones have been shown to affect nematode development (Davey, 1988). Juvenile hormone I and MF inhibited hatching of eggs of Haemonchus contortus (Rogers, 1978) and application of JH III (3.4 mM) to third-stage larval females of the rodent hookworm, Nippostrongylus brasiliensis, resulted in a 50% reduction in egg production (Glassburg et al., 1983). Farnesol was found to inhibit larval development of the nematode, Trichinella spiralis (Meerovitch, 1965). In the few studies done, these compounds did not appear to effect plant parasitic nematodes (Johnson and Viglierchio, 1970; Rogers, 1978).

Juvenile hormone III may have other possible biological roles, such as the protection of the plant against pathogens such as fungi and bacteria.

**Objectives**

The temporal and tissue distribution of JH III in *C. iria* was determined to
establish its levels and sites of accumulation in the plant. A radioimmunoassay developed by Goodman et al. (1990, 1995) for detection of JH in insect haemolymph was optimized for analysis of plant tissue. This sensitive technique enabled quantification of JH III in 10.0 mg of plant material. This has made it possible to measure JH III levels in different parts of the plant e.g. root, culm (stem), leaf, bract, inflorescence and nutlet (fruit) tissues. Knowledge of the developmental distribution of JH III in C. iria was necessary for the subsequent investigation of its possible biological functions in the plant. Physiologically relevant concentrations of JH III were used to determine its effects in seed germination and seedling growth bioassays and in antifungal assays.

**Biosynthesis of insect juvenile hormone III**

In the cockroach, *Diploptera punctata*, JH III is biosynthesized in retrocerebral endocrine organs, the corpora allata (CA) (Tobe and Stay, 1985). The sesquiterpenoid skeleton of this compound is formed through the terpenoid biosynthetic pathway from acetyl-CoA (Fig. 1). The early steps of this pathway involve the sequential condensation of three acetyl-CoA molecules (3 x 2C) to form the biosynthetic intermediate mevalonate (MVA, 6C). 3-Hydroxy-3-methylglutaryl CoA reductase, which catalyzes the formation of MVA, is thought to be the rate-limiting enzyme in the pathway (Monger, 1985), although its regulatory importance has been questioned (Sutherland and Feyereisen, 1996). In the next series of steps, MVA undergoes three phosphorylations (net two) and a decarboxylation to generate the important isoprene intermediate, IPP (5C); isomerization of this compound forms dimethyl allyl diphosphate (DMAPP, 5C). Through covalent linkages of DMAPP and IPP, prenyl diphosphate intermediates are formed which give rise to the terpenoid classes, such as the monoterpenes (10C), sesquiterpenes (15C),
Fig. 1. Classical mevalonate pathway generating the important isoprene intermediate isopentenyl diphosphate (IPP).
2 x Acetyl-CoA

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{O} \\
& \quad \text{CH}_2 \\
\text{S-CoA} & \quad \text{S-CoA} \\
& \quad \text{H}^+ \\
\end{align*}
\]

\[\text{Acetoacetyl-CoA} + \text{Acetyl-CoA}\]

(3S)-3-Hydroxy-3-methylglutaryl-CoA

\[
\begin{align*}
\text{HO} & \quad \text{CH}_2 \\
& \quad \text{COO}^- \\
\text{S-CoA} & \quad \text{HS-Enz} \\
& \quad \text{H}_2\text{O} \\
\text{H}_3\text{C} & \quad \text{CH}_2 \\
\text{S-CoA} & \quad \text{S-Enz} \\
\end{align*}
\]

\[2 \text{ NADPH} + \text{H}^+ \quad 2 \text{ NADP}^+\]

(3R)-Mevalonic acid

\[
\begin{align*}
\text{HO} & \quad \text{CH}_3 \\
& \quad \text{OH} \\
\text{COO}^- & \quad \text{ATP} \\
\text{ATP} & \quad \text{ADP} \\
\text{ATP} & \quad \text{ADP} \\
\text{ATP} & \quad \text{ADP} + \text{Pi} \\
\end{align*}
\]

\[\text{CO}_2 \quad \text{OPP} \quad \text{COO}^-\]

Isopentenyl diphosphate
diterpenes (20C) and sterols. Insects lack the enzymes to synthesize higher terpenoids (Svoboda et al., 1975) and JH III is synthesized from the fifteen-carbon farnesyl diphosphate (FPP). In vitro labelling of JH III in the CA of D. punctata has confirmed that the C-15 skeleton is derived from 3 moles of MVA (Feyereisen et al., 1981a). In these studies, 6 moles of [2-14C]acetate were incorporated per molecule of JH III which reflects the expected incorporation of three acetate molecules (3 x 2 C), followed by the loss of a labeled carbon dioxide (14CO2) for every isoprene unit (5C) synthesized.

From FPP, a phosphatase or pyrophosphatase catalyzes the removal of the pyrophosphate group, generating farnesol (Fig. 2). The next two steps, oxidation of this alcohol to farnesal and then farnesoic acid, are catalyzed by one or two NAD+-dependent dehydrogenase(s) (Baker et al., 1983). In the cockroach, D. punctata, methylation of farnesoic acid forms methyl farnesoate (MF), followed by epoxidation at C10,C11 producing JH III (Schooley and Baker, 1985; Cusson et al., 1991).

S-Adenosyl-methionine: farnesoic acid o-methyltransferase (EC 2.1.1.-), a cytosolic enzyme found in the CA, has been shown to catalyze the methylation reaction in adult female locusts, L. migratoria, the cockroach, D. punctata and adult female tobacco hornworms, M. sexta (Reibstein and Law, 1973; Feyereisen et al., 1981b; Wang et al., 1994). In a developmental profile, o-methyltransferase activity paralleled JH III biosynthesis in the CA of final larval instars and adult females of D. punctata (Szibbo et al., 1982; Tobe et al., 1985; Wang et al., 1994). The subsequent epoxidation reaction is catalyzed by a methyl farnesoate reduced flavoprotein: oxygen oxidoreductase (EC 1.14.14.-), which in the cockroach, Blaberus gigantus, and locust, L. migratoria, is associated with microsomal fractions in CA homogenates (Hammock, 1975; Hammock and Mumby, 1978; Feyereisen et al., 1981b). In Locusta, this enzyme was further shown
Fig. 2. Juvenile hormone biosynthetic pathway in the cockroach, *Diploptera punctata* (adapted from Cusson *et al.*, 1991. Reprinted with permission from Elsevier Science).
Farnesyl diphosphate \[\xrightarrow{\text{Phosphatase or pyrophosphatase}}\] Farnesol \[\xrightarrow{\text{Farnesol dehydrogenase, NAD}^+}\] Farnesal \[\xrightarrow{\text{Farnesal dehydrogenase, NAD}^+}\] Farnesolic acid \[\xrightarrow{\text{Methyl transferase, S-adenosyl-methionine}}\] Methyl farnesoate \[\xrightarrow{\text{Epoxidase, O}_2, \text{NADPH}^+H^+}\] Juvenile Hormone III
to be a NADPH-dependent cytochrome P450 monooxygenase with an apparent $K_m$ of 7.7 x $10^{-6}$ M, although the solubility of the substrate (methyl farnesoate) limited the rate of the reaction (Feyereisen et al., 1981b).

This pathway, or variations of it, occurs in other insect species. The ethyl branches of the higher JH homologs found in the Lepidoptera (see Chapter 1, Fig. 1) are derived from isoleucine and valine which are first metabolized to propionate and then incorporated, instead of acetate, in the early steps of biosynthesis (Brindle et al., 1987; Brindle et al., 1992). The sequence of the final steps of JH biosynthesis may also be different in Lepidoptera. In the CA of some Lepidoptera, epoxidation of farnesoic acid to JH acid occurs before final methylation (Law, 1981; Schooley and Baker, 1985; Cusson et al., 1991). Larval stages of the tobacco hornworm, M. sexta, and adult males of the silkworm, Hyalophora cecropia, synthesize JH acids in the CA and release these compounds into the haemolymph which then undergo methylation in the imaginal disks or accessory sex glands, respectively (Peter et al., 1981; Sparagana et al., 1985).

**Terpenoid biosynthesis in Cyperus iria**

In plants, the sesquiterpenoid backbone of JH III may be biosynthesized either through the classical mevalonate pathway outlined above, or through a unique pathway, the 2-C-methyl erythritol 4-phosphate (MEP) pathway. This latter pathway has been characterized in bacteria and the thylakoids of cyanobacteria and recently demonstrated in plastids of higher plants (Rohmer et al., 1993; Bach, 1995; Eisenreich et al., 1996). In this alternate pathway, a pyruvate derived activated acetaldehyde undergoes a transketolase-type reaction with glyceraldehyde 3-phosphate to generate 1-deoxyxylulose-5-phosphate (Fig. 3) (Lichtenthaler et al., 1997). This compound
Fig. 3. The 2-C-methyl erythritol 4-phosphate (MEP) pathway (adapted from Lichtenthaler et al. (1997) with permission from Elsevier Science).
Thiamine diphosphate

\[
\text{Pyruvate} \rightarrow \text{COOH} \quad \text{CO}_2 \quad \text{TPP} \quad \text{OH} \quad \text{THP} \quad \text{TPP} \quad \text{CH}_3 \quad \text{TPP} \quad \text{HO} \quad \text{TPP} \quad \text{CH}_2\text{OP}
\]

Glyceraldehyde 3-phosphate

D-1-deoxyxylulose 5-phosphate

Isomerization

\[
\begin{array}{c}
\text{OH} \\
\text{O} \\
\text{OH} \\
\text{P}
\end{array}
\]

Reduction

2-C-methyl-D-erythritol 4-phosphate

Isopentenyl Diphosphate
undergoes intramolecular isomerization and reduction to form 2C-methyl-D-erythritol 4-phosphate which after a series of biosynthetic steps generates the 5C isoprene unit, IPP. This is further complicated by recent evidence of limited interchange of IPP subunits between the plastid and cytosol (McCaskill and Croteau, 1995) In sesquiterpenoids from chamomile, labelling studies indicate that the first two C5 subunits of this compound are biosynthesized through the MEP pathway and the last C5 unit is derived either from MEP or MVA pathway (Adam and Zapp, 1998). Therefore, either there is some interchange of IPP subunits between the plastid and the cytosol or both pathways may be localized in the same compartment. In the lima bean, *Phaseolus lunatus*, the volatile terpenoid DMNT is synthesized de novo following treatment of freshly cut plantlets with the elicitor jasmonic acid (Piel et al., 1998). Labelling with deuterated intermediates demonstrate that jasmonic acid-induced DMNT is synthesized through the MVA pathway. However, if the MVA pathway is inhibited by mevastatin or cervastatin, labelled precursors can be incorporated into DMNT through the MVA pathway. The authors suggest that this flexibility allows the plant to increase the amount of IPP precursors necessary for the biosynthesis of DMNT or defensive molecules such as phytoalexins during times of stress, such as herbivore or pathogen attack. It is not known which of these pathways or if both pathways contribute to the sesquiterpenoid skeleton of JH III in *C. iria*.

**Juvenile hormone III biosynthetic pathway in *Cyperus iria***

Late intermediates known from the insect biosynthetic pathway of JH III have been isolated from *C. iria* and related sedges. For example, methyl farnesoate, the immediate biosynthetic precursor to JH III in insects (Fig. 2), has been identified in *C. iria, C. microiria* Steud, *C. monophyllus* Vahl., *C. pilosus* Vahl. and *C. serotinus* Rottb.
Another biosynthetic intermediate, farnesol (Fig. 2), has also been isolated from the last four plant species (Iwamura et al., 1978a, c; Iwamura, 1979; Komai et al., 1981). These common intermediates, found in both plant and insect, indicates that the late steps of JH III biosynthesis may be similar.

**Objectives**

A cell suspension culture of *C. iria* was established to elucidate the biosynthetic pathway of JH III in the plant. Suspension cultures provide a rapidly growing, relatively homogeneous biomass which is ideal for the investigation of metabolite pathways (Charlwood and Charlwood, 1991; Banthorpe, 1994). Their major disadvantage is that production of secondary metabolites is usually not reflective of the whole plant. Characterization of the JH III biosynthetic pathway in *C. iria* was conducted on the suspension cell cultures through precursor feeding and enzyme inhibition studies.

**References**


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Hammock, B.D. and Mumby, S.M. (1978) Inhibition of epoxidation of methyl farnesoate to juvenile hormone III by cockroach corpus allatum homogenates. *Pest.*


Iwamura, J., Komaki, K., Komai, K. and Hirao, N. (1979) [Studies on constituents in Cyperaceae. Part IV. The constituents of essential oil from *Cyperus pilosus* Vahl.]


Composition and location of phytoecdysteroids in *Ajuga reptans* in vivo and in vitro cultures. *Phytochemistry* 31, 1585-1591.


Chapter III. Quantification of Juvenile Hormone III in the Sedge, *Cyperus iria* L.: Comparison of High Performance Liquid Chromatography and Radioimmunoassay

Abstract

Juvenile hormones are sesquiterpenoids involved in the regulation of insect development and reproduction (Wigglesworth, 1985; Gilbert et al., 1996). Of these compounds, juvenile hormone III (JH III) appears to be ubiquitous in insects and has also been isolated from the sedges, *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf and Kük (Toong et al., 1988). The objective of this study was to compare the quantification of JH III extracted from the sedge *C. iria* by two analytical techniques, high performance liquid chromatography (HPLC) and radioimmunoassay (RIA). The RIA, developed originally to determine insect haemolymph titers (Goodman et al., 1995), was optimized for analysis of plant tissues. The antiserum 31867 displayed minimal cross-reactivity with precursors of JH III from the insect biosynthetic pathway or with extracts from related plant species. There was no significant difference between the ability of HPLC or RIA to quantify JH III levels in standards or plant extracts (paired t-test). However, the increased sensitivity of the RIA enabled less plant material to be used for analysis. The tissue distribution of JH III was determined in mature *C. iria* plants by RIA. While substantial JH III levels were detected in the aerial plant tissues (0.2 to 1.2 μg/g fresh weight (FW)), the majority was predominantly located in the roots (43.5 μg/g FW).
**Introduction**

Insect juvenile hormones are sesquiterpenoids involved in the regulation of physiological processes associated with metamorphosis and reproductive maturation (Wigglesworth, 1985; Gilbert *et al.*, 1996). To date, six naturally occurring juvenile hormones have been identified; they share a common sesquiterpenoid backbone, a methyl ester on C-1 and an epoxide function between C-10 and C-11 (Fig. 1). Of these compounds, juvenile hormone III (JH III), methyl-10R,11-epoxy-3,7,11 trimethyl-2E,6E-dodecadienoate, appears to be ubiquitous and has been isolated from many insect Orders, including Lepidoptera (moths and butterflies), Coleoptera (beetles), Hymenoptera (sawflies, wasps, ants and bees), Orthoptera (grasshoppers, locusts, katydids, crickets), Dictyoptera (cockroaches) and Isoptera (termites) (Schooley, 1977; Tobe and Feyereisen, 1983). In most of these Orders, JH III is the only juvenile hormone present.

One defensive strategy of plants against insect herbivory is the production of secondary metabolites that interfere with insect physiological functions (Rosenthal and Berenbaum, 1991 and references therein). Some plant compounds have been shown to mimic juvenile hormone activity or to inhibit its biosynthesis (Bede and Tobe, 1999 and references therein). Toong *et al.* (1988) isolated JH III and its biosynthetic precursor in insects, methyl (2E,6E) farnesoate, from the sedges *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf and Kük. This is the first and only example to date of the extraction and identification of an insect juvenile hormone from a plant. The JH III level was reported to be 151 μg/g fresh weight (FW); approximately 200 times the maximal JH III level found in whole body extracts of the
Fig. 1. Structure of insect juvenile hormone III.
adult female cockroach, *Diploptera punctata* (day 4) (Tobe *et al.*, 1985).

One of our objectives was to determine the distribution of JH III in the sedge *C. iria*.

To do so, an accurate means of JH III extraction and subsequent measurement had to be established. At present, quantification of JH III is possible using either high performance liquid chromatography (HPLC) or a radioimmunoassay (RIA) that was developed to determine juvenile hormone levels in insect haemolymph (Goodman *et al.*, 1990, 1995).

Other methodologies for JH III measurement include gas chromatography-mass spectrometry (chemical ionization or electron impact), but this method often requires extensive derivatization of the epoxide group and specialized, costly equipment (Schooley, 1977). Furthermore, the sensitivity of this method is not required for the analysis of this plant tissue.

The present study optimized the RIA for the detection of JH III extracted from *C. iria*, and compared HPLC and RIA analysis of plant tissues.

**Materials and methods**

**Plants**

Sedges were grown either in a growth chamber (conditions: light 2800 lux at 26 ± 2°C in a 16:8 light-dark cycle) or in a greenhouse from seeds obtained from Y.C. Toong (Penang, Malaysia). Plants were grown in individual pots in organic potting mix (Home Gardener) and the soil kept continuously moist by maintaining the plants in 2-5 cm water. Voucher specimens of the following plants are available from the Royal Ontario Museum Herbarium, Canada (TRT): *C. iria, C. eragrostis, C. polystachyos* and *Finbriystylis dichotoma*. For this study, individual plants were analyzed after maturity (flowering).
Chemicals

All solvents were of high performance liquid chromatography (HPLC) grade from Burdick and Jackson unless otherwise noted.

Plant extraction

High performance liquid chromatography

Preweighed plant tissues were ground in acid-washed sand (Sigma) and liquid nitrogen and extracted overnight in pentane at room temperature (RT). The extract was evaporated to near dryness and subjected to column chromatography over a hydroxyapatite column (HA) (BioRad) to remove chlorophylls and other contaminants. The anhydrous sodium sulphate (BDH) HA column prepared in a Pasteur pipette was prewashed with ethyl ether and iso-octane. This was followed by addition of the sample and elution with pentane-ether (70:30). The solvent was evaporated under nitrogen (Canox) and the sample resuspended in hexane for HPLC analysis.

Radioimmunoassay

In comparative studies between HPLC and RIA, the plant samples were prepared as above (see high performance liquid chromatography). In other experiments, plant tissues were ground in liquid nitrogen, weighed and extracted by acetonitrile (CH₃CN) or pentane.
**Acetonitrile extraction**

Juvenile hormone III was extracted from the plant by a modification of the purification procedure from insect haemolymph (Goodman et al., 1995). Plant material was extracted in CH₃CN (1 ml) at 60°C. After a 3 min incubation, samples were vortexed, centrifuged (450 g x 5 min) and the supernatant removed. This process was repeated. Brine (4% NaCl, 1 ml (Sigma)) and 1 ml pentane were added to the pooled CH₃CN, vortexed and centrifuged (450 g x 5 min) to separate the two phases. The pentane phase was removed and the pentane extraction repeated. Organic phases were pooled, evaporated under a stream of nitrogen and resuspended in toluene (ACS, BDH) for analysis by radioimmunoassay (RIA).

**Pentane extraction**

After tissue maceration, plant samples (approximately 0.1 to 0.4 g) were weighed and extracted in pentane (2 ml) overnight at RT. Following incubation, the samples were vortexed, centrifuged (450 g x 5 min) and the pentane removed. Pellets were washed again with pentane, vortexed, centrifuged and the supernatants pooled. Samples were dried under a stream of nitrogen and resuspended in toluene for analysis by RIA.

**Synthesis of juvenile hormone and biosynthetic precursors**

**Farnesal**

Farnesal was synthesized through the allylic oxidation of trans, trans-farnesol (Aldrich, 96%, 11.4 mmol) by activated manganese dioxide (MnO₂) (228 mmol, Aldrich) in the presence of anhydrous sodium carbonate (228 mmol, BDH) and hexane (125 ml), according to a
modification of the protocol of Corey et al. (1968) by Xiao and Prestwich (1990). The mixture was stirred for 22 h at 0°C, filtered through a layer of celite (Fisher) and then concentrated in vacuo (Buchler Instruments) to yield the aldehyde (2.1 g, 9.53 mmol, 85% pure (proton nuclear magnetic resonance; PNMR), 71.1% yield). Farnesal was further purified by thin layer chromatography on silica plates (Merck) in 10% ethyl acetate (HPLC grade, BDH) in hexane and 5% triethylamine (Sigma) ($R_f = 0.88$) after focusing in methanol (MeOH) (Sen and Garvin, 1995). The final sample contained two major peaks (31.5 % and 51.6 %) representing two farnesal isomers, as well as three minor peaks (personal comm., P. Teal, USDA, Gainesville, FL).

