



Azadirachtin, a scientific gold mine

E. David Morgan *

Chemical Ecology Group, Lennard-Jones Laboratory, Keele University, Keele, Staffordshire, ST5 5BG England, United Kingdom

ARTICLE INFO

Article history:

Received 27 August 2008

Revised 7 November 2008

Accepted 17 November 2008

Available online 9 December 2008

Keywords:

Antifeedant
Botanical pesticide
Deterrence
Growth disruption
Limonoid
Natural pesticide

ABSTRACT

Azadirachtin is a highly interesting compound both for its chemical structure, which required 18 years to solve, and its synthesis, which required another 22 years, and for its biological properties as a feeding deterrent for many insects and a growth disruptant for most insects and many other arthropods. Its mode of action, structure–activity relationships, and its biosynthesis still require much research. A valuable natural pesticide, it has very low toxicity for vertebrates, and yet it has still not achieved a prominent place among pesticides and in many countries it is not yet licensed for use. An attempt is made to understand its failure to capture a larger market, 40 years after its discovery.

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1. Introduction

Azadirachtin (**1**) (Fig. 1) is a triterpenoid of the class of limonoids, found in three species, the trees *Azadirachta indica*¹ (Rutales: Meliaceae), *A. excelsa*², and *A. siamensis*.³ It is chemically interesting because of its complex structure and the challenge its synthesis provided; and biologically interesting because it is a feeding deterrent for some insects and a growth disruptant for most insects, and many other arthropods and species in related phyla. It is, moreover, remarkably non-toxic to vertebrates. It has been the object of intense research by chemists and biologists for 40 years. The subject has been reviewed many times, from both chemical, and biological viewpoints. This review considers it from a new aspect and in this 40th anniversary of its first isolation.⁴

2. History

It was well known that swarming desert locusts eat most growing plants but do not eat the leaves of *A. indica*, from this point known as the *neem* tree, its most common name. A project to isolate the substance unattractive or deterrent to locusts was begun at Keele University in 1966, as part of a wider study on locusts. By a stroke of serendipity, the leaves, which contain very little azadirachtin, were not easily available, but the seeds, the only good source of the compound, were a commercial product in India. By isolation, monitored by feeding tests with the desert locust

Schistocerca gregaria, the pure compound most potent in feeding deterrence was isolated and named azadirachtin, from the botanical name of the plant, and work commenced on determining its chemical structure. In response to a paper claiming the discovery of the feeding deterrent in the seeds, a compound of quite different structure and of inferior deterrence, called meliantriol⁵ (**2**) (Fig. 2) a brief paper on the isolation of azadirachtin from the seeds of neem was published.⁴ This was followed by details of its isolation and feeding deterrent effect.⁶

Using material from the original isolation, it was shown that azadirachtin acted systemically,⁷ being taken up by leaves from the soil, and more importantly, it had a growth-disruptant effect for those insects which were not deterred from eating it.⁸ The publication of Rachel Carson's book 'Silent Spring' in 1962 bringing to the attention of the world the problems of persistent pesticides, and the banning of DDT in the USA in 1972 intensified the interest in such 'natural' pesticides. The discovery of azadirachtin and its properties therefore came at an opportune time.

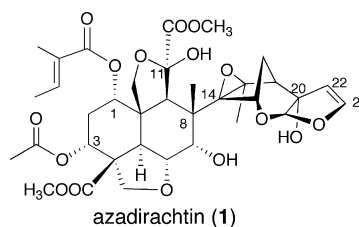


Figure 1.

* Tel.: +44 1782 680712; fax: +44 1782 712378.

E-mail address: e.d.morgan@chem.keele.ac.uk.

