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Azadirachtin—A Botanical Insect Growth Inhibitor and Its Relation to Biosemiotics

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Insects like any other living organism must, for their survival, interact with their environment. A signal-mediated strategy of inter-individual communication is called "biosemiotics" and plays an integral role in ecology. Honeybees with their high social colony structure represent the zenith of chemisociality. Many insects make, besides other signals, use of species-specific plant metabolites (kairomones) as distance and contact-perceivable semiochemicals. Azadirachtin is a botanical insect growth inhibitor which induces dramatic changes in insect growth, development and reproduction. Here too, signal-mediated communication is affected by the compound, as demonstrated with *Rhodnius prolixus*, the vector for transmittance of Chagas disease. The azadirachtin-treated insect is no longer attractive to or viable for the parasitic flagellate, *Trypanosoma cruzi*. Azadirachtin has a highly oxidized triterpenoid structure and its total synthesis has not yet been achieved. Calculations on quantitative structure-activity relationships support the hypothesis that the whole complicated molecular structure is involved in its biological activity. Based on the available experimental data a mode of azadirachtin action is discussed finally.

Key Words: Azadirachtin, Honeybee, Biosemiotics, Insect growth inhibitor, Kairomone, QSAR, *Rhodnius prolixus*, *Trypanosoma cruzi*

Introduction

Biochemistry, at the beginning and up to the first half of this century, was based on bio-organic chemistry and consequently on gaining pure natural compounds and elucidating their chemical structure. Biocatalysts were isolated by use of sophisticated bioassays and classified as vitamins, hormones, semichemicals, drugs, poisons. Having by this procedure identified most of the contents of life's tool box, biochemistry began to concentrate on dynamic aspects of metabolism and bioenergetics which finally ended up in the climax of today's molecular biology. Each biochemistry textbook reflects the reductionistic attitude of this field of biosciences and therefore comes to the same construction of biomolecules, mode of enzyme action, metabolism, and gene expression. The search for "lead structures" in bioactive natural products helps in the designing of new drugs or pesticides. However, is this the real "chemistry of life" already or are we missing some vital aspects which also should attract the chemist's interest? No doubt about the fact, that biology and primarily medicine are confronting us with such complicated and therefore unresolved problems as cancer, new diseases like AIDS, the rapidly increasing tolerance of pest insects and rodents against any type of toxic chemicals, or the reaction of our ecosystem on climatic changes. Can we expect some solution of these urgent problems from an explanation of biology by chemical and physical mechanisms? Are we able to deal with "information-driven systems" (Albrecht-Buehler, 1990) on the basis of applied physics of molecular interactions, or do these open problems simply demonstrate a dead end in our biochemical approach of life?

What shall be discussed to some extent in the following, has nothing to do with an epistemological view of nature and especially of biochemistry. I shall much more consider a biochemist's daily problems with the interpretation of his experimental results in the present reductionistic way of life sciences, which admittedly is our only chance for finding some solutions at least. We have experienced for quite a long time the solid basis of linear theories like the Darwinistic view of evolution with its essence of a convincing advantage for such species which through purely accidental variation in their DNA-based genetic program have become superior in their fitness to competitive species. In evolution, therefore, nothing but genetic engineering or is it much more complicated, as Lovelook (1991) argues in his GAIA hypothesis, the results from a feedback interaction of each organism with its total environment? The evolutionary process has come out as a total palet of internal momentums like self organization, iterative feedback loops, chaos, and coincidences of non-linear processes (Wesson 1991).

The Biosemiotic Concept

We have recently presented biosemiotics as paradigm of biology with the emphasis on biological signalling on the verge of deterministic chaos (Eder & Rembold 1992). Biosemiotics offers an interdisciplinary approch to the diversity and irregularity of living systems. The basic tenet is that biology, on all levels from molecular biology to ecosystems, can be viewed and investigated as communication, and that life processes can be defined as sign-mediated interaction. Biology, according to the biosemiotic view, is in itself and in all its aspects, natural semiotics with a pronounced proximity to deterministic chaos. The signal becomes a viable key and a practically feasible access to the understanding of life. For the biochemist this means to come behind nature's secret in balancing its fantastic biological diversity. Only after we have learnt to really understand the secret behind an evolutionary step that we can make use of this knowledge for our own profit. There are many models available which can guide us in this direction. Some of them may be mentioned briefly form our own fields of research.

