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THE AZADIRACHTINS – POTENT INSECT GROWTH INHIBITORS*

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In the course of their coevolution with insects, plants have learnt to protect themselves by chemical means. Semiochemicals act as antifeedants or deterrents, others by disrupting growth and development. By use of the Epilachna varivestis *bioassay we isolated from* Azadirachta indica *seed a group of triterpenoids which interfere with larval growth and development in ppm range. Main components are the azadirachtins A and B with identical biological activity. Various other azadirachtins were obtained, either as minor seed components or by chemical modification of the naturally occurring compounds. Structure vs. activity relation studies enabled us to postulate a basic structural element that should still be biologically active and with much simpler chemical structure than natural compounds.*

What underlies the biological activity of these insect growth inhibitors? Their interference with the hormonal regulation of development and reproduction has been studied in Locusta migratoria *and* Rhodnius prolixus. *In addition, tritiated dihydroazadirachtin A was used. With this approach, a precise correlation between administered dose, resulting effects, and retention of the compound was established. The azadirachtins either interrupt, delay, or deviate whole developmental programs. Results from these studies provide another chemical probe for studies in insect endocrinology and physiology.*

Azadirachtin is a feeding inhibitor and growth disrupting compound for most insect orders. It is present in the seeds of the neem tree, *Azadirachta indica* A. Juss (Butterworth & Morgan, 1968; Ruscoe, 1972; Steets & Schmutterer, 1975; Rembold et al., 1980b; Schmutterer & Rembold, 1980; Kubo & Klocke, 1986). Its former structure as proposed by Zanno et al. (1975) has recently been reassigned by three laboratories (Bilton et al., 1987; Kraus et al., 1987; Turner et al., 1987). This structure (Fig. 1) now unequivocally gave the basis for structural elucidation of the other isomeric azadirachtins by nmr spectroscopy.

A bioassay for detection of a whole group of natural insect growth inhibitors, as present in neem (Schmutterer & Rembold, 1980), has to combine high sensitivity for growth disruption with high tolerance for antifeedants. The Mexican bean beetle, *Epilachna varivestis*, combines these two attributes under simple test conditions. Two tests have been described for routine assays, a Petri dish test for individual larvae and a cage test for groups of larvae (Rembold et al., 1980b). The test insects are reared on bean leaves, *Phaseolus vulgaris*,