**Methyl farnesoate**

Methyl farnesoate (MF) was synthesized from farnesal (9 mmol) incubated with activated MnO$_2$ (284 mmol), sodium cyanide (46.1 mmol, BDH) and glacial acetic acid (0.76 ml, ACS, BDH) in 200 ml MeOH (Latli and Prestwich, 1991). The mixture was stirred for 14 h at RT, filtered through celite and then concentrated in vacuo to yield the methyl ester (1.5 g, 6 mmol, purity 80% (PNMR), 53.3 % yield). Methyl farnesoate was further purified by thin layer chromatography on silica plates in toluene-ethyl acetate-acetic acid (85:15:4 drops) ($R_f = 0.66$) after focusing in MeOH. The final sample contained two major peaks (21 % and 78 %) representing two MF isomers (personal comm., P. Teal, USDA, Gainesville, FL).

**Juvenile hormone III**

Juvenile hormone III (methyl-10$R$,11-epoxy-3,7,11-trimethyl 2$E$,6$E$-dodecadienoate) was
extracted from the plant as detailed above and purified by HPLC. The structure was confirmed by PNMR (personal comm., W.G. Reynolds, Department of Chemistry, University of Toronto). The concentration of stock solutions was determined from spectrophotometric measurements (Hewlett Packard 8454A with a diode array detector) at 218 nm using Beer's Law equation.

Radiolabelled juvenile hormone III

Tritiated JH III was synthesized through incubation of radiolabelled methionine with the corpora allata (CA) from the cockroach, Diploptera punctata, followed by purification by HPLC (Tobe and Clarke, 1985; King and Tobe, 1988). The CA from day 5 mated female cockroaches were incubated in methionine-free TC 199 medium, containing 2% Ficoll (Pharmacia, Biotech), 1.3 mM CaCl₂·2H₂O (Analar, BDH) 40 μM farnesolic acid (70%) and L-[³H-methyl]-methionine (Amersham, specific activity 3.11 TBq/mmol) overnight at 27°C. The aqueous media was extracted with iso-octane (2 x). Pooled organic fractions were eluted over a silica column (Sep-pak, Millipore) and the resulting eluent evaporated under a stream of nitrogen and resuspended in hexane. Final purification of enantomerically pure [³H-methyl]-10R,11-JH III was by HPLC.

Comparison of separation techniques

Pentane extracts of C. iria bract and inflorescence subjected to thin layer chromatography (TLC) or column chromatography over HA and HA/C₁₈ (Waters) columns were compared to direct pentane extractions.
**Thin layer chromatography**

Thin layer chromatography (TLC) separation of the samples was modified from Goodman *et al.* (1995). Plant samples (*C. iria* bracts and inflorescence) were extracted overnight in 2 ml pentane at RT. Samples were spiked with Sudan Black B, divided into two aliquots (sample and control) and were subjected to TLC according to Goodman *et al.* (1995). Sudan Black B is composed of two major blue bands which during chromatography bracket the JH III and mark its separation from slower migrating diglycerides and polar lipids and the faster moving triglycerides and nonpolar lipid components. The putative juvenile hormone-containing bands were scraped from the TLC plate and extracted. Samples were then analyzed by RIA; an aliquot of these samples was measured spectrophotometrically at $\lambda_{492}$ to determine losses during chromatography. The presence of any pigments in the plant extract was corrected for by subtraction of the control from the absorbance of Sudan Black B in the sample.

**Hydroxyapatite chromatography**

Pentane extracts of plant samples were subjected to HA chromatography in columns prepared as described above (plant extractions).

**C$_{18}$ chromatography**

After chromatographic separation on HA columns, some samples were subjected to C$_{18}$ chromatography in Pasteur pipette columns. These columns were washed with pentane, ether and then CH$_3$CN. Following application of the sample, compounds were eluted with pentane.
**High performance liquid chromatography**

Plant extracts were quantified by HPLC (Varian LC5020) by normal phase chromatography on a spheri-5 silica column (Brownlee, 250 x 4.6 mm). The mobile phase was 10% half-water saturated ethyl ether in hexane at a flow rate of 1 ml/min. Juvenile hormone III was monitored at 219 nm (SpectroMonitor III) and the concentration of extract calculated from the peak area by comparison to external standards.

**Radioimmunoassay Procedure**

**Glassware**

Glassware was treated following recommendations outlined in Granger and Goodman (1988). Where noted in the procedure below, glassware was coated with polyethylene glycol (PEG, 15,000 - 20,000 MW, Sigma) to reduce JH binding (Giese et al., 1977). Also, whenever possible, plastic was avoided. Microcap pipettes (Fisher) and glass syringes (Hamilton) were used to prepare JH III standards.

**Radioimmunoassay**

The RIA procedure was modified from Goodman et al. (1990, 1995). Antiserum was diluted in 0.1 M sodium phosphate buffer (Sigma), pH 7.2, containing 0.1% bovine serum albumin (protease-free, Sigma), 0.1% immunoglobulin G (Sigma), 0.02% sodium azide (Fisher) (buffer A). Tritiated JH III standard was prepared in buffer A in a PEG-treated vial. Both the antiserum and radiolabelled JH III standard were gently agitated for at least 30 min at 4°C.
**Standard curve**

A range of stock JH III standard (0.1 μg/ml in toluene) and the unknown samples were added to tubes (60 x 5 mm, Kimble, borosilicate culture tubes) which had been PEG-treated. The volume of all samples was adjusted to 100 μl with toluene. After the solvent was removed by lyophilization, 1 μl of ethanol (EtOH, Commercial Alcohols Inc.) was added to each tube and vortexed. Tritiated JH III stock and antiserum were added to each tube followed by vortexing. Samples were incubated overnight at 4°C. The assay was terminated by addition of 100% saturated ammonium sulphate (Sigma), pH 7.2 at 4°C. After 20 min incubation, tubes were centrifuged (450 g x 15 min), and the supernatant aspirated. Pellets were dissolved in 50% ammonium sulphate, pH 7.2 containing 0.1% BSA. Tubes were recentrifuged and the supernatant aspirated. The resultant pellet was dissolved in 20 μl water. Following addition of scintillation fluid and vortexing, samples were radioassayed (Beckmann LS6500). The standard curve was generated by a four parameter logistic curve (Graphpad Prism, version 1.0). Concentrations of unknown samples were determined from the standard curve. Assays were performed in triplicate.

**Quantification of juvenile hormone III in the sedge Cyperus iria.**

Juvenile hormone III in the plant was quantified using the optimized RIA procedure. Tissues from mature plants were divided into the roots (below-ground portion) (n = 6), the culm (stem) (n = 5), leaves (n = 5), bracts (modified leaves which subtend the inflorescence) (n = 6), inflorescence (pedicels, peduncles and inflorescence) (n = 6) and nutlets (fruits) (n = 6).
Plant material was prepared as outlined in the above section (plant extraction, pentane extraction) and JH III measured by RIA and HPLC.

Statistics

Analyses were performed using the statistical package SPSS 7.5.

Results

Optimization of plant tissue extraction

Extraction of JH III from insect haemolymph by CH₂CN and brine is necessary to disrupt the interactions between the hormone and its binding protein. This is followed by partitioning of JH III into pentane. However, the extraction efficiency of JH III from C. iria tissues in CH₂CN was low (Table I). In comparison, a higher yield was observed by direct extraction into pentane (Table II). Juvenile hormones are lipophilic compounds that strongly adsorb to many substrates (Giese et al., 1977). To determine the extent of JH III binding to plant material or glassware, ^3^H-JH III was added to plant tissues followed by extraction with pentane. No significant difference was observed between the control and plant tissues (Table III). These results confirm that a high extraction efficiency of JH III from C. iria tissues can be achieved using pentane (Table II and III). All subsequent plant extractions were performed with this solvent.

Comparison of chromatographic clean-up procedures

After CH₂CN extraction, insect haemolymph samples were subjected to thin layer
chromatography (TLC) to eliminate lipophilic compounds that interfere with the RIA (Goodman et al., 1995). A comparison of the three clean-up procedures on pentane extracts from plant tissues was made (Table IV). There was no significant difference in JH III amounts in samples which were directly extracted in pentane or those which had undergone TLC. Sudan Black B was used as an internal control during TLC, allowing for correction of losses. A decrease in JH III amount was observed in samples subjected to hydroxyapatite (HA) or HA/C₁₈ chromatography as compared with controls. An internal standard for these procedures was not used. Observed differences in JH III between the pentane control and samples subjected to further chromatographic procedures reflect slight losses during manipulations. In subsequent experiments, pentane extracts of the plant tissue were analyzed directly by RIA.

**Comparison of related plant species**

Plant material may interfere with the RIA through the binding of antiserum or JH III to plant compounds such as lignins and tannins or through cross-reactivity of the antiserum to compounds other than JH III. To determine the extent of these non-specific interactions, three related plant species were tested; *C. eragrostis* and *C. polystachyos* are in the same genus as *C. iria*, and *Fimbristyliis dichotoma* belongs to the same subfamily, Cyperoideae. Juvenile hormone was not detected in extracts from these plants, allowing their use to determine the amount of plant material that would cross-react with the RIA. Results indicate that more than 20 mg of plant tissue could interfere with the assay (Table V). In the second experiment, tissue extracts were spiked with JH III and analyzed. Again, it was observed that amounts greater than 25 mg of material interfered with the RIA, in this case, preventing detection of JH
Table I. Optimization of juvenile hormone III extraction procedure in acetonitrile.

<table>
<thead>
<tr>
<th>Extraction efficiency</th>
<th>% ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml CH₃CN</td>
<td>8.3 ± 1.6</td>
</tr>
<tr>
<td>CH₃CN + 2 ml water</td>
<td>38.4 ± 5.3</td>
</tr>
<tr>
<td>CH₃CN + 2 ml 2% brine</td>
<td>33.6 ± 0.8</td>
</tr>
<tr>
<td>CH₃CN + 2 ml 4% brine</td>
<td>27.1 ± 1.5</td>
</tr>
</tbody>
</table>

Acetonitrile (CH₃CN) was spiked with [³H-methyl]-JH III. Triplicate samples were treated as outlined in the table and extracted twice with 1 ml pentane. The radioactivity in the sample was determined and compared to the initial amount.
Table II. Optimization of juvenile hormone III extraction from plant tissues.

<table>
<thead>
<tr>
<th>Relative extraction efficiency</th>
<th>% ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentane</td>
<td>100 ± 15.2</td>
</tr>
<tr>
<td>CH$_3$CN</td>
<td>60.6 ± 12.3</td>
</tr>
</tbody>
</table>

Cyperus iria tissues (bracts and inflorescence) were ground in liquid nitrogen and extracted with either pentane (2 x 1 ml) or acetonitrile (CH$_3$CN) (2 x 1 ml). One ml of brine (4% NaCl) was added to the CH$_3$CN supernatant and extracted twice with pentane (1 ml). Triplicate samples were subject to thin layer chromatography and appropriate bands extracted and analyzed by radioimmunoassay with antiserum 31867 (1:2000) and $[^3]$H-methyl-10R-JH III. Paired t-test (one tail, p = 0.049) demonstrated that there is a significant difference between the extraction efficiency of these two solvents.
Table III. Pentane extractions of juvenile hormone III from plant tissues.

<table>
<thead>
<tr>
<th>Plant Tissue</th>
<th>Extraction efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 1.9</td>
</tr>
<tr>
<td>Bracts</td>
<td>92.7 ± 7.4</td>
</tr>
<tr>
<td>Inflorescence</td>
<td>87.0 ± 7.2</td>
</tr>
<tr>
<td>Roots</td>
<td>100.1 ± 2.9</td>
</tr>
<tr>
<td>Culm</td>
<td>97.7 ± 24.6</td>
</tr>
</tbody>
</table>

Tritiated JH III was added to triplicate plant samples and extracted in pentane (2 x 1 ml). No significant difference was observed in the extraction efficiency between the control and the plant tissues (one factor ANOVA, p = 0.636). Blanks (plant tissues incubated without radioligand) contained less than 25 dpm per sample.
Table IV. Comparison of chromatographic procedures used in juvenile hormone III
extractions from plant tissues.

<table>
<thead>
<tr>
<th>Method</th>
<th>Relative extraction efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentane extraction</td>
<td>100 ± 33.1</td>
</tr>
<tr>
<td>Pentane extraction/TLC</td>
<td>87.1 ± 30.9</td>
</tr>
<tr>
<td>Pentane extraction/HA</td>
<td>75.2 ± 36.4</td>
</tr>
<tr>
<td>Pentane extraction/HA/C_{18}</td>
<td>81.2 ± 19.8</td>
</tr>
</tbody>
</table>

*Cyperus iria* tissues (bracts and inflorescence) were ground in liquid nitrogen and extracted
with either pentane (2 x 1 ml) and directly analyzed by radioimmunoassay (RIA) or subjected
to clean-up chromatographic procedures such thin layer chromatography (TLC),
hydroxyapatite chromatography (HA) and C_{18} chromatography (C_{18}) as outlined in the table.
Triplicate samples were analyzed by RIA with antiserum 31867 (1:2000) and \[^{3}H\text{-methyl}]^{-10R-JH}\text{ III}. No significant difference was observed in the extraction efficiency between the
methods (one factor ANOVA, p = 0.706).
Table V. Radioimmunoassay of closely related plant species.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Cross-reactivity observed at:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fimbristylis dichotama</em></td>
<td>&gt; 20 mg of plant tissue</td>
</tr>
<tr>
<td><em>Cyperus polystachyos</em></td>
<td>none detected, at up to 200 mg of plant tissue</td>
</tr>
<tr>
<td><em>C. eragrostis</em></td>
<td>&gt; 150 mg of plant tissue</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plant + JH III</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. dichotama</em></td>
<td>&gt; 25 mg, interferes with RIA</td>
</tr>
<tr>
<td><em>C. eragrostis</em></td>
<td>&gt; 25 mg, interferes with RIA</td>
</tr>
</tbody>
</table>

Cross-reactivity of extracts from three plant species related to *C. iria* were compared by radioimmunoassay (RIA) using antiserum 31867 (1: 1750) and [1H-methyl]-10R-JH III as the radiotracer. In the second experiment, pentane extracts were spiked with 500 pg of JH III and analyzed by RIA.
III. Therefore, in all subsequent experiments with C. iria, less than 10 mg of tissue was assayed.

*Cross-reactivity of the biosynthetic precursors of juvenile hormone III*

In the corpora allata of the Pacific beetle cockroach, *Diploptera punctata*, JH III biosynthesis from farnesyl diphosphate occurs through five enzymatic steps (Cusson *et al.*, 1991). The competitive displacement curves of three of these biosynthetic intermediates, farnesol, farnesal and methyl farnesoate, with the antiserum 31867 demonstrated that the antiserum has little affinity for these compounds (Fig. 2); the ED<sub>50</sub> values of these compounds relative to JH III indicates that the antiserum has greater than a two thousand fold higher affinity for JH III than for the biosynthetic precursors tested (Table VI).

*Analysis of juvenile hormone III from Cyperus iria tissues; comparison of high performance liquid chromatography and radioimmunoassay*

Juvenile hormone III standards and extracts from plant tissue were analyzed by HPLC and RIA (Fig. 3a and b). No significant difference in JH III levels was observed using these two techniques, confirming that they can be used interchangeably with confidence. However, the RIA technique is more sensitive to JH III levels than analysis by HPLC; the antiserum 31867 displays an ED<sub>50</sub> in the range of 323 pg against 10R-JH III, using 3H-JH III as the radiotracer (Table VI), whereas the detection limit of HPLC is in the ng range. Therefore, the RIA allows quantification of JH III in as little as 10 mg of plant material.
Fig. 2. Competitive displacement curves for the biosynthetic precursors of juvenile hormone III (JH III), farnesol, farnesal and methyl farnesoate. The crossreactivity of farnesol (△), farnesal (▲) and methyl farnesoate (●) with the antiserum 31867 (1:1750) was determined by radioimmunoassay using [3H-methyl]-10R-JH III. Each point represents the mean ± standard deviation of triplicate experiments. Compound structures are illustrated in the insert.
The graph shows the relationship between Log Competitor (g) and Percent Bound for different substances. Different compounds are represented by different symbols and are labeled as follows:

- Farnesol: △
- Farnesal: ◀
- Methyl farnesolate: ●
- Juvenile hormone III: ■

The x-axis represents the log of the competitor concentration, while the y-axis represents the percent bound. The graph indicates a decrease in percent bound as the log of the competitor concentration increases.
Table VI. Summary of displacement values for juvenile hormone III and its biosynthetic precursors.

Competitor dose required to displace 50% (ED$_{50}$) of the [3H-methyl]- 10R-JH III.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10R-Juvenile hormone III</td>
<td>0.323</td>
</tr>
<tr>
<td>Farnesol</td>
<td>89590</td>
</tr>
<tr>
<td>Farnesal</td>
<td>21290</td>
</tr>
<tr>
<td>Methyl Farnesoate</td>
<td>735</td>
</tr>
</tbody>
</table>
Fig. 3. Comparison of high performance liquid chromatography and radioimmunoassay. A. Juvenile hormone III standards. Two juvenile hormone III standards (8.7 μg/ml and 17.4 μg/ml) were analyzed in triplicate by HPLC and RIA. Bars represent means ± standard deviation. The results were compared by a paired t-test followed by a sequential Bonferroni test; no significant difference was found between these two techniques (two tail, p = 0.084 (8.7), p = 0.964 (17.4)). B. Cyperus iria samples. Extracts of C. iria tissues (culm and root) were assayed in triplicate by HPLC as outlined in the materials and methods. Triplicate samples were also analyzed by RIA which had been optimized for plant tissues. The RIA was performed with antiserum 31867 (1:1500) using [³H-methyl]-10R-JH III. Bars represent means ± standard deviation. Results were compared by a paired t-test followed by a sequential Bonferroni test; no significant difference was observed between these two techniques (two tail, p = 0.034 (culm), p = 0.823 (roots))
Distribution of juvenile hormone III in mature plants of Cyperus iria

The distribution of JH III in the sedge, C. iria, were determined using the optimized RIA (Fig. 4). Juvenile hormone III levels in aerial tissues ranged from 0.2 to 1.2 μg/g fresh weight (FW), whereas in the root tissue, the average level was 43.5 μg/g FW, representing over 90% of the total JH III in the plant. These levels are approximately 3.5 fold lower than those previously reported (Toong et al., 1988). There is also a six fold difference between the level of JH III detected in these whole plant extracts and those reported from leaf extracts of 2-month-old C. iria plants (Schwartz et al., 1998). These discrepancies are attributed to changes in JH III levels in the plant throughout development as well as differences in cultivars and in the environmental conditions under which the plants were grown.

Discussion

These results demonstrate that the amount of JH III in C. iria can be determined by HPLC or RIA and that there is good correlation between these two assay techniques (Fig. 3a and b). The RIA was developed for the analysis of JH III titers in insect haemolymph where it is necessary to extract the compound in CH₃CN, partition into pentane and then separate this compound from the interfering lipids using TLC (Goodman et al., 1995). In C. iria, direct extraction of JH III from plant tissues with pentane was more efficient than CH₃CN (Table II), and subsequent clean-up of the extracts is unnecessary because of the lower lipid content (Table IV). Minimal cross-reactivity of the antiserum with biosynthetic precursors of JH III (insects) or extracts from closely related plant species was observed (Fig. 2, Table V and VI). However, these results did indicate that analysis of concentrated plant extracts (> 25 mg/ml...
Fig. 4. Distribution of juvenile hormone III in mature plants of *Cyperus iria*. Juvenile hormone III in the tissues of mature plants was measured by RIA with antiserum 31867 (1:2000) using \[^{3}H\text{-methyl}\]-10R-JH III as the radiotracer. Plant tissues were divided into the nutlets (n = 6), inflorescence (Inf.) (pedicels, peduncles and inflorescence) (n = 6), bracts (n = 6), culm (stem) (n = 5), leaves (n = 5) and roots (n = 6). The total amount of JH III in the plant (ttl. plant) was also determined (n = 5). Bars represent means ± standard error.
toluene) interfered with the RIA (Table V).

