STUDIES ON THE STRUCTURE OF THE INSECT FEEDING INHIBITOR AZADIRACHTIN

by

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ABSTRACT

Azadirachtin $C_{35}H_{44}O_{16}$, a locust feeding inhibitor, was first extracted from neem seeds by Butterworth in 1967. Butterworth identified two acetate esters, two carboxymethyl groups, a tiglate ester, two hydroxyl groups and a 2,3-dihydrofuran ring in the molecule.

During the course of this present work an additional tertiary hydroxyl group and a tertiary methyl group have been detected, while a ketone carbonyl function and an ether linkage at the 2-position of the dihydrofuran system have been tentatively assigned. In this way all sixteen oxygen in the molecule have been accounted for. Furthermore, selenium dehydrogenation of an azadirachtin derivative has yielded methyl-napthalenes indicating the presence of two fused six-membered carbocyclic rings in the parent molecule. Various pieces of evidence are presented which suggest that azadirachtin contains a structural unit equivalent to the intact A and B rings of a limonoid, indeed it is suggested that azadirachtin may be a highly oxidised member of this naturally occurring class of compounds.

A survey of the n.m.r. spectra of azadirachtin and several derivatives is presented. Thirty-three of the protons in the parent molecule are now definitely assigned and the n.m.r. signals for the remaining eleven protons are now recognised.

In the later stages of this work attention was given to

obtaining a crystalline derivative of azadirachtin which might be amenable to X-ray studies as it was felt that this would be the route by which the total structure and stereochemistry of the molecule would be solved. So far no crystalline derivative has been prepared despite a variety of approaches.

Further work on the physiological activity of azadirachtin against a number of insect species is also reported.

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Unless otherwise stated, all the work reported in this thesis was carried out by the Author under the supervision of Dr. E. D. Morgan.

CONTENTS

INTRODUCTION

Increased awareness of the dangers from the widespread use of toxic insecticides has led to interest in feeding deterrents as a possible alternative method of control. Insect feeding deterrent, feeding inhibitor, and antifeedant are synonomous terms for materials which discourage the feeding of insects and therefore could be used to bring about death indirectly through starvation. A non-toxic antifeedant chosen for its specific activity against a pest organism could be used in the field to protect crops without danger to predators, pollinators, or mammals.

For some time, a number of synthetic and naturally occurring substances have been known which possess an overall repellent effect towards insects.[1,2] However it is only recently that systematic work has been performed to find compounds which show antifeeding properties rather than gross repellent effects. Plants that possess natural resistance to infestation by insects are the most obvious potential source of antifeedants and several groups of workers have recently undertaken chemical fractionation monitored by some form of feeding test bioassay, in order to isolate inhibitors from such plants.

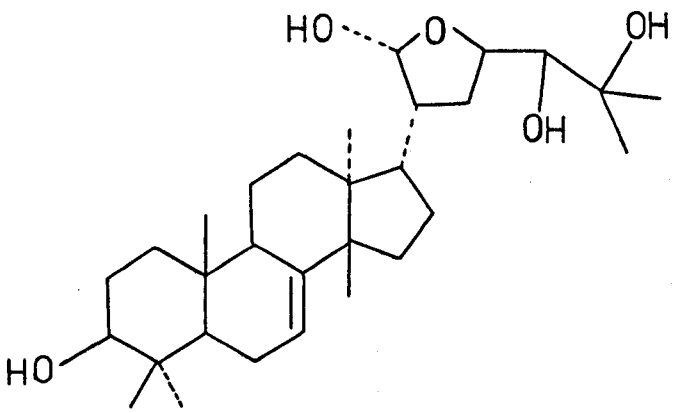
In 1964-65 Mrs. Shpan-Gabrielith,[3,4] working at the Anti-Locust Research Centre in London, screened a number of sub-tropical plants to determine their palatability to the desert locust Schistocerca

gregaria Forsk. The results obtained showed that Melia azedarach L. (Persian lilac, chinaberry or bakayan) of the Meliaceae family was completely unpalatable to the insect. Several other workers[5,6] had previously shown that certain parts, or various crude extracts of M. azedarach inhibited the feeding of S. gregaria and the african migratory locust Locusta migratoria migratorioides. Similarly another tree of the Meliaceae family Azadirachta indica A. Juss (syn.[7] Melia azadirachta L., Melia indica Brandis, Melia parviflora Moon), commonly known as the neem tree (also nim, nimba, Indian lilac, margosa), had been reported to possess locust antifeeding properties.[7,8]

Shpan-Gabrielith developed a feeding-test bioassay which later made possible more systematic work to isolate the compound or compounds responsible for the locust antifeeding activity displayed by the two species from the Meliaceae family. The test involved impregnating filter papers with a solution of an extract under test, drying, spraying with 0.25M sucrose solution, and drying again. The papers were then presented to mid-stadium fifth instar nymphs of the desert locust which had been starved for 24 hours previously. Extracts were scored as to their antifeeding activity according to the weight of paper eaten compared to the weight eaten of a control paper which had only been sprayed with sucrose solution.

Using this bioassay technique to monitor the extraction, fractionation, and purification, Lavie et al[9] in 1967 isolated the

active constituent Meliantriol (1) from the fruit of M. azedarach. Pure meliantriol, which they also claimed was present in neem seed oil, showed 100% antifeeding activity at a dose of 8µg./cm.^{2} on the filter papers used in the test.

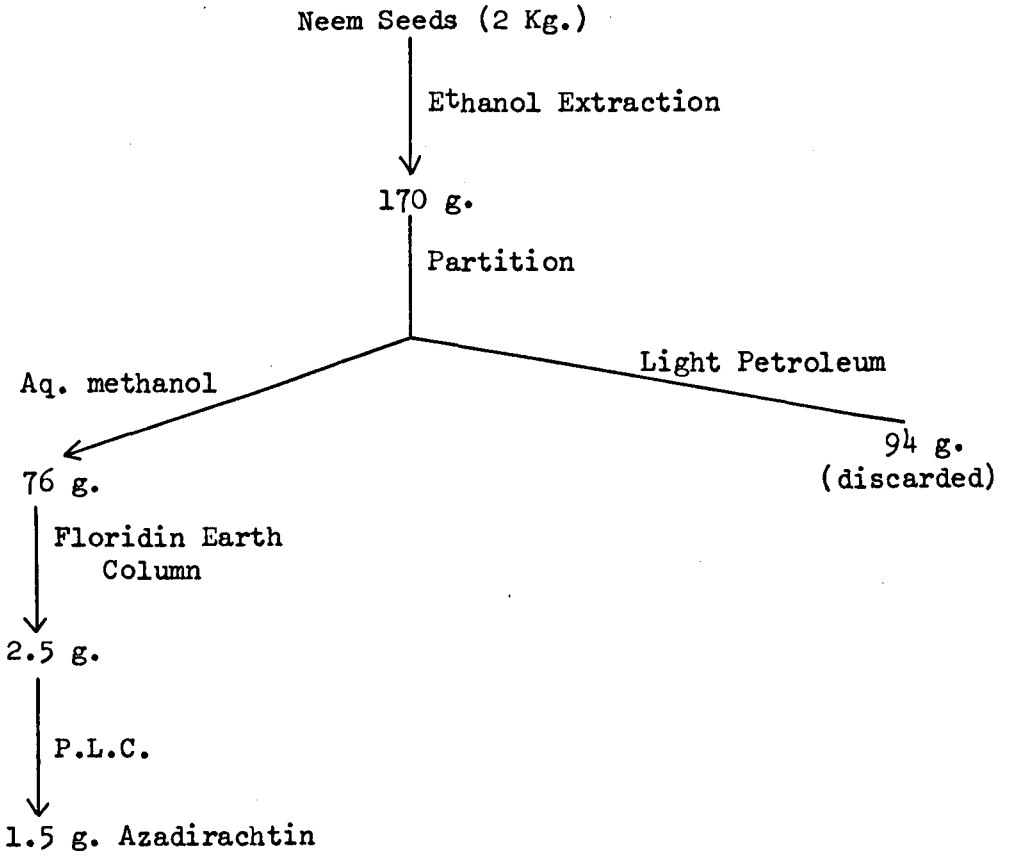


(1)

In 1966 Butterworth and Morgan undertook the fractionation of an ethanol extract of neem seeds and the previously described feeding test bioassay, employing the desert locust, was used to monitor the proceedure. Various solvent partitions and chromatographic techniques led to the isolation of the feeding deterrent azadirachtin, reported[10] in 1968. Spectral evidence clearly indicated that azadirachtin was not closely related to the triterpene alcohol

meliantriol and bioassay showed that the limiting activity of azadirachtin was at a dilution almost ten thousand times greater than that at which the limiting activity of meliantriol occurred. A test solution of azadirachtin was shown to be 100% active down to a concentration of 0.04 mg./l.

Initial work towards the structure of azadirachtin was restricted by the small amounts of material available. However an improved isolation proceedure was devised which was monitored by thin-layer chromatography (t.l.c.) as a more convenient alternative to bioassay. This proceedure has been fully described[11] and is summarised below:

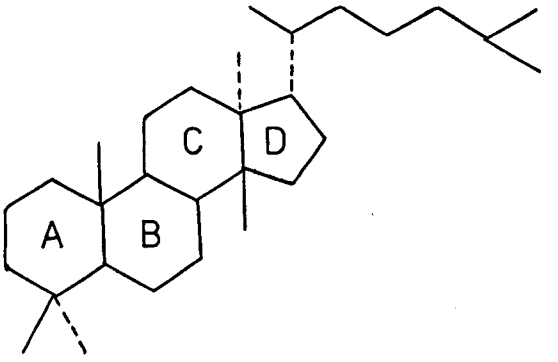


Azadirachtin was obtained as a colourless, amorphous powder, insoluble in light petroleum and ether, slightly soluble in carbon tetrachloride, soluble in benzene and toluene, and very soluble in chloroform, acetone, ethanol and methanol. The homogeneity of the material was established by t.l.c. in several eluant mixtures. Micro-analysis showed that the molecule contained no nitrogen but the figures for carbon and hydrogen content were variable due to retention of traces of solvent or impurity.

