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Bioassay of Anti Juvenile Hormone Compounds: An Alternative Approach

THOMAS C. SPARKS[1], R. MICHAEL ROE[2], ADRIAN BUEHLER[3], and BRUCE D. HAMMOCK[4]

[1]Department of Entomology, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70803
[2]Department of Entomology, North Carolina State University, Raleigh, NC 27695
[3]Abteilung fuer Zoophysiologie, Universitaet Bern, Erlachstrasse 9a, CH-3012 Bern, Switzerland
[4]Department of Entomology, University of California, Davis, CA 95616

During the last larval stadium of the cabbage looper, *Trichoplusia ni*, Hubner, the prepupal burst of juvenile hormone (JH) stimulates the appearance of juvenile hormone esterase (JHE) which, in turn, degrades the JH. Disruption of this prepupal burst of JH by anti-juvenile hormones (AJHs) such as fluoromevalonolactone, result in a variety of teratogenic effects including delayed tanning and/or pupation, malformed larvae and reduced JHE activity. Although general toxicants and esterase inhibitors may also cause malformed larvae, there is usually no delay in tanning and/or pupation. These observations provide the basis for a simple, rapid AJH bioassay using an economically important pest insect (*T. ni*). A simple 'key' was devised to help facilitate the use of this bioassay.

At the heart of any search for bioactive molecules is the need for effective bioassays. Several bioassays have been developed for the identification of compounds with anti-juvenile hormone (AJH) activity. The most common of these AJH bioassays involves the treatment of young larvae or nymphs with the potential AJH by incorporation into the diet or contact application, and then waiting for several days (or, in some cases, weeks) for precocious development (or other AJH response) to occur (1,2). Alternatively, AJH activity can be determined using *in vitro* assays such as corpora allata cultures or epidermal cell cultures to monitor for inhibition of juvenile hormone (JH) biosynthesis (3-6) or blockage of the JH induced inhibition of pupal commitment (7), respectively.

Although the above assays have all proven useful, there are a number of disadvantages associated with each of them. For example, the contact and feeding bioassays can require a large quantity of compound, compared to a typical topical bioassay, and it usually takes several days before the AJH effects are observed. The *in vitro* assays are tedious, require special skills in microsurgery, dedicated sterile facilities, are unsuitable for screening large numbers of compounds, detect a limited number of possible mechanisms,

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and in vitro activity may not translate into in vivo activity due to in vivo metabolism (8). Finally, the insects most commonly used for AJH bioassays, the tobacco hornworm, Manduca sexta (L.), and the large milkweed bug, Oncopeltus fasciatus (Dallas), are of limited economic importance, and both species seem to be hypersensitive such that compounds active on them (e.g. ETB, precocene) display little activity on major pest insects, especially among the Lepidoptera (2,9,10).

The cabbage looper, Trichoplusia ni Hubner, is a pest of a wide variety of agricultural crops (11-13) and represents a major family of insect pests, the Noctuidae, which also includes the corn earworm, Heliothis zea (Boddie), the tobacco budworm, Heliothis virescens (F.), the soybean looper, Pseudoplusia includens (Walker), the beet armyworm, Spodoptera exigua (Hubner), the fall armyworm, Spodoptera frugiperda (J. E. Smith) and others. There is also a growing body of knowledge concerning the endocrinology of T. ni (14,15). Recent studies on JH and JH esterase (JHE) regulation in wandering last stadium larvae and prepupae of T. ni (16-18) suggested an alternative approach to bioassaying compounds for AJH activity that circumvents some of the disadvantages associated with other AJH bioassays. From a more fundamental point of view, this report illustrates how key xenobiotics can be used to dissect developmental processes during critical phases of insect development.

The Bioassay

The bioassay presented herein is based on what appears to be a consistent set of teratogenic morphological effects that are produced in response to disruptions of the JH mediated regulation of development during the late last stadium of T. ni. To facilitate the use of this bioassay, a simple key has been devised and is presented below along with hypotheses for the physiological basis for each step.

Since the responses observed in this bioassay (and others) can be the result of a variety of effects, not only on the endocrine system, but also on the nervous and other systems (19), a selected group of JHs, AJHs and pesticides (Table I) were used in developing this bioassay in an effort to test and eliminate responses due to non-AJH compounds. Likewise, it is important in any successful bioassay to simply and rapidly eliminate inactive and inappropriate compounds (i.e. non-AJHs). Thus, the first steps in our bioassay attempt to exclude non-AJHs and inactive compounds, leaving the later steps for the confirmation of AJH activity to those few compounds that are most likely AJHs.