The advantage of RIA over HPLC is the increased sensitivity of the RIA allowing
detection of pg amounts of JH III. This permitted accurate measurement in 10.0 mg amounts
of plant tissue. In the distribution profile of aerial tissues, the highest amounts of JH III were
localized in the leaves, inflorescence and culm (Fig. 4). However, the vast majority of JH III
was detected in the roots. This suggests that the roots may be the site of biosynthesis.
Alternatively, it is possible that JH III is synthesized in leafy tissues and transported to
the root tissue for storage.

Although JH III has only been isolated from C. iria and C. aromaticus (Toong et al.,
1988), structurally similar compounds have been isolated from the roots of C. iria and related
Cyperus species (Iwamura et al., 1978b; 1978c; 1979; Iwamura, 1979; Komai et al., 1981).
For example, methyl farnesoate, the biosynthetic precursor of JH III in insects, has been
isolated from C. iria, C. monophyllus, C. pilosus and C. serotinus (Iwamura et al., 1978a;
1978c; 1979; Komai et al., 1979; Toong et al., 1988), as well as from grape skins (Versini et
al., 1994) and the bark of Polyalthia viridis Craib (Kijjio et al., 1990). Other structurally
related linear sesquiterpenoids have been isolated from C. microiria (Iwamura et al., 1978b)
and C. polystachyos (Iwamura, 1979). This suggests that JH III, MF and other similar
compounds may be present in a diverse array of plant species. However, in this and another
study (Schwartz et al., 1998), JH III was not detected by RIA in the following plant species:
C. albostriatus, C. alternifolius, C. eragrostis, C. esculentus, C. miliifolius, C. papyrus, C.
polystachyos and Fimbristylis dichotama.

The relatively high levels of JH III in the plant suggests that this compound plays an
important role(s) in *C. iria*. At present, its biological relevance is a matter for speculation (Bede et al., 1998; Bede and Tobe, 1999), but possibilities include plant-insect, plant-plant and plant-nematode interactions. There are a few studies in which *C. iria* has been shown to interfere with insect development, and these effects are likely attributable to the presence of JH III (Meneses and García de la Osa, 1988; Toong et al., 1988; Schwartz et al., 1998). However, other reports indicate that some insect species are able to feed on the plant without adverse effects (Naresh and Smith, 1984; Dela Cruz, 1986). These may be examples of insect species that are able to avoid plant tissues rich in JH III or to metabolize any ingested compound (Dowd et al., 1983). Juvenile hormone III may also function to reduce plant competition by inhibiting the germination and growth of plants in the immediate vicinity.

Two of the biosynthetic precursors of JH III, methyl farnesoate and farnesol, have demonstrated potent allelopathic activity (Komai et al., 1981; Wardle and Short, 1982) which may, in part, explain the invasive nature of *C. iria* (Holm et al., 1977). Juvenile hormone III may also serve to protect the plant against phytoparasitic nematodes. Juvenile hormone or biosynthetic precursors have been used to arrest the development of nematodes (Meerovitch, 1965; Rogers, 1978; Glassburg et al., 1983; Davey, 1988). However, an effect on plant parasitic nematodes has not yet been demonstrated (Johnson and Viglierchio, 1970; Rogers, 1978).

**References**


Tobe, S.S. and Clarke, N. (1985) The effect of L-methionine concentration on juvenile hormone biosynthesis by the corpora allata of the cockroach, Diploptera punctata.


Chapter IV. Developmental Distribution of Juvenile Hormone III
in the Sedge, *Cyperus iria* L.

Abstract

In insects, juvenile hormones (JHs) regulate physiological processes such as metamorphosis and reproduction, but their presence in plants is not understood. The temporal and tissue distribution of insect juvenile hormone III (JH III), methyl-10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate, was determined in the sedge, *Cyperus iria* L., over an eight month developmental time course, from the seedling to the senescent plant. Juvenile hormone III increased in immature plants until flowering, at which time a transient decrease in all plant tissues was observed. Levels subsequently increased in the mature plant until senescence when, again, the amount of this compound fell in aerial tissues. This pattern was not observed in the root tissue which remained viable. Over 85% of the total JH III in the plant was present in this tissue, suggesting that the roots may be a site of synthesis and/or storage. The developmental distribution of JH III in *C. iria* prompts speculation about the possible ecological role(s) of this unique compound.
**Introduction**

Insect juvenile hormones (JHs) are structurally-related sesquiterpenoids that regulate developmental processes such as metamorphosis and reproduction (Gilbert et al., 1996). The insect hormone ecdysone and its metabolites are responsible for the induction of the moultting process, whereas the titer of JH in the insect haemolymph prior to the moult determines the nature of the moult (Gilbert et al., 1996).

One defensive strategy of plants against insect herbivory is the production of secondary metabolites that interfere with insect physiological functions (Rosenthal and Janzen, 1979; Rosenthal and Berenbaum, 1991). Plants may contain compounds that either mimic JH activity, such as juvabione isolated from balsam fir, *Abies balsamea* (L.) Miller (Bowers et al., 1966), or that act as antagonists by inhibiting JH biosynthesis, such as the precocenes found in *Ageratum houstonianum* Miller (Bowers et al., 1976). In 1988, insect juvenile hormone III (JH III), methyl-10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate (Fig. 1), and its metabolic precursor in insects, methyl farnesoate, were first reported in the sedges *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf and Kilk (Toong et al., 1988).

In the present study, the distribution of JH III in the sedge *C. iria* was investigated over an eight month period using a radioimmunoassay (RIA), originally developed for quantification of JH in insect haemolymph (Goodman et al., 1995) that has been optimized for the analysis of plant tissue (Bede et al., 1999). This technique allowed measurement of JH III in small amounts of plant material (e.g. 10 mg) permitting separate analysis of the roots, culm (stem), leaves, bracts, inflorescence and nutlets (fruit) tissues. Investigation of the distribution of JH III in these plant tissues may lead to a better understanding of the role that this compound plays in the plant.
Fig. 1. Structure of insect juvenile hormone III, methyl-10R-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate.
Results and discussion

**Cyperus iria**

*Cyperus iria* is a tufted, annual sedge native to Eurasia (Holm *et al.*, 1977). The red fibrous roots and long lower bract are characteristic of this species. It also appears to be biochemically unique as it produces the insect JH III (Toong *et al.*, 1988). This study examined the temporal and spatial distribution of JH III in *C. iria* over an eight month period. Over this time course, plant fresh weight (FW) increased until maturation, when a cessation of growth was observed likely reflecting the shunting of plant resources into reproductive tissue (unpaired t-test, 3 month vs 4 month plants, p = 0.264) (Fig. 2). After flowering, the plant weight continued to increase until seven months after which senescence of aerial tissues was observed (unpaired t-test, 7 month vs 8 month plants, p = 0.697).

**Juvenile hormone III levels in whole plants of Cyperus iria**

Juvenile hormone III levels increased in immature plants of *C. iria* from 17.6 nmoles/g FW in two-week-old plants to 87.8 nmoles/g FW over the first three months. At flowering, a cessation of plant growth and a dramatic decrease of JH III in all tissues were observed (Fig. 3a and b). One possible explanation is that at maturation, the plant directs its resources into developing reproductive tissues rather than vegetative growth and the biosynthesis of this secondary metabolite. Juvenile hormone III levels subsequently increased over the next three months to approximately 1.24 µmoles in the mature plant. In eight-month-old plants, levels of JH III were highly variable between plants, which may reflect differing states of senescence.

**Juvenile hormone III levels in aerial tissues**

Juvenile hormone III levels in the aerial tissues are illustrated in figures 4a and 4b. In immature plants, aerial tissues consisted of the culm and leaf which were not separated
Fig. 2. Growth of *Cyperus iria* plants. The fresh weight of *Cyperus iria* plants was measured over an eight month period. Four individuals were sampled at each time point, except 0.5 month old plants (n = 8), 3 month old plants (n = 3), 5 month old plants (n = 3) and mature 6 month old plants (n = 5). Bars represent mean weight ± standard error.
Fig. 3. Time course of juvenile hormone III levels in *Cyperus iria* plants. The developmental distribution of JH III was measured by radioimmunoassay using antiserum 31867 (1:1750) and [3H-methyl]-10R, 11-JH III as the radiotracer. **A. Juvenile hormone III levels in *C. iria***. Four individuals were sampled over an eight month period, except immature 0.5 month-old plants (n = 8) and mature 5, 6 and 8 month-old plants (n = 3). Bars represent means ± standard error. **B. Amount of juvenile hormone III in *C. iria***. Juvenile hormone III was measured in plant tissues as defined above. Bars represent means ± standard error.
at these stages. After flowering, plants were divided into the culm (stem), leaf, bracts and inflorescence tissues. In newly mature plants (4 to 4.5 months), the inflorescence tissue also included the fruit; in older plants (5 to 8 months), the JH III content of the nutlets was measured separately.

Juvenile hormone III levels in the aerial tissues of immature plants did not significantly change until flowering (Fig. 4a) (one-factor ANOVA, \( p = 0.355 \)), although the total amount of JH III increased from 3.0 to 8.6 nmoles (one-factor ANOVA, \( p = 0.006 \)). As the plant matured, JH III levels in these tissues decreased, then increased again over the next three months until senescence.

Further analysis of plant tissues revealed that JH III levels remained low in culm and leaf tissues until three months after flowering at which time a significant increase in JH III was observed (Fig. 4a) (one factor ANOVA, \( p = 0.004 \) (culm); \( p < 0.001 \) (leaf)). Total JH III amounts decreased in both tissues as the plant became senescent. Most of the JH III detected in the culm was localized in the lower third of the stem (data not shown).

A similar developmental profile was observed in the bracts and inflorescence (Fig. 4b). In mature plants, JH III in these tissues increased to their highest levels in seven month old plants and then declined in senescent tissue. There was no distinguishable difference in JH III levels between these two tissues (paired t-tests, \( p = 0.928 \) (4 months), \( p = 0.138 \) (4.5 months), \( p = 0.060 \) (5 months), \( p = 0.337 \) (6 months), \( p = 0.666 \) (7 months), \( p = 0.516 \) (8 months)). However, the amounts of JH III found in these tissues were low and represented less than 20% of the total amount detected in the aerial parts. Juvenile hormone III levels in the nutlets did not change significantly over the period of study (one factor ANOVA, \( p = 0.226 \)) (Fig. 4b).
Fig. 4. Time course of juvenile hormone III distribution in aerial tissues of *Cyperus iria*. The developmental distribution of JH III was measured by radioimmunoassay using antiserum 31867 (1:1750) and $[^{3}H$-methyl]-10R, 11-JH III as the radiotracer. **A. Juvenile hormone III levels in the culm, leaves and total above-ground tissues.** The amount of JH III was determined per gram of plant tissue (fresh weight (FW)). In immature plants (before flowering), the JH III in the total above-ground tissue was measured. After flowering, culm and leaf tissues were separated and analyzed. Bars represent the means ± standard error of three to five individuals. **B. Juvenile hormone III levels in the bracts, inflorescence and seeds.** In mature plants, the concentration of JH III was determined in the flowering tissues which were divided into the bracts, which are modified leaves surrounding the inflorescence, the inflorescence, which is comprised of the flowers, pedicels and peduncles, and the nutlets. Prior to five months, nutlets were not analyzed separately from the inflorescence. Bars represent the mean ± standard error of three to five individuals. **C. Total juvenile hormone III in aerial tissues.** Juvenile hormone III was measured in the plant tissues defined as above. Bars represent the mean ± standard error of three to five individuals.
Juvenile hormone III levels in subterranean tissues

Most of the total JH III in the plant (> 85%) was sequestered in the fibrous roots. In immature plants, JH III levels increased until flowering (Fig. 5a); the decrease observed in two month old plants is partially explained by an increase in plant FW at this time. Again, it was observed that JH III levels dropped in the root tissue as the plant matured and then increased over the next three months. Juvenile hormone III levels in the root tissues did not significantly change in mature seven month and eight month old plants (Fig. 5b) (unpaired t-test, p = 0.799 (concentration); p = 0.557 (amount)). This differs from the observation in aerial tissues in which a significant decrease in JH III levels were measured at this time (Fig. 4a and 4b). Aerial tissues become senescent after seed production in these plants. Therefore, we believe that there is an inhibition of JH III biosynthesis and/or transport of the compound out of these senescening tissues. However, since vegetative propagation in these plants also occur through tillers, the root tissue remains viable and shows high levels of JH III at this time.

During the eight month period of study, the highest levels of JH III were detected in the root tissue, suggesting that the roots are the site of biosynthesis. However, JH III might be synthesized in aerial tissues, such as leaves, and transported to the roots. The cellular site of accumulation of this acyclic compound is unknown. In plants, such lipophilic secondary metabolites are often sequestered in specialized cells or resin ducts or covalently modified by the addition of sugars or phosphate groups for transport or storage in the vacuole (Fahn, 1979; Hösel, 1981; Grebenok et al., 1994). Secretory oil cells have been characterized in some taxa in the family Cyperaceae (Kern, 1972-6; Metcalf, 1971). However, the presence of JH III in these cells in the sedge C. iria has not yet been demonstrated.
Fig.5. Time course of juvenile hormone III distribution in the roots of *Cyperus iria*.

The developmental distribution of JH III was measured by radioimmunoassay using antiserum 31867 (1:1750) and [3H-methyl]-10R, 11-JH III as the radiotracer. **A. Levels.** The amount of JH III was determined per gram of plant tissue (fresh weight (FW)). Bars represent the means ± standard error of three to four individuals. **B. Amount.** Total JH III was measured in root tissue. Bars represent the mean ± standard error of three to four individuals.
Juvenile hormone III levels in a plant cluster

The above profiles represent the developmental distribution of JH III in individual C. iria plants. However, tillers may be present as early as 2 months after germination of primary plants. These secondary plants were not included in the above analyses. In general, the amount and concentration of JH III in these plantlets reflected the developmental profile observed in individual plants in which accumulation of this secondary metabolite increased in tissue over time and was more prevalent in inflorescence and leaf tissues than in the culm (Fig. 6).

Conclusions

In the original report identifying JH in a plant, JH III was isolated by steam distillation and identified following chemical derivitization by electron impact mass spectroscopy (Toong et al., 1988). These authors determined that three month old mature plants contained 151 µg JH III/g FW. This is comparable to concentrations found in a subsequent study in which 193 and 143 µg JH III/g FW were detected in leaves of one month and two month old plants, respectively (Schwartz et al., 1998). These values are approximately seven times the whole plant levels detected in the present investigation, though plant analyses in this and the last study studies were performed using a radioimmunoassay which was able to detect pg quantities of JH III. Discrepancies in JH III concentration are attributed to the different cultivars and to environmental conditions under which the plants were grown.

It is tempting to speculate that the high level of JH III in C. iria may be involved in the protection of the plant against insect herbivory. Insect JH titer is precisely regulated during development. During the final larval stadium, there is a drop in haemolymph titer prior to the moult to an adult (Szibbo et al., 1982) and topical application of JH or synthetic analogues at this stage results in the inappropriate retention of juvenile
Fig. 6. Distribution of juvenile hormone III in aerial tissues of individual plants from a plant cluster of *Cyperus iria*. Juvenile hormone III (JH III) content was determined in the aerial tissues of individual plants from a plant cluster. In this example, there are four mature plants and two immature plants which are numbered sequentially from 1 to 6, where 1 represents the oldest individual which had flowered 3 months previously. In immature plants, total above-ground tissue was assayed. In mature plants, the tissue was divided into the culm, leaves and inflorescence (anthelodia, inflorescence and nutlets). Juvenile hormone content was measured by radioimmunoassay using antiserum 31867 (1:1750) and [\(^{3}\)H-methyl]-10\(R\), 11-JH III as the radiotracer. The amount of JH III was determined per gram of plant tissue (fresh weight (FW)). Bars represent the JH III concentration in a single individual.
characteristics at the next moult (Sehnal, 1983). Also, application of these compounds to insect eggs can disrupt embryonic development (Riddiford, 1972). This can range from immediate ovicidal effects to delayed developmental effects. The levels of JH III observed in the plant are significantly higher than the amounts found in insects. In the female cockroach, *Diploptera punctata*, JH III concentrations reached their maximum 4 to 5 days after mating. In whole body extracts, the level of JH III is approximately 504 ng/g (day five) (Tobe et al., 1985). By comparison, seven month old *C. iria* plants contained 327.7 μg JH III; over forty times the levels found in day five, mated female cockroaches.

There have been few investigations on the interactions between *C. iria* and associated insects. In laboratory studies, third stadium grasshopper nymphs, *Melanoplus sanguinipes*, were fed a diet of wheat seedlings or *C. iria*; no difference in growth was observed (Toong et al., 1988). However, following the moult to adult, 90% of those fed exclusively *C. iria* showed deformed wings and other morphological changes consistent with development in the presence of excess JH, and the adult female grasshoppers were infertile. Leaves of *C. iria* exhibited a larvicidal effect when added to water containing larvae of the mosquito, *Aedes aegypti* (Schwartz et al., 1998). In field studies, eggs of the Dipteran leafminer, *Hydrellia* sp., did not hatch when laid on leaves of *C. iria* (Meneses and García de la Osa, 1988). It is assumed that these above effects are related to the JH III content of the plant. However, in other reports, *C. iria* had no effect on insect development (Naresh and Smith, 1984; Dela Cruz, 1986). It is unclear whether these insects were able to avoid adverse developmental effects by metabolizing or sequestering the ingested JH or by avoiding tissues that contained high concentrations of the compound. However, other biological functions of JH III, such as its potential role as an allelopathic agent in plant-plant interactions or in plant defense against nematode, fungal or bacterial attack, should also be examined.
Materials and methods

Plants

Sedges were grown in the greenhouse (Dept. of Botany, University of Toronto, Jan. to Oct. 1997, photoperiod ranged from 9.00:15.00 (Jan.) to 10.29:13.31 (Oct.) (light:dark)) from seeds obtained from Y. Toong (Penang, Malaysia). Plants were grown in individual pots in organic potting mix (Home Gardener) and kept continuously moist by maintenance in 2-5 cm water. Greenhouse temperatures ranged from approximately 22°C in the winter months to ambient during the summer. Voucher specimens of C. iria have been deposited at the Royal Ontario Herbarium, Canada (TRT).

Plant analysis

Immature plants were assayed at 0.5, 1, 2 and 3 months (one month = 4 weeks) post-germination. At approximately 4 months, the bracts opened, exposing the inflorescence. Plants at this stage were defined as mature and assayed at 4, 4.5, 5, 6, 7 and 8 months. Immature plants were divided into aerial and subterranean (root) tissues. After flowering, mature plants were divided into the root tissues, culm (stem), leaves, bracts (modified leaves which subtend the inflorescence) and the inflorescence which comprises of compound multiple spikes, flowers and nutlets (fruits). A month after flowering, the nutlets were analyzed separately from the rest of the inflorescence.

Extraction

Plant tissues were ground in acid-washed sand (Sigma) and liquid nitrogen. After maceration, between 0.1 to 0.4 g of material from individual plants was weighed and extracted overnight in 2 ml pentane (HPLC grade, Burdick & Jackson) at RT. In most cases, sufficient tissue was available for triplicate samples. After incubation, the mixture was vortexed and centrifuged (450 g x 5 min) to pellet the insoluble plant material. The supernatant was removed and the extraction repeated. Combined supernatants were
evaporated under a stream of nitrogen. Samples were brought to volume in toluene (ACS, BDH) for analysis by radioimmunassay (RIA).

Radioimmunoassay
The RIA used for the detection of JH III in *C. iria* was previously described in Bede *et al.* (1999). From the standard curve (four parameter logistic curve, Graphpad Prism, version 1.0), concentrations of unknown samples were interpolated. Assays were performed in triplicate.

Statistics
Analyses were performed using the statistical package SPSS 7.5. One way Analysis of Variance (ANOVA) tests were conducted to evaluate the relationship between JH III levels in plant tissue over the developmental time course. Unpaired student's t-tests determined JH III differences between two time points.