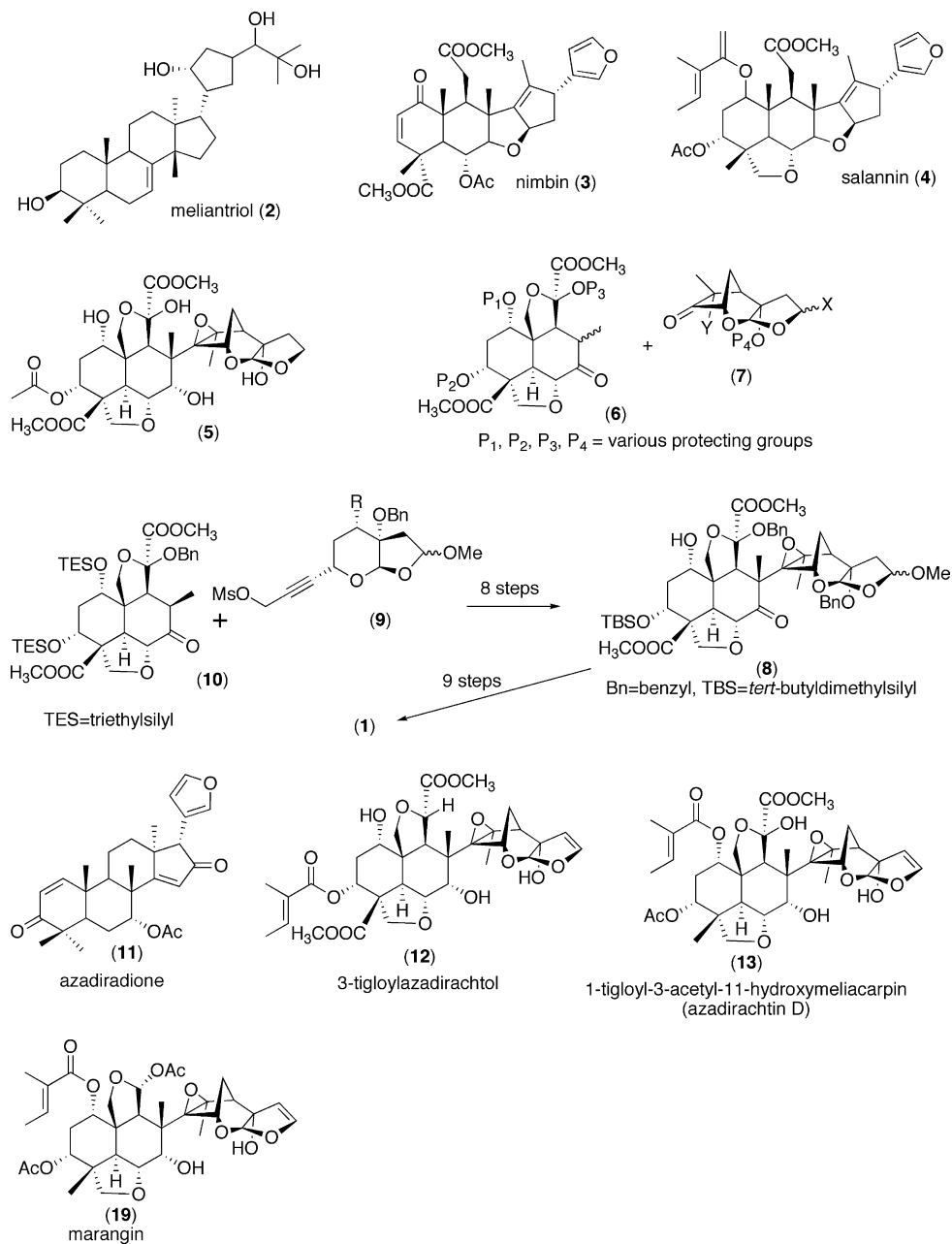


Figure 2.

3. Chemistry

Azadirachtin was initially obtained, by repeated fractionation of a solvent extract of powdered neem seeds, as a microcrystalline powder, mp 154–158 °C (UV absorption λ_{max} 217 nm, ϵ 9000) soluble in polar organic solvents, and slightly soluble in water. It had no molecular ion, initially leading to an incorrect formula,⁴ but accurate mass determination on a trimethylsilyl ether gave the formula $C_{35}H_{44}O_{16}$. In 1990, by repeated preparative HPLC, very pure crystalline azadirachtin was obtained⁹ with mp 160 °C, later corrected to mp 174 °C with $[\alpha]_D^{25} -71.4$ (c 0.21, CHCl_3).¹⁰ From its available chemical, chromatographic and spectral properties it was concluded that the compound belonged to the tetranortriterpenoids, and was related to the compounds nimbin (3) and salannin (4) (Fig. 2) already known from the seeds of neem. The presence of 16 stereocentres in azadirachtin, seven of these quaternary and nine secondary, and 16 oxygen atoms,

arranged in four ester groups, two hydroxy-groups, a hemiacetal, an epoxide and a dihydrofuran group, made structure progress slow.¹¹ It was only with developing techniques in NMR spectrometry, particularly two-dimensional correlation methods, that better progress could be made. A structure for azadirachtin was proposed in 1976¹² but though it was wrong,¹³ it was difficult to prove it so. A crystal structure for a partly degraded hydrogenated product (5) of azadirachtin was finally obtained, which enabled the correct structure of azadirachtin (1) to be proposed.¹⁴ It was confirmed with further NMR information,¹⁵ with two other research groups publishing the same structure simultaneously.^{16–18} The absolute configuration was determined by high-field NMR application of the Mosher method and confirmed by X-ray crystallographic analysis.¹⁹ A later crystal structure determination on the highly purified (1) confirmed what had already been agreed.²⁰ The chemistry of azadirachtin has been reviewed by Ley et al.^{21,22} and Akhila and Rani.²³

The synthesis of azadirachtin provided a challenge equal to that of its structure elucidation. Its complex chirality, internal hydrogen bonds, and sensitivity to alkali, strong acid and light, place many limits on what strategy could be used (Fig. 2). It was possible to construct the 'left-hand' decalin portion of its structure (**6**) and the 'right-hand' tricyclic part (**7**), or similar structures, but after many efforts it proved impossible to join the two between atoms 8 and 14 to make azadirachtin. The synthesis was finally completed in what is described as a relay method. The compound (**8**) had already been produced from azadirachtin in six steps, and it had been re-converted back into azadirachtin in nine steps.²⁴ Therefore (**8**) was the goal sought. Both enantiomers of (**8**) could be used in the synthesis of (**1**). The right-hand portion was constructed, starting from galactose, until the pyran fragment (**9**) was obtained, and then coupled to a suitably protected tetralin fragment (**10**), through a C-8 ether, a crucial step. The relay compound (**8**) was attained in a further 8 stages.²⁵ After 22 years of effort by Ley and his group at Imperial College, University of London and later at Cambridge University, the synthesis of azadirachtin was completed in 71 steps and 0.00015% yield.²⁶ The synthesis also made it possible to produce five other naturally-occurring products closely related to azadirachtin.²⁷ Ley's successful synthesis was not the only attempt at it. Considerable progress was made elsewhere, notably by the groups of Murai,²⁸ Nicolaou²⁹ and Watanabe.³⁰ In all, Vietch et al.²⁵ list some 20 papers on the synthesis of azadirachtin.