No molecular ion was seen in the mass spectrum of azadirachtin and consequently the molecular weight was in doubt until a bis(trimethylsilyl)-ether derivative was made.[12] This was more amenable to mass spectrometry, and accurate mass determination on the peak at highest $^{m}/e$ indicated the composition $C_{41}H_{60}O_{16}Si_2$. Assuming that this was the molecular ion of the derivative, Butterworth postulated the molecular formula $C_{35}H_{44}O_{16}$ for azadirachtin itself, with molecular weight 720.

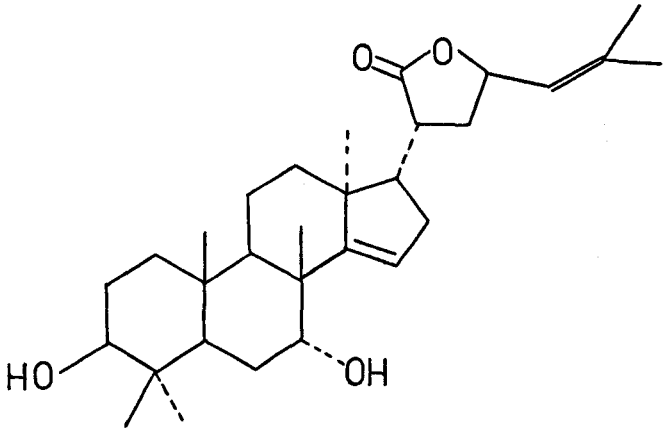
This molecular formula, and the absence of characteristic features of any other class of compound, suggested that azadirachtin might be related to the group of highly oxygenated triterpenoids which has been isolated from neem and other Meliaceae species. These compounds vary greatly in structural complexity but it is postulated that they all fall on a biosynthetic pathway of increasing oxidation and rearrangement based on the tirucallane skeleton (2), oxidised at the 3-position. Meliantriol (1) for example could be produced

from the proposed precursor by oxidation in the side-chain.



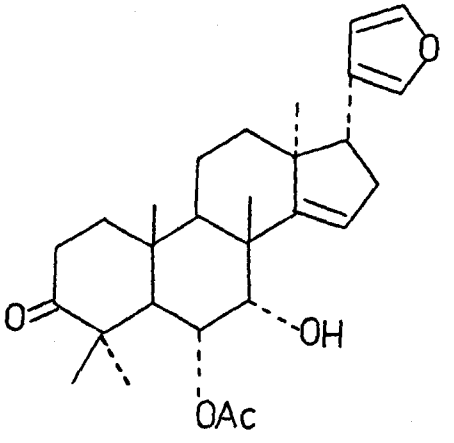
(2)

A further stage on the proposed biogenetic pathway involves the shift of a methyl group from C14 to C8 to give compounds with an apo-euphol ring structure and a tirucallane side chain.[13-15] The compound (3) isolated[16] from Melia azedarach by Lavie and Levy in 1969 has such a structure.



(3)

Another important step on the proposed biosynthetic route is the oxidation of the side-chain to a β-substituted furan ring[15, 17-19] with loss of four carbon atoms to give the tetranor-triterpenoid series. These compounds, often called limonoids because of their structural relationship to limonin,[20] occur widely in Meliaceae and Rutaceae species and reviews by Dreyer[21] and Rao[22] have been published. Limonoids isolated from neem at this oxidation level include meldenin[23] (4), epoxy-azadiradione (5) and related compounds,[24] and vepenin[25] (6).



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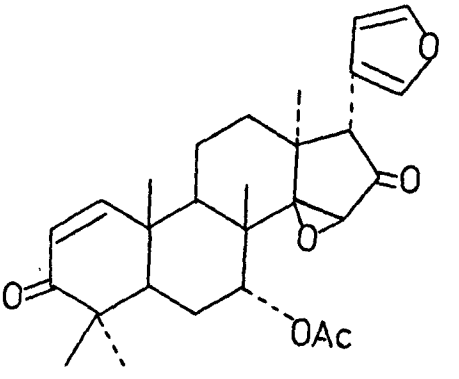

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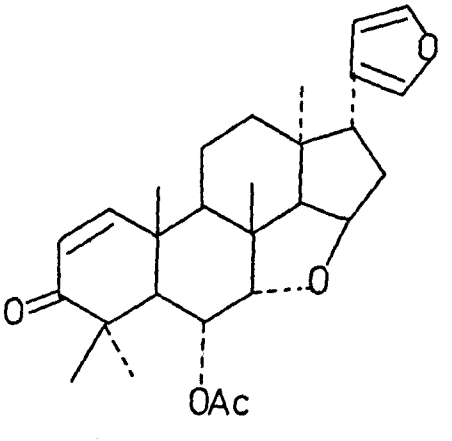
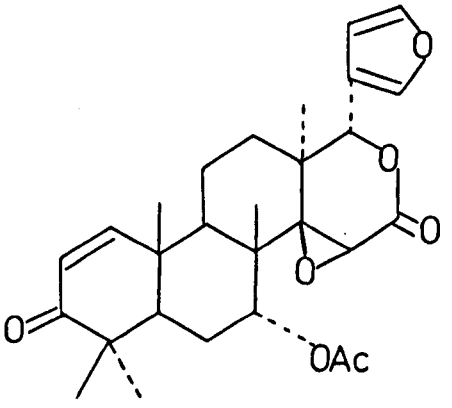


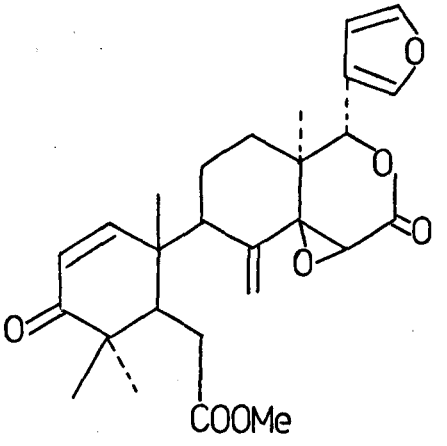
(6)

Further oxidation may occur in the D ring to give a δ-lactone structure and gedunin[26] (7) is a typical compound isolated from neem at this level of oxidation.



(7)

Further biosynthesis in many Meliaceae species produces B-seco limonoids, such as andirobin[27] (8) isolated from Carapa guayanensis Aubl., and it is thought that these may undergo cyclisation[21] between the C-2 and C-30 positions to give the bicyclononanolide compounds such as xylocarpin[28] (9) isolated from Xylocarpus granatum Koen.



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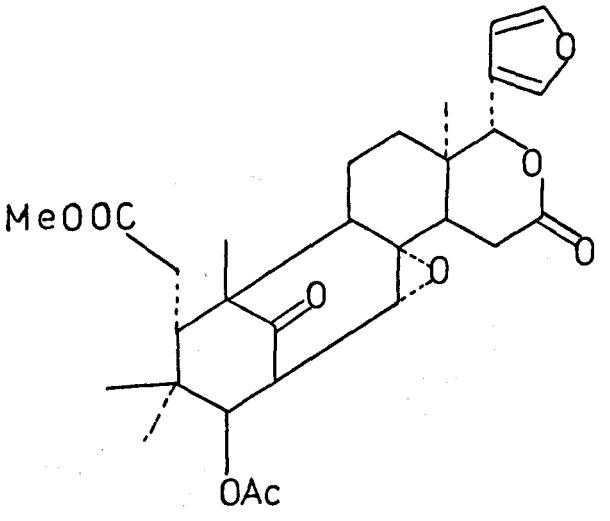
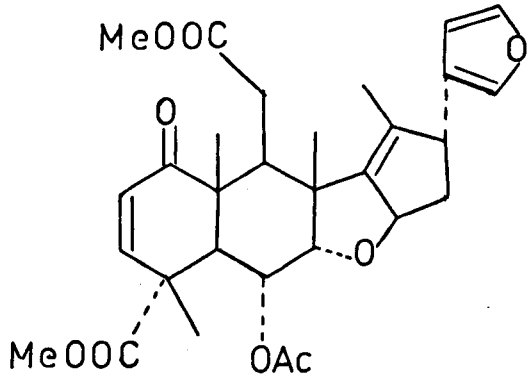


(9)

However, on the evidence of compounds so far isolated, biosynthesis in A. indica leads to the production of C-seco limonoids such as nimbin[29](10) and salannin[30](11), with the B-ring left intact.