References


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Chapter V. Activity of juvenile hormone III: seed germination and seedling growth

Abstract

Insect juvenile hormone III (JH III), methyl-10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate, has been identified in two sedge species, *Cyperus iria* L. and *C. aromaticus* (Ridley) Maff. & Kük. (Toong *et al*., 1988). Potential allelopathic activity of this compound and the structurally related sesquiterpenoid farnesol was investigated using seed germination and seedling growth assays with radish, lettuce and rice. Treatment of seeds with JH III delayed lettuce seed germination and potently inhibited rice shoot growth. Both farnesol and JH III inhibited growth of *C. iria* seedlings. The antimicrobial activity of JH III was also tested on a taxonomic and ecologically diverse range of fungi. Using the classic cytotoxic disk assay, JH III had no effect on the fungal species tested.
Introduction

In the constant struggle against vertebrate and invertebrate herbivory and pathogen attack, sessile plants have evolved a diverse array of defensive strategies. These mechanisms can be divided into constitutive defenses, which function independently of injury, or induced defenses, plastic traits produced upon attack (Karban and Baldwin, 1997). Plant secondary compounds involved in protection may fall into either category. One defensive strategy of plants against insect herbivory is the production of secondary metabolites that interfere with the physiology of the insect. In particular, it is believed that targeting the endocrine system may make it difficult for the insect to develop counteradaptive strategies (Bowers, 1991). In nature, there are many examples of plant compounds that interfere with insect endocrine functions either by mimicking hormones such as the juvenile hormone (JH) mimic, juvabione, originally isolated from *Abies balsamea* (L.) Miller, or the phytoecdysteroids, isolated from over a 100 plant families, or by interfering with the biosynthesis of these hormones, for example the precocenes, isolated from *Ageratum houstonianum* Miller, that destroy the endocrine site of JH production (Bowers *et al.*, 1966; Bowers *et al.*, 1976; Adler and Grebenok, 1995). Recently, insect juvenile hormone III (JH III), and its biosynthetic precursor in insects, methyl farnesoate, were identified in the sedges, *Cyperus iria* L. and *C. aromaticus* (Ridley) Maff. & Kük. (Toong *et al.*, 1988). This was the first report of the presence of an insect JH in a plant.

In insects, the role of juvenile hormones (JHs) in the regulation of metamorphosis and reproduction is well defined, although the mechanisms of action are not completely understood (Gilbert *et al.*, 1996). Insect JH titers are closely regulated during
development. Therefore, topical application of JH or synthetic analogues to susceptible insects can result in the inappropriate retention of juvenile characteristics at the next moult (Sehnal, 1983).

The presence of JH III in C. iria suggests that one function of this compound may be to protect the plant against insect herbivory. In no-choice experiments, the growth of third stadium nymphs of the grasshopper, Melanoplus sanguinipes, reared on wheat seedlings or C. iria did not differ (Toong et al., 1988). However, at the imaginal (adult) stage, grasshoppers reared on C. iria exhibited morphological abnormalities (e.g. twisted wings, colour changes), indicative of disrupted metamorphosis. Adult female grasshoppers were also infertile. Addition of C. iria leaves to water containing larvae of the mosquito, Aedes aegypti, increased mortality (Schwartz et al., 1998). This activity was attributed to the JH III content of the plant, although the larvicidal activity of the leaf was greater than the pure compound alone. Therefore, the plant may contain compounds that synergize the action of JH or protect JH III from degradation.

Application of JH or JH analogues to insect eggs can cause immediate ovicidal or delayed developmental effects (Sehnal, 1983). Eggs of the Dipteran leafminer, Hydrellia sp., did not hatch following oviposition on leaves of C. iria (Meneses and García de la Osa, 1988). Therefore, plant JHs may affect insect egg development and, indirectly, subsequent herbivory.

In other studies, association with the plant had no apparent effect on insect development. Nymphs of the planthoppers, Nisia strovenosa and N. nervosa, were reared successfully on C. iria and C. aromaticus, although more insects reached maturity on the latter species (Dela Cruz, 1986). In a feeding preference study, the rice stink bug,
Oebalus pugnax, fed on C. iria (Naresh and Smith, 1984). It is unclear if the insects in these last examples avoided the detrimental developmental effects of JH III through metabolism or sequestration of the ingested hormone or by avoiding plant tissues containing high concentrations of this compound (Dowd, et al., 1983).

Sedges such as C. iria are nutritionally poor and contain a high silica level which is believed to deter insect herbivory (Vicaria and Bazely, 1993). The basal meristematic tissue of these plants also allow them to tolerate herbivore grazing. The ability of C. iria to interfere with insect development, presumably as a consequence of the presence of JH III, has been clearly demonstrated (Toong et al., 1988; Schwartz et al., 1998). However, conclusive evidence that JH III protects the plant from insect herbivory has not been forthcoming. The objective of the present study was to explore other potential biological activities of JH III in the plant.

Cyperus iria is a tufted, annual sedge with a widespread distribution throughout Asia and Africa (Holm et al., 1977; Catling, 1992). In many areas, this plant is an invasive weed of crops, particularly rice. In a search for allelopathic compounds, Komai et al. (1981) isolated methyl farnesoate and farnesol from the closely related sedge, C. serotinus Rottb. These compounds exhibited potent inhibitory activity on shoot growth of lettuce and rice seedlings and are also biosynthetic intermediates of JH III in insects (Schooley and Baker, 1985; Cusson et al., 1991). The structural similarities between these acyclic sesquiterpenoids suggest that JH III may play a similar role in C. iria. Therefore, we compared the allelopathic activity of JH III on seed germination and seedling growth of radish, lettuce and rice with the structurally related sesquiterpenoid farnesol, as its biological activity is well documented (Wardle and Simpkins, 1980;
Wardle and Short, 1982; Wardle et al., 1986). The activity of these compounds on the growth of *C. iria* seedlings was also measured. In the second part of the study, the antimicrobial activity of JH III on a range of fungal species, representing plant pathogens, coprophilous and soil fungi, was determined.

**Materials and methods**

**Plant material**

Sedges were grown from seeds obtained from Y.C. Toong (Penang, Malaysia) in the greenhouse. Plants were grown in organic potting mix and kept continuously moist. Temperatures ranged from approximately 22°C (winter) to ambient (summer). Voucher specimens of *C. iria* have been deposited at the Royal Ontario Herbarium, Canada (TRT).

**Chemicals**

All solvents used were HPLC grade from Burdick & Jackson, unless otherwise noted.

**Extraction of juvenile hormone III from roots of Cyperus iria**

Juvenile hormone III, methyl-10R,11-epoxy-3,7,11-trimethyl 2E,6E-dodecadienoate, was extracted from two to three month old plants. Root tissue was ground in acid-washed sand (Sigma) and liquid nitrogen and then extracted overnight at room temperature. The extract was evaporated to near dryness and subjected to column chromatography over an anhydrous sulphate (BDH)-hydroxyapatite column (HA, BioRad) to remove chlorophylls and other contaminants. A Pasteur pipette HA column was prewashed with ethyl ether (5 ml) and iso-octane (5 ml), before adding the sample, then washed with 5 ml pentane and
eluted with pentane-ether (70:30). Following solvent evaporation under nitrogen (Canox), the sample was resuspended in hexane for final purification.

**High Performance Liquid Chromatography (HPLC)**

Juvenile hormone III was purified from root extracts by HPLC (Perkin-Elmer 410 liquid chromatograph) by normal phase chromatography on a spheri-5 silica column (Brownlee, 250 x 4.6 mm). The mobile phase was 10% half-water saturated ethyl ether in hexane at a flow rate of 1 ml/min. Juvenile hormone III was monitored at 219 nm (Applied Biosystems 1000S Diode Array Detector) and identified on the basis of retention time relative to standards (Sigma, racemic, approx. 88% pure).

**Stock solutions**

Juvenile hormone III fractions collected from HPLC were pooled and the solvent evaporated. Juvenile hormone III stock solution was prepared in hexane and re-analyzed by HPLC to confirm the concentration. Stock solutions of farnesol (96% purity, mixture of isomers, Aldrich) were prepared in hexane.

**Tritiated juvenile hormone III**

Radiolabelled JH III was biosynthesized through the incubation of $^3$H-methionine with corpora allata (CA) from the cockroach, *Diploptera punctata*, followed by purification by HPLC (Tobe and Clarke, 1985; King and Tobe, 1988). The CA from day 5 mated female cockroaches were dissected and incubated in methionine-free TC 199 medium, containing 2% Ficol (Pharmacia, Biotech), 1.3 mM CaCl₂·2H₂O (Analar, BDH), 40 μl
farnesoic acid (a gift of Dr. F.C. Baker, 70% pure) and L-[3H-methyl]-methionine (Amersham, specific activity 3.11 TBq/mmol) overnight at 27°C. The aqueous medium was extracted twice with iso-octane. Pooled organic fractions were eluted through a silica column (Sep-pak, Millipore) and the resulting eluent evaporated under nitrogen and resuspended in hexane. Final purification of enantomerically pure [3H-methyl]-10R-JH III was by HPLC. Again, the solvent was evaporated and the stock solution prepared in toluene. When used in an assay, 3H-JH III was added to a polyethylene glycol (PEG) (MW 15,000-20,000, Sigma)-treated scintillation vial (20 ml, VWR Canlab). The toluene was evaporated under nitrogen and the solution prepared in hexane.

**Solubility of juvenile hormone III**

Tritiated JH III stock solution was prepared in hexane, an aliquot placed in a 2 ml vial with cold JH III (Sigma, 0 to 8.8 mM) and the solvent evaporated. After the addition of 0.5 ml sterile distilled water, vials were tightly capped and sonicated for 3 hours in a water-bath (Cole-Parmer, ultrasonic cleaner, 8845-4) to form finely dispersed micelles. The solution was allowed to settle for 3 days at room temperature. Four aliquots of the aqueous solution were removed using a disposable glass Accupette pipette (Canlab). Scintillation fluid (Cytoscint, ICN) was added and the radioactivity of the sample measured using a Beckmann LS 6500 Scintillation Counter (n = 3). The experiment was repeated twice.

The aqueous solubility of JH III was estimated to be 1.3 mM. This is in agreement with the predicted solubility of JH III which is 0.86 mM (Advanced Chemistry Development) and the literature in which the solubility has been reported to be > 200 µM.
in 5 mM Tris-HCl, pH 8.3 (Kramer et al., 1976). It is also consistent with reports on the solubility range of oxygenated terpenoids (Weidenhamer et al., 1993). The biological assays in this paper were performed using JH III solutions between 0.01 to 1.0 mM, concentrations below its limit of solubility in an aqueous solution. It should also be noted that JH III may be present in even higher concentrations in specialized compartments such as oil bodies in planta.

**Osmotic potential and pH**

Osmolarity and pH may affect seed germination and seedling growth (Anderson and Loucks, 1966; Reynolds, 1975a; b). The osmolarity and pH of JH III and farnesol solutions was measured using a Vapro vapour pressure osmometer 5520 (n = 3) and pH meter (n = 1) and compared to the hexane control. The osmotic potential of these solutions was 31.5 ± 1.5 and the pH was 7.05 ± 0.04. Therefore, any allelopathic activities observed were not the result of differences in osmolarity or pH between the test solutions and the control.

**Allelopathic assays**

The effects of farnesol and JH III on seed germination and seedling growth were tested on radish (Raphanus sativus L. var. Cherry Belle, McKenzie), lettuce (Lactuca sativa L. var. Grand Rapids, McKenzie) and rice (Oryza sativa L. cultivar AI-NAN-TSAO, seeds obtained from Dr. D. Saini (Montreal, Quebec)). Activities were also tested on C. iria seedling growth (seeds originally obtained from Y.C. Toong (Penang, Malaysia)). Under sterile conditions, 0.5 ml of sterile distilled water and 50 μl of test solution (hexane
(control), farnesol or JH III) were added to seeds on sterile filter paper (Ahlstrom) in 6 well plates (Nunc). The following numbers of seeds (mean ± SEM, median) were added to each well: radish (52.5 ± 0.7, 51.5), lettuce (54.5 ± 0.9, 53.0), C. iria (102.1 ± 7.0, 85.0) and rice (13.2 ± 0.1, 13.0). Fifty μl of test solution (hexane (control), farnesol or JH III) was added and the plates incubated in a growth chamber under a 14:10 light-dark cycle (22,000 lux) at 26 ± 2 °C. Percentage seed germination (defined as extension of the radicle 2 mm from the seed coat) was monitored over the next 3-5 days. For each plant species, six replicates were performed.

At days 3, 4 and 5 for radish, lettuce and rice, respectively, shoot and root tissues were excised, dried at 50°C overnight and dry weights recorded. At day 6, the entire C. iria seedling was dried and weighed.

Affinity of juvenile hormone III for plastic

Juvenile hormone III adsorbs strongly to plastic (Giese et al., 1977). Its affinity for the plastic plates used in the allelopathic experiments was determined, using the procedure established for the seed germination and seedling growth assays. Tritiated JH III was added to the 6 well plate containing filter paper and an aqueous solution of cold JH III (0.1 to 1.0 mM). After three days, the medium was removed and extracted twice with iso-octane. The organic phases pooled, evaporated and resuspended in 100 μl of iso-octane. After addition of scintillation fluid, the radioactivity of the sample was measured (n = 3). Blanks were subtracted. The experiment was repeated twice. At all the concentrations tested (0.01 to 1.0 mM JH III), > 83% of the JH III was recovered. Therefore, in the
assay, it is assumed that the concentration of JH III approximately represents the amount to which the seeds are exposed.

**Antifungal activity**

Five μl of test solution (JH III (1.0 mM or 10.0 mM) or hexane (control)) was added to a sterile disk (Ahlstrom, grade 601) placed 3 cm from the edge of a Petri plate (Fisher, 100 x 15 mm) containing 20 ml modified Leonian's agar (Malloch, 1981). Fungal specimens were added by point inoculation approximately 3 cm from the disk. A diverse range of fungal species were tested: Phylum Oomycota: *Pythium aphanidermatum* (root rot agent); phylum Zygomycota: *Mucor piriformis* (fruit rot), *Absidia* sp. (soil saprophyte), *Micromucor* sp. (soil saprophyte); subphylum Basidiomycotina (Dikaryomycota): *Coprinus patouillardii* (coprophilous), *Schizophyllum commune* (wood decay fungus), *Trametes versicolor* (white rot of timber), *Cryptococcus albidus* (basidiomycetous yeast); Dikaryomycota, subphylum Ascomycotina: *Alternaria alternata* (foliar blight pathogen), *Cladosporium cladosporioides* (ubiquitous foliar blight), *Epicoccum nigrum* (foliar blight pathogen), *Microsphaeropsis olivaceus* (saprophyte), *Trichophyton mentagrophytes* (agent of human, "ringworm"), *Arthodera quadrifidum* (soil inhabiting keratinophilic fungus), *Ascochistus crenulatus* (coprophilous), *Iodophanans carneus* (coprophilous), *Botrytis cinerea* (blight and rot disease of grape), *B. allii* (soft rot pathogen of onion), *Sclerotinia sclerotiorum* (ubiquitous foliar and vacular plant pathogen), *Sclerotium cepivorum* (white rot of onion), *Beauveria* sp. (insect pathogen), *Trichoderma viride* (mycoparasitic soil fungus), *Fusarium oxysporum* (crown rot and vascular wilt pathogen), *Stachybotrys chartarum* (cellulophilic indoor contaminant), *Penicillium chrysogenum*.
(food spoilage agent). *P. funiculosum* (mycoparasitic soil fungus), *Sordaria fimicola* (coprophilous) and *Podospora araneosa* (coprophilous). The zone of inhibition around the disk was measured 5-21 days post-inoculation, depending on the rate of fungal growth \((n = 2)\).

**Statistics**

Analyses were performed using the statistical package SPSS 7.5. One-way Analysis of Variance (ANOVA) was used to compare the effects of JH III on shoot, root and seedling growth. Statistical significance was determined using a Tukey HSD post-hoc test. Repeated measures ANOVA was used to analyze seed germination and fungal mycelial growth.

**Results**

*Effect of farnesol and juvenile hormone III on seed germination*

Farnesol significantly delayed germination of radish, lettuce and rice seeds (Fig. 1a, 1c and 1e). In the presence of 1 mM farnesol, seed germination of radish, lettuce and rice was inhibited by 56.7%, 54.1% and 18.4% on days 2, 3 and 2.5, respectively. However, on days 3, 4 and 5, respectively, there was no significant inhibition of radish, lettuce or rice seed germination at any of the farnesol concentrations tested compared to the hexane control.

JH III did not affect radish or rice seed germination at the concentrations tested (Fig. 1b and 1f). A delay of lettuce seed germination was observed (Fig. 1d). In the presence of 1 mM JH III, lettuce seed germination was reduced by 43.9% at day 4.
Effect of farnesol and juvenile hormone III on seedling root growth.

Application of farnesol to radish or lettuce seeds had no significant effect on root growth at the concentrations tested (Fig. 2a and 2c) whereas a concentration-dependent decrease in root growth was observed in the case of rice (Fig. 2e). Treatment of rice seeds with 1.0 mM farnesol resulted in a 28.0% inhibition of root growth.

Treatment of radish, lettuce and rice seeds with JH III did not significantly affect root growth, compared with hexane controls, at the concentrations tested (Fig. 2b, 2d and 2f).

Effect of farnesol and juvenile hormone III on seedling shoot growth.

Neither farnesol or JH III significantly affected shoot growth of radish or lettuce at the concentrations tested (Fig. 3a-d), whereas both reduced rice shoot biomass (Fig. 3e and 3f); 1.0 mM farnesol and JH III inhibited growth by 36.8% and 24.0%, respectively.

Effect of farnesol and juvenile hormone III on Cyperus iria seedling growth.

*Cyperus iria* seedlings were left intact and not separated into root and shoot tissue. Both farnesol and JH III inhibited the growth of these seedlings (Figs. 4a and b). At concentrations of 1.0 mM, growth of seedlings was only inhibited 5.1 % and 6.7 % by farnesol and JH III, respectively, relative to control values. Although there was an inhibition of the growth of *C. iria* seedlings, this may be offset by an accelerated
Fig. 1. Percent of radish (A and B), lettuce (C and D) and rice (E and F) seed germination after treatment by hexane control (○), 0.01 mM (▲), 0.1 mM (▼) or 1.0 mM (◆) of farnesol or juvenile hormone III. Each point represents the mean of six experiments ± standard error.

<table>
<thead>
<tr>
<th></th>
<th>Farnesol</th>
<th>Juvenile hormone III</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radish</strong></td>
<td>Inhibition</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>((df = 23, F = 4.919; p = 0.010))</td>
<td>((df = 23, F = 1.139; p = 0.357))</td>
</tr>
<tr>
<td><strong>Lettuce</strong></td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td></td>
<td>((df = 23, F = 3.143; p = 0.048))</td>
<td>((df = 23, F = 4.468; p = 0.015))</td>
</tr>
<tr>
<td><strong>Rice</strong></td>
<td>Inhibition</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>((df = 23, F = 6.513; p = 0.003))</td>
<td>((df = 23, F = 516.663; p = 0.055))</td>
</tr>
</tbody>
</table>
Fig. 2. Activity of the sesquiterpenoids farnesol and juvenile hormone III (JH III) on radish (A and B), lettuce (C and D) and rice (E and F) seedling root growth. The compound was applied to the seed and the root biomass (dry weight) was measured at 3, 4 and 5 days after treatment for radish, lettuce and rice, respectively. Each bar represents the mean of six experiments ± standard error. Bars with different letters are significantly different (one way ANOVA, p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Farnesol</th>
<th>Juvenile hormone III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radish</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>(df = 23, F = 0.881; p = 0.467)</td>
<td>(df = 23, F = 1.289; p = 0.305)</td>
</tr>
<tr>
<td>Lettuce</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>(df = 23, F = 0.823; p = 0.496)</td>
<td>(df = 23, F = 0.221; p = 0.881)</td>
</tr>
<tr>
<td>Rice</td>
<td>Inhibition</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>(df = 23, F = 4.672; p = 0.012)</td>
<td>(df = 23, F = 2.825; p = 0.065)</td>
</tr>
</tbody>
</table>
Fig. 3. Activity of farnesol and juvenile hormone III (JH III) on radish (A and B), lettuce (C and D) and rice (E and F) seedling shoot growth. The compound was applied to the seed and the shoot biomass (dry weight) was measured 3, 4 and 5 days after treatment for radish, lettuce and rice, respectively. Each bar represents the mean of six experiments ± standard error. Bars with different letters are significantly different (one way ANOVA, p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Farnesol</th>
<th>Juvenile hormone III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radish</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>$(df = 23, F = 0.575; p = 0.638)$</td>
<td>$(df = 23, F = 0.651; p = 0.592)$</td>
</tr>
<tr>
<td>Lettuce</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>$(df = 23, F = 1.369; p = 0.281)$</td>
<td>$(df = 23, F = 0.105; p = 0.956)$</td>
</tr>
<tr>
<td>Rice</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td></td>
<td>$(df = 23, F = 7.16; p = 0.002)$</td>
<td>$(df = 23, F = 7.343; p = 0.002)$</td>
</tr>
</tbody>
</table>
Fig. 4. Activity of farnesol (A) and juvenile hormone III (JH III) (B) on *Cyperus iria* seedling growth (dry weight). The compound was applied to the seed and the seedling was measured on day 6 after treatment. Each bar represents the mean of six experiments ± standard error. Bars with different letters are significantly different (one way ANOVA, p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Farnesol</th>
<th>Juvenile hormone III</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cyperus iria</em></td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td></td>
<td>$(df = 23, F = 9.988; p &lt; 0.001)$</td>
<td>$(df = 23, F = 8.828; p = 0.001)$</td>
</tr>
</tbody>
</table>
sprouting of seeds that was observed only in the case of JH III-treated seeds (personal observation).