4. Other triterpenoids

The neem tree must be one of the most intensively studied sources of natural products. The seeds, fruit, flowers, leaves, twigs, bark and roots have all been examined, chiefly for triterpenoids, and usually with the hope in mind of finding other antifeedants or natural pesticides. Akhila and Rani record the properties of 150 triterpenoids from the neem tree.²³ Compounds are also listed by groups, in their probable biosynthetic order, by Kraus.^{31,32}

Almost all of the triterpenoids identified in the plant belong to the limonoid group, that is, tetranortriterpenoids where four terminal carbon atoms are lost from the side chain of a parent triterpenol, and the remaining four atoms are linked in a furan ring. The most abundant limonoid found in the seeds is usually azadiradione (**11**), first isolated from the seed oil,³³ and later synthesised.³⁴ Among the more polar limonoids, extracted with azadirachtin, salannin (**4**) is the most abundant,³⁵ followed by azadirachtin itself.³⁶ Others are present in decreasingly smaller amounts in the seeds. Butterworth and Morgan⁶ recognised that there were other feeding deterrents present in the seeds in smaller quantities. The next most abundant is 3-tigloylazadirachtol (**12**) identified by Kraus et al.³⁷ Unfortunately Rembold introduced the names azadirachtin A, B, C and D, describing these as isomers.^{38,39} Azadirachtin A is identical to azadirachtin, so the original name stands. Since the structure of azadirachtin B was determined by Kraus et al. and named 3-tigloylazadirachtol (**12**), that name stands. Azadirachtin C was never fully described, so the name is unused. Azadirachtin D is correctly 1-tigloyl-3-acetyl-11-hydroxymeliacarpin (**13**).⁴⁰ These three compounds belong to different compound groups and are not isomers. Later authors have isolated, chiefly by repeated preparative HPLC, still further compounds of the azadirachtin, azadirachtol and meliacarpin groups, naming them as 'azadirachtin E, F, G,' etc. For the correct names of all the related compounds from neem, see Kraus.^{31,32} A huge number of minor triterpenoids have been isolated from various parts of the plant by the group of Siddiqui.^{23,31,32,41} The isolation of meliantriol⁵ has not yet been reproduced.

5. Analytical

Azadirachtin and related limonoids are obtained from the seeds by solvent extraction, but because the seeds also contain ~40% oil, this must be removed at an early stage, either by petrol extraction of the oil,⁴² or by a petrol–aqueous methanol partition,⁶ or some equivalent. To avoid destroying some of the azadirachtin, the aqueous methanol must be removed at low temperature, to give a crude extract of 2–6% of triterpenoids. This must be separated further by various chromatographic methods^{6,42–45} or repeated preparative HPLC.⁹ Supercritical fluid extraction of azadirachtin has been explored, but does not give complete recovery.^{45–47} Pure samples of azadirachtin for research have only been obtainable through a lengthy procedure,⁶ by preparative HPLC,^{9,48} high speed counter-current chromatography,^{49,50} medium pressure liquid chromatography,⁵¹ and microwave-assisted extraction.⁵² We have made efforts to devise a simple method not requiring expensive equipment and have produced a flash chromatography procedure giving azadirachtin and 10 other limonoids in mg quantities from seeds.⁵³

Since azadirachtin is non-volatile and highly polar, reverse-phase HPLC with UV detection would appear to be the obvious technique for analytical quantification, but its absorption maximum is at very short wavelength where most solvents absorb strongly. Various protocols have been published;^{43–45,54–56} one uses methanol–water at 214 nm,⁴³ another uses acetonitrile–water at 218 nm;⁴⁴ with retention times near 10 min. Unless the sample has been taken through an extensive and time-consuming clean-up, two major problems arise. First, the quantification may not be possible because of overlapping peaks from other compounds in the extract; and secondly, the column must be thoroughly flushed between runs to remove the non-polar triterpenoids. Use of anisole as internal standard provided an improvement and quantification in the range 4×10^{-3} – 0.02×10^{-3} (w/v).⁵⁷ A normal-phase supercritical fluid method was developed that is an improvement because all the non-polar triterpenoids are eluted with the solvent peak, peak shapes and resolution are better and azadirachtin is eluted with supercritical carbon dioxide–methanol in 6.9 min.^{58,59} Supercritical fluid chromatography equipment is not available in many laboratories, but the growing availability of LC–MS has provided another alternative with the advantages of sensitivity about 1000 times greater than HPLC. The ability to quantify azadirachtin in the presence of overlapping compounds by the choice of a few characteristic ions reduces need for initial clean-up.^{60–63} The latest method⁶³ records a limit of detection below $1 \mu\text{g kg}^{-1}$. An enzyme-linked immunosorbant assay method, with similar sensitivity (detection of 1 ppb) but lower specificity, is also available but is unlikely to compete with LC–MS methods now.^{64,65}

The yields recorded for azadirachtin are highly variable. The original isolation gave 0.075% of the weight of seeds.⁶ One source of Nigerian seed gave 0.19%.⁶⁶ Various authors, in describing alternative isolation procedures give yields. Uebel et al.⁶⁷ give 0.018%, Yamasaki et al.⁴⁴ give 0.056%, and Schroeder and Nakanishi⁶⁸ give 0.15%. Effects of ecotype, climate, soil, rainfall, season and annual variation are essentially unknown,^{69–72} but conditions of storage of the seeds certainly have an effect.⁷³ Isolated yields may vary from less than 0.01% to 0.9% of weight of seed kernels.

The amounts of limonoids found in other parts of the plant are generally not recorded. Sunderam analysed by HPLC samples of seed, bark, leaves, root and stem wood, all from the same group of trees from south India.⁷⁴ He found the following results, based on dried weight: seed kernels, 0.03%; leaves, $0.9 \times 10^{-3}\%$; bark, $0.5 \times 10^{-3}\%$; root, $0.3 \times 10^{-3}\%$; stem $0.2 \times 10^{-3}\%$. The leaves are a very inferior source of azadirachtin, as confirmed by early work, which gave an extract of 111 mg from 1 kg of leaves that still con-

tained many components and had poor antifeedant properties.⁶⁶ Yet investigators continue to try to find azadirachtin in leaves.