The Insect and Assays. Last (fifth) stadium (L5) larvae of T. ni were used throughout. Larvae were reared on a 14L:10D photoperiod (lights on at 5 AM) at 27°±2°C, and staged as described previously (31). Larvae were treated with the desired compounds either by injection along the mid-dorsal line of abdominal segments 1 & 2 (1 µl in distilled water), or by topical treatment (1-2 µl in acetone or ethanol) on the dorsum of the last thoracic segment. Controls received acetone or ethanol (topically, 1-2 µl), or water (injected, 1 µl). Obviously, the method of treatment will be a

function of the solubility of the test compound and/or its suspected ability to penetrate the insect cuticle. Injection is easily accomplished using either a sharpened 10 µl Hamilton syringe or a finely-drawn glass capillary tube. The capillary tube has the advantage of practically eliminating any bleeding after injection. Treated larvae were examined for behavioral and developmental changes or abnormalities.

A pooled (3-5 larvae) hemolymph sample was collected by clipping either anal or thoracic legs of the larvae. JHE activity in the hemolymph was monitored as described previously (32,33) using JH III (H^3 at C_{10}, 11 Ci/mmol., New England Nuclear) as the substrate (5×10^{-6} M). All assays were run in duplicate on at least two (usually three or more) occasions.

The Key. The bioassay is divided into 5 steps which form a key for the identification of AJH activity. At each step examples are provided of compounds that exhibit the possible responses. Since these compounds were used to develop the key, they do not necessarily follow the same logical progression that one would see if the bioassay was being used to evaluate their AJH activity.

Identification of AJH Activity

1. Are there larval-pupal intermediates or malformed larvae?
   Treatment: L5D3 (last stadium, day 3) larvae treated ca. 9 AM; 200 nmol./larva.
   Expected Result: A majority (>50%) of the treated larvae become tanned, malformed larvae without displaying any obvious toxic response (see explanation below). Pupation/tanning in controls generally occurs on L5D4 ca. 3 PM (ca. 30 hr. posttreatment). Results are expressed as the percentage of larvae displaying the above effect relative to the controls (solvent only).
   A) YES - GO TO 2. Examples: FMev, EPPAT, DEF (Table I).
   B) NO - Increase dose and/or change the time of treatment or mode of application and try again; otherwise STOP. Examples: Epofenonane, hydroprene, ETB, precocene II, ethoxy-precocene, DFP, pipernoyl butoxide, chlordimeform, TFT, carbaryl (Table I).

Explanation: The appearance of tanned, malformed larvae following treatment with an exogenous compound can be the result of AJH activity or a response on the part of the insect to an external stress such as an insecticide. Since a variety of molecular structures are likely to be tested for AJH activity, it is important to eliminate compounds that are general toxicants at the beginning of the bioassay.

If the compound is causing the insect to be stressed or intoxicated, the insect larva (in this case a prepupa) may have trouble casting the old cuticle, yet be capable of tanning. Thus, the insect becomes a tanned larva or what appears to be a half pupa/half larva (due, in part, to incomplete ecdysis). For example, larvae treated with paraoxon (200 nmol./larva; data not included in Table I due to high mortality) caused the formation of tanned larvae in all larvae treated. However, all of these larvae had obviously been adversely affected (intoxicated) by the paraoxon

Table I. Compounds[1] used to Develop and Test the AJH Bioassay, and Their Effect on *T. ni*.

Compound	Dose used for key (nmol.)	Previously tested for AJH activity	% Malformed Larvae[2]	T_{50} for tanning (hrs.)[3]	JHE Activity (% Control)[4] L5D3	L5D1
Used in the Development of the AJH Bioassay						
1. Juvenile hormone I	200	--	0	4.0+	304±83	105±23
2. Juvenile hormone III	200	20	0	0.0	164±54	112±9
3. Epofenonane: 1-(4'-ethylphenoxy)-6,7-epoxy-3-ethyl-7-methylnonane	200	--	0	1.5-	445±226	110±31
4. Hydroprene: ethyl (2*E*,4*E*)-3,7,11-trimethyl-2,4-dodecadienoate	200	9, 21	0	1.5+	365±32	100±25
5. FMev: tetrahydro-4-fluoromethyl-4-hydroxy-2*H*-pyran-2-one	200	4, 9, 18, 22-26	100	7.8-	45±21	99±7
6. ETB: ethyl 4-(2-(*tert*-butylcarbonyloxy)butoxy)benzoate	200	2, 7, 9 22, 24, 26 28	0	1.0+	365±139	87±26
7. Precocene II: 6,7-dimethoxy-2,2-dimethylchromene	200	1, 2, 3, 10 (for review), 28	0	0.1-	96±19	87±25

Table 1. Con't.