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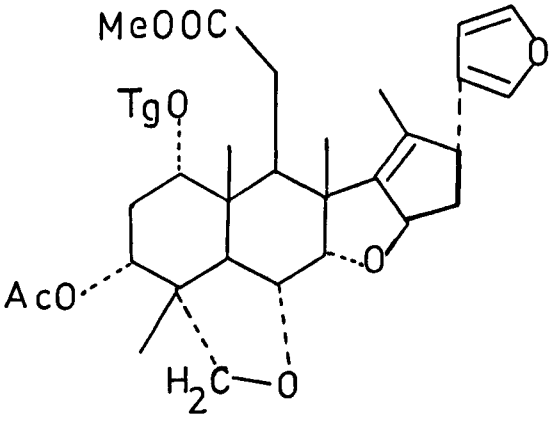
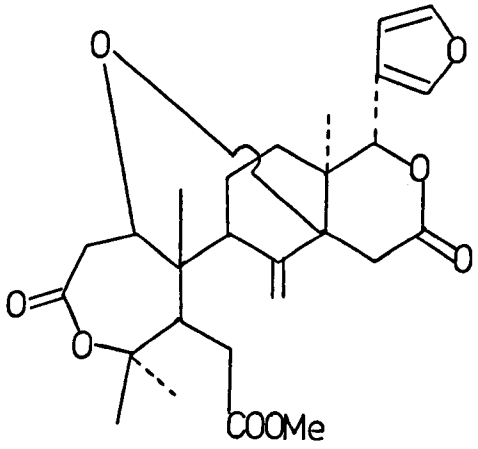


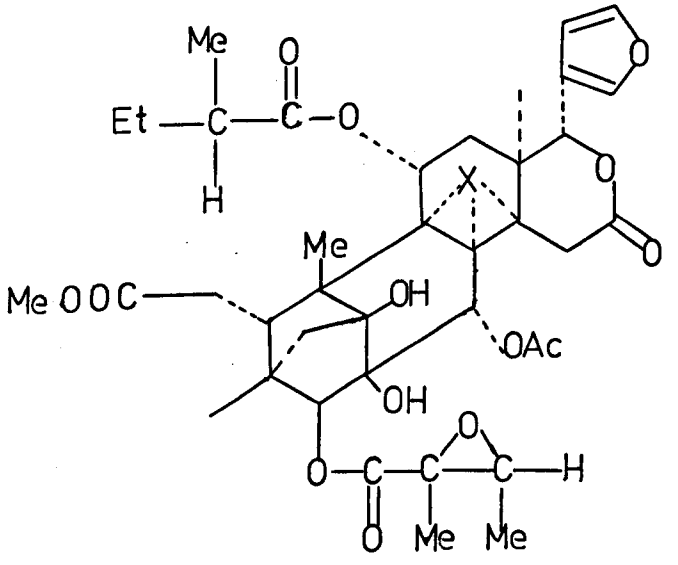
(11) Tg = Tigloyl

Compounds from the Meliaceae which have an expanded A ring were unknown until the isolation of methyl ivorensate[31](12), from Khaya ivorensis in 1969, but no such compound has yet been reported from neem.



(12)

Utilin (13) isolated[32] from Entandrophragma utile (Meliaceae) shows a very high degree of rearrangement and it is interesting to note that the structure[33] of this compound was elucidated by X-ray crystallography: a technique which is finding increasing application in this field.

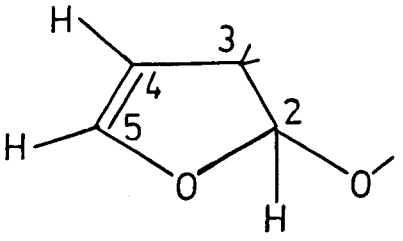


(13) X = orthoacetate

It was apparent however that azadirachtin did not fit directly into the biogenetic scheme outlined above. This was clear from the nuclear magnetic resonance (n.m.r.) spectrum (Fig. 1) of the compound which did not show the methyl singlet signals in the region τ8.5 to 9.3 which are a common feature in the spectra of triterpenoids and tetranor-triterpenoids. Spectral evidence also showed that there was no characteristic β-substituted furan ring system in the molecule.

By chemical and spectral evidence Butterworth[12] recognised the presence of two carboxymethyl groups, one tiglate and two acetate ester entities, and two hydroxyl groups in the azadirachtin molecule. He also tentatively suggested that azadirachtin contained one tertiary

methyl group. Furthermore he proposed the partial structures shown (14, 15) for the molecule.



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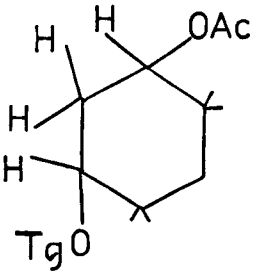


(15) Tg = Tigloyl

The 2,3-dihydrofuran system which Butterworth proposed for azadirachtin is rare among naturally occurring substances. However all such compounds containing this grouping which have been reported,[34-38] show spectral and chemical properties in accord with those of azadirachtin as far as this structural entity is concerned.

Overall, Butterworth definitely assigned twenty-three of the protons seen in the n.m.r. spectrum of azadirachtin and he gave a tentative assignment to nine more. His work left three oxygen atoms unassigned though it was proposed that one of these was attached at the 2-position of the 2,3-dihydrofuran ring system. When the molecular formula of azadirachtin was considered in the light of the structural entities elucidated, it was clear that six double bond equivalents remained to be assigned.

It was thought that azadirachtin might represent a triterpenoid oxidised to a greater extent than any other such compound so far isolated from neem. This interesting possibility, as well as the high degree of biological activity shown by the compound, suggested that studies on the structure and antifeeding effects of azadirachtin should be continued. Therefore the work described later in this thesis was undertaken.

A bioassay similar to that employed by Butterworth was used by Haskell and Mordue[39] to show that azadirachtin had 100% antifeeding activity against Schistocerca gregaria down to a test solution concentration of 1×10^{-6}%, i.e. 0.01 mg./l. However Mulkern[40] treated a specially prepared artificial diet, with solutions of azadirachtin of concentration up to 5×10^{-3}% and found no feeding deterrent effect against two species of the grasshopper Melanoplus. Further tests on Schistocerca by Mrs. Mordue[41] using Mulkern's technique have shown that azadirachtin was not deactivated by the special diet, so clearly the North American grasshoppers do not respond to the compound at the concentration quoted. Further work by Haskell & Mordue[39] has shown that the A_3 receptors (classification Thomas[42]) in the mouth-parts of the desert locust are the principle sites at which detection of azadirachtin occurs, though other areas can display a degree of sensitivity.

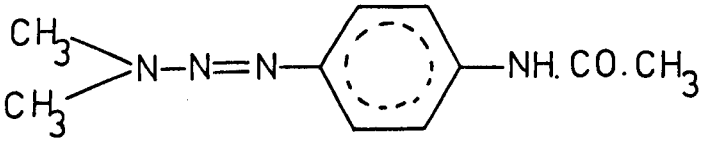
Azadirachtin has been tested by several other groups of workers for anti-feeding activity against various insects. It has been shown[43]

to be active against larvae of the cabbage white butterfly Pieris brassicae, the diamond back moth Plutella maculipennis, and the silver-Y moth Plusia gamma though the feeding of these species was only reduced during the 24 hour test by 50%, 25%, and 40% respectively when a deposit of 1.6 mg. of azadirachtin per square cm. of leaf was employed. Behavioural and electrophysiological tests on azadirachtin by Schoonhoven[44] have revealed activity against the larvae, and in some cases the adults, of the Colorado potato beetle Leptinotarsa decemlineata Say, the cabbage white butterfly P. brassicae, the leafroller Adoxophyes reticulana, the peach aphid Myzus persicae, and the cotton bug Dysdercus koeningi but quantitative details are not yet available. Work at the Forest Products Research Laboratory has established[45] that solutions of azadirachtin up to a concentration of 2.4 g./l. spread on a sample of the host-wood Pycnanthus angolensis (ilomba) do not deter feeding by the termite Reticulitermes santonensis.

As well as its resistance to the feeding of the desert locust, M. azedarach is apparently undesirable to most insects since few, if any, feed on it. McMillan et al[46] have shown that a crude chloroform extract of chinaberry leaves not only inhibits the feeding of the corn earworm Heliothis zea Boddie and the fall armyworm Spodoptera frugiperda J.E. Smith, but also can retard the growth of these species or cause death.

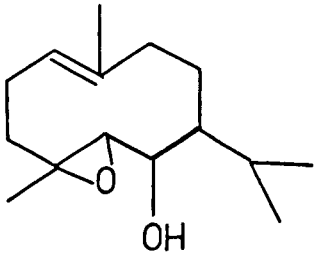
Hartley and West[2] in their book published in 1969 mention

only one feeding deterrent and this is indicative that interest in such compounds has arisen very recently. The one example they give is the non-toxic, synthetic compound 4-(dimethyl-triazeno)-acetanilide (16) which deters the feeding of the southern armyworm.



(16)

Feeding deterrents belonging to several classes of compounds have recently been isolated from various plants (short review: Munakata[47]), and some of these compounds are oxygenated triterpenoids. Munakata et al[48, 49] have reported that shiromool (17), shiromodiol diacetate (18a) and shiromodiol monoacetate (18b), isolated from Parabenzoin trilobum Nakai, are active as antifeedants against Prodenia litura F. and Abraxas miranda Butler.