6. Biosynthesis

Azadirachtin is one of the most highly oxygenated triterpenoids yet isolated. Little biosynthetic study has been attempted on it, but there has been much speculative proposal. Radiolabelled azadirachtin, nimbin and salannin have been produced in the seeds using labelled acetate and mevalonate, but the position of the label has not been located.⁷⁵ Labelled nimbin, salannin and other limonoids have been obtained from the leaves.⁷⁶ The large number of triterpenoids known to be produced by the neem tree makes it possible to suggest very probable routes by which it is formed, but the starting triterpenol is not known.^{21–23,31,32,77} Euphol (**14**) (Fig. 3) has been shown to be incorporated into nimbolide (**15**) much more efficiently than butyrospermol, tirucalol or Δ^7 -tirucalol.⁷⁸ The isolation of simple triterpenols derivatives from seed kernels, for example, (**16**)⁷⁹ and (**17**),⁸⁰ however, suggest a butyrospermol (**18**) origin. Loss of the four terminal carbon atoms from the side chain of a simple triterpenoid like (**16**) or (**17**) and ring closure at C21 and C23 gives the furan ring of a typical limonoid like azadiradione (**11**). Opening of the C ring is a characteristic

of neem limonoids,²² to give compounds such as nimbin (**3**). Oxidation at C29 and C6 with ring closure gives compounds of the salannin type (**4**) and nimbolide (**15**). It is possible to speculate how further oxidations lead to meliacarpin **13**, azadirachtol **12** and azadirachtin types, but the sequence cannot be predicted. Biosynthetic studies have been made on some simpler neem limonoids with isotopic tracers.^{81,75,82}

7. Botany

A. indica A. Juss. 1830 (synonyms *Melia azadirachta* L. 1753, *Melia indica* (A. Juss.) Brandis (1874) *Antelaea azadirachta* (L.) Adelb.), known most generally as the neem tree, is a native of the Indian sub-continent, but it is now distributed widely in the drier tropics, from Indochina and Indonesia, westward across Africa, Central and South America.¹ It has also been taken to China, Northern Australia, Fiji, and Mauritius. It was, for example, introduced to Ghana about 1920 by the colonial governor, Sir F.G. Guggisberg, who had known it when he was in India, and from there it has spread widely in sub-Saharan Africa. Neem trees were introduced into Yunnan Province of China in 1995 with a plan to produce 26,000 ha of plantations.

It is a valuable plant, quite apart from its ability to produce azadirachtin. It is a small-to-medium sized tree, growing rapidly

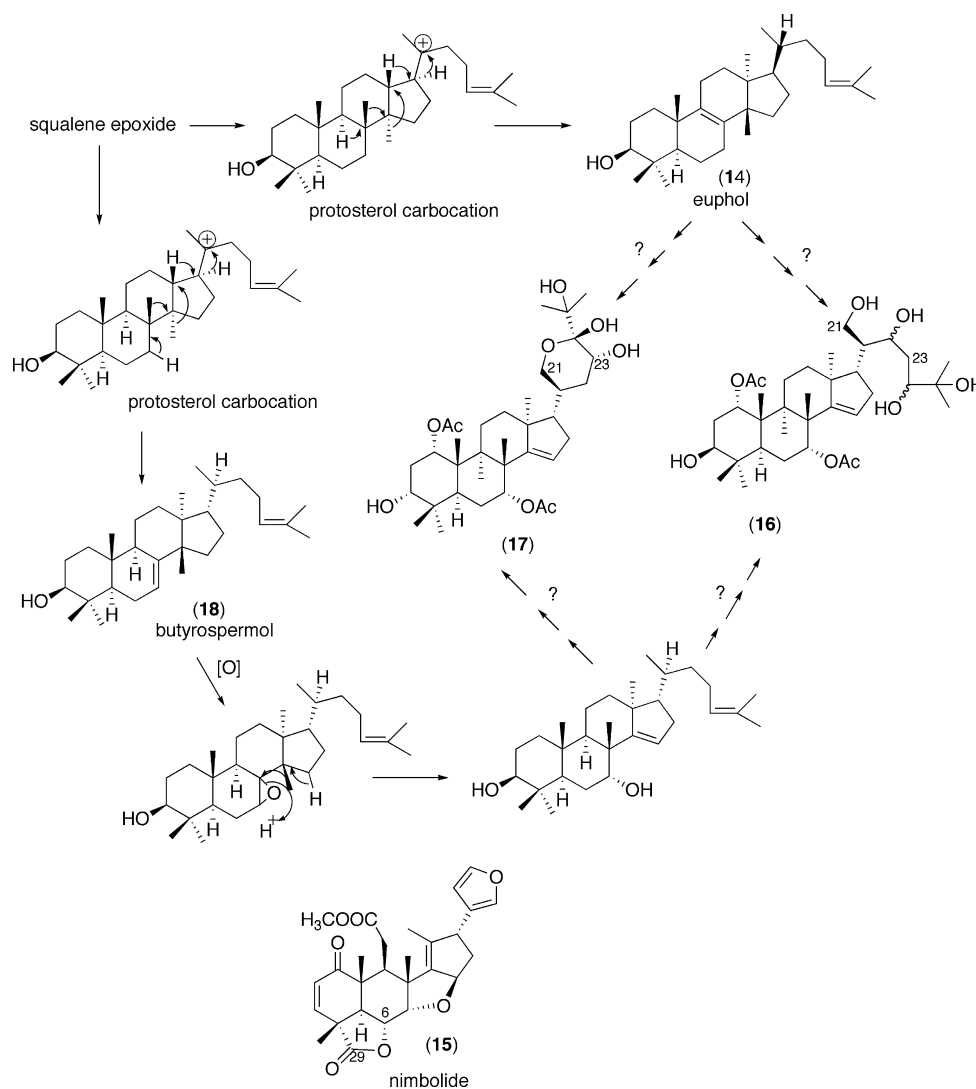


Figure 3.