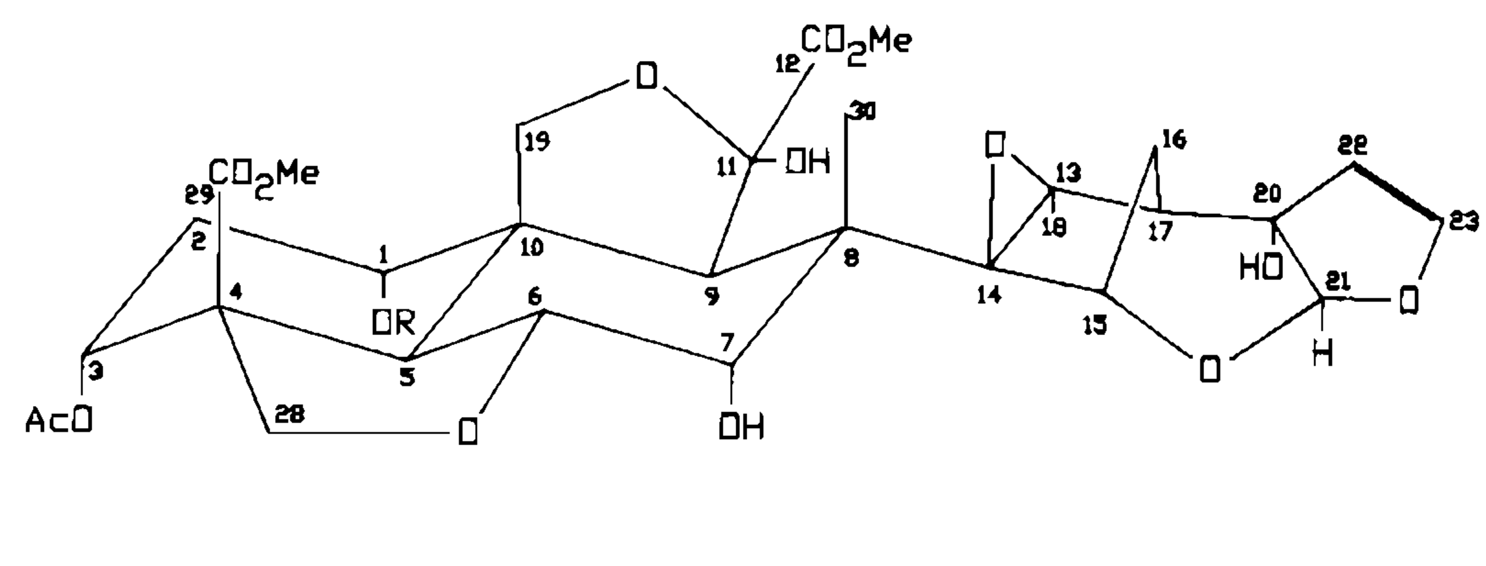

Fig. 1: Structure of azadirachtin A.

and their weight gain and survival is followed, during the first two days on the treated, and then on untreated bean leaves.

The azadirachtins

Azadirachtins is difficult to isolate and the yields are usually low. Azadirachtins A (Fig. 1) and B (Rembold et al., 1984) were obtained, out of 27 kg neem seed, in an amount of 3.5 and 0.7 grams, respectively, after extensive chromatographic purification by HPLC (Rembold et al., 1987; Forster, 1988). A series of minor bioactive compounds was further obtained in milligram quantities in pure form and named azadirachtins C - G (Forster, 1988). They all have in common a high structural similarity to the main compound, azadirachtin A, and a similar biological activity in the *Epilachna* assay. They all induce three different biological effects, depending on the amount of

*Dedicated to Professor Dr. h.c. mult. A. Butenandt on the occasion of his 85 th birthday.

substance applied, namely, (a) toxic effects if applied in high concentrations of more than 1000 ppm, (b) in concentrations between 10 and 100 ppm they are very active phagorepellents, combined with growth disrupting activity; (c) in concentrations between 1 and 10 ppm, all the azadirachtins interfere with growth without any phagodeterring effect. In this concentration range their mode of action can be studied as that of an ideal insect growth inhibitor. Azadirachtin seems to inhibit feeding at much lower concentrations in hemimetabolous than in holometabolous insects.

All the neem compounds which are biologically active in the *Epilachna* assay below 10 ppm have several structural features in common (Forster, 1988) which will be discussed briefly.

(I) The type of substitution at the decalin rings A and B is critical; free hydroxyl groups at C-1 an C-3 increase the growth inhibitory activity of the azadirachtins. However, there is a fundamental difference between the ecdysteroid and the azadirachtin structures. The ecdysteroids have the two decalin rings *cis*-connected, whereas they are in *trans*-configuration in the azadirachtins.

(II) The 22,23-double bond which is present in all the natural azadirachtins isolated so far, can be hydrogenated – or tritiated, as will be shown in the following – resulting in even a slight increase in biological activity. The labelled azadirachtins can therefore be used for studies on the mode of biological action without any reservation.

(III) Structural variation at position 11 also affects the growth inhibitory activity. It seems to be important not to have a group at this position which sterically hinders this area.

(IV) The most critical structural element is the epoxy group at position 13, 14. Removal of this groups ends up with compounds which are completely inactive in the *Epilachna* bioassay.

Mode of azadirachtin action

The azadirachtins seem to have a higher repelling effect against hemi- than against holometabolous insects. Even in the heteropteran, *Rhodnius prolixus*, only high doses of azadirachtins A and B had an antifeedant effect when given through a blood meal, whereas molt inhibition was observed at hundred- to thousandfold lower doses already (Garcia et al., 1984). On the other hand, all the four lepidopteran pest larvae, *Heliothis zea, Heliothis virescens, Spodoptera frugiperda*, and *Pectinophora gossypiella* were inhibited from feeding at lower azadirachtin doses than needed for growth inhibition (Kubo & Klocke, 1982). There must be two different receptor types, therefore, which interact with azadirachtin. What do we know about its effect on insect growth?

