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AZADIRACHTIN

A study in the methology of structure determination

by

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Continuing research into the triterpenoids of the Meliaceae still produces compounds of considerable chemical and biological interest. The most famous of these is azadirachtin, which was first isolated by Morgan in 1968 from the seed of *Azadirachta indica*, the Indian neem tree[1].

Azadirachtin is remarkable both for its chemical complexity and for its biological activity. Investigations of the insecticidal activity, started by Morgan, have shown that it is probably the most active of a whole new class of insecticides which have been isolated from neem and related plants. These compounds have become the centre of a considerable industry, with work in many laboratories being co-ordinated into international neem conferences, and leading to scientific discoveries in chemistry, insect physiology and related subjects. It is still too early to say whether there will also be significant commercial advances; in the opinion of the present author it is unlikely that azadirachtin itself will be readily enough available for really wide spread use, and possibly the most hopeful prospect is for the development of simple synthetic analogues.

The chemistry of azadirachtin has also generated much activity. Determination of the structure is of course a necessary preliminary to the biological investigation of any simpler analogues; but it has also proved to be of considerable technical interest, and has become something of a test bed for modern methods. These are now so highly developed that it is commonly thought, even among structure chemists, that once a compound has been isolated, determination of the structure is rather routine. It has been said that determination of the structure of a newly isolated compound is often simpler than searching the literature to find whether it is already known. However, in the case of azadirachtin this has not been so. The early work of Morgan soon showed that the structure was complex, and this first stage culminated in 1975 with the proposal of a structure by Nakanishi[2]. The effect of this was to silence opposition, rather than to carry conviction. Many chemists had more or less well founded doubts about the proposal, but none had hard evidence to contradict it. As a consequence, subsequent structural work has been a matter of interest to teams of international standard, and during 1985 activity rose to an almost feverish level, with three strong teams working on the subject and several alternative structures under consideration.

The results of these three teams, who now agree upon a structure which is supported by compelling data, are presented in the following papers. It is not the purpose of this review to provide a precis of these results, which must be

read in the original form to be appreciated, but to seek to investigate why the structural work was so troublesome, and to see what lessons may be learnt for use in future investigations.

The usual stand-by of structure determinations is crystallography. However, as is of course well known this is not universally applicable, because not all compounds are crystalline nor do they all readily give crystalline derivatives. Azadirachtin is micro crystalline, and does not give crystals suitable for x-ray, and no simple derivatives were found to be crystalline. Eventually however, Ley and his colleagues found a suitable derivative, and a structural determination was made. However, there are still intrinsic disadvantages in the x-ray approach. One is that the preparation of derivatives may lead to unsuspected molecular rearrangements which can cause confusion, another is that each structure determination has to be carried out from first principles, so that the structure of one compound is not of great assistance in determining the structure even of a closely related derivative.

It is claimed by its adherents that mass spectroscopy may in the future provide a universal method. It is certain that the physical state of the sample is unimportant, that the sample size is normally much lower than for any other method, and that a vast amount of information can readily be obtained. The doubts lie in the processing of this information. It may be true that in general a structure can be determined, even if only by mathematical methods of permutations and combinations. In a real case, this requires much data-processing capacity, even in an age of computers, and there are unresolved doubts as to how far it is practical, supposing that it be possible. Unfortunately, it is very easy to draw unfounded or erroneous conclusions from mass spectroscopic data, and there are many superficial uses of the method in the literature. For these reasons, the present author remains unconvinced and no further consideration will be given to mass spectroscopic methods here. In the case of azadirachtin moreover, mass spectroscopy was little used in reaching the final structure. Although a considerable study was made of the data by Ley, and earlier by Morgan, this was not taken to a conclusion before the answer was obtained in other ways and it remains a matter for conjecture how far this would have been possible.

These critical remarks are directed at the general case. There is no doubt that in special cases, of which some indole alkaloids are good examples, mass spectroscopy may be extremely useful, because of the previously existent background of special information available.

N.m.r. spectroscopy provides a method that is closer to the organic chemist's heart. This is no doubt because he can use his own specialist knowledge in the structure elucidation; but more fundamental reasons are that the process is universally applicable, given only that the compound in question is soluble; and that structural knowledge of related compounds can be fully utilised, so that for example many derivatives of azadirachtin can, now that azadirachtin is known, be speedily identified. It should be emphasised for an uninitiated reader that such subsidiary studies are normally quite reliable in their conclusions, and far quicker than any other method. This is of course not to say that mistakes cannot be made, but they should not be.