in poor or degraded soil, and semi-arid conditions (a minimum of 400 mm rainfall per annum). It can tolerate high temperatures and remains green long after other trees have dropped their leaves in drought. It has been recommended as a source of firewood in the drier tropics.⁸³ It is a valuable plant to form windbreaks, to halt desertification and as a shade tree. Cultivation of the tree^{84–86} and its pests and diseases^{87–89} have been reviewed. The seeds are rich (~40%) in a bitter-tasting non-edible oil which, as yet, has not found a use, except to a small extent for soap. The extracted seed cake is also bitter and unpalatable to animals, though rich in proteins.⁹⁰ It still has insecticidal properties and has been used with great effect for that and as a soil improver by Saxena.⁹¹

A. excelsa (Jack), known as marango, is a tall tree of the wetter climate of Malaysia, Thailand, Indochina and Indonesia. Its seeds have been shown to contain azadirachtin⁹², and several new relatives, marrangin (**19**),⁹³ or azadirachtin L and others labelled azadirachtins M to Q.⁹⁴

The Thai neem, *A. siamensis*, is closer in appearance to *A. indica* and can hybridise with it. The seeds contain azadirachtin and 1-tigloyl-3-acetylazadirachtol (that is, differing from azadirachtin only in lacking the C11-OH group).⁹⁵

By far the greatest part of research has been on *A. indica* and azadirachtin itself, obtained from it. Young neem trees begin to bear fruit from the third to fifth year. Mature trees produce up to 50 kg of dried seeds per season.⁹⁶ The triterpenoids are contained in secretory cells, which comprise about 5% of the cells of the cotyledons, and this corresponds to a total triterpenoids extractable of near 5% of the weight of the kernels.^{97,98} The secretory cells begin to differentiate about 40 days after flowering, in the small green fruits, and formation is complete 20 days later.⁹⁷ Different systems of timing make comparison difficult. Azadirachtin begins to form in the green fruit and reaches its maximum amount about 10 days later,³⁶ or, in another report, it begins 60 days after bud formation and reaches a maximum 56 days later.⁹⁹ Thereafter the seeds accumulate oil, with a consequent slight fall in the percentage of azadirachtin in the seeds.^{36,99} A recent report suggests that the yield of azadirachtin in the seeds can be increased by artificial infection with arbuscular mycorrhiza.¹⁰⁰

8. Insecticidal effects

A feeding deterrent or antifeedant has been defined as a chemical which inhibits feeding but does not kill the insect directly, the insect often remaining near the treated plant and possibly dying through starvation.¹⁰¹ Although hundreds of plant-derived feeding deterrents have been discovered,^{102–105} none that are alone antifeedant (unlike azadirachtin), have yet been exploited commercially. The subject of insect feeding deterrents has been reviewed many times.^{102–104} (chemical), ^{106,107} (biological)

The dramatic feeding deterrent effect of azadirachtin on the desert locust *S. gregaria* may result from the evolution of the locust together with the neem tree. While azadirachtin is deterrent to *S. gregaria* at 0.04 ppm,⁶ *Schistocerca americana* and eight North American grasshoppers are insensitive to it at low concentrations.¹⁰⁸ If the sensory mouthparts of *S. gregaria* are cauterised, they will feed on material treated with azadirachtin, but will show the same developmental toxicity as other insects.

The real importance of azadirachtin is as an insecticide, following the report of Ruscoe.⁸ Insecticidal effects are found at much lower doses than required for feeding deterrence.¹⁰⁹ Insects ingesting azadirachtin and related minor compounds in the seed kernels do not die immediately, but soon stop feeding. Non-lethal effects include reduced feeding, delays in development of larvae and nymphs, permanent larvae, incomplete ecdysis, malformed pupae and adults, sterile eggs and reduced fecundity.

Little is known about structure–activity relationships. Small changes in structure make small decreases in feeding deterrent activity.^{21,66,110} The greater the change in structure, the greater the decrease in activity. The effects of change of structure on growth disruption has not been systematically studied.

Heinrich Schmutterer, an entomologist and plant pathologist, impressed by the swarming locusts which refused to feed on the leaves of neem trees in Sudan, began intensive study of its interaction with insects, and organised three international conferences, the first in 1980, on the subject of the neem tree and its insecticidal properties.^{111–113} The insecticidal effects of azadirachtin and limonoid extracts of neem seed kernels have been examined in scores of papers, and the subject has been reviewed many times.^{114–116} Azadirachtin has been tested on 600 or more insect species, on which it has been found active at 1–10 ppm.¹⁰ More than 500 insect pest species are listed as sensitive to neem seed extracts.¹¹⁷ Application rates are 100–500 g active ingredient per hectare.¹¹⁸ The most comprehensive survey is found in several chapters of Schmutterer's book *The Neem Tree*,^{119,120} dealing with insect orders in turn. Effects on pests of vegetables and fruit trees,¹²¹ ornamental trees, shrubs and flowers,¹²² stored grain insects,¹²³ and insects affecting man and animals,¹²⁴ have all been covered. A report compiled by the US Department of Agriculture in 1979 listed 81 insects on which azadirachtin or neem seed extract had been tested, in only nine cases were the results negative.⁸⁹ The report also included five species of nematode on which it is effective. Unfortunately, many studies have used neem seed extracts or neem seed oil of unknown azadirachtin content, which has sometimes led to disagreement about activity or application rates.