Effect on ecdysis of the insect larva

Treatment of the insects or of their food with azadirachtin causes growth inhibition, malformations, mortality, and reduced fecundity (Steets & Schmutterer, 1975; Schmutterer & Rembold, 1980; Redfern et al., 1981; Rembold et al., 1981). A detailed study of these effects on *Locusta migratoria* larvae showed a typical dose-dependence on the responding animals (Sieber & Rembold, 1983). At a dose of 2 μg per gram animal, no larva was able to undergo or to terminate ecdysis, whereas at 0.6 μg/gram only about 10% of the larvae reacted. The intermolt of azadirachtin-injected larvae varied between 8 and 60 days, whereas the control larvae showed a period of 6 (fourth instar) resp. 9 (fifth instar) days. Similar results were found after injection of 1-2 μg azadirachtin per gram fresh weight into *Bombyx mori* fifth-instar larvae (Koul et al., 1987). Even more dramatic effects are induced in *Rhodnius prolixus* after fed on a blood meal (Garcia & Rembold, 1984). The effective dose that prevented ecdysis in 50% of the nymphs was $4x10^{-4}$ μg/ml of blood and doses higher than 1 μg/ml inhibited ecdysis by 100%. A single treatment with azadirachtin was enough to inhibit any molt even after a period of five months.

Effects on the adult insect

After a single injection of 10 μg azadirachtin into a *Locusta migratoria* female between days 2 and 13 after emergence, about 60% died during the following four days and all of them lost weight in the range of about 50%. If injected between days 2 and 10 during the phase of previtellogenesis, no maturation of the terminal oocytes was observed. Injection between days 10 and 13 resulted in ovaries with almost mature oocytes. Similar to the effects in the larva, there is also a sensitive phase visible during oocyte development. Most of the treated locusts had no oviposition and only ecdysteroid traces were present in the ovaries (Rembold & Sieber, 1981). Similar effects can be induced by injection of anti-brain-antibodies. Such antibodies to brain material from *Locusta migratoria* females were raised in rabbits. *In vivo* injection into young locusts also inhibited ovary development, indicating a possible blockade of allatotropic or gonadotropic activity (Rembold et al., 1980a).

Similar azadirachtin effects are reported from other insects. In *Oncopeltus fasciatus* it affects longevity, fecundity and hatchability of eggs from the treated parents (Dorn, 1986). In *Schistocerca gregaria*, after injection of 2 μg/g of the compound into newly hatched females, it completely inhibited growth. No substantial increase in ovary weight was observed (Subrahmanyam & Rao, 1986).

Effects on the endocrine system

What is the endocrine basis for the effects of azadirachtin on the growing larva? Such reactions like inhibition of metamorphosis indicate an interaction of the compound with the hormone system of the treated larva. There is a pronounced effect on control of ecdysteroid titer as first demonstrated in fifth instar *Locusta migratoria* (Sieber & Rembold, 1983). The authors explain the effect with an interference of azadirachtin with the neuroendocrine system. This argument is supported by histological studies which show an increase of neurosecretory material in the *pars intercerebralis* of azadirachtin treated last-instar locusts (Rembold et al., 1981; Sieber & Rembold, 1983). Concomitantly with the ecdysteroid also the juvenile hormone synthesis is affected by azadirachtin (Rembold, 1984; Uhl, unpulb. results).

The effect of azadirachtin on neural control centers is also indicated by changes in behavior. As discussed, application to early larval stages of *Locusta migratoria* extends duration of the larval stage to several weeks (Sieber & Rembold, 1983). Such larvae show a sexual behavior like adults (Shalon & Pener, 1984) and flight pattern formation, the flight muscle activity resembling the flight motor pattern of young locusts (Kutsch, 1985). Azadirachtin treatment of *Leucophaea maderae* shortens the period length of the locomotor activity rhythm in the circadian rhythm and induces splitting of this rhythm into two components (Han, 1986).