- The model *par excellence* for a biosemiotic treatment are the social insects. Insect societies represent a climax of biological integration. Semiotic aspects are at the heart of any social grouping, and social organization is always based on some sort of language-mediated communication. Within the languages and dialects used in insect societies, the chemical language seems to be a universal one, an aspect that has already been included within the term *chemisociality* (Blum & Fales 1988). Honey bees clearly represent the zenith of chemisociality. A large variety of behavioral modes has been demonstrated to be regulated by pheromonal signals originating from diverse exocrine glands of either worker or queen. The caste system above all and the resulting division of labour display a communicative elegance which points to a close linkage between biosemiotic structures and social organization (Wenner 1968, Rembold 1987).

- Within the context of interspecific chemical communication, sign-mediated relationships between phytophagous insects and their host plants represent an outstandingly rewarding topic. In the course of insect/plant co-evolution, a number of plants have learned to protect themselves by means of semiochemicals which can be successfully investigated within a biosemiotic perspective. Repelling substances in leaf exudates are distance-perceivable or contact signals. Modifying the behaviour of pest insects they are resistance factors and contribute to a partial protection of the plant. Both crop plants and their insect pests are often imbedded in changing agroclimatic conditions and respond to stress factors in their specific ways. The emergence of kairomones was a significant achievement for the benefit of the insect. Plant volatiles are important cues directing the insect towards its host plant. In the absence of visual orientation, they are essential for night-active phytophagous insects and, in some cases, appear to control their reproduction (Rembold 1988).

- Insects are often extremely specific vectors of tropical diseases. Chagas disease is caused by a parasitic flagellate, *Trypanosoma cruzi*, which develops and reproduces in an insect as intermediate host. The host-parasite relationship between the kissing bug, *Rhodnius prolixus* and *T. cruzi* is a distinctly biosemiotic phenomenon, with the insect's hormones and their titer changes acting as signals. As will be discussed later, azadirachtin affects the bug's hormone system and also disrupts the carefully synchronized arrangement between the parasite and its intermediate host in a lasting way. An azadirachtin-treated insect is affected in such a fundamental way, that it loses its biosemiotic identity and, for this reason, is no longer attractive to or viable for the parasite (Rembold & Garcia 1989).

Azadirachtin—Lead Structure of a New Insecticide Generation?

Plants are, like any other higher organism, artists in chemical synthesis. To give a general idea, each plant produces around 2,000 different chemical structures of low molecular weight, and about as many in the high molecular range. Very few of them are biologically active against other organisms. For the simple reason that, if the plant product were of general biological activity or even toxic, the host had to develop a careful protection system against its own product. Alkaloids, for example, are often stored in special organs or are present in the plant as inactive precursor molecules, like glycosides, peptides or esters of mineral acids which become toxic after fermentation only. Many natural products cannot pass the barrier of the root or the intestine. The plants, during their co-evolution with the insect kingdom for millions of years, have developed species-specific secondary metabolites which first had the task to repel phytophagous insects from oviposition or from feeding. Some insects in turn have become adapated to such plant signal and learned to use plant-born defence compounds as kairomones for their own host finding strategy or even as precursors for their sex or aggregation pheromones.

The seeds of the neem tree, *Azadirachta indica* A. Juss, are rich in oil and in limonoids with highly insect-specific effects. The most active component is a whole group of azadirachtins (Rembold 1989) which, for some insect species, are feeding repellents, for others growth inhibitors at low doses already. No toxic effect against mammals has been found, not even at very high doses. Inhibition of feeding and of growth are two effect which are completely independent from each other. The desert locust, *Schistocerca gregaria*, will not even touch a plant treated with azadirachtin in trace amounts, whereas the Mexican bean beetle, *Epilachna varivestis*, will feed on relatively high azadirachtin doses without any visible starvation. But this species is extremely sensitive against the growth-inhibitory effect of these compounds.