**Differences between farnesol and juvenile hormone III activity.**

Farnesol and JH III are structurally related acyclic sesquiterpenoids (see Table I). In these assays, distinct biological activities for farnesol and JH III on seed germination and seedling growth were observed. Both compounds inhibited the germination of lettuce seeds and the growth of rice seedlings shoots and *C. iria* seedlings. Farnesol and JH III had no effect on the root and shoot growth of radish or lettuce seedlings. However, farnesol has a broader range of activity and also inhibited radish and rice seed germination and the root growth of rice seedlings.

**Antifungal activity of juvenile hormone III.**

No clear antimicrobial activity of JH III was observed on any of the fungi tested. However, variable inhibition of mycelial growth was observed in *Ascobolus crenulatus* and *Sclerotinia sclerotiorum* raising the possibility that there is a general inhibition of growth by this volatile compound rather than a discrete zone of inhibition.

**Discussion**

The observed allelopathic activities of farnesol were consistent with reports in the literature. The inhibition of lettuce seed germination was also observed by Wardle and Short (1982) and Komai *et al.* (1981) showed that 1.0 mM farnesol did not affect lettuce seedling root elongation. However, contrary to our results, Komai *et al.* (1981) found that
Table I. Linear sesquiterpenoids isolated from the roots of *Cyperus* species.

<table>
<thead>
<tr>
<th>Compound</th>
<th>C. <em>iria</em></th>
<th>C. <em>monophyllus</em></th>
<th>C. <em>pilosus</em></th>
<th>C. <em>microiria</em></th>
<th>C. <em>polystachyos</em></th>
<th>C. <em>difformis</em></th>
<th>C. <em>serotinus</em></th>
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</thead>
<tbody>
<tr>
<td>Juvenile hormone III:</td>
<td></td>
<td></td>
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<tr>
<td>methyl 10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate</td>
<td>+</td>
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<tr>
<td><img src="image1.png" alt="Chemical Structure" /></td>
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<tr>
<td>methyl 3,7,11-trimethyl-11-hydroxy-2E,6E,9Z-dodecadienoate</td>
<td>+</td>
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<td>+</td>
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<tr>
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</tr>
<tr>
<td>methyl 3,7,11-trimethyl-11-hydroxy-2E,6E,9E-dodecadienoate</td>
<td>+</td>
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<td></td>
<td>+</td>
<td>+</td>
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<tr>
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<tr>
<td>methyl 3,7,11-trimethyl-2E,6E,9E,11-dodecatrienoate</td>
<td>+</td>
<td></td>
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<td></td>
<td>+</td>
<td>+</td>
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</tbody>
</table>
The compound structure is illustrated below the chemical name. Bolded names indicate biosynthetic precursors of JH III in some insect species (Cusson et al., 1991). References: Iwamura et al., 1978a, b, c; Iwamura, 1979; Iwamura et al., 1979; Komai et al., 1981; Toong et al., 1988.

<table>
<thead>
<tr>
<th>Compound</th>
<th>+</th>
<th></th>
<th>+</th>
<th></th>
<th>+</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>methyl 3,7,11-trimethyl-2E,6E,9Z,11-dodecatraenoate</td>
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<td>methyl 3,7,11-trimethyl-2E,6E,10-dodecatrienoate</td>
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<td>trans, trans farnesol:</td>
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<td>3,7,11-trimethyl-2,6-10-dodecatrien-1-ol</td>
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1.0 mM farnesol had no effect on rice root elongation and at higher concentrations, stimulation of root growth was observed. This discrepancy may have resulted from differences in rice cultivar, experimental conditions or the measurement parameters used. We measured seedling dry weight as an indicator of growth whereas Komai et al. (1981) directly analyzed root elongation. Wardle and Short (1982) found that barley root elongation was stimulated in the presence of farnesol (83 µM) which was attributed to an increase in root meristem tissue (Wardle et al., 1986). However, a reduction in root hair formation was also observed. Therefore, farnesol may affect root elongation and dry weight differently. Komai et al. (1981) also reported that 1.0 mM farnesol inhibited elongation of lettuce hypocotyls. Again, this differed from our results and discrepancies are attributed to differences in cultivars and/or experimental methodology.

*Cyperus iria* is an aggressive weed of many crops and this may, in part, be attributable to the presence of JH III. The distribution of JH III has been characterized in *C. iria* over an eight month period, from seedling to senescent plant (Bede et al., 1999). In immature whole plants, total JH III increased over time to approximately 440 nmoles per plant until the opening of the bracts and the exposure of the inflorescence. At this point, cessation of plant growth and a transient decrease in JH III content in all tissues was observed. In older plants, the levels of this compound increased until eight months. At this time, the aerial tissues became senescent and JH III levels in these tissues, again, declined. However, the JH III levels in the root tissue, which remained viable, did not change significantly during the final two months of the study. Over the period of observation, the highest levels of JH III were consistently found in the root tissue, in which 102 nmoles/g fresh weight was detected in seven month old mature plants.
In the present study, JH III delayed lettuce seed germination. This inhibition may confer an ecological advantage to *C. iria* by affecting the germination of potential competitor plant species in its immediate vicinity. Juvenile hormone III also had a potent inhibitory effect on the shoot growth of rice seedlings; biomass was inhibited by over 24% in the presence of 1.0 mM JH III. Mature root tissue may contain more than 1.2 μmoles JH III. Therefore, there is, potentially, sufficient compound to confer allelopathic activity either through a delay in seed germination or through an inhibition of seedling growth or both. Such actions may play an important ecological role with respect to the ability of this weed to invade rice fields. However, until root leachate studies are conducted, the questions of the amount of JH III and biotransformation of JH III in the immediate plant microenvironment remain.

Using the classic cytotoxic disk assay, JH III demonstrated no antimicrobial activity on the fungi tested. However, a minor decrease was observed in the growth of *Ascobolus crenulatus* and *Sclerotinia sclerotiorum*. The latter fungus has been isolated from the related sedge species, *C. rotundus* (Singh and Singh, 1986) and is the causative agent of a number of economically important plant diseases (Agrios, 1988).

Structurally similar acyclic sesquiterpenoids have been isolated from several related *Cyperus* species (Table 1) as well as unrelated plants. For example, the insect biosynthetic intermediate methyl farnesoate has been isolated from *C. iria, C. microiria* Steud., *C. monophyllus* Vahl., *C. pilosus* Vahl. and *C. serotinus* Rottb., as well as from grapes and the bark of *Polyalthia viridis* Craib (Iwamura et al., 1978a, b, c; Iwamura, 1979; Iwamura et al., 1979; Toong et al., 1988; Kijjoa et al., 1990; Versini et al., 1994). The linear sesquiterpenoid methyl \((E,E)-10,11\text{-dihydroxy}-3,7,11\text{-trimethyl}-2,6-\)
dodecadienoate has been isolated from a South American canopy tree, *Hortia regia* Sandwith., and an African rainforest tree, *Cleistopholis patens* (Benth.) Engl. and Diels and *Cl. staudtii* (Engl. & Pierre) (Waterman and Muhammad, 1985; Jacobs et al., 1987; Tane et al., 1988). This compound is identical to JH III except for the hydration of the epoxide to a *vicinal* diol. Because of the susceptibility of the epoxide to hydrolysis in the presence of trace amounts of acid or base, this compound may be an artifact produced from JH III by contaminants in the extraction solvent. Therefore, we believe that JH III may be present in other plant species, but that the labile nature of this compound has prevented detection.

The isolation of JH III from two sedge species, its presence in *C. iria* throughout development and the identification of structurally similar compounds from a diverse range of plant species, suggests that this compound may have important biological roles in plants. This investigation has demonstrated that treatment of seeds with JH III resulted in a delay of lettuce seed germination and the potent inhibition of rice seedling growth. Therefore, JH III may, at least in part, be responsible for inhibition of the germination and growth of surrounding plant species, giving *C. iria* an ecological advantage. However, other potential biological functions of JH III, such as in plant defense against insect herbivory, bacterial or nematode invasion or synergistic biological activities with other compounds, cannot be discounted.

**References:**


Dela Cruz, C.G. (1986) Host plant range of the planthopper *Nisia atrovenosa.* *Int. Rice*


Chapter VI. Production of Juvenile Hormone III in Cell Suspension Cultures of the Sedge, *Cyperus iria* L.

Abstract

Juvenile hormones are sesquiterpenoids that regulate metamorphosis and reproduction in most insect species. There has been one report of the identification of an insect juvenile hormone in plants: Juvenile hormone III (JH III), methyl-10R,11-epoxy-3,7,11-trimethyl-2E, 6E-dodecadienoate, has been isolated from two sedge species, *Cyperus iria* L. and *C. aromaticus* (Ridley) Mattf and Kük. This is the first report of callus and cell suspension cultures derived from *C. iria*. Farnesol and methyl farnesoate, two biosynthetic intermediates of JH III in insects, as well as JH III have been identified in suspension culture cell extracts by gas chromatography-mass spectroscopy. Therefore, these cultures provide an excellent *in vitro* model to investigate the biosynthesis of JH III in the sedge, *C. iria*. 
Introduction

Juvenile hormones (JHs) are a group of structurally related sesquiterpenoids that regulate insect development and reproduction (Gilbert et al., 1996). Six naturally occurring insect JHs have been isolated (see Bede and Tobe, 1999, and references therein) which share common structural features of a methyl ester moiety on C-1 and an epoxide function. Of these compounds, juvenile hormone III (JH III), methyl-10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate (Fig. 1), appears to be ubiquitous and in many insect orders is the only JH present (Tobe and Feyereisen, 1983). In immature insects, these hormones are required for the maintenance of juvenile characteristics (Tobe and Stay, 1985). In adult female insects, JHs regulate reproductive processes such as vitellogenin uptake and oocyte maturation (Tobe and Stay, 1985).

In insects, the isoprenoid skeleton of JHs is synthesized through the mevalonate pathway (Monger, 1985). A phosphatase or pyrophosphatase catalyzes the removal of the pyrophosphate group from the sesquiterpenoid intermediate farnesyl diphosphate (Fig. 1). Successive oxidation of farnesol to the aldehyde (farnesal) and carboxylic acid (farnesoic acid) is catalyzed by one or two NAD^+-dependent dehydrogenase(s) (Baker et al., 1983). In the cockroach, D. punctata, methylation of farnesoic acid generates the methyl ester, methyl farnesoate (MF), followed by an epoxidation at C-10, C-11 to produce JH III (Cusson et al., 1991)

One defensive strategy of plants against insect herbivory is the production of secondary metabolites that interfere with the physiology of the insect (Rosenthal and Janzen,
Fig. 1. Final steps of the biosynthetic pathway of juvenile hormone III in the cockroach, *Diploptera punctata* (adapted from Cusson *et al.*, 1991. Reprinted with permission from Elsevier Science).
Phosphatase or pyrophosphatase

Farnesyl diphosphate

Phosphatase or pyrophosphatase

Farnesol

Farnesol dehydrogenase, NAD

Farnesal

Farnesol dehydrogenase, NAD

Farnesoic acid

Methyl transferase, S-adenosyl-methionine

Methyl farnesoate

Epoxidase, O₂, NADPH+H⁺

Juvenile Hormone III
1979; Rosenthal and Berenbaum, 1991). It has been suggested that it is difficult for insects to evolve counteradaptive strategies to phytochemicals which act at the same physiological targets and in the same manner as their endogenous hormones (Bowers, 1991). In fact, there are many examples of plants that produce phytoecdysteroids, compounds which are structurally identical to or which mimic the insect moulting hormone, 20-hydroxyecdysone (Adler and Grebenok, 1995). However, there has been only one report of the identification of an insect JH from plants: Juvenile hormone III and MF have been isolated from the sedges Cyperus iria L. and C. aromaticus (Ridley) Mattf and Kük (Toong et al., 1988). The objective of the research described here was to develop a cell culture of C. iria to permit investigation of the JH III biosynthetic pathway in the plant.

In vitro inflorescence cultures have been established for two members of the Cyperaceae, Cyperus rotundus L. and Carex flacca Schreb. (Smith, 1968; Ram and Batra, 1970). The aim of these studies was to investigate factors involved in floral development and did not involve the undifferentiation and growth of plant tissue. Micropropagation of another member of the Cyperaceae, Caustis dioica R. Br., has also been achieved but, again, did not involve callus induction (Rossetto et al., 1992). The only report of the generation of undifferentiated cell cultures from a plant in the Cyperus genus was by Fisher (1977), who successfully established callus and suspension cultures of C. rotundus on Schenk and Hildebrandt (SH) basal medium in the presence of the auxin growth regulator, naphthaleneacetic acid.

In this study, callus cultures were initiated from sterile explants of C. iria on a variety
of tissue culture media. Once callus and suspension cultures were established, the presence of farnesol, MF and JH III in these cells was confirmed by gas chromatography-mass spectrometry (GC-MS).

Materials and methods

Materials

All chemicals were purchased from Sigma unless otherwise noted.

Plants

Seeds of *C. iria* were obtained from Y.C. Toong (University of Malaysia, Penang). Voucher specimens of this plant have been deposited at the Royal Ontario Museum Herbarium, Canada (TRT).

Explants

*Cyperus iria* seeds were surface sterilized by submersion in 70% ethanol (Commercial Alcohols) for 1 min, followed by 5.25% sodium hypochlorite (Queen Bleach Co.) containing a few drops of Tween 20 for 20 min and then rinsed three times with sterile distilled water. Seeds were placed on sterile, water-saturated filter paper in Petri dishes (Sarstedt, 60 x 15 mm). After germination, seeds were aseptically transferred to 20 ml vials (Kimble) containing the following medium: \( \text{NH}_4\text{NO}_3 \) (165 g/l), KNO\(_3\) (190 g/l), MgSO\(_4\)-7H\(_2\)O (37 g/l), KH\(_2\)PO\(_4\) (17 g/l), casein hydrolysate (0.5 g/l) with 3% sucrose (BDH) and 0.8% agar (Difco-bacto).
The medium was adjusted to pH 5.8 ± 0.1 with 1 M KOH (BDH) and autoclaved for 28 min at 121°C, then poured into 20 ml vials under sterile conditions. Excised shoots and roots from the resultant aseptically-grown seedlings were used as explants and cultured in plastic Petri dishes under a variety of media regimes.

**Media**

Because the appropriate nutrient conditions for the initiation of callus from *C. iria* seedlings were unknown, explants were cultured on Murashige and Skoog's medium (MS) (Murashige and Skoog, 1962) supplemented with the following organic compounds (numbers in brackets indicate the range of concentrations tested): sucrose (2-6%), thiamine (0.1-40.0 µM), pyridoxine (0.6-6.0 µM), niacin (4.0-40.0 µM), casein hydrolysate (0.01-0.5 mg/l) and myo-inositol (0.1-0.6 mM). To these media, either the synthetic auxin growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5-5.0 mg/l) or a cytokinin growth regulator, kinetin (0.1-1 µM) or zeatin (0.5-50.0 µM) was added. Explants were also cultured on the following media: B₅ (Gamborg *et al.*, 1968), Schenk and Hildebrandt (SH) (Schenk and Hildebrandt, 1972), White's (White, 1963) as well as 1/2 MS salts, supplemented with 3% sucrose, 3.0 µM thiamine-HCl, 2.4 µM pyridoxine-HCl, 4.0 µM nicotinic acid, 0.5 g/l casein hydrolysate, 0.6 mM myo-inositol and 2.5 mg/l 2,4-D. The pH of each medium was adjusted to pH 5.8 ± 0.1 with 1 M KOH or 1 N HCl (Caledon) and autoclaved for 28 min at 121°C. All chemicals used for tissue culture media were obtained from Sigma, except cupric sulphate (CuSO₄·5H₂O) (Fisher) and cobaltous chloride (CoCl₂·6H₂O)(BDH). For each treatment, three replicates
were prepared.

**Callus culture**

Under aseptic conditions, explants were placed in various orientations (vertical, horizontal) on the agar plates and the plates incubated in a growth chamber at 26 ± 2°C under either low intensity illumination (2800 lux) with a 16:8 (light:dark) photoperiod or in dark conditions. After 3 weeks, callus tissue was selected and grown in the same fresh medium. After a number of subcultures, the optimal conditions for growth were determined. Callus was established on three media: MS, SH and B, supplemented with 3% sucrose, 3.0 μM thiamine-HCl, 2.4 μM pyridoxine-HCl, 4.0 μM nicotinic acid, 0.5 g/l casein hydrolysate, 0.6 mM myo-inositol and 2.5 mg/l 2,4-D. Callus on SH and B, media were maintained in the light-dark cycle while callus on MS media grew best under dark conditions. At 3-week intervals, the callus was subcultured onto fresh media.

**Suspension cultures**

Suspension cultures were initiated from established SH and MS callus cultures by adding the cells to 100 ml medium in 250 ml Erlenmeyer flasks. Medium for suspension cultures was prepared exactly as that for callus culture but without inclusion of agar. Suspension cultures were maintained in a shaking incubator at 26 ± 2°C in the dark with rotary gyration at 125 rpm. Cells were subcultured at 18-day intervals (approximately 1:30 dilution).
**Chemical analysis**

SH suspension cells were collected by filtration and homogenized in pentane (HPLC grade, Burdick & Jackson). The organic phase was evaporated under vacuum in a rotovaporator (Buchler Instruments) to approximately 0.2 ml and added to an anhydrous sodium sulphate (BDH, approximately 0.5 g) hydroxyapatite (BioRad, approximately 0.4 g) Pasteur pipette column that had been prewashed with 5 ml ethyl ether (HPLC grade, Burdick & Jackson) and iso-octane (HPLC grade, Burdick & Jackson). After addition of sample, the columns were washed with 5 ml pentane and the sample was eluted with 5 ml pentane-ether (70:30).