Simpler neem seed limonoids like azadiradione (**11**) and nimbin (**3**) are insecticidally inactive though some of them have feeding deterrent effect,¹²⁵ but all the compounds of the azadirachtin, azadirachtol and meliacarpin types are active. Feeding deterrent or toxicity data is given in several tables in Kraus,^{31,32} and in other surveys.^{10,126} Salannin is intermediate in activity. It is therefore contrary to sense to purify azadirachtin for commercial use. The crude extract of highly oxygenated limonoids is both cheaper and more efficient. There is evidence of synergistic action between unknown components in the seed extract.¹²⁷ Moreover, although azadirachtin is degraded by light, some photo-oxidation products of the marginally active salannin are almost as active as azadirachtin itself.^{127–130} The relative amounts of some of the active limonoids of neem seeds are shown in Figure 4.

The term 'neem extract' has unfortunately been used loosely for different products from different parts of the plant. The term

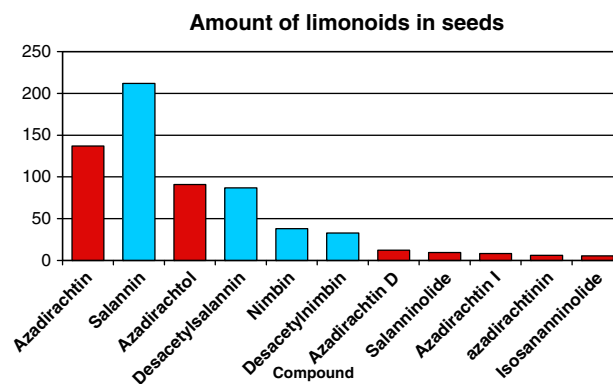


Figure 4. Relative amounts of the principal insecticidal limonoids in an azadirachtin product. The proportions vary with the sample; as shown they represent the average from a number of samples. The active compounds are shown in red, inactive in blue, though salannin is intermediate in activity. See text. 'Azadirachtol' is 3-tigloylazadirachtol (**12**).

azadirex has been coined for the insecticidally active extract of neem seeds, however obtained, containing azadirachtin as its principal active component, with other biologically active limonoids.¹³¹

Little has been made of the systemic effect. When used on bean plants *Phaseolus vulgaris*, systemic activity initially was at 0.010 ppm in dry soil, fell to 10 ppm after 15 days, but was still effective at 25 days at 10 ppm.⁷ Protection of barley from nematodes was also noted.⁷ Seeds soaked in azadirachtin or azadirex solution were protected from locust damage for one week after germination,⁷ and from western flower thrips.^{132,133} Azadirex is actively translocated in the leaves of young trees.^{74,134}

There are reports that azadirex does not affect beneficial insects (honeybees, wasps) and useful parasitoids.^{115,135} This may be partially true, since these insects do not feed on treated plants, but azadirachtin is toxic to bee larvae, though less toxic to adults.¹³⁶ The effects of neem seed products on egg, larval and pupal parasitoids of pest species are generally positive, and have been recently summarised.¹³⁷ Effects on predatory insects, mites and spiders are positive.¹³⁷ It is better if parasitoids and predators do not come into direct contact with the pesticide. This can be helped by the timing of spraying. While toxic effects are clearly shown on predators and bees in the laboratory, far less drastic effects are observed in the field.¹³⁸ Azadirex is wholly compatible with bacterial and virus pesticides.¹³⁷

9. Mode of action

In 1993 it could still be said that the mode of action of azadirachtin was entirely unknown.¹¹⁵ Ruscoe suggested the effect of azadirachtin on normal development might be an interference with ecdysteroid function because of similarity of structure.⁸ This was a misunderstanding because there is little resemblance between their structures. Much early work was devoted to exploring the effects of azadirachtin on moulting and juvenile hormones.^{115,139} Effects on reproduction noted have been blockage of vitellogenesis and reduction of testes development. Azadirachtin, salannin and other limonoids from the seeds inhibit ecdysone 20-mono-oxygenase, the enzyme catalysing the last step from ecdysone to the active hormone 20-hydroxyecdysone.¹⁴⁰ The effects found are very important for defining the overt effects seen in the whole animal on inhibition of growth, moulting defects and sterility, however their effects are probably secondary ones caused by the main mode or modes of action on dividing cells and microtubule formation in cells. Of all the effects of azadirachtin on insect physiology recorded, probably the most fundamental is the effect on actively reproducing cells, for example, wing discs in developing larvae, fat body, ovaries and testes.^{139,141} Blocking of cell proliferation and RNA synthesis was also noted after azadirachtin treatment in a protozoa *Tetrahymena thermophilae*.¹⁴² Recent work indicated that the action of azadirachtin, at the cellular level in actively dividing cells, was to block microtubule formation.¹⁴³ It specifically blocks microtubule formation in a single stage of the malarial parasite *Plasmodium berghei*.¹⁴⁴ This appears not to be the only function however.¹¹⁶

A possible azadirachtin binding protein has been identified in *Drosophila* Kc167 cells.¹⁴⁵ This work has demonstrated a dose-dependent inhibition of *Drosophila* Kc167 cell proliferation within 48 h and direct nuclear damage within 6 h. A large complex (590 kDa) within the Kc167 cells bound azadirachtin-hemisuccinate conjugated to bovine serum albumin. Heat shock protein 60 (HSP60) was identified by peptide mass fingerprinting as one of the components of this binding complex.¹⁴⁵ Additionally, azadirachtin inhibited polymerisation of isolated pig tubulin.¹⁴³ There have also been reports implicating actin as the target of azadirach-

tin,¹⁴⁶ though this latter report is difficult to reconcile with other observations. More work is required finally to identify the cellular mode of action. Until this target has been identified, progress on rational structure design based on the azadirachtin parent molecule, and structure–activity relationship studies will be hampered. The successful synthesis of azadirachtin^{24,25} and the knowledge accrued during its elucidation is a major impetus towards determining the cellular target of azadirachtin.