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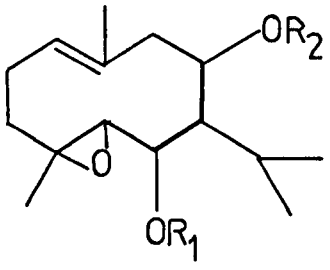
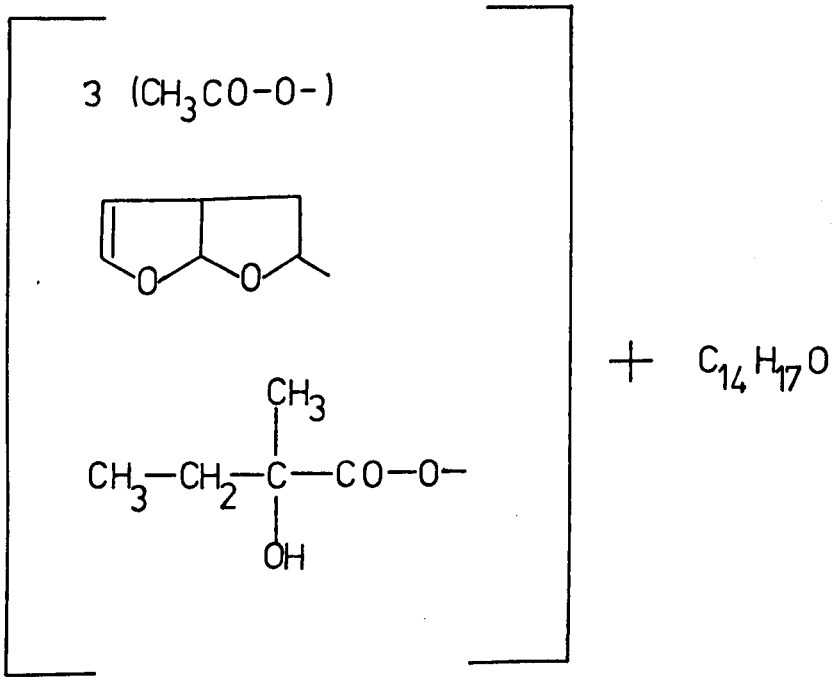


(18a) $R_1 + R_2 = Ac$

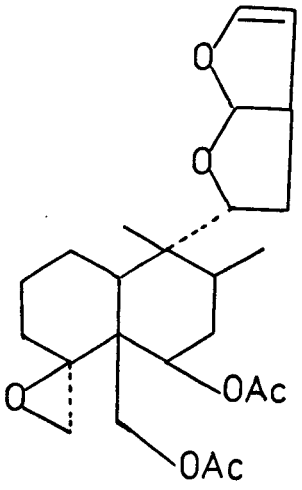
(18b) $R_1 = H$, $R_2 = Ac$

Munakata et al have also reported [47] the isolation of two compounds, clerodendrin A and B, from Clerodendron tricotomum Thumb which inhibit the feeding of P. litura. The partial structure proposed for clerodendrin A, $C_{31}H_{42}O_{12}$, is shown (19) and further work is proceeding by X-ray analysis.



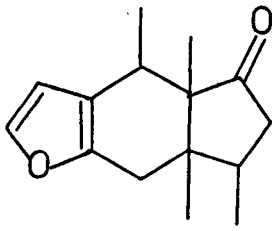
(19)

Although the overall structures of azadirachtin and clerodendrin A may not be related, some interesting comparisons can be made between some of their respective structural entities. For example, clerodendrin A contains a 2-hydroxy-2-methylbutyrate ester entity which is formally constructed by addition of a molecule of water to the double bond of a tiglate ester group. Also the compound contains a 2,3 dihydrofuran ring substituted by an ethereal oxygen function at the 2-position. A similar structural entity may well be present in azadirachtin, though fully substituted at the 3-position, and has also been identified in clerodin[35] (20) from Clerodendron infortunatum.



(20)

A survey of a number of terpenoid substances by Wada and Munakata[50] has shown that pinguison (21) and absinthin (22) are also active antifeedants against the cotton leaf worm P. litura. It is interesting to note that two limonoids, nomilin[51] and limonin,[20] were found to be inactive.



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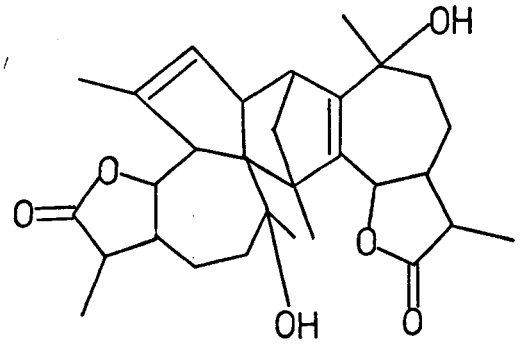

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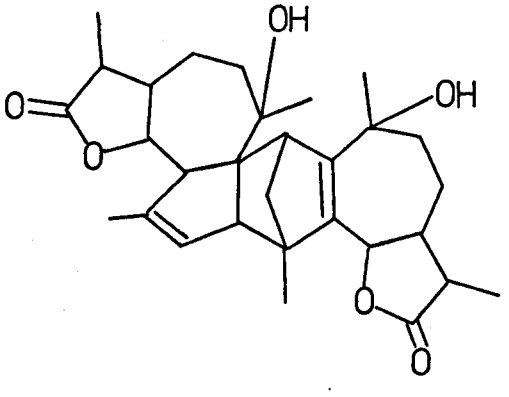


(22) (alternative possible structures)

DISCUSSION

The Isolation of Azadirachtin from Neem Seeds

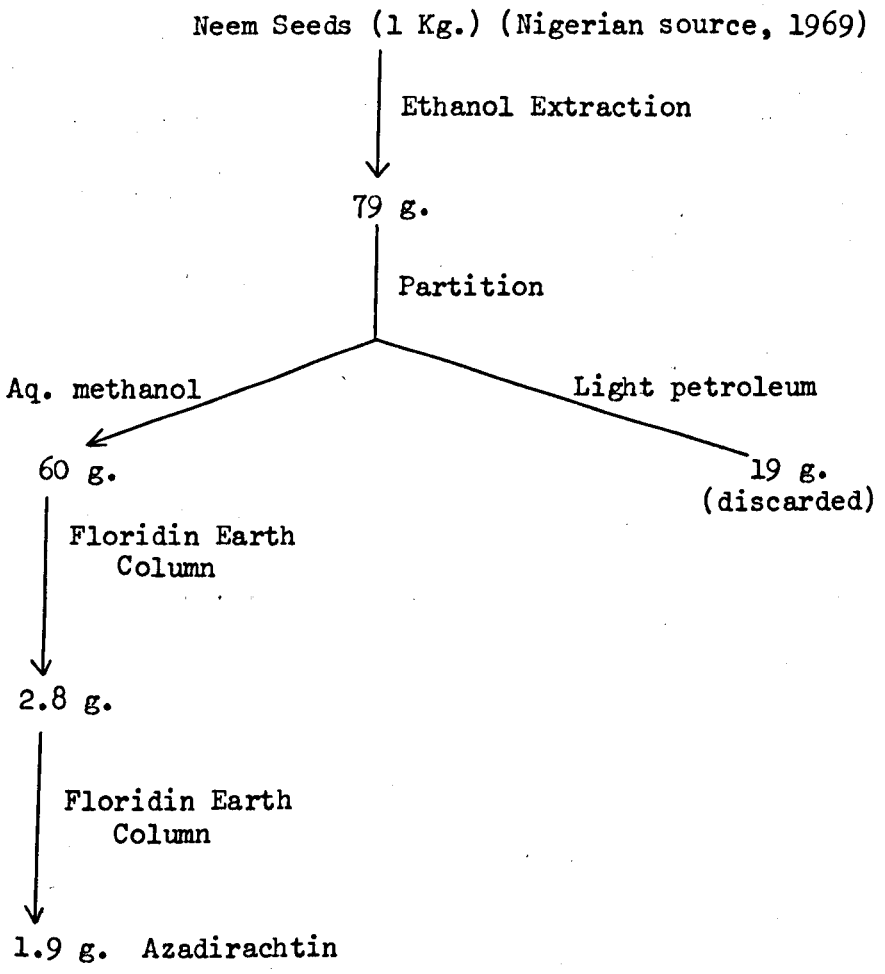
Since this work began in 1969, neem seeds have been obtained from a number of sources. Most batches of seeds were supplied by commercial sources in India through the Tropical Products Institute (T.P.I.) in London. These seeds were probably picked at ripeness when the commercially important seed oil (neem oil) content was at a maximum. One batch of seeds used as a source of azadirachtin was obtained from Nigeria through the T.P.I. and another came from a private source in India. There has been a considerable variation from one batch of seeds to another both in the quantity of azadirachtin extracted, and in the ease with which it has been isolated. As a result the procedure described by Butterworth[12] for the isolation of azadirachtin has been modified as circumstances required.

Neem seeds obtained from commercial sources in India through the T.P.I. were the sole source of azadirachtin used by Butterworth. The isolation procedure he employed, which is summarised in the Introduction, routinely involved 2 Kg. of seeds at a time. Maceration of such a quantity of seeds with 95% ethanol, filtration and evaporation of the solvent generally yielded about 170 g. of a brown gum which was then partitioned between light petroleum and aqueous methanol. Evaporation of the methanol left a brown gum (76 g.)

which was dissolved in toluene and the resulting solution was applied to the top of a column of Floridin Earth (950 g.) made up in toluene. The column was eluted with ether-acetone (95:5) and the fractions (250 ml. each) obtained were investigated by t.l.c. and n.m.r. spectroscopy. Those fractions which contained azadirachtin were combined to give usually about 2.5 g. of a yellow solid. This solid was purified by P.L.C. eluting twice with ether-acetone (95:5) to give approximately 1.5 g. of azadirachtin with melting point 149-153°. This procedure was efficient for all the batches of Indian neem seeds obtained by Butterworth through the T.P.I. from 1966 to 1969.

Variation from the general outline described by Butterworth was first noticed when a batch of Nigerian neem seeds obtained through the T.P.I. in 1969 proved to be a more than usually convenient source of azadirachtin. Following the extraction procedure described above, the azadirachtin eluted from the large Floridin Earth column was accompanied by such a small quantity of impurities that chromatography on a second, smaller column of Floridin Earth was sufficient to give a sample of azadirachtin as pure, according to t.l.c. and n.m.r. spectroscopy, as that obtained by Butterworth after P.L.C. The substitution of a column chromatography stage for the P.L.C. stage used by Butterworth considerably reduced the material losses from the high level normally experienced during the use of preparative chromatography plates. This fact,

coupled with the richness of the source, led to a high yield (1.9 g.) of azadirachtin from 1 Kg. of these particular seeds. The separation procedure employed for these Nigerian seeds is summarised below.