Compound	Dose used for key (nmol.)	Previously tested for AJH activity	% Malformed Larvae[2]	T_{50} for tanning (hrs.)[3]	JHE Activity (% Control)[4] L5D3	L5D1
8. Ethoxy-precocene: 7-ethoxy-6-methoxy-2,2-dimethylchromene	200	10, (for review), 29	0	1.1-	87±14	91±10
9. EPPAT: O-ethyl S-phenyl phosphoramidothiolate	200	--	72	1.5-	17±20	11±8
10. DFP: O,O-diisopropyl phosphorofluoridate	200	--	3	0.8-	98±7	105±15
11. DEF: S,S,S-tri-n-butyl-phosphorotrithiolate	200	--	71	1.8-	34±13	81±22
12. Piperonyl butoxide: 3,4-methylenedioxy-6-propylbenzyl n-butyl diethylglycol ether	200	2, 3, 9 20	0	0.1-	111±29	94±27
13. Chlordimeform: N,N-dimethyl-N'-[2-methyl-4-chlorophenyl]-formamidine	200	--	0	4.4-	50±19	98±15
14. TFT: 1,1,1-trifluorotetra-decan-2-one	200	--	0	0.8-	122±28	89±24

Continued on next page

Table 1. Con't.

Compound	Dose used for key (nmol.)	Previously tested for AJH activity	% Malformed Larvae[2]	T_{50} for tanning (hrs.)[3]	JHE Activity (% Control)[4] L5D3	L5D1
15. Carbaryl: 1-naphthyl N-methylcarbamate	200	--	7	0.8-	111±21	89±5
Evaluated Using the AJH Bioassay						
16. Methyl compactin: methyl 7-[1,2,6,7,8,8a-hexahydro-2-methyl-8-(2-methylbutyryloxy) naphthylenyl-1]-3,5-hydroxy-heptanate	100	5, 6, 29	0	1.3-	-	-
17. L-643,049-01K01: 7-[1(S),2(S), 6(R),7,8(S),8a(R)-hexahydro-2,6-dimethyl-8-(2,2-diethyl-butyryloxy)-naphthalenyl-1]-3(R),5(R)-hydroxyheptanic acid sodium salt	100[5]	--	89	5.7-	57±9	103±17
18. L-643,049-00H03: 6(R)-[2-[8(S)-(2,2-diethylbutyryloxy)-2(S),6(R)-diemthyl-1,2,6,7,8, 8a(R)-hexahydronaphthyl-1(S)]-ethyl]-4(R)-hydroxy-3,4,5,6-tetrahydro-2H-pyran-2-one	56[5]	--	10	1.7-	-	-

Table I. Con't.

Compound	Dose used for key (nmol.)	Previously tested for AJH activity	% Malformed Larvae[2]	T_{50} for tanning (hrs.)[3]	JHE Activity (% Control)[4] L5D3	L5D1
19. L-643,737-00S03: 6(R)-[2-[8(S)-(2-ethyl-2-methylbutyryl-oxy)-2(S),6(R)-diemthyl-1,2,6,7,8,8a(R)-hexahydronaphthyl-1(S)]ethyl]-4(R)-hydroxy-3,4,5,6-tetrahydro-2*H*-pyran-2-one	58[5]	--	83	3.8-	68±41	124±52

1. Sources of the compounds were as follows; 1,2 Calbiochem-Berhing; 3, P. Masner, Dr. Maag Ltd.; 4-6, G. Quistad, D. Schooley and G. Staal, Zoecon Corporation; 7,8, Sigma; 9, P. Magee, Chevron Chemical Company; 10,11, Aldrich; 11,12,13,15, Chem Service; 14, synthesiszed as described in (30); 16-19, R. Dybas, Merck, Sharp & Dohme.
2. Tanned and/or malformed larvae due to incomplete ecdysis or other causes.
3. Time for 50% of the treated larvae to tan and/or pupate relative (+ = before; - = after) to the controls.
4. JHE activity measured *in vitro* from larvae treated on L5D3 or L5D1. Values are means ± their standard devations.
5. Compound was injected. All others were applied topically.

treatment (i.e. flaccid, hemolymph accumulation in the abdomen, failure to migrate to the top of the rearing containers, dehydration). Likewise, chlordimeform modified the behavior of the larvae in the form of abnormal spinning and wandering behavior. Thus, neither of these compounds (at the dose tested) would be considered as a potential AJH. It should be remembered, however, that for some insect species the effective dose for a particular AJH may be near the toxic dose for that compound (see 22). One disadvantage of any *in vivo* bioassay, including this one, is that they will not detect the AJH activity of toxic molecules which, in an *in vitro* system, would function as AJHs.