The azadirachtin effect on molting processes and ecdysteroid titers has also been confirmed in *Rhodnius prolixus* (Garcia et al., 1984). ATP, a phagostimulant, if added to the blood together with azadirachtin, reverses its antifeedant activity. Azadirachtin, if injected into 4th-instar nymphs of *Rhodnius prolixus* after a blood meal, affects molting, mortality, ecdysteroid titers and consequently also the mitotic index of the cuticle (Garcia et al., 1986).

One can generalize from these findings that azadirachtin irreversibly or at least for an extended period of time blocks and sometimes changes developmental programs, also such which are normally expressed in the next instar only. Some examples are the adultoid characters of permanent larvae of *Locusta migratoria* (Shalom & Pener, 1984; Kutsch, 1985), of *Oncopeltus fasciatus* (Dorn et al., 1986) and of *Manduca sexta* (Schlüter et al., 1985). Nothing is known about the molecular basis of these irreversible events. Tracer studies with tritiated 22,23-dihydroazadirachtin A only showed, that practically all of the excreted radioactivity was identical with the administered compound and the same holds true for the radioactivity which is retained in the insect (Rembold et al., 1984).

The amount of azadirachtin retained in the insect is extremely low. It is selectively bound to membranes, as shown with the example in Fig. 2. The section through the Malpighian tubules shows by the deposited silver grains a significant radioactivity in the basal region and the nuclear membrane only. No radioactivity is seen in the lumen of this excretory organ which indicates a high-affinity binding of the adminstered labelled azadirachtin to specific membrane sites. Whether highly specific membrane receptors in the brain region, which are responsible for the controlled release of such organotropic signals like PTTH or ATH, are blocked by azadirachtin, is still an open question which is being studied in our laboratory.

Conclusions

It has become clear from our studies, that azadirachtin shifts and decreases ecdysterone, juvenile hormone, and vitellogenin peaks to a later time concomitantly. What does that mean in terms of endocrine regulation? The two metamorphic hormones are under control of the *pars intercerebralis* and all the experimental facts indicate an interference of azadirachtin with the neuroendocrine control of metamorphosis. That could be achieved by feedback control of the neurosecretory system by the hormones synthesized at the periphery and circulating in the haemolymph. A structural homology of azadirachtin and ecdysone was dis-

Fig. 2: Localization of ^{3}H-dihydroazadirachtin A in the Malpighian tubules of *Locusta migratoria* by autoradiography. Heavy accumulation of silver grains is predominantly on the basal region and the nuclear membrane. The picture shows a cross section of the tubule from a locust injected 2.5 μg of 22, 23-dihydroazadirachtin (spec. act. 15.38 Ci/mM) per gram body weight. The compound was injected into a three day old female and the Malpighian tubules fixed for autoradiography five days later. Four micron sections were exposed for 25 days to Kodak NTB 2 emulsion. B: basal region; L: lumen; N: nucleus. Courtesy of Dr. B. Subrahmanyam.

cussed to be responsible for blockade of the binding sites for the hormone (Kåuser & Koolman, 1984). However, such an "anti-ecdysteroid" function can be ruled out for simple stereochemical reasons, as has been discussed already. There are some structural similarities between all the azadirachtins. Due to the results from tracer experiments, the amount of azadirachtin which is retained in the insect is extremely low. Such a trace of inhibitor can be enough in terms of very special receptor sites which trigger the many endocrine, and as a consequence of that also the morphological and behavioral effects. These can last for long, in some cases like in *Rhodnius*, even for the whole lifetime. More and more indirect proofs are coming up for a very central target of azadirachtin binding and it will be one of the most stimulating future tasks of basic studies to find the molecular basis for this target. This answer will also stimulate more research in new strategies of chemical insect control.

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