The structural work on azadirachtin is thus a case study in the use of n.m.r. spectroscopy in structural elucidation.

The original 1975 structure for azadirachtin, proposed by Nakanishi, was based on the hypothesis of a relation to nimbin and salannin, known but less complex compounds isolated from neem; on n.m.r. evidence; and on a curious and unique reaction whereby azadirachtin was acetylated by refluxing in acetic anhydride for ten minutes. This gave a tertiary monoacetate, and it was suggested that this was produced by acetyl transfer from a secondary hydroxyl, which could then not reacetylate due to steric hindrance. This ingenious explanation was completely wrong, and furnishes a good example of the peril of using the evidence from unusual reactions in structural elucidation. Frequently, as in the present case, these are only understood subsequently when the structure is known from other, less equivocal, methods. A similar consideration can apply to the preparation of derivatives for x-ray crystallography.

The n.m.r. evidence used was mainly derived from a study of the partially relaxed Fourier transform (PRFT) spectrum, at that time a new technique, in which ^{13}C peaks of different proton coupling have positive or negative signs, thus greatly simplifying the spectrum in crowded regions. Use was also made of proton-decoupling techniques, which with assistance from shift reagents, revealed five different spin systems. A long-range coupling was observed between H-9 and a methyl group, which was considered to be on C-10; and a nuclear Overhauser effect was observed between H-9 and 3H-18 (Me-13); which was considered to show that this methyl group was on the α-face of the molecule. The use of one-bond carbon-carbon connectivity plots gave no useful information, because of the number of ether links, and more recent pulse sequences which might have helped were not then available, and indeed have never been applied to azadirachtin.

There is no doubt that at the time the proposal of this structure was a considerable tour-de-force; and the general reaction was a rather stunned respect. However, as this cleared it was seen that hard evidence for much of the structure was lacking.

The carbon skeleton and the substitution in rings A and B was based on analogy to nimbin and salannin. This appeared inherently probable, and could be accepted on the basis of close spectral analogy to known compounds. The dihydrofuran, which was new, was supported by good spectroscopic evidence. However the other ether links, the spectral assignment of some of the carbon and hydrogen atoms, and much of the stereochemistry could only be described as not proven.

A very strange feature was the appearance of a methyl group resonance at δ2.06; not due to an acetate methyl. This very low-field shift is presumably due to deshielding by adjacent oxygens, but is much greater than normally encountered.

It should be particularly noted that the evidence for the basic structure, which turned out to be correct, was analogy with previously known limonoids and identification of the various isolated spin systems. Thus if azadirachtin had been the first limonoid isolated, the 1975 structure could not have been

proposed on the basis of the other evidence available at the time, because of the lack of information on the relation of these spin systems.

Particularly doubtful was the assignment of the oxidised methyl in the acetal ring to C-30 (= Me-8). It is not now apparent why this assignment was made, since the structure determination leans so heavily on precedent, for C-30 is not oxidised in limonoids, though frequently in quassinoids. A much more likely structure for a limonoid would have C-19 oxidised. The evidence available did not distinguish between C-19 and C-30, which are symmetrically disposed about the acetal carbon, so that the coupling observed to H-9 could arise from a methyl group in either position.

An observation which was made, but not followed up until after the structure was known, was that azadirachtin has a temperature dependent spectrum. This is likely to mean, as in prieurianin[3], that the molecule consists of two sections joined only by a single bond, which exhibit restricted rotation. When this single bond in prieurianin derivatives is reinforced by an ether link, the rotation stops and the temperature dependence disappears. The only significant possibility for rotation in azadirachtin seems to be about the 8-14 bond, which implies there is no ether link between C-11 and the right hand section of the molecule. If this idea had been followed up in 1975, when the facts about prieurianin were already known, the C-19, C-30 uncertainty might have been resolved, and the correct structure deduced much earlier.

Given these doubts, further study of azadirachtin was undertaken. This study used long-range couplings to determine the proximity of various parts of the molecule, study of the connectivity pattern to confirm the basic skeleton, and especially study of the nuclear Overhauser effect to approximate structural elements one to another. In simple cases, n.O.e. measurements are an ideal way of determining stereochemistry of substituents when these are the only unknown feature in the molecule. In the present case the evidence was much more complex, and led to confusion which is considered in more detail later.