When discussing chemical structures and their relation to biological activity, one first has to define the type of biological activity. This depends on the test insect's sensitivity as well as on its selectivity to a certain compound. Some insects may be able to degrade the substance concerned, and others will not even resorb it when added to their food. Sometimes it will even be necessary to inject the compound in order to bypass an intestinal barrier or chemoreception at the mouthpart of the insect. Biological activity against an organism must be carefully defined therefore. In the followig the state of structural work will be presented based on the standardized and reproducible *E. varivestis* bioassay and on statistically significant data from natural azadirachtins and from a series of azadirachtinoids which are chemically modified azadirachtins.

Biological Effects of Azadirachtin

With the help of last instar *E. varivestis* larvae for bioassay under standard conditions, twelve azadirachtins could be isolated from neem kernel extract. Most of them were structurally identified and could then be compared in their growth inhibitory activity and structural differences—main component being azadirachtin A (figure 1). All the representatives A-M of the azadirachtin group induced a 50% growth inhibition in the range of 0.3-2.8 ppm under the standard *Epilachna* test conditions. Tritium-labelled azadirachtin demonstrates a rapid excretion by the insect within very few hours after injection and as unmetabolized, intact molecule. The residual radioactivity is concentrated in the neurolemma of the insect's brain and in its corpus cardiacum, the neurohemal organ which excretes hormonally active neuropeptides into the bloodstream. The extremely insect-specific mode of action induces a dramatic change in the neuroendocrine control of hormone levels and subsequent disturbance of growth, development and reproduction of the treated insects. Most of the treated insects will die due to their reduced physiological

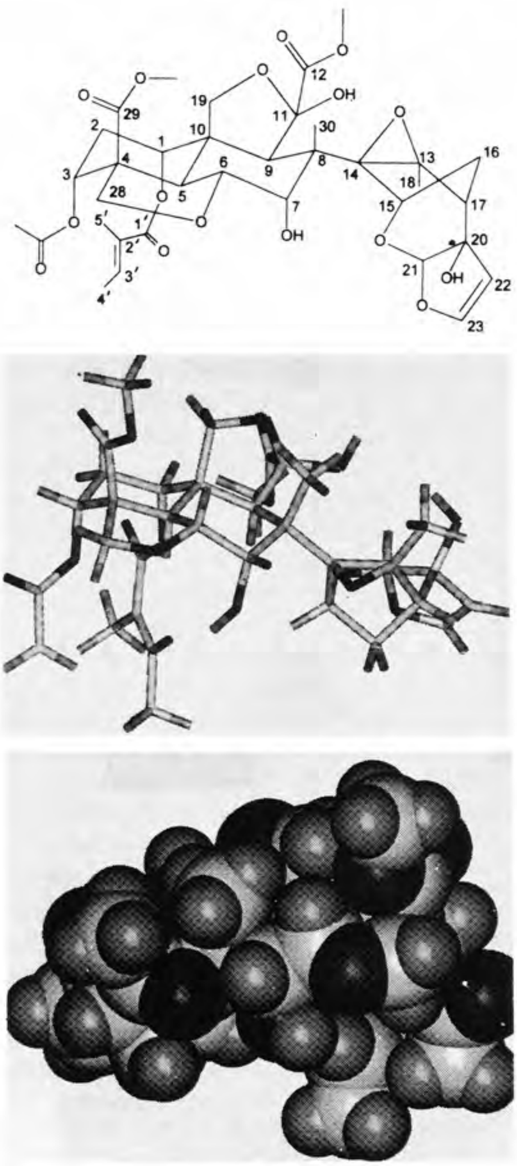

Figure 1 Chemical structure of azadirachtin A, the main representative of this whole group of insect growth inhibitors from the neem tree. The usual structural representation is given on top with the two six-membered rings from C_1-C_{10} and the furane ring moiety on the right side with C_{13}-C_{23} and the C_8-C_{14} link of the two moieties of the molecule. An energetically minimized computer model of the same limonoid is represented by the lower structures.

fitness, difficulties in shedding off their cuticle during molt, or due to malformations. As mentioned already, the kissing bug, *R. prolixus*, even fails as an intermediate host for its traditional parasite, *T. cruzi*. The extremely target-specific mode of azadirachtin action releases a whole cascade of events on the cellular level.