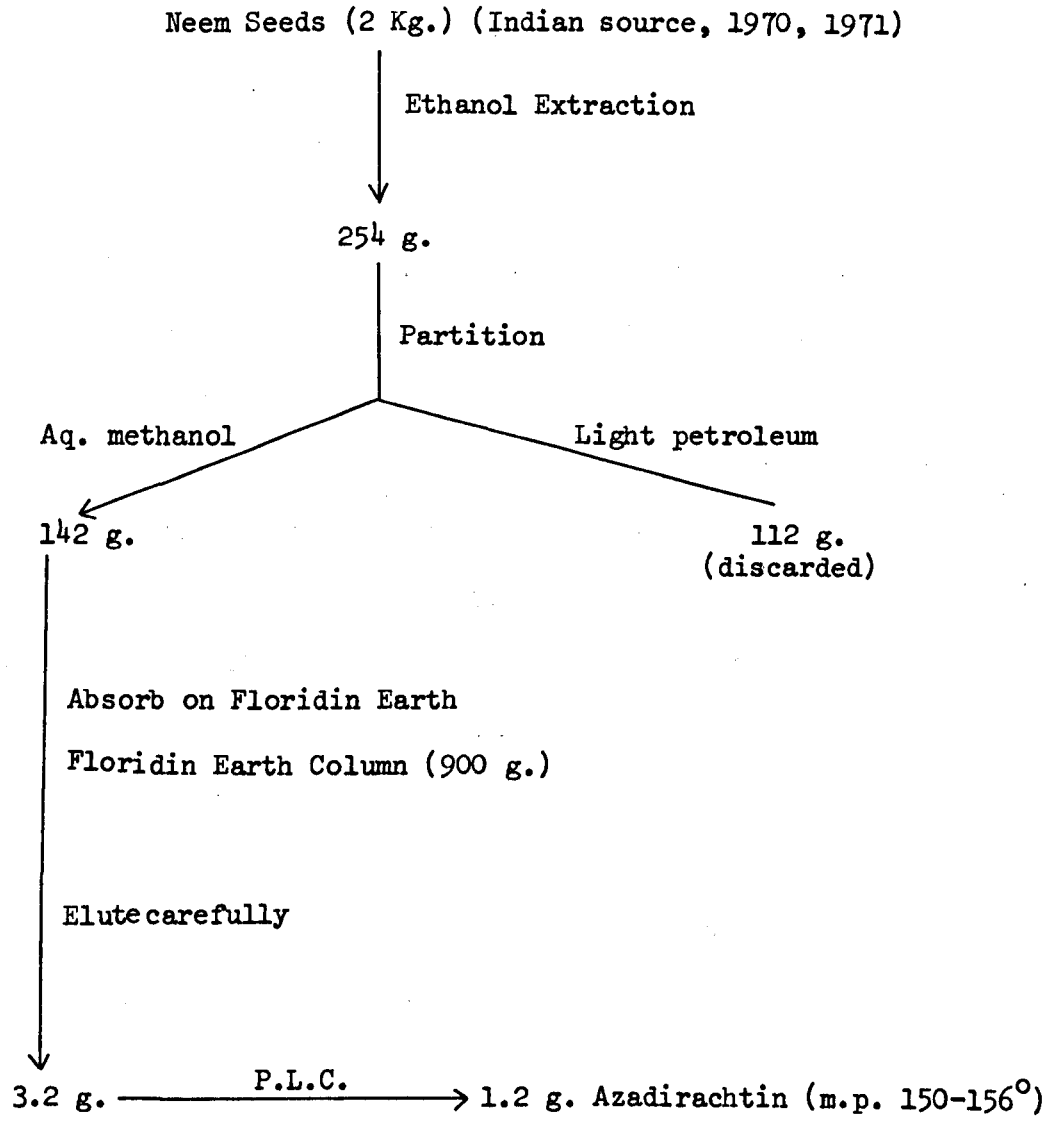
Unfortunately this batch from Nigeria only consisted of 2.5 Kg. of seeds and no seeds since obtained have been so rich in azadirachtin or so convenient as a source of the compound.

The batch of seeds obtained from private sources in India in 1970 were unripe and consequently still retained the outer

fleshy coating around the kernel. These seeds proved to be a particularly inconvenient source of azadirachtin and two P.L.C. stages in addition to two column chromatography procedures were required to obtain azadirachtin of acceptable purity. The yield too was very poor: 400 mg. of the compound from 2 Kg. of these seeds, and this may have been due to a low concentration of azadirachtin in this batch. However the low yield may have been due, in part at least, to losses incurred during the increased number of chromatographic procedures necessary to isolate azadirachtin from the large quantities of other materials which ran close to it both on columns and on preparative plates.

Batches of Indian neem seeds obtained through the T.P.I. during 1970 and 1971 were the major source of azadirachtin used in this work. These seeds proved to be very different from those used by Butterworth and a revised procedure for the isolation of azadirachtin was developed. This new procedure, described in the experimental section, is summarised below.

It is to be noted that whereas for the seeds used by Butterworth only 76 g. of material remained after the solvent partition, these later seeds yielded 142 g. of gum after this stage. Furthermore, the gum was only partly soluble in toluene and so it was dissolved in methanol and absorbed onto Floridin Earth (270 g.) to give a free-flowing powder which was then applied to the top of the column.



It was found that immediate elution of the column with ether-acetone (95:5) as described by Butterworth yielded azadirachtin containing fractions heavily contaminated with other materials, mainly less polar than the required compound. Therefore the column was first eluted with toluene, followed by toluene-ether mixtures, and finally with ether-acetone mixtures.

In this way the fractions containing azadirachtin were contaminated with much smaller amounts of other materials. A single P.L. C. stage eluting twice with ether-acetone(85:15) was then usually sufficient to afford pure azadirachtin. Occasionally a sample of the compound was obtained which had retained a small quantity of impurities. In such a case further purification by P.L.C. eluting twice with chloroform-acetone(3:1), yielded azadirachtin of high purity.

Gradient elution of a small chromatographic column, used in conjunction with a fraction collector was investigated with the possibility that if this technique was a success it could be used instead of the P.L.C. stage which involves a low recovery of azadirachtin. However a Davison silica gel (grade 923, 100-200 mesh) column eluted with ether-acetone mixtures or with toluene-methanol mixtures, and a Floridin Earth column eluted with ether-acetone mixtures all failed to rid azadirachtin of its close running impurities. Similarly attempts to purify column fractions by precipitating the azadirachtin from a carbon tetrachloride solution all failed. In general azadirachtin was only obtained pure if P.L.C. was used as the final stage in the isolation.

Neem leaves as a possible source of Azadirachtin

The leaves of neem have long been used in India to protect stored clothes and books from insect attack[7]. It was therefore decided to subject a crude ethanol extract of the leaves to the

standard feeding test bioassay. It was found that the extract was 100% active at a concentration equivalent to 100 g. of leaves in a litre, and 80% active at 10 g. leaves/l. This was considerably less active than a crude ethanol extract of neem seeds which Butterworth reported was 100% active at a concentration equivalent to 0.5 g. seeds/l. However the figures for the crude leaf extract may have been complicated by the presence of a feeding stimulant which could have partially masked the antifeeding activity. Therefore it was decided to fractionate the extract to ascertain whether azadirachtin was present in the leaves.

Details of the procedure employed are given in the Experimental section. An initial ethanol extraction of the leaves was followed by fractionation of the extract on a column of Floridin Earth by elution with toluene, toluene-ether mixtures, and ether-acetone mixtures. All the fractions which contained material with a similar R_F on t.l.c. to that of azadirachtin were combined and subjected to P.L.C. eluting twice with ether-acetone (92:8). Materials designated A, B, and C were isolated from each of three bands with R_F close to that of azadirachtin. Investigation of these materials by t.l.c. showed that they were all inhomogeneous and furthermore their n.m.r. spectra did not show any of the characteristic features of azadirachtin such as methyl singlets around τ6.2 and τ8. Indeed the n.m.r. spectra of all three of these materials consisted mainly of ill-defined absorptions in the region τ8 to τ9. It would seem

from n.m.r. evidence that if azadirachtin was present in any of these materials it was as a minor constituent, probably less than 10%.

In order to determine what proportion of azadirachtin, if any, was present in these three materials, each one was subjected to a feeding test. The results, which are tabulated on page 166 showed that the lowest concentration at which 100% activity occurred was 50 mg./l. in all three cases, though material A did display 99% activity at 5 mg./l. Azadirachtin is known to be 100% active at 0.05 mg./l. so if A, B, or C contained the compound at all it could only have been at a level of one part in one thousand. There was in the order of 100 mg. of each of these materials isolated from 1 Kg. of leaves so on this evidence azadirachtin was present at a concentration of about one part in ten million in the leaves if it was there at all. However, by a similar comparison, the figures from the feeding test on the crude leaf extract suggested a possible concentration of azadirachtin in the leaves of about one part in two million. This disparity may not be very meaningful though the crude extract may have contained a feeding stimulant without which the disparity might have been greater.

It is possible therefore that there is some feeding deterrent, other than azadirachtin, present in the leaves which gives the crude extract a higher activity than one might expect from the activity shown by the material in the chromatographic region of azadirachtin. This possibility could only be checked by a full-

scale extraction monitored throughout by bioassay rather than a specific search for a known compound as was undertaken here. Above all, it is clear that if azadirachtin is present, an extraction starting with only 1 Kg. of the leaves yields an amount of the compound too small to be detected by chemical or spectral means.