**Sample preparation**

Extracts were further purified by thin layer chromatography on silica plates with fluorescent dye indicator (Merck) in toluene (ACS, BDH)-ethyl acetate (HPLC grade, BDH)-acetic acid (ACS, BDH) (85 ml:15 ml:4 drops, v/v) after focusing in methanol (HPLC grade, Burdick & Jackson). Bands containing MF (R_f = 0.66) and JH III (R_f = 0.59) were scraped and extracted in ether. The solvent was evaporated under nitrogen and the residue dissolved in hexane (GC-MS grade, Burdick & Jackson). The samples were further purified by silica gel chromatography. The silica gel (Silica gel 60, 230-400 mesh, EM Science) was washed twice with hexane followed by three washes with acetone (HPLC grade, Burdick & Jackson) prior to slurry packing in a 20 x 1.6 cm stainless steel HPLC column fitted with 10 μm frits and 7.5 mm end fittings. The column was attached to the injector of a Varian 1400® gas chromatograph (GC) and helium was passed through the column at 30 ml/min. The oven
temperature was increased to 300 °C for 24 h. Column chromatography was performed using Pasteur pipettes fitted with a glass wool plug and containing a 1 cm bed of silica gel. The silica column was then preconditioned with 2 ml hexane (GC grade, Burdick & Jackson). After application of the sample in hexane, the column was washed with 1 ml hexane. Famesal and MF were eluted with 500 μl of 2.5% ethyl acetate (Burdick & Jackson, glass distilled) in hexane. Juvenile hormone III was eluted with 500 μl of 10% ethyl acetate in hexane. All eluting solvent contained 500 pg of famesyl acetate (Sigma) as an internal standard.

**Gas chromatography**

Samples were analyzed by capillary gas chromatography (GC) using a 30 m x 0.25 mm (id) SE30 (apolar phase) (Alltech Assoc.) analytical column connected to the cool-on-column injector with a 10 cm length of 0.5 mm (id) deactivated fused silica. Two min after sample injection onto a cool-on-column injector (-60°C), oven and injector temperature increased by 7.5°C/min until a final temperature of 180°C was reached. The helium carrier gas linear flow velocity was 18 cm/sec. Samples were also analyzed by GC on a polar 30 m x 0.25 mm Carbowax 20 M column (Alltech Assoc.), under the same conditions as above. Standards were obtained from Sigma, with the exception of methyl farnesoate that was chemically synthesized from famesol according to Latli and Prestwich (1991). Recoveries of standards after chromatographic procedures were in excess of 80% for famesol and 85% for MF and JH III.
Gas chromatography-mass spectroscopy

Compounds were identified based on retention time and comparison of their spectra (60-350 amu) to standards after separation on a 30 m x 0.25 mm analytical DB5-MS® column (J & W) interfaced to a Finnigan Matt ITS40® ion trap mass spectrometer operated in the chemical ionization mode (CI). The initial injector temperature was -40°C which after the first 120 sec., increased at 170°C per minute until 270°C was reached. The initial column temperature was 40°C which after 5 min increased at 5°C per min to 210°C. The helium carrier gas linear flow velocity was 24 cm/sec and the GC-MS transfer line temperature was 230°C. Isobutane (Matheson, 99.9%) was the reagent gas used for chemical ionization.

Results and discussion

Cell cultures

Two to three weeks after initiation, undifferentiated tissue protuberances were visible from the sterile explants. The orientation of the explants did not seem to affect callus formation. After 3 months, callus cultures were established on MS, B₃ and SH media, each supplemented with 3% sucrose, 3.0 μM thiamine, 2.4 μM pyridoxine, 4.0 μM nicotinic acid, 0.5 g/l casein hydrolysate, 0.6 mM myo-inositol and 2.5 mg/l of the synthetic auxin growth regulator 2,4-D. These cultures produced compact friable callus. Whitish cells formed on the MS medium and knobby and gray-yellow callus predominated on the B₃ and SH media. Initially, callus growth appeared better on the B₃ and SH plates, which may reflect the lower ammonia concentrations in these media. Establishment of callus was unsuccessful on White's
Callus cultures have been maintained for over five years on SH and MS media.

From the callus cultures, cell suspension cultures were initiated in SH and MS media. Cells in both media were elongated and ovoid. Cells grown in MS media were creamy white and grew rapidly in small clusters, whereas the cells in the SH media were yellow, formed larger clumps and exhibited slower growth. A typical growth curve was observed in these cell suspension cultures; there was an initial lag phase, followed by the logarithmic growth phase and the cells entered the stationary phase 18 days after subculturing. High levels of the auxin, 2,4-D, were necessary for initiation and maintenance of the tissue culture; in fact, root formation was induced when these cultures were transferred to auxin-free media. Suspension cell cultures in these media have been maintained for over four years.

Secondary metabolites

Pooled extracts of suspension culture cells were analyzed by gas chromatography-mass spectroscopy (GC-MS). Juvenile hormone III and two of its biosynthetic precursors in insects, methyl farnesoate (MF) and farnesol, were detected (Fig. 2a-c). The MF and JH III content of individual cell suspension cultures (100 ml, day 18) and of callus cultures (day 21) were analyzed (Table I). These compounds accounted for less than 0.1% of the detectable ions in the samples. Farnesol was also present, but in amounts too low to quantify. The total MF in suspension cultures (cells and media) was higher in the SH than in MS media, while approximately equal amounts of MF were detected. Callus cultures grown on MS plates produced more MF than MS suspension cultures. This was reversed for the SH callus and
Fig. 2. Identification of juvenile hormone III and its biosynthetic precursors in the extract from *Cyperus iria* suspension culture cells.

A. Mass spectrum of famesol.

B. Mass spectrum of methyl famesoate.

C. Mass spectrum of juvenile hormone III.
A. Farnesol

B. Methyl Farnesoate

C. Juvenile Hormone III
Table I. Identification of compounds from *Cyperus iria* cell suspension and callus cultures.

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<th>Callus culture</th>
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<td></td>
<td>MS</td>
<td>SH</td>
<td>MS</td>
<td>SH</td>
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<td>(pg/g FW)</td>
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<tr>
<td>Methyl farnesoate</td>
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<td>5012.1</td>
<td>1070.9</td>
<td>133</td>
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<tr>
<td>Juvenile hormone III</td>
<td>125.6</td>
<td>80.19</td>
<td>1.72</td>
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Methyl farnesoate and juvenile hormone III levels (pg/g fresh weight (FW)) were measured in *C. iria* cell suspension cultures (individual flasks, day 18) and in callus cultures (day 21) by gas chromatography-mass spectroscopy. Data represents the content of combined cell and media extracts from individual flasks of cell suspension cultures and of cell extract of callus cultures and compares the amounts produced in Murashige and Skoog (MS) or Schenk and Hildebrandt (SH) media.
suspension cell cultures. The average amount of MF detected in these SH suspension cultures was approximately 5 ng/g fresh weight (FW). This is much higher than the quantity of JH III produced by these cultures (~0.08 ng/g FW). The quantities of JH III produced in SH or MS suspension cultures were similar and approximately fifty times greater than the amounts found in the respective callus cultures. Again, little difference was seen in the amount of JH III in the cells or media. The differences in MF and JH III production between callus and suspension cultures and between SH and MS media reflect the commonly observed effects of factors such as the nature of the explant, the degree of differentiation, light and nutrients on the biosynthesis of plant secondary metabolites in vitro (Charlwood and Charlwood, 1991; Banthorpe, 1994).

**Juvenile hormone III biosynthetic pathway**

At present, little is known about the biosynthesis of JH III in the sedge, *C. iria*. In insects, the sesquiterpenoid backbone is synthesized through the classical mevalonate pathway (Monger, 1985). In plants, this also appears to be the predominant pathway of isoprenoid biosynthesis, particularly of higher sterols (Bach, 1995). However, in bacteria and in the thylakoids of cyanobacteria, a unique pathway synthesizes the isoprene C₅ units through the condensation of a pyruvate-derived activated acetaldehyde with a triose phosphate derivative, followed by skeletal rearrangement (Rohmer et al., 1993). Recently, evidence for this pathway has been found in higher plants (Bach, 1995 and references therein; Eisenreich et al., 1996). It is not known which of these pathways provides the sesquiterpenoid backbone of
JH III in *C. iria*.

Methyl farnesoate, the immediate biosynthetic precursor of JH III in the insect, has been identified in four *Cyperus* species: *C. iria*, *C. monophyllus*, *C. pilosus* and *C. serotinus* (Iwamura *et al.*, 1978; Iwamura *et al.*, 1979; Komai *et al.*, 1981; Toong *et al.*, 1988). Another biosynthetic intermediate, farnesol, has also been isolated from the roots of the latter three species (Iwamura *et al.*, 1978; Iwamura, 1979; Komai *et al.*, 1981). These common intermediates found in both the plant and insect suggest that the late steps in the JH III pathway may be similar.

Cell suspension cultures provide a rapidly growing, relatively homogeneous biomass that is ideal for the investigation of metabolic pathways and could provide an *in vitro* model to elucidate the pathway of JH III biosynthesis in the sedge, *C. iria*. One problem with this approach is the frequent absence of secondary metabolite production in undifferentiated tissue. This may be attributed to changes in carbon fluxes in rapidly dividing cells and in the gene(s) expression and enzyme activity in these cells, as well as to the absence of morphological differentiation which may be required for enzyme activity or for the sequestration of secondary metabolites, particularly cytotoxic compounds (Charlwood and Charlwood, 1991; Banthorpe, 1994).

*Cyperus iria* cell suspension cultures are able to produce the secondary metabolites farnesol, MF and JH III. These compounds are also found in the insect JH III biosynthetic pathway suggesting that the plant and insect pathways may be similar. Therefore, these cell suspension cultures provide an excellent model to investigate the JH III biosynthetic pathway.
in *C. iria* and to study its regulation. Future work will focus on increasing the yield of these compounds in the SH suspension cultures and the investigation of their biosynthetic pathway.

Although there are many examples of phytochemicals that interfere with insect physiological processes (Rosenthal and Janzen, 1979; Rosenthal and Berenbaum, 1991), the selective pressures driving the evolution of these compounds are unclear (Rhoades, 1979; Futuyma and Keese, 1992). It has been argued that many secondary metabolites that presently play a protective role against insect herbivores originally evolved as a response to vertebrate herbivory (Hay and Steinberg, 1992). However, the production in *C. iria* of JH III, a compound that specifically disrupts endocrine-related processes in insects, may be an example of a phytochemical that has evolved to protect the plant against insect herbivory. Although it is tempting to speculate that this is the case, the biological role(s) of this compound in the plant is unknown at present.

References


dehydrogenase(s) in corpora allata of the tobacco hornworm moth, *Manduca sexta*.

*J. Lipid Res.** 24: 1586-1594.


Chapter VII. Biosynthetic pathway of juvenile hormone III in the sedge, *Cyperus iria* L.

Abstract

Juvenile hormones regulate metamorphosis and reproduction in most insect species. In the insect, these sesquiterpenoids are synthesized by small retrocerebral endocrine organs, the corpora allata, via the classical MVA acid pathway. One of these compounds, juvenile hormone III (JH III), has also been identified in the sedge, *Cyperus iria* L. In higher plants, biosynthesis of the sesquiterpenoid backbone may proceed through two distinct pathways: the classical mevalonate (MVA) pathway or the 2-C-methyl erythritol 4-phosphate (MEP) pathway or through a combination of both. *Cyperus iria* cell suspension cultures were used as a model to elucidate the biosynthetic pathway of JH III in the plant. Enzyme inhibition and precursor feeding studies indicate that the sesquiterpenoid backbone of JH III is, at least partially, synthesized via the MVA pathway. Precursor feeding studies also suggest that the later steps of the biosynthetic pathway are similar to the insect pathway.
Introduction

Juvenile hormones regulate critical physiological processes such as metamorphosis and reproduction in most insect species (Gilbert et al., 1996). The biosynthetic pathways of these sesquiterpenoids have been characterized in insect models (Cusson et al., 1991). Juvenile hormone III, methyl-10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate, has also been identified in a plant, the sedge Cyperus iria L (Toong et al., 1988). The biosynthetic pathway of this compound in the plant is not known. In the present study, the biosynthetic pathway of JH III was investigated in cell suspension cultures of C. iria.

In insects, juvenile hormone III (JH III) biosynthesis occurs via the classical mevalonate (MVA) pathway (Cusson et al., 1991). The pathway is presented in detail in Chapter II. Briefly, through the MVA pathway, the fifteen carbon intermediate farnesyl diphosphate (FPP) is generated. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl CoA to MVA and is considered to be a key regulatory step in controlling isoprenoid metabolism via this pathway (Monger, 1985). Juvenile hormone III is biosynthesized from FPP in five steps. Farnesol is generated by the removal of the pyrophosphate group and undergoes two dehydration reactions forming the aldehyde (farnesal) then the acid (farnesolic acid). In the cockroach, Diploptera punctata, methylation of farnesolic acid by an S-adenosyl methionine-dependent methyl transferase is followed by epoxidation at C10, C11, generating JH III (Schooley and Baker, 1985; Cusson et al., 1991).

In plants, sesquiterpenoids, such as JH III, may be formed through two biosynthetic pathways; the classical MVA pathway or the alternate 2-C-methyl erythritol
4-phosphate (MEP) pathway (Rohmer et al., 1993; Bach, 1995; Lichtenthaler et al., 1997). In this second pathway, the isoprene C5 units are formed through the condensation of a thiamine-activated acetaldehyde with glyceraldehyde 3-phosphate followed by skeletal rearrangement (Rohmer et al., 1993). There are also examples of terpenes which contain isoprene units derived from both pathways (Adam and Zapp, 1998; Piel et al., 1998). It is unknown which of these two pathways or if both pathways contribute to the sesquiterpenoid skeleton of JH III in C. iria.

Farnesol and methyl farnesoate have been isolated from C. iria and a number of related plant species (Iwamura et al., 1978a, b, c; Iwamura et al., 1979; Komai et al., 1981; Toong et al., 1988); these two compounds are also intermediates in the insect JH III biosynthetic pathway, suggesting that the later steps in the pathway may be similar between plants and insects.

_Cyperus iria_ suspension cultures provide a relatively homogeneous biomass which could be experimentally manipulated under defined, controlled conditions and allow the investigation of JH III biosynthesis. Preliminary investigations of the biosynthesis of the sesquiterpenoid backbone of JH III were conducted through enzyme inhibition studies using the fungal metabolite mevinolin, a potent inhibitor of the MVA pathway. Further characterization of the biosynthetic pathway was investigated through precursor feeding studies using the intermediate farnesoic acid.

**Materials and methods**

**Suspension cultures**

Suspension cultures were maintained on Schenk and Hildebrandt medium (SH) (Schenk and Hildebrandt, 1972) supplemented with 3% sucrose, 3.0 μM thiamine-HCl, 2.4 μM
pyridoxine-HCl, 4.0 μM nicotinic acid, 0.5 g/l casein hydrolysate, 0.6 mM myo-inositol and 2.5 mg/l 2,4-D (Chapter VIII, Bede et al., 1999a). All chemicals used for tissue culture media were obtained from Sigma, except cupric sulphate (CuSO₄·5H₂O) (Fisher), cobaltous chloride (CoCl₂·6H₂O)(BDH). The pH of each medium was adjusted to pH 5.8 ± 0.1 with 1 M KOH and autoclaved for 28 min at 121°C. Cells were subcultured at 18-day intervals (approximately 1:30 dilution) and maintained in a shaking incubator at 26 ± 2°C in the dark with rotary gyration at 125 rpm.

**Extraction and radioimmunoassay**

SH suspension cells were collected by filtration and homogenized in pentane (HPLC grade, Burdick & Jackson). The media (100 ml) was extracted overnight with 50 ml of pentane. The organic phase were evaporated under vacuum in a rotovaporator (Buchler Instruments) to near dryness. The remainder of the solvent was evaporated under a gentle stream of nitrogen and resuspended in toluene for analysis by radioimmunoassay (RIA) as outlined in Chapter III (Bede et al., 1999b). As a control, SH media alone was extracted and, as expected, JH III was not detected.

**Enzyme inhibition and feeding precursor experiments**

The HMG-CoA reductase inhibitor, mevinolin (Sigma) (final concentration: 10 or 50 μM) was added to day 0 suspension cultures of C. iria. After 48 hours, cells and media were filtered and extracted in pentane as described above and the JH III content analyzed by RIA. In complementary experiments, mevalonate lactone (Sigma) (final concentration 5 mM) or farnesolic acid (purity, 70%, M. Feldlaufer)(final concentration: 0.65 mM) were added to cultures containing mevinolin 24 hours after the addition of the inhibitor. If
these compounds are biosynthetic precursors to JH III, then production will be restored in mevinolin-inhibited cultures. Mevalonate lactone was preincubated with 10 mM NaOH for two hours on ice to generate the free acid. After an incubation period of 24 hours, cultures were filtered and extracted as above.

**Statistics**

Statistical analyses were performed using the statistical package SPSS 7.5.

**Results and discussion**

*Juvenile hormone III accumulation in Cyperus iria cell suspension cultures*

A typical growth curve was observed in these cell suspension cultures (Fig. 1). At subculturing, cells are introduced into new medium (day 0). After a brief lag phase (day 0 to 4), the exponential phase (day 4 to 7) and the linear growth phase are observed (day 7 to 16). The progressive deceleration of suspension culture growth occurred at approximately day 16 to day 19, followed by the stationary phase. Suspension cultures were subcultured on day 18.

Generally, biosynthesis and accumulation of secondary metabolites occurs in the stationary phase of the suspension culture growth curve (Charlwood and Charlwood, 1991; Banthorpe, 1994). During the exponential and linear phases, the carbon flux is directed towards primary metabolites necessary for growth. As cells enter the stationary phase, biomass production declines and production of secondary metabolites is often observed.

During the lag phase, JH III was detected in cells and media (Fig. 2a). Unexpectedly, on day 0, substantial amounts of JH III was present in the cells, but mainly
Fig. 1. Time course of cell biomass of *Cyperus iria* suspension cultures. Cell suspension cultures were grown in 100 ml of Schenk and Hildebrandt media in 250 ml Erlenmeyer flasks (Schenk and Hildebrand, 1972) and maintained at 26 ± 2°C in the dark on a rotary gyrator at 125 rpm. On day 18, suspension cultures were subcultured into new medium. This graph represents the cellular fresh weight of *C. iria* suspension cultures from the time of subculturing (day 0) to day 24. Each point represents the mean of at least 3 determinations ± the standard error.
Fig. 2. Time course of juvenile hormone III accumulation in *Cyperus iria* suspension cultures. Juvenile hormone III was measured by radioimmunoassay using antiserum 31867 (1:1750) and [3H-methyl]-10R, 11-JH III as the radiotracer. A. Amount of juvenile hormone III in cells and media. Bars represent the means ± standard error of three determinations. B. Cellular concentration of juvenile hormone III. Bars represent the means ± standard error of three determinations. C. Extracellular concentration of juvenile hormone III. Bars represent the mean ± standard error of three to four determinations.
Juvenile hormone III concentration in media (pmoles/g fresh weight of cells)

Juvenile hormone III concentration in cells (pmoles/g fresh weight)

Juvenile hormone III per flask (pmoles)
found in the media. In comparison, on day 4, approximately the same total amount of JH III was observed, but it predominated in the cells rather than the media. The JH III content of the suspension cultures decreased as the cells enter the exponential growth phase and steadily increased in the linear growth phase to its highest levels in day 17 cultures. As the suspension cultures entered the progressive deceleration phase, a decrease in JH III was observed but the amount of JH III increased once again during the stationary phase.

Comparison of levels of JH III in the cells and media over the suspension culture growth interval revealed that the cellular level was highest during the lag phase (Fig. 2b). As predicted, the level of JH III in the cells sharply declined as the suspension cultures entered the growth stages (exponential and linear phases). During the progressive deceleration phase, a rise in cellular level was observed which then decreased in the stationary phase. This general profile was also observed with respect to the extracellular concentration of JH III. However, variability was greater in the media (Fig. 2c).

The time course of JH III accumulation in the suspension cultures illustrates the dynamic temporal fluctuations of this compound. To account for these changes, there must be cellular synthesis, transport, excretion into the media and degradation, either intra- or extracellularly or both. With the exception of day 0, changes in the amount of cellular JH III reflected the total JH III levels; the quantity of JH III in the media was highly variable.

The high concentration of JH III detected in day 0 cells and media was surprising. On day 18, suspension cells were subcultured into new media at approximately a one to thirty dilution. The JH III detected in these suspension cultures was over 20 fold times the amount expected considering the dilution. Therefore, this is not residual JH III produced
in the day 18 cultures and introduced into the new media, but rather must represent predominately newly biosynthesized compound. This stimulation of synthesis may be attributable to the introduction of cells to new nutrients or the stress of subculturing on the suspension culture.