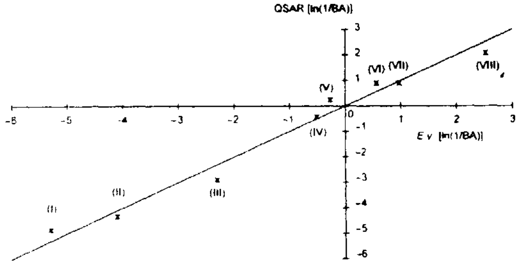

Figure 2 Data from a quantitative structure activity relationship (QSAR) calculation and from the *Epilachna varivestis* (E.v.) bioassay show a convincing correlation of chemical structure and biological activity for a selected grop of seven azadirachtins and azadirachtinoids (*BA*, biological activity)

Azadirachtin Structure and Biological Activity

Which of the structural elements are responsible for the biological activity within this 40-carbon complicated molecules? All the eight structurally identified natural substances show more or less the same activity. They all differ only in their peripheral structural elements, whereas both the two-ring decalin and the furane moiety (comp., figure 1) are present in all the eight azadirachtins. In order to find the smallest chemical structure which still maintains the typical biological effect, the natural azadirachtin A had to be modified in such a way that it lost its growth inhibitory effect in the *E. varivestis* bioassay either partially or completely. From a whole series of azadirachtinoids a small group could be identified which demonstrates three critical elements which must be present in the azadirachtin structure for inducing the typical effect on the insect endocrine system. The molecule must have free rotation of the two moieties around the C_8-C_{14} link which is necessary for the optimal reactive configuration. The epoxide ring is the alkylating agent of the molecule and therefore the second important structural element. Thirdly, the hydroxyl groups at positions 1 and 3 (figure 1) are the anchors which are binding to a highly specific receptor. Calculations on quantitative structure-activity relationships (QSAR) are in line with the biological effects (figure 2) and are as such supporting our hypothesis on the mode of action of this new type of insecticide.

According to this hypothesis, the bulky molecule shields the epoxide off from any chemical attack and the azadirachtin therefore can maintain its sensitive structure, as long as the *p*H is near to 7. When it comes to its target which seems to be a highly specific receptor in the neurolemma of the brain and where feedback receptors are to be expected, azadirachtin binds with its decalin ring moiety to its specific receptor. Then the furane moiety brings the epoxide thrugh rotation around the C_8-C_{14} axis into the vicinity of a proton donor which is followed by an immediate alkylation by the epoxide. The receptor consequently is inactivated and no more available for its controlling function.

Azadirachtin and Its Relation to Biosemiotics

Azadirachtin is a natural product which almost ideally represents an insect-specifi control agent. Its highly reactive epoxide ring is stable during its transport inside the insect with the consequence, that the molecule can be detected unmetabolized even weeks after application. And it attacks a highly specific target molecule, which due to its non existing mammalian toxicity is not present in mammals. By switching off one component in the whole regulatory network, the endocrine situation is being modified in an azadirachtin--specific way. All the many signals which directly or indirectly are responsible for the maitenance of homeostasis in the treated insect, are changed in their titers, as exemplified by the serotonin pools or by a dramatic change in the polypeptide pattern of the brain primarily. Azadirachtin has come out for the insect endocrinologist as an extremely sensitive chemical probe which now can unlock many of the secrets which we have to solve when thinking of a less aggressive, much softer control of insect pests and of transmittable diseases.

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