As with the insecticides, FMev (a highly active AJH for the Lepidoptera; 22) also causes the formation of tanned larvae. While these tanned larvae may also be the result of general teratogenic or stress effects, the removal of the corpora allata (site of JH biosynthesis and release) also causes similar effects (34). Although the presence of tanned larvae seems to consistently correlate with AJH activity, it is imperative that the formation of tanned larvae not be used as the sole criterion for the determination of AJH activity. Rather, it is a single marker that is only of significance when coupled with Step 2 (below).

2. Is there a delay in time of pupation?
   Treatment: L5D3 larvae (9 AM); 200 nmol./larva.
   Expected Result: Treated larvae display a distinct (ca. 4 hr.) delay in the time of pupation/tanning. Results are expressed as the advancement '+' or delay '-' (in hrs) in the time of tanning/pupation relative to the controls (solvent only). Controls usually pupate/tan about 30 hr. posttreatment (L5D4, ca. 3 PM).
   A) YES - Compound may possess AJH activity - GO TO 3 for verification. Examples: FMev, chlordimeform (Table I).
   B) NO - STOP. Examples: JH I, JH III, epofenonane, hydroprene, ETB, precocene II, ethoxy-precocene, EPPAT, DFP, DEF, piperonyl butoxide, TFT, carbaryl (Table I).

Explanation: In many lepidopterans, including *T. ni*, the presence of JH in the prepupa seems to accelerate the time of ecdysis to the pupa (18,35-38). Conversely, a reduction in the JH titer due to an AJH causes a delay in the time of ecdysis to the pupae and/or the tanning process (18,37). This particular effect can be prevented, in part, by the coapplication of JH I (18). Thus, a delay in the time of tanning/pupation seems to be related to the ability of a compound to block JH biosynthesis/release or action, and hence act as an AJH.

Verification of AJH Activity

3. Is there reduced JHE activity in L5D4 larvae?
   Treatment: L5D3 (9 AM) larvae; 200 nmol./larva.
   Expected Result: Larvae are assayed 24 hr. posttreatment (L5D4, 9 AM, larvae are now prepupae) for JHE activity. A compound with AJH activity should cause the level of JHE activity to be lower than normal. Results are expressed as a percentage of the JHE activity present in larvae treated with only the solvent.

A) YES - GO TO 4. Examples: FMev, EPPAT, chlordimeform, DEF (Table I).
B) NO - STOP. Not likely to be an AJH for *T. ni*. Examples: JH I, JH III, epofenonane, hydroprene, ETB, precocene II, ethoxy-precocene, DFP, piperonyl butoxide, TFT, carbaryl (Table I).

Explanation: Hemolymph JHE activity in late last stadium larvae of *T. ni* is directly regulated by the JH titer (16-18). Thus, any compound that affects JH biosynthesis /release or action at JH receptor sites will cause a change in the prepupal peak of JHE activity. Compounds with JH activity stimulate JHE production, often to greater than normal levels (e.g. JH I, JH III, epofenonane, hydroprene and ETB; Table I). For an AJH, a reduction in the JHE activity is the expected outcome. However, a reduction in JHE activity can also be the result of an inhibitor that interacts directly with the JHE (see below).

4. Normal JHE activity in L5D1 larvae?
Treatment: L5D1 larvae (3 PM); 200 nmol./larva.
Expected Result: Treated larvae are assayed for JHE activity 1-2 hr. posttreatment. An AJH should have no effect on JHE activity in this short of a time period. Results are expressed as a percentage of the normal (control) JHE activity.
A) YES - Compound is an AJH. GO TO 5 for determination of mode of action. Example: FMev (Table I).
B) NO - STOP. Compound is a general toxicant and/or JHE inhibitor, and is not an AJH. Example: EPPAT (Table I).