Attempted Isolation of Meliantriol from Neem Seeds

In 1967 Lavie, Jain and Shpan-Gabrielith[9] reported the isolation of the antifeedant meliantriol (1) from the fruit of Melia azedarach and from neem seed oil. The isolation procedure had been monitored by feeding test bioassay employing the desert locust and pure meliantriol was claimed to show 100% antifeeding activity at a dose of 8 $\mu g./cm^2$ on the test papers. Tests at Keele had indicated that azadirachtin possessed 100% antifeeding activity down to a test solution concentration of 0.05 mg./l. It was found that one 5.5 cm. filter paper absorbed about 0.5 ml. of test solution and this was calculated to give a concentration of azadirachtin of 1 $ng./cm^2$ on the test paper. So it appeared that azadirachtin gave 100% antifeeding activity at a concentration eight thousand times less than that required in the case of meliantriol. It should be noted that on a similar basis a crude ethanol extract was 100% active at a dose of 1 $\mu g./cm^2$, that is it showed higher activity than pure meliantriol.

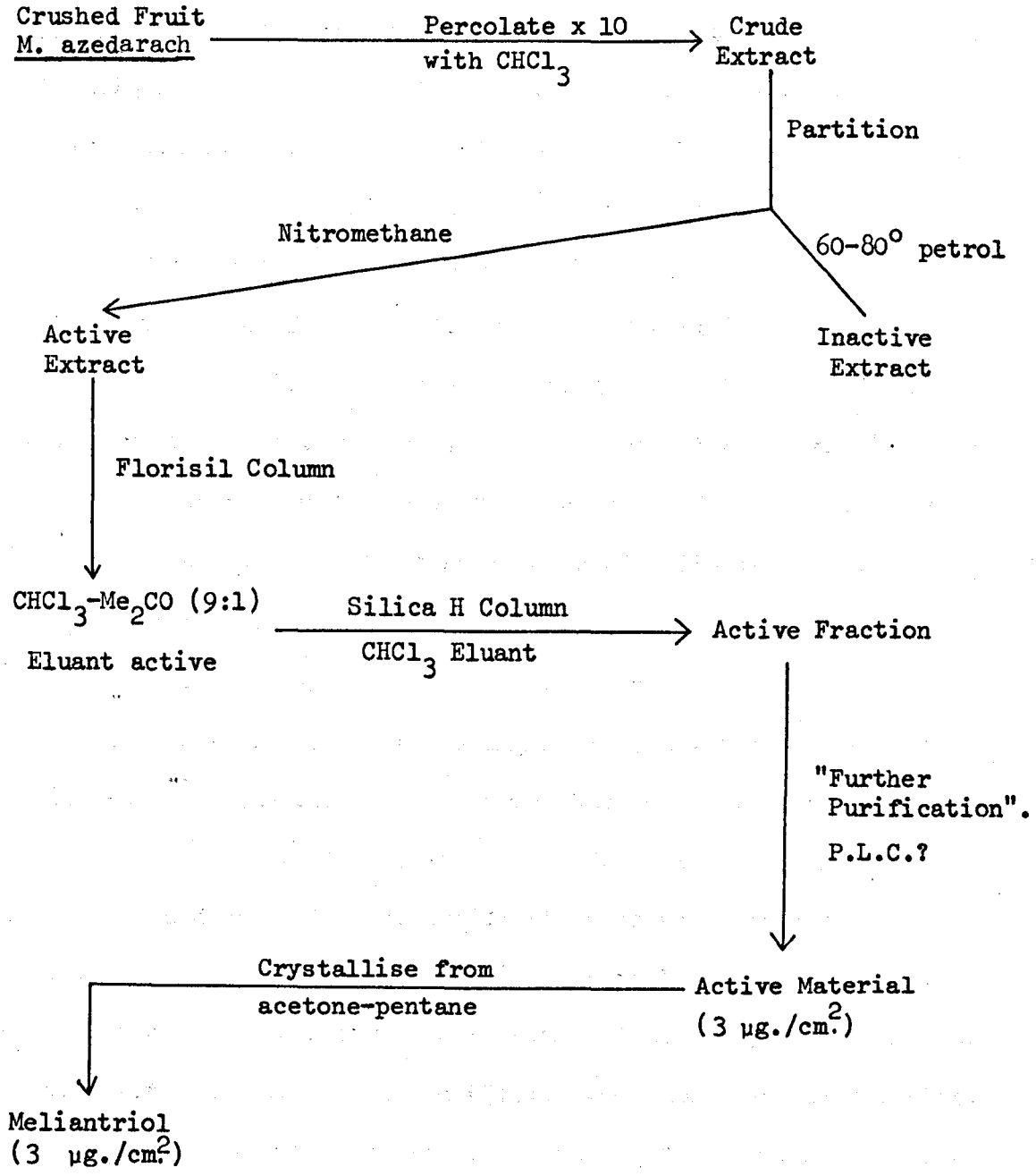
The R_F of meliantriol in three solvent systems was obtained from a section of a thesis by Jain[52] (kindly supplied by Professor D. Lavie) and these are shown in the table below alongside the corresponding data for azadirachtin.

Solvent System	Meliantriol	Azadirachtin
Chloroform-acetone (7:3)	R_F 0.33	R_F 0.40
Ethyl acetate-benzene (4:1)	R_F 0.47	R_F 0.41
Ethyl acetate	R_F 0.62	R_F 0.60

These figures suggested that the activity shown by meliantriol might have been due to the presence of a small quantity of azadirachtin as an impurity in the sample. Such a situation would explain Lavie's observation that a crude sample of meliantriol was 100% active at a lower concentration (3 $\mu g./cm^2$) than that required for pure meliantriol (8 $\mu g./cm^2$) to show 100% activity, though such a result may have been due to natural variation in the bioassay. Lavie's claim that meliantriol synthesised from melianone showed activity comparable to that of naturally occurring meliantriol appeared to contradict the proposal that this activity was due to traces of azadirachtin in the sample. In order to clarify this situation an attempt was made to isolate meliantriol so that a direct comparison could be made with azadirachtin under exactly

the same feeding test and t.l.c. conditions.

Neem seed oil was not available at the time, so attempts were made to isolate meliantriol from whole neem seeds by following the published procedure for the isolation of the compound from chinaberry fruit. This procedure is summarised below.



The progress of the extraction using neem seeds is described in detail in the Experimental section. After the Florisil column stage in the extraction of chinaberry seeds Lavie took only the chloroform-acetone (9:1) eluant on to the next stage. However in the neem seed extraction the fractions eluted from the column by chloroform-acetone mixtures in the range (9:1) to (2:3) all contained materials with R_F close to that quoted for meliantriol on t.l.c. eluting with chloroform-acetone (7:3). Therefore all these fractions were combined and applied to the silica gel H column. This column was eluted successively with chloroform and chloroform-acetone mixtures but none of the fractions obtained contained components with the characteristic R_F of meliantriol in all three of the solvent systems quoted above. The n.m.r. spectra of selected fractions were determined but none of these showed the group of methyl resonances in the region τ8.6 to 9.3 characteristic of meliantriol. However an appreciable quantity of azadirachtin was recognised in the fractions eluted from the column by chloroform.

After the silica gel H column, Lavie undertook further purification of the chinaberry extract but no description of this procedure was given. In the absence of further details it was decided to undertake further purification of the neem extract by P.L.C. Selected fractions containing material running on t.l.c. near to the R_F of meliantriol were combined and subjected to P.L.C. eluting with chloroform-acetone (7:3). Again none of the materials isolated showed the characteristic features of meliantriol on t.l.c.

or by n.m.r. spectroscopy. Attempts to crystallise meliantriol from pentane-acetone solutions of selected fractions from before and after the P.L.C. stage were all unsuccessful.

After the failure to isolate meliantriol from neem seeds by following Lavie's procedure, selected fractions from before and after the P.L.C. stage of an extraction of neem seeds according to the Butterworth procedure were investigated by t.l.c. and n.m.r. spectroscopy. None of these fractions appeared to contain material displaying the t.l.c. properties or spectral features of meliantriol.

No definite explanation of this failure to isolate meliantriol from neem seeds is offered. However it is possible that meliantriol was present in this particular batch of seeds at too small a concentration for it to be detected by the means used. No indication of the concentration of meliantriol in neem, or M. azedarach, has been given either in Lavie's publication[9] or in Jain's thesis[52], though Lavie has stated[53] that the extraction of meliantriol is "a very tedious process and only small quantities have been secured." Therefore it is not possible to predict the weight of meliantriol which could have been present in 2 Kg. of neem seeds. Lavie was unable to supply a sample of meliantriol and so the problem of the relative activities of meliantriol and azadirachtin was left unresolved.

It is interesting to note that Lavie did not discover azadirachtin in neem oil and there seem to be two possible reasons

for this. Firstly he may have monitored the extraction of neem by t.l.c., using a reference sample of meliantriol from the M. azedarach source, rather than by the bioassay technique which would surely have led to the discovery of azadirachtin. Secondly, it is not known if neem oil contains azadirachtin. It is possible that the compound is not expressed from the seeds in quantity, if at all, during the production of the oil.