The EC₅₀ values for various cultured insect cell lines vary from 10⁻¹⁰ to 10⁻⁹ M, by which it is classed as highly toxic, whereas for all mammalian cell lines, values of EC₅₀ are 10⁻⁵–10⁻³ M, which places it in the mildly toxic to non-toxic class, and gives a margin of safety in excess of 100-fold between insect and mammalian cells.¹⁴¹ There is evidence that the difference in toxicity may be due to the ability of mammalian cells to remove the azadirachtin (R.H.C. Strang, personal communication).

Studies with labelled azadirachtin found that when 1.5 µg/g body weight of (22,23-³H₂)dihydroazadirachtin, which has biological activity approximately equivalent to azadirachtin, was injected into female *Locusta migratoria*, 0.4–0.5 µg/g was recovered unchanged after 5 days. Most (74%) of the tritiated dihydroazadirachtin that was retained in the body was in the Malpighian tubules,^{147,148} but this was not confirmed in another study.¹⁴⁹ The uptake was specific and saturable, the retained label was largely unmetabolised.¹⁴⁹ Autoradiography of *S. gregaria* testes showed the labelled azadirachtin localised in the tails of developing sperm.¹⁵⁰ About half of 1.0 µg of tritiated dihydroazadirachtin injected into larvae of *Heliothis virescens* either orally or into the haemocoel was excreted, while 25% was retained in the body converted to a more polar form, and this was sufficient to disrupt pupation.¹⁵¹

10. Commercialisation

The publication of the initial papers on azadirachtin^{4,6} precluded a possible composition-of-matter patent on the compound. A provisional application was made for a use patent in 1971,¹⁵² but for complex reasons, this was never taken to a full patent. R.O. Larson, importing timber from India to the USA, also provided neem seeds for research, and became interested in the subject.¹⁵³ He used his company Vikwood Ltd to pay for the initial necessary animal testing to show that a simple extract containing azadirachtin, other limonoids and some oil was non-toxic to mammals, fish and birds.¹⁵³ He obtained a US patent for a product, Margosan-O, of improved stability.¹⁵⁴ Though it might be thought of limited breadth and usefulness, the patent has maintained its position commercially and in the courts,¹⁵⁵ and has passed from company to company, still in force. There are subsequent US patents and also a number of Indian patents.¹⁵⁶

It was the enlightened policy of the US Environmental Protection Agency (EPA) to ease the regulations for 'botanical' pesticides, like Margosan-O, compared to those for synthetic pesticide. The regulations designed for synthetics remain in force in many countries, in which data on the toxicity of every compound in the pesticide must be provided. This is impossible, or at best prohibitively expensive, for a botanical pesticide like azadirex. Therefore, in many countries azadirex products still cannot be used, but there are about 40 countries where regulations do permit it.

Margosan-O was licensed in the USA from 1989 to 2004, and for greenhouse use from 1990, and then replaced by Neemix (4.5% azadirachtin) and Azatin (3% azadirachtin), still available. It is noteworthy that Margosan-O had a maximum content of 0.4% azadirachtin, little more than is found in seeds, suggesting a lot of the azadirachtin was lost or destroyed during extraction. NeemAzal T/S (10 g l⁻¹) and NeemAzal F (5% azadirachtin) were developed

by Trifolio-M GmbH in Germany, and are available in Germany, Austria, Italy, Spain and The Netherlands. Their Indian partners, EID Parry, sell in India and the USA. Over 20 commercial neem pesticides are available in India¹⁵⁷ and many others in other countries.¹⁵⁸ The list of commercial products is long.^{118,159}

11. Natural pesticides

The use of chemicals from plants to treat sickness or control pests goes back at least two millennia in the records of ancient China, Egypt and Greece. These treatments were crude extracts of unknown activity. In the nineteenth and early twentieth centuries, through advances in chemistry, better defined plant extracts, such as derris, pyrethrum or nicotine came into use. The arrival of synthetic pesticides in the 1940s changed the control of agricultural and garden pests, and that has changed again with the unforeseen problems of persistence and resistance. It is noteworthy that most botanical pesticides (e.g., azadirax, pyrethrum, nicotine, derris) consist of a mixture of compounds, in contrast to single-compound synthetics. This has the advantage of preventing the onset of insecticidal resistance. Performance of new synthetic pyrethroids has been clouded by the rapid development of resistance in some pests, while natural pyrethrum has maintained its activity. It has been shown that resistance to pure azadirachtin developed quickly in the aphid *Myzus persicae*, but there was no detectable resistance to azadirax over 40 generations.¹⁶⁰

Derris and pyrethrum have maintained some place among pesticides throughout the period of the synthetics, and their sales have grown again with increasing restrictions on persistent pesticides and the growth of the organic food and farming movement. The demand for pyrethrum is much greater than world production. East

Africa used to be the main source. Output there is heavily dependant upon weather conditions, and varies from 2000 to 10,000 tonnes *per annum*. The Australian Government has made an estimated Aus\$25 million investment in creating a prosperous pyrethrum-growing industry in Tasmania which now provides 45% of world supply.¹⁶¹ In 2005 a grant of Aus\$700,000 was made for research on pyrethrum storage. Neem products, by comparison, are not in the same league. Because azadirax belongs to everyone, no-one has been willing to chance investing a lot of money in it.