In day 0 versus day 4 suspension cultures, the cellular concentration of JH III was approximately the same. However, the JH III concentration in the media is thirteen times greater on day 0 than on day 4. One possible explanation for this difference is that the synthesized JH III is released into the medium where degradative enzymes metabolize the excreted compound and that a greater quantity and/or more active enzymes are present in the day 4 than in day 0 cultures. In a control experiment, the extraction efficiency of sterile media spiked with JH III was greater than 80%, indicating that there are no endogenous factors in the media which catabolize JH III. Therefore, it is unlikely that extracellular degradative enzymes are present in the day 0 suspension cultures which have just been subcultured into fresh, sterile media.

From the profiles of JH III accumulation in the suspension cultures (Fig. 2a, b and c), two time points were selected; day 0 and day 16. These points seem to represent times of high rates of JH III biosynthesis and lower degradation rates and day 0 suspension cultures were selected for further biochemical studies.

**Biosynthesis**

3-Hydroxy-3methylglutaryl-CoA reductase (E.C. 1.1.1.34) (HMG-CoA reductase), a key regulatory enzyme in the classical MVA pathway, catalyzes the formation of mevalonate from 3-hydroxy-3-methylglutaryl CoA (Stermer et al., 1994; Chappell et al., 1995). The fungal metabolite mevinolin is a potent competitive inhibitor
of HMG-CoA reductase (Bach et al., 1990). Treatment of tobacco suspension cultures with low concentrations of mevinolin (10-30 μM) inhibited the MVA pathway as observed through a reduction in the incorporation of [14C]acetate into free sterols (Vögeli and Chappell, 1991). This compound is a highly specific inhibitor of HMG-CoA reductase; *in vitro* microsomal HMG-CoA reductase activity was inhibited by over 90% in the presence of 3 μM mevinolin. However, higher mevinolin concentrations (100 μM) inhibited the activity of sesquiterpene cyclase, which catalyzes the first branchpoint step in the formation of cyclic sesquiterpenoids, *in vitro*.

Treatment of suspension cultures with mevinolin did not effect cell growth at the concentrations tested (Fig 3). However, over 95% inhibition of JH III production was observed in the presence of 10 or 50 μM mevinolin (Fig. 4). The enzyme HMG-CoA reductase catalyzes the conversion of 3-hydroxy-3-methylglutaryl CoA to MVA. Addition of MVA to these mevinolin-inhibited cultures resulted in the rescue of JH III production (Fig. 5). These results confirm that biosynthesis of the terpenoid skeleton of JH III proceeds at least partially via the MVA pathway in *C. iria*.

Treatment of mevinolin-inhibited cultures with farnesoic acid, an intermediate in the JH III biosynthetic pathway of insects, resulted in an increase in JH III production in both cells and media (Fig. 6). Production of JH III in these farnesoic acid-treated cultures was 38 times higher than the amount observed in the control suspension cultures, representing approximately 0.7% conversion of the added farnesoic acid into JH III, suggesting that the enzymes in the final steps of JH III biosynthesis are not rate limiting. The cells were also brown and dead, possibly representing autotoxicity of JH III or farnesoic acid in these cultures. Therefore, it appears that farnesoic acid may be a biosynthetic precursor of JH III in the sedge *C. iria* and that the JH III biosynthetic
Fig. 3. Effect of mevinolin on *Cyperus iria* suspension culture cell growth. The fungal metabolite mevinolin is a reversible inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, a key regulatory enzyme in the mevalonate pathway. Cell growth in the presence of 10 or 50 μM mevinolin was not significantly different from controls (ANOVA). Bars represent the mean of three experiments ± standard error.
Fig. 4. Effect of mevinolin on juvenile hormone III (JH III) production. Juvenile hormone III production was significantly inhibited in *C. iria* suspension cultures treated with mevinolin, a potent inhibitor of the key regulatory enzyme in the mevalonate pathway, 3-hydroxy-3-methylglutaryl CoA reductase (student's t-test, \( p \leq 0.008 \)). Bars represent the mean of three experiments ± standard error.
Fig. 5. Effect of mevalonate lactone on juvenile hormone III production in mevinolin-inhibited *C. iria* suspension cultures. Addition of mevinolin to *C. iria* suspension cultures inhibited the enzyme 3-hydroxy-3-methylglutaryl CoA reductase and significantly reduced juvenile hormone III (JH III) levels (student's t-test, $p \leq 0.008$). This enzyme catalyzes the formation of mevalonate from 3-hydroxy-3-methylglutaryl CoA. Addition of mevalonate (MVA) to these mevinolin-treated suspension cultures restored JH III production. Bars represent the mean of three experiments $\pm$ standard error.
Fig. 6. Effect of farnesoic acid (FA) on juvenile hormone III production in mevinolin-inhibited *C. iria* suspension cultures. Treatment of *C. iria* suspension cultures with mevinolin significantly decreased juvenile hormone III (JH III) production by inhibiting the key regulatory enzyme in the mevalonate pathway, 3-hydroxy-3-methylglutaryl CoA reductase (student's t-test, *p* ≤ 0.008). Addition of farnesoic acid (FA) to these mevinolin-treated suspension cultures restored JH III production. Bars represent the mean of three experiments ± standard error.
pathways in plants and insects may be similar.

We are further characterizing the biosynthetic pathway of JH III in *C. iria* suspension cultures through [1-$^{13}$C]glucose labeling, enzyme inhibition and precursor feeding studies.

References


In insects, juvenile hormones regulate key developmental processes in insects such as metamorphosis and reproduction (Gilbert et al., 1996). Juvenile hormone III (JH III), methyl-10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate, has also been identified in two sedge species, Cyperus iria L. and C. aromaticus (Ridley) Mattf and Kük (Toong et al., 1988). Little is known about the presence of this compound in these plants and the objective of this thesis was to provide insight into its distribution and possible biological function in C. iria as well as to elucidate its biosynthetic pathway.

Although JH III has only been detected in two plant species, C. iria and C. aromaticus, structurally similar sesquiterpenoids have been isolated from the roots of C. iria and a number of related Cyperus species (see Chapter 5, Table 1) (Iwamura et al., 1978a, b, c; Iwamura et al., 1978b; Iwamura, 1979; Iwamura et al., 1979; Komai et al., 1981; Toong, et al., 1988). Juvenile hormone III, methyl farnesoate and structurally similar compounds are present in diverse plant species and thought to be more prevalent than previously thought.

Dynamic temporal and spatial fluctuations of JH III were observed in C. iria throughout development (Chapter IV). Juvenile hormone III increased in the plant until flowering at which time a decrease in all tissues was observed. Levels subsequently increased in the mature plant until senescence when, again, the amount of this compound declined in aerial tissues. This fluctuation was not observed in root tissue which remained viable. It is noteworthy that JH III was found primarily in the roots; over 85% of the JH III detected in the plant was localized in this tissue and amounts as high as 1.2 μmoles were measured in root tissue of 7 month old, mature C. iria plants. The opposite
distribution pattern was observed in the related sedge, *C. aromaticus*. In this plant, JH III predominates in the aerial tissues with the highest levels found in the bracts and leaf tissue (W. Bowers, personal comm.).

The subcellular site of JH III localization in the root tissue may provide insight into its physiological role. JH III is a lipophilic compound and may be compartmentalized in the vacuole if conjugated to hydrophilic molecules such as glycosides or phosphate residues or sequestered in specialized structures which sequester lipophilic compounds. Such oil bodies have been reported in *C. iria* roots (Kern, 1972-6; B. Bendena, personal comm., 1998). In collaboration with B. Bendena (Queen's University), experiments, using antiserum to JH III, are currently underway to determine if JH III accumulates in these structures.

Sequestration into specialized compartments would translate into higher levels of JH III in specific microenvironments. In groundsel, *Senecio vulgaris* L., the majority of the pyrrolizidine alkaloids are distributed in the aerial tissues with significant amounts associated with the inflorescence (Hartmann and Zimmer, 1986). In the vegetative tissue, the alkaloids accumulate in the peripheral layers of the stem. In *Lupinus polyphyllus*, the quinolizidine alkaloids are also sequestered in leaf epidermis tissues (Wink, 1986). These localized areas are believed to concentrate the alkaloids at the surface tissues where they are easily detected and deter insect herbivory.

Following stress or attack, plants may respond by altering certain traits (Karban and Myers, 1989). These are known as an induced responses. Alternately, some plant defenses are constitutive. These responses are independent of injury. In the defense against insect herbivory, plants use both these defense responses. Induced defenses are thought to be less costly to plant fitness, allowing resources to be directed towards
growth and reproduction until needed. They also allow temporal and spatial variability, decreasing the insects ability to develop counteradaptations to the plant defenses. Their disadvantage is the lag time between the herbivore attack and the appearance of a response, leaving the plant vulnerable. Therefore, constitutive defenses provide an advantage to plants in response to constant herbivore pressure, whereas induced defenses are optimal when plants are subject to low, unpredictable herbivory (Dam et al., 1993).

The fir tree, Abies grandis, possesses a number of synthases which catalyze terpenoid cyclization (Phillips and Croteau, 1999). Expressed constitutively are multi-product synthases which exhibit "relaxed" substrate and product specificity, generating a diversity of terpenoid structures. In response to wounding, single-product synthases are induced (Bohlmann et al., 1998). Therefore, the number and diversity of terpenoids form the general defensive arsenal of the fir. In times of stress, however, specific defensive compounds are synthesized. In this investigation, induction of JH III in C. iria in response to wounding and insect herbivory was not examined.

The high levels of JH III found in the root tissue suggests that this is the site of biosynthesis. Alternatively, this compound may be synthesized in leaf tissue and transported to the roots for storage, providing a sink for photosynthate. There are many examples of this long-distance transport of secondary metabolites. Pyrrolizidine alkaloid N-oxides are synthesized in root tissues and transported to shoots via the phloem (Hartmann et al., 1989). In tobacco, Nicotiana sylvestris, leaf damage induces de novo synthesis of nicotine in the roots which is then transported to the aerial tissues via the xylem stream (Baldwin et al., 1996). In the Madagascar periwinkle, Catharanthus roseus, developmental and tissue specific expression is observed for genes involved in the biosynthesis of the indole alkaloid vindoline (St-Pierre et al., 1999). In the leaf, these
genes are expressed in young, actively growing tissue forming a basipetal gradient of expression. Early genes in the pathway are only detected in the upper and lower leaf epidermis, whereas expression of later genes is associated with idioblasts in the palisade and spongy mesophyll layers and with laticifers at the interface of these tissue types. Therefore, there appears to be movement of intermediates from the early vindoline pathway, which is associated with the epidermis of immature leaves and stems, into laticifers and idioblasts where vindoline is formed. This movement of intermediates between cells and/or tissues appears to be a common motif in the biosynthesis of secondary metabolites in plants.

The present study did not investigate the effects of abiotic conditions such as photoperiod, light intensity and nutrient levels on JH III amounts in C. iria. Though these factors may influence secondary metabolite levels in some plant species (Waterman and Mole, 1986). For example, a diurnal cycle of terpenoid release was observed in cotton plants infested with larvae of the beet armyworm, Spodoptera exigua; a ten-fold higher induction of acyclic volatiles was observed in the light compared to the dark phase (Loughrin et al., 1994). Cyclic terpenoids also released by the plant in response to insect herbivory did not exhibit this pattern. Light intensity also may affect terpenoid levels. Under high light intensity, leaves of Lima bean plants emitted significantly higher levels of (E)-β-ocimene (Takabayashi et al., 1994). The authors speculate that this is result of either the increased photolytic energy being converted into metabolites or to water stress inducing production of this compound. However, large differences in light intensity (13000 vs 7000 lux) were necessary to induce these changes in terpenoid levels.

Metabolic turnover of JH III in C. iria was not measured. The previous dogma that the terpenoids are metabolically unstable and undergo rapid turnover in plants has
recently been challenged (Gershenzon et al., 1993). Terpenoid pools were found to be stable in a range of taxonomically diverse plants, Melaleuca alternifolia, Tanacetum vulgare, Salvia officinalis and Pinus contorta. It is believed that previous results were artefacts due to the invasive manipulation of plants during the experimental procedure.

The isolation of JH III from C. iria and C. aromaticus and the extraction of structurally similar compounds from closely related species as well as other plant species suggests that this compound may play an important biological role(s) in plants. Since there is no evidence that JH III functions as a feeding deterrent, any antiherbivory activity that it demonstrated would stem from its ability to interfere with insect development. However, there is only a short, stage-specific window of sensitivity when treatment of an insect with JH would interfere with its development (Nijhout and Wheeler, 1982). Arrest of larval development may prolong the feeding stage of the insect which would be detrimental to the plant. In adult insects, JH III may interfere with reproductive development. In this case, there would also be a lag time before a reduction in herbivory was noted. In two laboratory studies, contact of insects with C. iria has been shown to disrupt development (Toong et al., 1988; Schwartz et al., 1998). These effects are attributed to the JH III content of the plant. In the plant, JH III is localized in the roots. In these tissues, JH III levels are over forty times the highest levels found in mated, female cockroaches, Diploptera punctata (Tobe et al., 1985). If this compound is sequestered in specialized cells at the tissue surface or at the tips of growing tissues, then insects that feed on these tissues may risk exposure to particularly high levels. Therefore, JH III in C. iria may play a role in plant-insect interactions, particularly in below-ground herbivory, or by effecting insect egg development.
Other studies indicate that some insect species undergo normal development when reared on a diet of C. iria. Insects possess a vast array of gut enzymes, especially mixed function oxidases which are involved in the detoxification of plant secondary metabolites (Dowd et al., 1983). Insects which develop resistance to some pesticides may also exhibit cross-resistance to JH analogues due to an upregulation of these enzymes (Cerf and Georgiou, 1972). Strategies of insect evasion of plant JH III either through avoidance, sequestration or detoxification could provide an understanding of the coadaptation between plants and insect herbivores.

Sedges, such as C. iria, are monocots, possessing basal meristematic tissue which allows them to tolerate herbivore grazing pressure. The poor nutritional quality of these plants and high silica content are believed to deter most insect herbivores (Vicari and Bazely, 1993). Is the presence of JH III in the plant an additional defence against insect herbivory or does it play another role in the plant?

Potent allelopathic activity of JH III on rice seedling growth has also been demonstrated (Chapter V). Cyperus iria is an extremely invasive weed, responsible for economic losses in many important crops such as rice (Holm et al., 1977; Catling, 1992). It is possible that this compound contributes to the aggressive nature of this plant by leaching out into the immediate environment and affecting the germination and growth of neighbouring seedlings. Further work characterizing the levels of JH III in the plant root leachates and the biotransformation of this compound in the microenvironment must be conducted before definitive conclusions about its biological function can be reached.

Other biological activities such as the antimicrobial and nematocidal activity of JH III should also be investigated. Induction of JH III in response to stimuli such as
wounding, insect herbivory or plant pathogens could also contribute to the understanding of its function in the plant.

In plants, terpenoids are a diverse class of compounds ranging from photosynthetic pigments (chlorophyll and carotenoids) to defensive molecules (phytoalexins) and growth regulators (gibberellins and abscisic acid). Despite the identification of over 30,000 isoprenoids, our current knowledge of the regulation of terpenoid biosynthetic pathways in higher plants is still prefatory (Bach, 1995; Wink, 1999). In fact, the presence of two distinct isoprene biosynthetic pathways has only been recently demonstrated in higher plants (Rohmer et al., 1993; Bach, 1995; Lichtenthaler et al., 1997).

Little is known about the biosynthesis of the sesquiterpenoid juvenile hormone III (JH III) in the sedge, C. iria. To elucidate this pathway in the plant, cell suspension cultures were established (Chapter VI). These cultures represent a relatively homogeneous plant biomass that can be experimentally manipulated under defined conditions. However, a common problem of these undifferentiated cultures is that secondary metabolite production is often substantially lower than in the whole plant. The presence of JH III in these suspension cultures was confirmed by gas chromatography-mass spectroscopy. Juvenile hormone III levels in these cultures is low (pmole range). Therefore, steps can be taken to try to increase production. Following subculturing, suspension cells undergo five stages of growth; the initial lag phase, followed by the exponential and linear growth phases then the progressive deceleration and stationary phases. During the growth phases, nutrients in the medium are principally utilized in primary metabolic pathways. When cell division declines, these metabolites and cofactors become available for secondary metabolite formation. Therefore, optimization of biomass
does not necessitate an increase in the production of JH III. A two-stage continuous culture would allow optimization of conditions for both growth and production of secondary metabolites. In the first phase, the medium is optimized to stimulate cell growth and biomass production. As the cells enter the stationary phase, they are transferred to a second medium that is optimized for metabolite production. This system is used effectively especially when the product of interest is produced in the stationary phase, as in the case of shikonin production in *Lithospermum erythrorhizon* suspension cultures (Fujita, 1988) and the diterpenes cryptotanshinone and ferruginol in *Salvia miltiorrhiza* cell cultures (Miyasaka *et al*., 1986). Variables which can be optimized include the organic and inorganic components of the media, the plant-growth hormones, pH and the addition of biosynthetic precursors.

The potential cytotoxicity of mono- and sesquiterpenes may account for their low yield in suspension cultures. In intact plants, these compounds are often compartmentalized in secondary structures such as resin ducts or glandular trichomes (Gershenzon and Croteau, 1991). In cell cultures, it is possible to decrease terpenoid toxicity and enhance production through the use of lipophilic phases such as XAD-2 and Miglyol 812. Mono- and sesquiterpenoid production in suspension cultures of *Pelargonium fragrans* increased approximately 500-fold in the presence of Miglyol (Charlwood and Brown, 1988) Nearly all (99%) of the essential oil produced was sequestered in the lipophilic trap. The essential oil profile also changed. Initially, there was an increase in the levels of oxygenated monoterpenes. After several subcultures, the sesquiterpenoid hydrocarbons, such as farnesene, formed the major constituents of the essential oil. A substantial decrease in growth rate was also observed in these cultures.
The addition of the plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) to culture medium is often necessary to induce callus formation, particularly in monocots. In *Salvia miltiorrhiza* suspension cultures, 2,4-D is required for growth. However, production of the diterpene ferruginol only observed in 2,4-D-free media (Miyasaka et al., 1986). In the absence of 2,4-D, *C. iria* suspension cells, undergo limited differentiation and form stumpy roots. Juvenile hormone content of these tissues has not been determined. Other plant growth regulators may also be required for terpenoid biosynthesis in these cell cultures. For example, abscisic acid was necessary for production of the sesquiterpene lactone altamisin in callus cultures of *ambrosia tenufolia* (Goleniowski et al., 1992). The addition of gibberellic acid to these cultures favoured the production of psilostachyinolides. Undefined culture media such as coconut milk have also been shown to affect secondary metabolite production.

The biosynthesis of JH III may in these cultures may also require tissue differentiation. A hairy root culture, which is generated through infection of plant tissue with *Agrobacterium rhizogenes*, could be used to induce JH III production. Close correlation has been shown between mono- and sesquiterpenoid production in the parent plant and hairy root cultures of *Lippia dulcis* Trev. (Sauerwein et al., 1991). Since the highest levels of JH III are found in the roots of *C. iria*, hairy root cultures may have the ability to synthesize and accumulate JH III. However, there are few examples of transformation of a monocot species by *A. rhizogenes* (Toivonen, 1993).

The absence of JH III may also be due to the divergence of biosynthetic precursors into higher steroid pathways. Inhibition of these pathways may redirect the carbon flux into JH III biosynthesis. Addition of 2-diethylaminoethyl-3,4-dichlorophenylether, an inhibitor of carotenoid biosynthesis, to *Catharanthus roseus*...
suspension cultures resulted in an increased accumulation of monoterpenoid indole alkaloids (Lee et al., 1988).

Production of JH III in C. iria suspension cultures was monitored over the growth cycle (day 0-18) (Chapter VII). At subculturing (day 0), there was an increase in JH III production which reflected de novo biosynthesis. This time was selected for characterization of the JH III biosynthetic pathway in these suspension cultures.

In plants, the isoprenoid skeleton of terpenoids may be synthesized through one of two different pathways; the mevalonate (MVA) or 2-C-methyl erythritol 4-phosphate (MEP) pathway or both pathways. Using enzyme inhibition studies, it has been conclusively demonstrated that the classical MVA pathway contributes to the sesquiterpenoid skeleton and studies are continuing to determine possible involvement of the MEP pathway (Chapter VII).