Structural Studies on Azadirachtin

Butterworth[12] prepared dihydro-azadirachtin by hydrogenation of the double bond of the 2,3-dihydrofuran ring in azadirachtin itself and the n.m.r. spectrum of this derivative is shown in Fig. 2. Hydrolysis of dihydro-azadirachtin in methanol solution by treatment with 2½% aqueous potassium hydroxide for one hour, followed by re-methylation with ethereal diazomethane, was shown by Butterworth to produce two products. The less polar of these products was identified as deacetyl-dihydro-azadirachtin, (n.m.r. spectrum, Fig. 3). The n.m.r. spectrum of the more polar product showed that an acetate group had been lost in the hydrolysis and that only one carboxymethyl group was present in the molecule. In the absence of an absorption in the I.R. spectrum characteristic of the hydroxyl group of a carboxylic acid, Butterworth postulated that a lactone had been formed between a carboxyl group produced by hydrolysis of a carboxymethyl group and a hydroxyl group liberated by hydrolysis of an acetate ester. The expected molecular weight

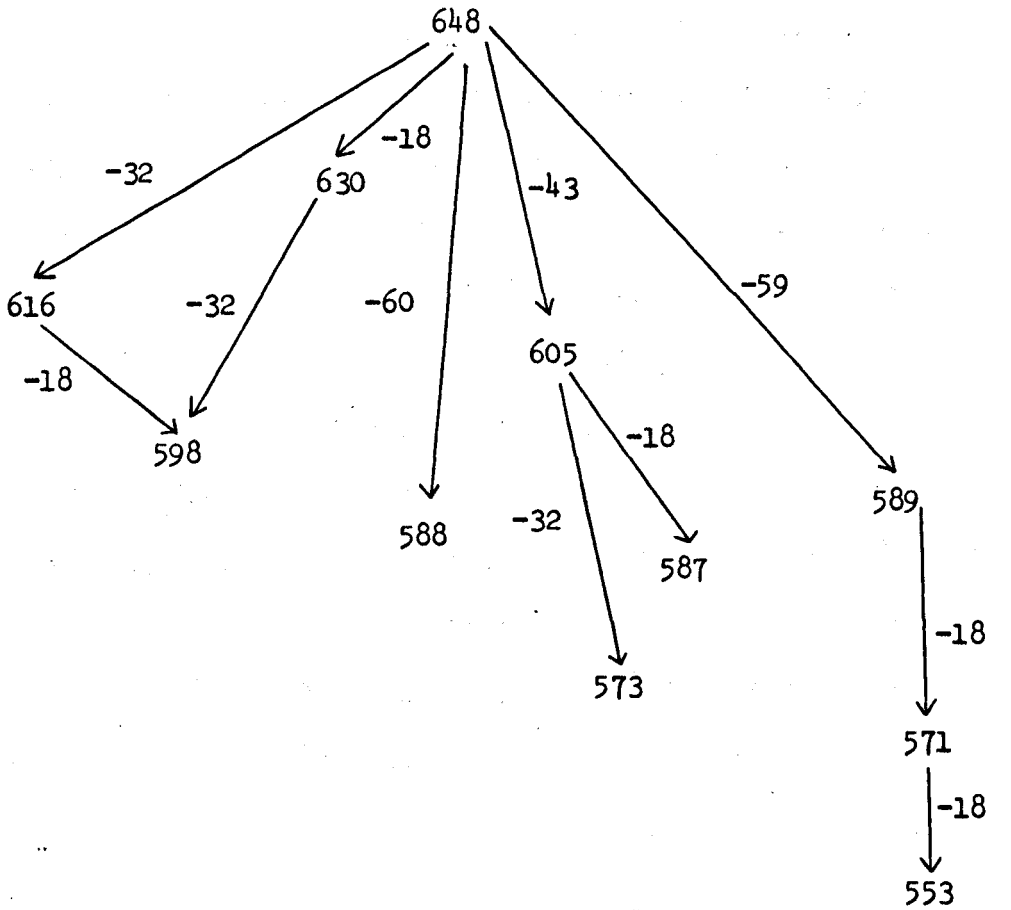
of such a derivative was 648 but the mass spectrum of this product showed ions up to $^{m}/e$ 704 with an intense ion at $^{m}/e$ 649. Butterworth concluded that further work was necessary to correlate this derivative with the parent compound.

It was therefore decided to prepare a further quantity of this suspected lactone. Alkaline hydrolysis of dihydro-azadirachtin was carried out according to Butterworth's procedure with the exception that the ethyl acetate extract obtained after acidification of the reaction mixture was left at 0^{o} overnight before removal of the solvent and subsequent treatment with diazomethane. It was hoped that this change would result in increased production of the lactone as compared with the uncyclised material. However, no improvement in the yield of the lactone was achieved.

As in Butterworth's procedure the crude product after remethylation was subjected to P.L.C. eluting twice with an ether-acetone mixture, but the sample of suspected lactone obtained was not pure. This was probably true for the material obtained by Butterworth and explains why the mass spectrum of his sample was anomalous. A second P.L.C., eluting twice with chloroform-acetone (7:3), yielded a homogeneous sample of the suspected lactone. Unfortunately no solvent system could be found which would afford pure suspected lactone from the crude product after only one P.L.C. stage.

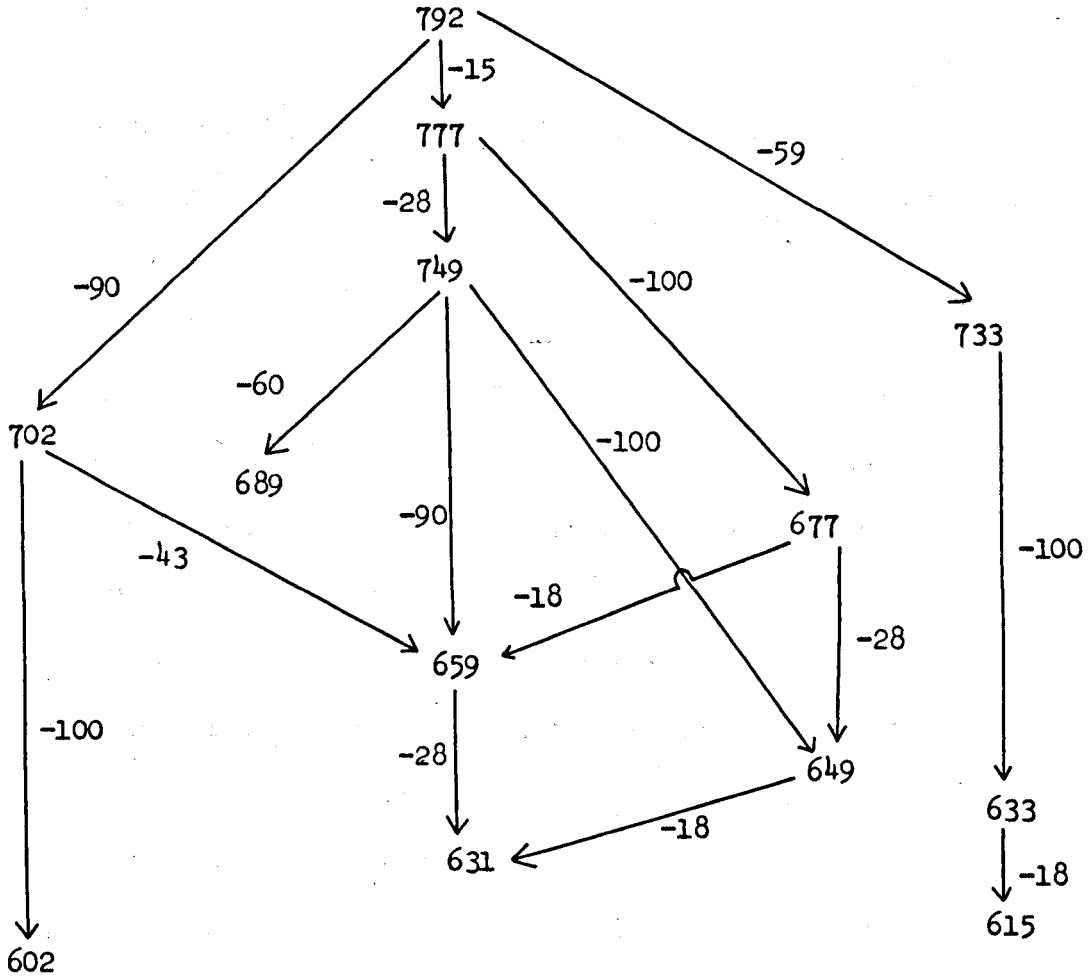
The mass spectrum of the pure sample showed ions up to

m/e 648 which is the molecular weight expected for the postulated lactone compound. Furthermore all the ions above m/e 550 could be accounted for by losses of known fragments in the molecule assuming a molecular weight of 648.



In order to check that the peak at m/e 648 represented the molecular ion and not an ion resulting from the fragmentation of this entity, it was decided to make a trimethylsilyl-ether derivative which would be more volatile than the parent compound in the mass spectrometer and less subject to loss of fragments in volatilization. Due to the small amount of suspected lactone

available, the silylation reaction was only carried out on a scale large enough to produce sufficient of the derivative for mass spectrometry. The proposed lactone was unaffected by B.S.A. in chloroform solution but when dimethylformamide was used as the solvent two products were isolated by preparative t.l.c. Only the less polar product was subjected to mass spectrometry as it was assumed that this product was silylated to the greater extent of the two and would therefore be the more volatile. This product proved to be a bis(trimethylsilyl)-ether with a molecular ion of mass 792.3192 which was within 2 p.p.m. of the calculated mass for $C_{38}H_{56}O_{14}Si_2$. The starting material therefore had a molecular formula $C_{32}H_{40}O_{14}$ and molecular weight 648 which was as expected for the lactone proposed by Butterworth. The ions above $^{m}/e$ 600 in the mass spectrum of the bis(trimethylsilyl)-ether derivative of the lactone were all accounted for by losses of fragments known to be present in the molecule as shown below. The loss of 90 mass units corresponds to the elimination of a molecule of trimethylsilanol.