Trifolio-M sell about 300 tons of their neem products, and there is about an equal amount sold by competitors, except for India, where because of the large number of producers, volume is difficult to estimate. California provides the best pesticide use statistics available. Usage there varies widely from year to year. Between 1996 and 2006 the average amount of azadirachtin used was 1300 kg per year, chiefly on tomatoes, lettuce and greenhouse flowers. In 2006, 8900 kg of pyrethrum was used there. Why the difference? Are they comparable products, or is pyrethrum so much superior? The answer to the first question is difficult to reach. Table 1 makes some comparison between pyrethrum and azadirax. The *scientific* interest in azadirax is great. A check of key words gives 900 papers on azadirachtin compared with 218 on pyrethrin since 1970, or 1,637 on neem against 352 on pyrethrum. Pyrethrum has been in use for many more years than azadirax, and a drop in scientific activity over pyrethrum is to be expected, but does not explain the great difference between interest and use.

Far too little attention was paid in early fieldwork to the conditions of stability of azadirax to light, heat and pH, with resultant irreproducible results, and discouragement of potential users. More is now understood about stability,^{61,74,162} use of UV

Table 1
Comparison of azadirax with pyrethrum, noting some of the advantages and disadvantages of azadirax in production and use

Property	Pyrethrum	Azadirax	Advantage?
Source	Flowers	Seeds	
Countries where grown	About 10	About 80	+
Soil requirements	Rich volcanic	Poor, low fertility	+
Water requirement	High	Low	+
Replanting	Every 4 years	Permanent plants	
Harvesting	Every 2 weeks	Once a year	–
Processing method	Solvent extraction	Solvent extraction	
Processing ease	Simple	Complex	–
Yield per hectare	55 kg dried flowers	2000 kg seeds ^a	+
Active concentration	1–2% in dried flowers	0.45% in seed kernels ^b	+
Actives per hectare	0.55–1.1 kg ha ⁻¹	9.0 kg ha ⁻¹	+
Price per kg	US\$1 dried flowers	US\$2 dry seeds	– –
Gross income per hectare	\$55–110	\$4000	++
Number of compounds	6	10+	
Effectiveness of compounds	Varies widely	Varies widely	
Range of insects	Very wide	Very wide	
Speed of action	Very rapid	Very slow	–
Systemic action in plant	No	Yes	+
Beneficial insects	Toxic	Non-toxic ^c	+
Toxicity to others	Very toxic to fish, slight to birds	Non-toxic	+
LD ₅₀	500–1000 mg/kg rats	>3540 mg/kg rats ^d	
Human toxicity	Very low	Very low	
Problem in use	Allergies possible	Aflatoxin testing ^e	
Stability in storage	Very good	Very good	
Stability on plants	Very low	Very low (?)	
Residues on food	None	None	
Stability in water	Rapidly hydrolysed in acid or base	Stable between pH 3.5 and 6	
Licensed for use	Most countries	Fewer countries	–
Value, annual production	US\$600 million	US\$55 million	–

Costs and values must be approximate in a period of rapidly rising prices and fluctuating exchange rates.

^a Estimate based on spacing of trees at 10 m.

^b Based on mean value for six Asian countries.¹⁴¹

^c Depending on how it is used, see text.

^d It is reported 1500 mg/kg/day was administered to rats for 90 days and gave no observable effect.¹⁴⁴ Intraperitoneal injection of 1000 mg kg⁻¹ had no effect.

^e Neem seeds subject to fungal infection, with aflatoxin production and loss of activity, if not carefully dried.

screens,^{74,163,164} and formulation, but these are beyond the scope of this review. The interesting suggestion has been made to use cyclodextrin complexes of azadirachtin to increase water solubility and stability.¹⁶⁵ The main obstructions appear to be the relatively high cost of the raw material, and resultant price of the users product, and the cost of licensing, well discussed by Isman.¹⁶⁶

The German aid organization, Gesellschaft für Technische Zusammenarbeit (GTZ) produced booklets in several languages explaining to third world farmers how they could make and use crude neem seed extracts to protect their crops.¹⁶⁷ Sadly, the word did not spread, and the response was disappointing. Farmers, showing prejudice against something cheap and available, continued to use expensive synthetics which gave rapid results, or used nothing at all.

The price of neem seeds must be reduced to be economic. This can be helped by mechanical harvesting, as has been tried experimentally in Australia, by finding a more efficient method of extraction of azadirachtin, and finding a by-product that reduces overall cost. Little use has been made yet of the extracted seed cake. Large quantities of oil are available that might be used for biodiesel, if the content of volatile sulphur compounds present¹⁶⁸ does not prove a drawback. There is a potential supply of 700,000 tons of neem oil available in India.¹⁶⁹

12. Other uses

It has been noted that azadirachtin is also useful against mites and some nematodes. It has been shown to be useful in the control of blowfly, hornfly and other pests of farm and domestic animals.^{170–172} A seed extract shows fungicidal properties, but no compound has yet been identified. There has been much interest in recent years in the use of various neem products in Ayurvedic and herbal medicine, such as fungicidal,¹⁷³ anti-inflammatory, antiulcer, spermicidal and dermatological effects.^{174–176} The proof of safety is largely lacking. There does not seem to be any long-term studies on use either internally or externally, and no clinical studies, so medical use of any preparations from the seed or oil cannot be recommended. There are reports of children dying after administration of neem oil, but this may have been the result of aflatoxin infection of the seeds.

13. Conclusion

After 40 years in the hands of scientists, there is much still to be discovered about azadirachtin. It has not yet reached most of its potential users. Botanical pesticide use is still tiny compared to that of synthetic products. Increase in the cost of petroleum starting materials may soon alter that. Progress towards greater use is slow, but slow progress is progress, and we can expect to learn more scientifically and see it better known outside the scientific world.

Acknowledgements

I thank Professor A. J. Mordue (Luntz) (together with A. Bowman and A. J. Nisbet) and Dr. R. H. C. Strang for discussion and guidance about mode of action, Dr. Hubertus Kleeberg for information about commercialisation, and a generation of students who have learned their skills working on this absorbing subject.

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