Explanation: Step 4 insures that the potential AJH is not merely acting as a JHE inhibitor. Since there is always the possibility of bioactivation, an *in vivo* test is used. AJHs will have no effect on JHE activity in the time-frame used in this step (1 hour) while this amount of time should be more than sufficient for JHE inhibitors to act (e.g. EPPAT; 19,39,40). If the compound under consideration makes it through this part of the bioassay key, then it has some AJH activity.

AJH Classification (Optional)

5. Does the AJH block the juvenoid induced increase in JHE activity?
Treatment: L5D3 (9 AM) larvae; selected doses of the AJH (100 nmol./larva is a good starting place) are coapplied with the juvenoid, epofenonane (100 nmol./larva).
Expected Result: Larvae are assayed for JHE activity on L5D4 (9 AM). The JHE induction should not be affected if the AJH acts by blocking JH biosynthesis. Results are expressed as a percentage of the JHE activity induced by epofenonane (100 nmol./larva) alone.
A) YES - AJH is probably a suboptimal JH or a JH receptor antagonist. Example: ETB (Figure 1).
B) NO - AJH probably functions by directly blocking JH biosynthesis and/or release. Example: FMev.

Explanation: This section is included in an effort to provide a means to gain some insights into the mode of action of the test compound. Epofenonane appears to be a JH agonist for T. ni that is somewhat less active than JH I (16,26). Unlike JH I, epofenonane is not susceptible to ester hydrolysis, which eliminates the problem of the JHE affecting the level of its own induction. Like epofenonane, the JH agonist-antagonist ETB was able to induce the appearance of the hemolymph JHE in T. ni, but only at much higher dosages (Figure 1). However, at lower doses ETB caused a dose dependent reduction in the JHE activity induced by epofenonane in T. ni (Figure 1). Since other tests demonstrated that ETB was not a direct JHE inhibitor (26), it appeared that ETB was acting as a JH receptor antagonist (26). Unlike ETB, FMev appeared to have no effect on the JHE induction by epofenonane (41). This observation is consistent with other studies that show it to function as an inhibitor of JH biosynthesis (22,23). Thus, this assay can potentially provide information on the mode of action of an AJH. Running similar tests in ligated abdomens would further insure that the action being observed was probably due to competition for JH receptors (probably the fat body), and not due to other interactions with the brain, corpora allata, etc.

Using the Key to Test for AJH Activity. Besides the compounds used to develop the key, methyl compactin and three of its analogues (Table I) were evaluated for AJH activity (through step 4). Of the 4 compounds tested, both L-643,049-01K01 and L-643,737-00S03 (tested at 100 and 58 nmol./larva, respectively; injected) caused the formation of tanned malformed larvae (89 and 83%, respectively; Step 1) and a large delay (ca. 4-6 hr.) in the time of tanning/ pupation (Step 2) (Table I). Thus, both L-643,049-01K01 and L-643,737-00S03 appeared to possess AJH activity and were tested further.

Treatment (100 and 58 nmol./larva, respectively; injected) of L5D3 larvae with either of these compounds resulted in a 30-40% decrease in the prepupal JHE activity peak (Step 3), while L5D1 larvae treated (as above) with either compound possessed normal levels of JHE activity one hour posttreatment (Step 4) (Table I). These results confirm that both L-643,049-01K01 and L-643,737-00S03 are AJHs for T. ni.

Discussion

The bioassay-key presented here provides a relatively simple approach to identifying compounds with AJH activity. Obviously, the sensitivity of the assay is, in part, a function of the number and magnitude of the dosages used. In using the bioassay and key, it is best to start with as high of a dose as possible to rapidly eliminate the inactive compounds. In our assays 200 nmol./larva is typically used as the starting dose. In doing so, however, it should be kept in mind that JH agonists/antagonists, such as ETB (2,9,26), may only display AJH activity over a narrow dose range which may be far below our 200 nmol./larva starting dose. At this high dose only JH like activity may be seen. For example, it has been demonstrated that ETB can antagonize the JH action of the juvenoid epofenonane in T. ni (26, Figure 1) and yet be