The first progress was made in the positive assignemnt of H-7 (δ4.75, d.2.7) and H-15 (δ4.67, d.3.4). In his original work[4], Morgan correctly assigned H-7, but later these two resonance signals were confused, and both were at times considered to be due to OH groups. The assignment was finally made by demonstrating the coupling of H-6 to H-5 and H-7. H-7 was linked by n.O.e. measurements, and by decoupling in DMSO, to the hydroxyl proton at δ3.07; which was thus 7-OH. It was then possible to locate the methyl and methylene groups to C-30 and C-19, by showing that one proton of the methylene group gave a positive n.O.e. with H-1 and H-2b, while the second gave a negative (three-spin) n.O.e. with H-1. In contrast, the methyl group gave no n.O.e. with H-1 or H-2, but a positive n.O.e. with H-7 and with H-15. This was only consistent with the location of the methylene taking part in the acetal bridge being attached to C-10, and the methyl group attached to C-8; and the structure was revised accordingly.

This new structure is still unable to explain the presence of a large n.O.e. between H-21 and H-7, or those between the low-field hydroxyl (δ5.05) and H-9 and 3H-30. Consequently a search had to be made for yet another structure. There are in azadirachtin 16 oxygen atoms. Eight of these are

accounted for in esters, and two in the dihydrofuran and the ring A/B tetrahydrofuran, leaving three hydroxy groups and three ether rings, including the suspected C-11 → C-19 acetal bridge, of uncertain location. The core of the azadirachtin structural problem is to decide which of the remaining nine carbon atoms attached to oxygen (three hydroxy, six in three ether rings) bear hydroxy groups. This was first, and most simply, done by Kraus, who deuterated azadirachtin in DMSO solution, and noted that the chemical shift of only three carbon atoms, C-7; C-11; and C-20 were altered by this. These carbons therefore bore hydroxy groups. It was a surprise to find a hydroxyl at C-11, so that the C-11 acetal was in fact a hemiacetal; but as already pointed out, this could have been foreseen from the temperature sensitivity of the spectrum. Given this location of the hydroxyl groups, and assuming the carbon skeleton to be now correct, which as previously mentioned is very probable at this point, this only leaves one structure possible for azadirachtin. A survey of the n.O.e. data then showed that they fitted this structure; thus providing confirmation of its correctness. The final point, of the stereochemistry of the epoxide ring, was demonstrated by the existance of a positive n.O.e. between H-7 and H-21 and between 7-OH and H-21.

This completed the n.m.r. structure determination, at about the same time, although published earlier, Ley arrived at the same structure by x-ray crystallography. Meanwhile, Nakanishi was attempting to identify the hydroxyl-bearing carbons by methylation of the hydroxyls, and this was completed a little later.

In practice, both the Kraus and Nakanishi methods are justified by their results, but both are open to criticism.

Methylation of aliphatic hydroxyl groups may require rather vigorous conditions, and it must be rather doubtful whether a compound as complex as azadirachtin will survive methylation without rearrangement; although it is true that any such rearrangement should be readily detectable in some spectral alteration. On the other hand, deuteration of hydroxyl groups does not always indicate which carbons are hydroxylated. In the case of naringenin, recorded by Wehrli and Nishida[5] C-5, which is hydroxylated, is shifted on deuteration, but C-7, which is also hydroxylated, is not. Moreover, C-4; which is not hydroxylated, is shifted on deuteration, probably because of a change in the structure of a hydrogen bond.

At the same time as this was going on, attempts were being made to confirm the carbon skeleton by spectroscopic measurements, and to deduce the structure in its entirety from the mass of n.O.e. data which had been built up.

Complete direct determination of the skeleton by n.m.r., as opposed to the indirect methods used by Kraus and Nakanishi, was not possible because of the numerous ether linkages which act as insulators to the more simple n.m.r. probes of connectivity which were employed. However, more recent methods[6,7] should be able to overcome this problem; since they have not been tried on azadirachtin they are not relevant in the present context.

Of greater interest are the n.O.e. studies. The assumption was made that if enough n.O.e. data could be assembled, then only one structure would fit them. This is doubtless true; but unfortunately it is much easier to test a given structure by n.O.e. measurements than to deduce one on this basis. Moreover,

the n.O.e. data were highly confusing, and different workers obtained different results. This is probably partly for instrumental reasons; n.O.e. measurements can be decidedly tricky in practice, and do not always give the expected results even when experienced workers are handling known compounds. In the case of azadirachtin there were more fundamental problems to be faced.