A comparison of compounds isolated from C. iria with biosynthetic precursors of JH III in the cockroach, Diploptera punctata, suggested that the later steps of biosynthesis from the intermediate farnesyl diphosphate may be similar in these organisms. The fungal metabolite mevinolin is a potent inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, a key regulatory enzyme in the early steps of the MVA pathway. Treatment of C. iria suspension cultures with mevinolin inhibited JH III production. Addition of farnesoic acid, a biosynthetic precursor of JH III in insects, to the mevinolin-treated suspension cultures resulted in the rescue of JH III production. This implies that farnesoic acid is a biosynthetic precursor of JH III in C. iria and that the biosynthetic pathway in plants and insects are similar.

We are continuing to characterize the later steps of the JH III biosynthetic pathway in the sedge, C. iria, through precursor feeding studies. Mevinolin-inhibited
suspension cultures will be treated with farnesol, farenol, methyl farnesoate and epoxyfarnesolic acid (an alternate biosynthetic precursor to JH III in Lepidoptera) and JH III production monitored. Similar inhibition experiments may be conducted on putative enzymes in the JH III biosynthetic pathway in vitro. For example, since the cytochrome P₄₅₀ enzyme, methyl farnesoate reduced flavoprotein: oxygen oxidoreductase (EC 1.14.14.-), catalyzes the final step in JH III biosynthesis (Feyereisen et al., 1981; Hammock, 1975; Hammock and Mumby, 1978), inhibitors of cytochrome P₄₅₀ enzymes, such as carbon monoxide, oxidized cytochrome c, clotrimazole, miconazole and piperonyl butoxide, may inhibit JH III biosynthesis in the plant. Further characterization of the biosynthetic pathway can be achieved through enzyme purification and gene identification. This would enable investigation into the regulation of this pathway.

Metabolons are noncovalent aggregations of enzymes which catalyze sequential steps in a metabolic pathway. The close association of these proteins facilitates the transfer of intermediates between sequential enzymes, thereby increasing their catalytic efficiency. Characterization of the biosynthetic pathway through enzyme purification and isolation of the respective genes would enable investigation into the regulation of the pathway and elucidation of putative metabolic complexes.

Through the use of immunocytochemistry and molecular biological techniques, localization of the pathway could be established and changes in enzyme level or activity or gene expression over development or in response to stimuli such as wounding or insect herbivory could be monitored. Cell suspension cultures also provide an excellent model to study the potential role of signal transduction in the regulation of JH III biosynthesis.

References


Hammock, B.D. (1975) NADPH dependent epoxidation of methyl farnesoate to juvenile hormone in the cockroach *Blaberus giganteus* L. *Life Sciences* 17: 323-328.


Tane, P., Ayafor, J.F. and Sodengam, B.L. (1988) A substituted cinnamoyl ester from


Appendix I. Chemistry of juvenile hormone III

Chemical data for JH III is summarized in Table I. In the infrared spectrum, the band at 1720 cm$^{-1}$ is characteristic of an ester carbonyl group (C=O) and the band at 1650 cm$^{-1}$ is reflective of the alkene nature of the molecule (C=C) (Bowers et al., 1965; Meyer et al., 1968)

**Mass spectroscopy**

Electron impact mass spectroscopy routinely uses a high energy beam of 70 eV; however, in the fragmentation pattern of JH III (Table II), important high mass ions are also observed in the low energy resolution spectrum (15 eV) (Trost, 1970; Liedtke and Djerassi, 1972; Dunham et al., 1976). The observed peak at m/z 248 results from the migration of two hydrogen atoms to the epoxide oxygen and the subsequent loss of water. Mass ions at m/z 234 and 206 characterize the methyl ester moiety; m/z 234 represents the loss of the methyl ester group (CH$_3$OH) and m/z 206 represents the loss of the methyl ester and the C=O from the acid. Fragmentation cleavage patterns give rise to mass ions at m/z 195, 114, 81 and 71 (Trost, 1970). Low mass ion fragments (m/z 135, 114, 81, 71 and 43), which are detected in both the high and low energy resolution spectra, arise from hydrogen migration patterns suggestive of McLafferty-type rearrangements (Silverstein et al., 1991). Scheme I illustrates the hydrogen rearrangement which generates the ion of m/z 71 and demonstrates the relationship between fragments produced at m/z 71 and 43 (Liedtke and Djerassi, 1972). The base peak ion (m/z 81) results from two carbon-carbon bond cleavages and the transfer of one hydrogen atom (Dunham et al., 1976) and can be generated through mass ions of m/z 195, as shown by Trost (1970), or m/z 153 (Liedtke
Table I. Chemical data for juvenile hormone III, methyl-10R-3,7,11-trimethyl 2E-6E-dodecatrienoate.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beilstein Registry Number</strong></td>
<td>1316317</td>
</tr>
<tr>
<td><strong>MW</strong></td>
<td>266.38</td>
</tr>
<tr>
<td><strong>C₁₆H₂₆O₃</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Boiling point: (ethanol)</strong></td>
<td>125-126°C (0.08 mm)</td>
</tr>
<tr>
<td><strong>30°C (0.02 mm)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Ultraviolet spectroscopy (ethanol):</strong></td>
<td>( \lambda = 221 \text{ nm}, \varepsilon = 14350 )</td>
</tr>
<tr>
<td><strong>Infrared spectroscopy (film):</strong></td>
<td>2950, 1720, 1215, 1130 cm(^{-1}).</td>
</tr>
<tr>
<td></td>
<td>1720, 1650 cm(^{-1}).</td>
</tr>
</tbody>
</table>

Table II: Mass spectral data for juvenile hormone III.

<table>
<thead>
<tr>
<th>Mass (m/z)</th>
<th>Ion Fragment</th>
<th>Base peak (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>266</td>
<td>M⁺</td>
<td>C₁₆H₂₆O₃</td>
</tr>
<tr>
<td>248</td>
<td>(M-H₂O)</td>
<td>C₁₆H₂₄O₂</td>
</tr>
<tr>
<td>234</td>
<td>(M-CH₃OH)⁺</td>
<td>C₁₅H₂₂O₂</td>
</tr>
<tr>
<td>206</td>
<td>(M-CH₃OH + CO)⁺</td>
<td>C₁₄H₂₂O</td>
</tr>
<tr>
<td>195</td>
<td>(M-C₄H₇O)⁺</td>
<td>C₁₂H₁₉O₂</td>
</tr>
<tr>
<td>163</td>
<td>(195-CH₃OH)</td>
<td>C₁₁H₁₅O</td>
</tr>
<tr>
<td>153</td>
<td>(M-C₆H₉O₂)</td>
<td>C₁₀H₁₇O</td>
</tr>
<tr>
<td>135</td>
<td>(153-H₂O)</td>
<td>C₁₀H₁₅</td>
</tr>
<tr>
<td>114</td>
<td>(195-CO + CH₃OH)</td>
<td>C₆H₁₀O₂</td>
</tr>
<tr>
<td>81</td>
<td>(M-C₁₀H₁₆O)</td>
<td>C₆H₉</td>
</tr>
<tr>
<td>71</td>
<td>(C₁₂H₁₉O₂)</td>
<td>C₄H₇O</td>
</tr>
<tr>
<td>43</td>
<td>(C₁₃H₁₉O₃)</td>
<td>C₃H₇</td>
</tr>
<tr>
<td>266</td>
<td>M⁺</td>
<td>C₁₆H₂₆O₃</td>
</tr>
<tr>
<td>248</td>
<td>(M-H₂O)</td>
<td>C₁₆H₂₄O₂</td>
</tr>
<tr>
<td>234</td>
<td>(M-CH₃OH)⁺</td>
<td>C₁₅H₂₂O₂</td>
</tr>
<tr>
<td>195</td>
<td>(M-C₄H₇O)⁺</td>
<td>C₁₄H₁₉O₂</td>
</tr>
<tr>
<td>163</td>
<td>(195-CH₃OH)</td>
<td>C₁₁H₁₅O</td>
</tr>
<tr>
<td>135</td>
<td>(153-H₂O) and (195-CO + CH₃OH)</td>
<td>C₁₀H₁₅</td>
</tr>
<tr>
<td>114</td>
<td>(M-C₁₀H₁₆O)</td>
<td>C₆H₁₀O₂</td>
</tr>
<tr>
<td>81*</td>
<td>(M-C₁₀H₁₇O₃)</td>
<td>C₆H₉</td>
</tr>
<tr>
<td>71</td>
<td>(C₁₂H₁₉O₂)</td>
<td>C₄H₇O</td>
</tr>
<tr>
<td>43</td>
<td>(C₁₃H₁₉O₃)</td>
<td>C₃H₇</td>
</tr>
</tbody>
</table>

Bolded masses represents ions unique to the low energy spectra. An asterix denotes the base peak.

Scheme I. Mass spectroscopy fragmentation pattern of juvenile hormone III.

(Adapted from Liedtke and Djerassi (1972). Reprinted with permission from the American Chemical Society).
\[
\text{H}_{\text{\(\text{H}^+\)}} \quad \text{m/e 43} \quad \text{- CO} \quad \text{m/e 71}
\]
and Djerassi, 1972) (Scheme II). The mass ion m/z 135 is generated through the
fragmentation of ions at m/z 153 and 195 and also m/z 163 (Liedtke and Djerassi, 1972).
Lastly, the intense ion peak at m/z 114, which is present in the spectrum of JH III and its
biosynthetic precursor methyl farnesoate may occur by hydrogen transfer through a
McLafferty-type rearrangement or by the migration of a hydrogen from C-4 to the
carbonyl oxygen followed by transfer of another hydrogen from C-8 or C-8' to C-4
(Thomas et al., 1969; Liedtke and Djerassi, 1972; Dunham et al., 1976).

Nuclear magnetic resonance

Proton nuclear magnetic resonance spectrum of juvenile hormone III

Table III compares the assignment of chemical shifts based on three reports in the
literature (Bowers et al., 1965; Trost, 1970; Meyer et al., 1970). The singlet at δ 3.72
represents the three protons on the methyl ester. The vinyl proton on C-2 is denoted by a
signal at δ 5.75. The sharp doublet at δ 2.18 represents a vinyl methyl group which is cis
and β to the carbonyl group. The vinyl proton on C-6 is indicated by δ 5.22. The methyl
on C-7 is shown by a singlet at δ 1.65. The unusual chemical shift at δ 2.74 represents the
epoxide proton on C-10 and the singlets at δ 1.27 and δ 1.32 are due to the two methyl
groups on C-11. The remaining protons on C-4, C-5, C-8 and C-9 are represented by
bands at δ 1.7 and δ 2.1.

13C nuclear magnetic resonance spectrum of juvenile hormone III

The 13C nuclear magnetic spectrum assignment for JH III is listed in Table IV
(Kuhnz and Rembold, 1981).
Scheme II. Mass spectroscopy fragmentation pattern of juvenile hormone III.

(Adapted from Liedtke and Djerassi (1972). Reprinted with permission from the American Chemical Society).
Table III. Proton nuclear magnetic spectra of juvenile hormone III:

<table>
<thead>
<tr>
<th>δ</th>
<th>Assignment</th>
<th>δ</th>
<th>Assignment</th>
<th>δ</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>double peak = two methyl groups attached to epoxide</td>
<td>1.27 &amp;</td>
<td>s, 2 x 3H, CH₃ at C-11</td>
<td>1.26</td>
<td>s, 3H, CH₃ at C-11</td>
</tr>
<tr>
<td>1.63</td>
<td>s, 3H, C-7</td>
<td>1.32</td>
<td>s, 3H, CH₃ at C-7</td>
<td>1.3</td>
<td>s, 3H, H-12</td>
</tr>
<tr>
<td>1.65</td>
<td>s, 3H, CH₃ at C-7</td>
<td>1.65</td>
<td>s, 3H, CH₃ at C-7</td>
<td>1.62</td>
<td>s, 3H, CH₃ at C-7</td>
</tr>
<tr>
<td>2.18</td>
<td>d, s, J = 1.5 Hz, CH₃ at C-3</td>
<td>2.18</td>
<td>d, s, J = 1.5 Hz, CH₃ at C-3</td>
<td>2.70</td>
<td>t, J = 6 Hz, 1H, H-10</td>
</tr>
<tr>
<td>2.5</td>
<td>epoxide proton</td>
<td>2.74</td>
<td>t, J = 6Hz, 1H, H-10</td>
<td>2.70</td>
<td>t, J = 6 Hz, 1H, H-10</td>
</tr>
<tr>
<td>3.61</td>
<td>carboxyl methyl</td>
<td>3.72</td>
<td>s, 3H, OCH₃</td>
<td>3.69</td>
<td>s, 3H, OCH₃</td>
</tr>
<tr>
<td>5.12</td>
<td>vinyl protons C-6</td>
<td>5.22</td>
<td>m, 1H, H-6</td>
<td>5.14</td>
<td>t, J = 6 Hz, 1H, H-6</td>
</tr>
<tr>
<td>5.59</td>
<td>vinyl protons C-2</td>
<td>5.75</td>
<td>m, 1H, H-2</td>
<td>5.67</td>
<td>br. s, 1 H, H-2</td>
</tr>
</tbody>
</table>

Solvent: CCl₃, CDCl₃, CDCl₃

Chemical shifts are reported as δ values in ppm. Tetramethyldisilane was the internal standard.

References: Meyer et al., 1970; Anderson et al., 1972; McCormick and Schafer, 1977; Kleijn et al., 1981; Rodriguez and Gros, 1990.
Table IV. $^{13}$C nuclear magnetic spectra of juvenile hormone III (CDCl$_3$):

<table>
<thead>
<tr>
<th>δ</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.9</td>
<td>OCH$_3$</td>
</tr>
<tr>
<td>167.2</td>
<td>C-1</td>
</tr>
<tr>
<td>115.7</td>
<td>C-2</td>
</tr>
<tr>
<td>159.9</td>
<td>C-3</td>
</tr>
<tr>
<td>41.1</td>
<td>C-4</td>
</tr>
<tr>
<td>26.1</td>
<td>C-5</td>
</tr>
<tr>
<td>123.8</td>
<td>C-6</td>
</tr>
<tr>
<td>135.5</td>
<td>C-7</td>
</tr>
<tr>
<td>26.5</td>
<td>C-8</td>
</tr>
<tr>
<td>27.7</td>
<td>C-9</td>
</tr>
<tr>
<td>64.2</td>
<td>C-10</td>
</tr>
<tr>
<td>58.2</td>
<td>C-11</td>
</tr>
<tr>
<td>25</td>
<td>C-12</td>
</tr>
<tr>
<td>25</td>
<td>CH$_3$ at C-11</td>
</tr>
<tr>
<td>16.2</td>
<td>CH$_3$ at C-7</td>
</tr>
<tr>
<td>18.9</td>
<td>CH$_3$ at C-3</td>
</tr>
</tbody>
</table>

Chemical shifts are reported as δ values in ppm. Tetramethylsilane was the internal standard. Numbering of juvenile hormone III as illustrated in Table III.

Chirality of juvenile hormone III

The absolute configuration of the epoxide of insect juvenile hormones was determined by spectroscopy and circular dichroism (Nakanishi et al., 1974). In the first method, perchloric acid was used to catalyze the hydrolysis of the epoxide in the presence of H$_2^{18}$O. The resulting vic-diol was analyzed by mass spectroscopy and definitively demonstrated the 10R,11S chirality of the epoxide (Meyer et al., 1971). The chirality was confirmed by circular dichroism. The epoxide was hydrolyzed by sulfuric acid and tetrahydrofuran and the circular dichroism cotton effect of the resulting acyclic glycol was measured in the presence of tris(dipivaloylmethanato)praseodymium (Nakanishi et al., 1971).

General chemistry of juvenile hormone III

Juvenile hormones are lipophilic molecules and relatively insoluble in aqueous solutions. The solubility of JH I is approximately 3 x 10$^{-5}$ M in 0.2 M Tris-HCl buffer, pH 7.5 (Giese et al., 1977); changes in pH, buffer and ionic strength had no effect on solubility. The addition of proteins such as bovine serum albumin (BSA) and immunoglobulin G increases the solubility of JH I; for example, in 5% BSA, a 1 mM solution of JH I can be made. Juvenile hormone III is more hydrophilic than JH I; in 5 mM Tris-HCl, pH 8.3, the solubility of JH III is greater than 2 x 10$^{-4}$ M (Kramer et al., 1976). However, its limited solubility must be recognized when preparing solutions.

The epoxide group on insect juvenile hormones is particularly susceptible to hydrolysis through a S$_N$2-type mechanism, generating the trans-dihydrodiol (van Tamelen, 1968; Meyer et al., 1970; Mumby and Hammock, 1979). Therefore, protic acids, in aqueous or methanolic solutions readily convert the JH to its diol or 11-
methoxy-10-hydroxy derivative, respectively (Schooley et al., 1976; Bergot et al., 1981). This reaction can be used to quantify juvenile hormones in insect haemolymph by generating derivatives which can either be analyzed directly or derivatized further and analyzed by gas chromatography-mass spectroscopy (GC-MS). Oxidation of the epoxide with neutral alumina or silica or heating to temperatures above 150°C results in an allylic alcholoh which will undergo cyclization at higher temperatures (180°C) to a tetrahydrofuran derivative (Meyer et al., 1970; Anderson et al., 1972). Therefore, in the past, the direct analysis of JH by GC was contraindicated by the tendency for thermolysis at the temperatures required for volatization (Anderson et al., 1972; Dunham et al., 1975). However, this can now be overcome through the use of a cool-on-line injector and programming of the oven after sample injection (P. Teal, personal comm.). Cyclization of JH III to mono- and bicyclic products also occurs readily in the presence of boron trifluoride or phosphoric acid (H₃PO₄) (van Tamelen, 1968). Transition metals may catalyze similar reactions in aqueous solution (Schooley, 1977). Inorganic salts, such as ferric chloride and zinc and magnesium sulfate, react with JH I, producing undetermined products (Mumby and Hammock, 1979). The methyl ester function on C-1 of JH III is resistant to saponification by strong base (Meyer et al., 1968).

One must also be aware of the binding affinity of juvenile hormones to different substrates. Juvenile hormones strongly adsorb to many commonly used plastic materials such as polystyrene, Millipore filters PSAC 02510, polyvinylchloride, polyethylene and plexiglass and to a lesser extent, glass and teflon (Giese et al., 1977). Therefore, if plastics are used, it is recommended that they should be tested to determine the degree of JH affinity. Glassware should be treated with siliconizing agents or polyethylene glycol (PEG) to block JH binding sites (Giese et al., 1977). Prior to this treatment, glassware
should be washed with a nonionic detergent, rinsed in distilled water and heated to at least 200°C for 5 hours (Meyer et al., 1970). The use of an acid, such as chromic acid, in the washing of glassware is contraindicated because of the susceptible nature of the epoxide group to acid hydrolysis (Meyer et al., 1970; Mumby and Hammock, 1979). Then the glassware can be treated with PEG or a siliconizing agent. Traces of acid catalyst in the PEG (Carbowax) may need to be removed before coating of the glassware, again because of the susceptibility of the epoxide ring to hydrolysis in the presence of acid. The solvents used should be of the highest quality and glass distillation is recommended in some cases (Meyer et al., 1970).

Pure juvenile hormones are oils which can be stored for a number of years at -20°C or lower (Schooley, 1977). Solutions of JH, which are made up in aprotic, relatively non-volatile solvents such as hexane or isooctane, can also be stored at this temperature (Schooley, 1977; Croston et al., 1987). In both solvents, the solution should be stored under an inert gas such as N₂ to prevent the oxidation of the double bonds.

**Extraction and quantification of the juvenile hormones**

Methods for the extraction and quantification of physiological levels of insect juvenile hormones by chromatographic techniques (thin-layer chromatography, high performance liquid chromatography, gas chromatography-mass spectroscopy (GC-MS), immunological techniques (radioimmunoassay) and the radiochemical assay will not be reviewed here. Analysis by electron impact GC-MS requires an initial derivatization(s), as a consequence of the susceptibility of the epoxide group to thermolysis, followed by detection through electron capture or single ion monitoring. This treatment is not necessary with chemical ionization GC-MS.
References