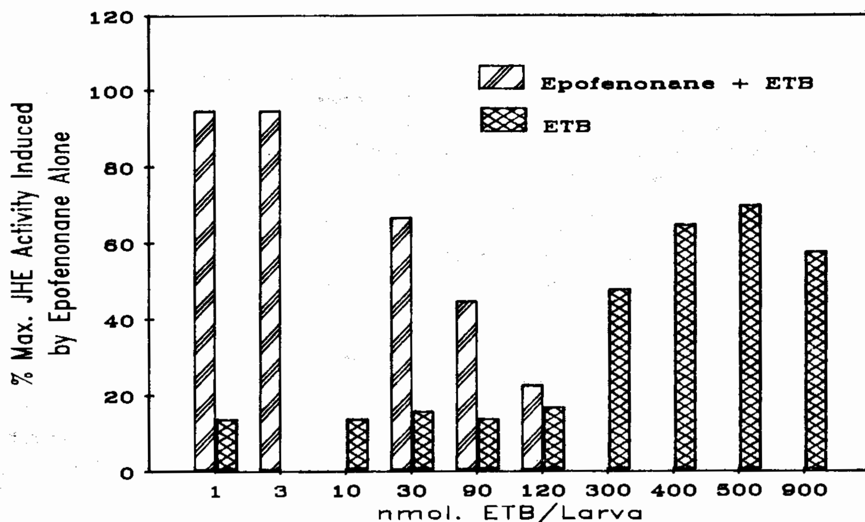

Figure 1: JHE activity in L5D3 (9 AM) larvae of _T. ni_ treated with epofenonane (100 nmol./larva) and selected doses of ETB (1-120 nmol./larva) compared to larvae treated with ETB (1-900 nmol./larva) alone. JHE activity is expressed as a percentage of the JHE activity produced by epofenonane (100 nmol./larva) alone. Data is adapted from (26).

devoid of AJH activity at doses tested in both the classical AJH bioassays as well as the one presented here (41 and see above). Likewise, ETB displays JH-like activity at high doses (≥ 200 nmol.; 18,26, Figure 1). In L5D3 larvae of *T. ni*, JH activity in the whole animal is manifested by normal wandering and prepupal activities leading to apparently normal pupae from which, however, there is no adult emergence (18). Thus, if apparently normal pupation occurs, but no adult eclosion is forthcoming, then it is possible that the compound, at the dose being tested, is acting as a JH.

With any new procedure, there is always the concern for its proper use. In our hands, the results of the first two steps of the bioassay, which easily can be combined into a single test, have provided a very consistent test of AJH activity that has, to date, always been confirmed by the later steps in the bioassay. However, care must be taken in the evaluation of compounds that display toxic effects. As already mentioned, pesticides like paraoxon can produce tanned larvae and this tanning process can be very greatly delayed relative to the controls (solvent only). However, since the larvae that display these effects were behaving in an abnormal/intoxicated manner (i.e. flaccid, lack of activity when probed, hemolymph accumulation, failure to spin, etc.), the results should be discounted. As a safety measure, however, it is important to test all suspected AJHs as described in steps 3 and 4. This process will insure that compounds that are general toxicants will not be mistakenly assigned as AJHs. Finally, it is suggested that appropriate controls (solvent only) be run at all times since there can be some day to day variation in the exact time of ecdysis to the pupa and/or tanning. If at all possible, a standard such as FMev should also be tested occasionally to insure that the assay is working properly.

Unlike some of the more classic AJH bioassays, the one presented here allows for the rapid (ca. 36 hrs) elimination of inactive compounds, saving the more intensive labor for only the more promising compounds. An additional advantage is that only a relatively small amount of material is needed for the bioassay. Since the test organism, *T. ni*, is a major economic pest insect with a broad host range, it can be assumed that, unlike *M. sexta* or *O. faciatus*, it has a rather well developed detoxification systems. Thus, compounds active on *T. ni* are more likely to be active on other economically important insect pests.

The bioassay presented here for *T. ni* is based on a growing body of knowledge that has been accumulated during the last decade. While few other insects have been so well studied in terms of endocrine regulation of metamorphosis, it may be possible to adapt this bioassay-key to other less studied, but economically more important insect pests, such as *H. virescens*, *S. frugiperda* and *P. includens*. Likewise, this bioassay should be easily adapted to *M. sexta* for which there is a wealth of endocrinological information (42,43).

Compactin exhibits AJH activity in several insects (5,6,29) and seems to function as an inhibitor of 3-hydroxyl-3-methylglutaryl-CoA reductase in both the rat (44,45) and *M. sexta* (5). Although the methyl ester of compactin was inactive as an AJH in our bioassay, two of its analogs (L-643,049-01K01 and L-643,737-

00S03) did display AJH activity. Thus, for *T. ni*, analogs of compactin would appear to be good candidates for continued exploration as potential AJHs.

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