One, which has not been systematically investigated, is the effect of solvent. Measurements were conducted in $CDCl_3$ or in DMSO, and it is unfortunately not always quite clear which solvent was used in all the measurements described. It seems that the degree of temperature-dependence of the spectra, and hence the concentration effects and the n.O.e. observations, are themselves dependant on the solvent. This is not unlikely since highly polar solvents, such as DMSO, which may be expected to hydrogen-bond to azadirachtin, will affect the degree of hindrance to rotation about the 8-14 bond, and thus influence the temperature-dependance of the spectrum.

A second, and major, problem was saturation transfer between hydroxyl groups, which meant that quite unexpected results might be evoked. In order to eliminate this, Ley made n.O.e. measurements at 270K, whereby transfer effects were almost completely eliminated, with much greater ease of interpretation.

The other major problem was the temperature sensitivity of the spectrum. At low temperatures, two sets of lines were obtained, many nuclei giving rise to split lines. This is probably due to restricted rotation of the molecule about the 8,14 bond. In order to eliminate this effect, Nakanishi made n.O.e. measurements at 333K. It is not clear why these workers found such different conditions to be optimum, and suggests there is no completely satisfactory answer to the problems.

In an earlier paper[8] Ley reported the existence of a strong n.O.e. between H-16a; a proton on the ring D methylene group, and Me-13 (3H-18), and hence deduced that the original structure was wrong in the proposed *trans* arrangement of these two groups. In more recent work, this n.O.e. is not reported, but instead Me-13 is found to have a positive n.O.e. with H-9, H-17, H-3', OH-7, and OH-20, all of which are consistent with the trans arrangement which appears in the final structure. The earlier result is now explained[9] as being due to the proximity of Me8(3H-30), 3H-4', and H-16a, which render the n.O.e. measurements unselective. This again shows the care which has to be taken with n.O.e. measurements in complex molecules.

A significant point is that whereas the Ley and Nakanishi groups determined n.O.e. effects by two-dimensional methods, Kraus relied on one-dimensional subtraction spectra. It seems clear that whereas the 2-D methods are quicker the reliability of the 1-D method is much greater.

An interesting experiment by Ley plotted the temperature dependence of the chemical shifts of the three hydroxyl protons, showing that OH-11 was much less sensitive than the other two, and hence considerably more hydrogen bonded. It is presumed to be this hydrogen bond, from OH-11 to the oxide oxygen, that is the main restriction on rotation of the azadirachtin molecule.

Although the structure of azadirachtin is now agreed, the structure of the Nakanishi acetate has not yet been elucidated. On the basis of the limited results available, it seems most probable that it is the 11-acetate; but this

should be checked, perhaps by applying Kraus' deuterium exchange experiment to the acetate.

In the course of this work, several new compounds related to azadirachtin have been isolated, the structures of which can be fairly easily deduced now that azadirachtin is known.

The nomenclature of these compounds is confusing, due to trivial names being applied to compounds of unknown structure. Two series of compounds are known, those from *Azadirachta indica*, which are named as derivatives of azadirachtin, or as derivatives of azadirachtol (11-deoxy, 1,3-dideacyl azadirachtin); or as derivatives of azadirachtinin, a rearrangement product of azadirachtin which will be returned to shortly. The other series, isolated by Kraus from *Melia azedarach* are similar, but lack oxidation at C-29, which is present as a methyl group, not oxidised to a methoxy carbonyl as in azadirachtin. These compounds are named more systematically as derivatives of meliacarpin, which is the 29-methyl analogue of azadirachtol, or meliacarpinin, analogous to azadirachtinin. So far the compounds related to azadirachtin which have been isolated from *Azadirachta indica* (A) or *Melia azedarach* (M) are the methanol addition product of azadirachtin, 22,23-dihydro-23β-methoxy azadirachtin (A); 3-deacetyl-3-cinnamoyl azadirachtin (A); 1-cinnamoyl-3-feruloyl-11-hydroxy meliacarpin (M); the 22,23-dihydro-23β-methoxy derivative of this last compound ((M); 1-tigloylazadirachtol (= 3-deacetyl-11-deoxy-azadirachtin) (A); 3-tigloyl-azadirachtol (A); and two rearranged derivatives, 1-tigloyl-3-acetyl-11-methoxy azadirachtinin (A), and 1-tigloyl-11-methoxy-20-acetyl meliacarpinin (M).

Of these, it is only necessary to discuss here the two tigloyl azadirachtols and the rearranged derivatives.

The first have been isolated on three occasions, by Kubo[10,11], by Kraus and by Ley. All three preparations have extremely similar spectral properties, but the samples of Kubo and Ley have mp 148° $[\alpha]_D$ -40°; while that of Kraus has mp 208° $[\alpha]_D$ -69°. In the Kraus sample, H-1 was found to show an n.O.e. with H-19A, and to be coupled to a secondary OH group. Hence it is a 3-tiglate.

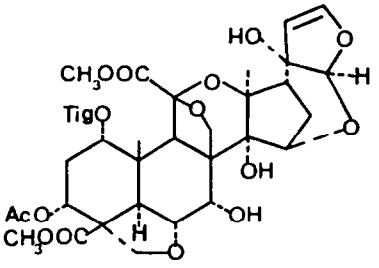
There can be little doubt that the compound isolated by Kubo and by Ley is the isomeric 1-tiglate, although there is no direct evidence of this. The expected n.O.e. from the presumed H-1 to H-19 could not be found, which is strange since the corresponding effect in azadirachtin itself was quite apparent. This may show once again the unreliability of 2D-n.O.e. methods. It is possible that one or the other of these isomeric tiglates is an artefact produced by tigloyl migration, but there is no evidence of this.

The structure of the two compounds with the alternative nucleus are more complex to determine. The relationship between azadirachtin and azadirachtinin, or the parallel one between meliacarpin and meliacarpinin, involves a rotation of the two halves of the molecule relative to the way that the azadirachtin structure is usually drawn. As a consequence, a correct projection of azadirachtinin is difficult to relate to azadirachtin, and the structure is here re-drawn the better to show the relationship. This is discussed in relation to azadirachtinin, meliacarpinin is entirely parallel.

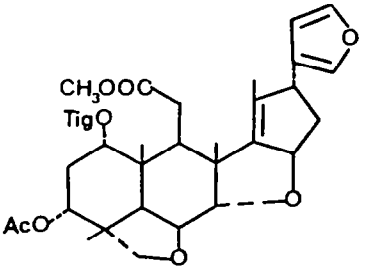
Extensive preliminary n.m.r. studies showed the presence of most of the structure of azadirachtin; the left hand half was intact except that 11-OH was methylated, and 7-OH was no longer present as a hydrozyl. In the right half of the molecule, the signals for C-13 and C-14 were drastically shifted down-

field, showing the absence of the epoxide, while Me-13 (3H-18) is shifted downfield by 8 ppm compared to azadirachtin, indicating a different stereochemistry. C-14 shows a 3J-coupling to 9-H; thus demonstrating the presence of the 8-14 link. The two hydroxy groups were located at C-14 and C-20 by the deuterium shift method, which leaves the final oxide bridge to be at C-7 to C-13. This was confirmed by the chemical shifts of C-7, C-13 and H-7. Finally, the stereochemistry of C-13, expected to be inverted from the change in the Me-13 shift, was shown by the n.O.e. to H-5, H-9, H-16a, H-17 and 11-OH. This completes the structure determination; it is interesting to see that the azadirachtinin nucleus is then the expected product from attack of 7-OH in azadirachtin on the epoxide C-13, with the usual Walden inversion.

I am grateful to the senior authors of the accompanying papers for information supplied during the course of the work, and especially to Professor Nakanishi for a visit to his laboratory in New York in 1985. The references are only listed when they involve earlier papers, references to the three accompanying papers, which are frequent, are not specifically drawn.



Azadirachtin (Nakanishi, 1975)



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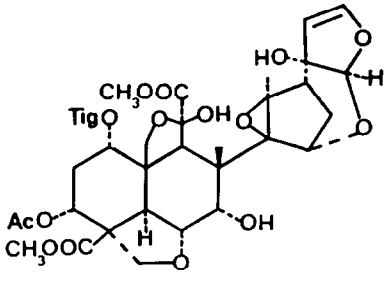
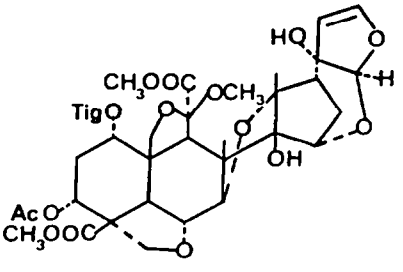


Azadirachtin (Kraus, Ley 1985)



1-tigloyl-3-acetyl-11-methoxy azadirachtinin

Azadirachtin and related compounds. Formulae re-drawn to show the relationship between azadirachtin and azadirachtinin.

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