The n.m.r. spectrum of the lactone in deuterochloroform ($CDCl_3$) is shown in Fig. 4. It was not possible to correlate all the signals with absorptions in the spectrum of dihydro-azadirachtin itself. The τ8.0 and τ6.2 regions of the lactone spectrum showed that an acetate ester and a carboxymethyl group had been lost in the hydrolysis of dihydro-azadirachtin. The triplet seen at τ4.5 in dihydro-azadirachtin, and azadirachtin itself, was moved upfield beyond τ5 in the spectrum of the lactone. This signal had been assigned by Butterworth to a proton on the carbon atom bearing the acetate ester group which is in a 1,3 relationship

to a carbon atom carrying the tiglate ester function in the molecule (see part structure 15). The upfield movement of this signal showed that it was this acetate group which had been lost from the dihydro-azadirachtin molecule in the production of the lactone. Butterworth had already shown, again from the upfield shift of the H-C-oAc signal from τ4.5, that this same acetate group was lost in the formation of deacetyl-dihydro-azadirachtin from dihydro-azadirachtin itself. There was no evidence in the spectrum to prove whether it was the new hydroxyl group left on removal of the acetate which was involved in lactone production, or if one of the other hydroxyl groups already present was involved.

The one proton singlet at τ4.4 in the spectrum of azadirachtin was assigned by Butterworth to a proton at the 2-position of the 2,3-dihydrofuran ring further deshielded by an adjacent oxygen function (see part structure 14). In dihydro-azadirachtin this signal moves upfield to τ4.7 and it is interesting to note that in the lactone it is further shifted to τ4.98. No corresponding further shift upfield in this signal was seen in the spectrum of deacetyl-dihydro-azadirachtin.

Although only three reactions namely hydrogenation, hydrolysis, and re-methylation were necessary to prepare the lactone from azadirachtin itself the overall yield of pure lactone was very poor (~5%), in part due to the three P.L.C. stages involved. Therefore, having established the molecular formula of the lactone,

further work on this interesting derivative in order to fully interpret its n.m.r. spectrum had to be abandoned. Further work which could have been attempted had the yield of lactone not been so poor includes the preparation of this compound from azadirachtin by saturation of the 2,3-dihydrofuran double bond with deuterium followed by alkaline hydrolysis and remethylation with deutero-diazomethane[54]. In this way a sample of the lactone could have been produced which would have shown a simplified n.m.r. spectrum in the regions τ5.9 and 7.7 (α and β methylenes in the tetrahydrofuran ring) and the absence of a carboxymethyl group singlet just above τ6. Such a spectrum may have lent itself more easily to interpretation than that displaying the usual complement of ^{1}H resonances associated with this compound.

Alkaline hydrolysis of azadirachtin in methanol solution by treatment with 2½% aqueous potassium hydroxide for one hour at room temperature was shown by Butterworth[12] to produce deacetyl-azadirachtin (n.m.r. spectrum in $CDCl_3$, Fig. 5) by loss of the acetate ester attached β to the tiglate ester. No product corresponding to the lactone produced by alkaline hydrolysis of dihydro-azadirachtin was produced by hydrolysis of azadirachtin itself.

The yield of deacetyl-azadirachtin obtained by Butterworth was poor (~12%) and attempts were made to improve this by using a 1% solution of potassium hydroxide. However no outstanding improvement was realised and on the three occasions that 1%

potassium hydroxide was used the yields of deacetyl-azadirachtin were 9% (reaction time 2½ hours), 17% (reaction time 3 hours), and 14% (reaction time 3½ hours). Further small scale hydrolysis reactions were carried out on azadirachtin using various concentrations of potassium hydroxide down to 0.1% and aliquots of the reaction mixtures were removed at intervals up to 5 hours. Investigation of the products by t.l.c. indicated no obvious improvement in the yield of deacetyl-azadirachtin as against the quantity of material appearing as a streak on the plate.

Butterworth[12] had shown that azadirachtin itself was not attacked by Cornforth's Reagent[55] (chromium trioxide-pyridine-water) over a period of two days at room temperature. It was thought possible therefore that the additional hydroxyl group liberated in deacetyl-azadirachtin could be oxidised specifically by this reagent. A quantity of deacetyl-azadirachtin accumulated from a number of hydrolysis experiments was therefore treated with Cornforth's Reagent at room temperature. The material isolated after 71 hours still contained some of the starting compound but that which had reacted had produced a complex mixture of materials from which no pure component could be isolated by P.L.C. eluting with chloroform-acetone (85:15). In view of the small amounts of deacetyl-azadirachtin available, further attempts at the oxidation of this compound were not undertaken.

The presence of at least one hydroxyl group in the

azadirachtin molecule was shown by a broad absorption in the I.R. spectrum at 3460 cm^{-1}. In the n.m.r. spectrum of the compound in $CDCl_3$ (Fig. 1) a broad singlet integrating for one proton and exchangeable with D_2O was seen at about τ7, its exact position varying from sample to sample. Butterworth assigned this resonance to an OH proton. He also tentatively suggested the presence of an OH signal at about τ4.9 though uncertainty remained about this area of the spectrum.

Butterworth treated azadirachtin with B.S.A. in chloroform solution at room temperature and obtained bis(trimethylsilyl)-azadirachtin. The n.m.r. spectrum of this compound in $CDCl_3$ (Fig. 6) showed two singlets, each integrating for nine protons, at τ9.83 and τ9.89 ($-SiMe_3$ groups). The mass spectrum of the bis(trimethylsilyl)-ether of azadirachtin showed an ion at highest $^m/_e$ with accurate mass 864.3438 corresponding to the composition $C_{41}H_{60}O_{16}Si_2$ (calculated mass 864.3420). Butterworth assumed that this was the molecular ion of the derivative and on this basis postulated that the molecular weight of azadirachtin itself was 720 with composition $C_{35}H_{44}O_{16}$.

The I.R. spectrum of bis(trimethylsilyl)-azadirachtin in carbon tetrachloride solution showed a weak absorption at 3570 $cm.^{-1}$ Butterworth assumed that this was due to adsorbed moisture in the sample and that the derivative did not contain any hydroxyl groups. He concluded therefore that azadirachtin itself contained

two hydroxyl groups and he proposed that one of these was secondary and the other tertiary from the evidence gained on acetylation of azadirachtin.

Butterworth prepared acetyl-azadirachtin by treating azadirachtin with boiling acetic anhydride and the n.m.r. spectrum of this derivative in $CDCl_3$ is shown in Fig. 7. The resonance at τ4.4 in the n.m.r. spectrum of azadirachtin, assigned by Butterworth to a proton attached to the 2-position of the 2,3-dihydrofuran ring, was not moved by acetylation. Butterworth concluded, therefore, that the oxygen atom also attached at the 2-position (part structure 14) was not part of a hydroxyl group because such a group would have undergone acetylation and the resonance at τ4.4 would have been moved downfield (cf: melianone[56], H21 moves from τ4.62 to τ3.80 on acetylation at C21). However a one proton triplet was shifted from τ5.3 in the spectrum of azadirachtin to τ4.8 in that of acetyl-azadirachtin. This absorption Butterworth assigned to a proton attached to a carbon atom bearing the hydroxyl group which was acetylated in the derivative. One hydroxyl group in the parent molecule was therefore assigned as secondary in character and the failure to form a diacetate derivative suggested that the other hydroxyl group was tertiary.

In the course of this present work, an investigation of the resonances in the τ4.9 region in the n.m.r. spectrum of

azadirachtin was undertaken in order to clarify the assignments in this area. The one proton doublet at $\tau 4.95$ assigned by Butterworth to the vinyl proton at the 4-position of the 2,3-dihydrofuran ring (part structure 14) appeared to integrate for two protons in the spectra of some samples and Butterworth had suspected the presence of an OH resonance in this position, though no further evidence was available at the time. Accordingly the n.m.r. spectrum of a pure sample of azadirachtin was recorded over a range of concentrations from 560 mg./ml. to 40 mg./ml. in $CDCl_3$. An exchangeable singlet integrating for one proton was seen at $\tau 4.59$ in the most concentrated sample and this signal was quite distinct from the vinyl proton doublet resonance at $\tau 4.95$. On dilution the exchangeable singlet moved downfield and broadened slightly. In the range of concentrations normally used for routine n.m.r. spectrum determinations (60 to 160 mg./ml.) the exchangeable signal was located beneath the vinyl proton doublet at $\tau 4.95$ and accounts for the integration for two protons often observed at this position. In the most dilute samples investigated the exchangeable signal was seen as a distinct broad singlet at a higher field than the vinyl doublet with an upper limit of about $\tau 5.05$. The behaviour of this signal indicated that it represented a hydroxyl proton which was susceptible to inter-molecular hydrogen-bonding.

Two hydroxyl protons, $\nu\tau 4.9$ and $\nu\tau 7.0$, were now assigned in the n.m.r. spectrum of azadirachtin in $CDCl_3$. However,

two pieces of evidence suggested the possible existence of a third hydroxyl group in the molecule. Firstly, the broad absorption at 3550 to 3570 cm^{-1} in the I.R. spectrum of bis(trimethylsilyl)-azadirachtin was of considerable intensity in all samples of the compound investigated, suggesting the continued presence of an unsilylated hydroxyl group in the molecule. Secondly, this same derivative showed a broad absorption, integrating for one proton and exchangeable with D_2O, at τ7.2 in its n.m.r. spectrum in $CDCl_3$ characteristic of an O<u>H</u> proton. It was decided therefore to determine the n.m.r. spectrum of azadirachtin in dimethylsulphoxide (DMSO) solution in order to gain further evidence about the hydroxyl groups in the molecule.

In 1964 Chapman and King[57] reported the use of DMSO as a solvent for n.m.r. spectroscopy. They showed that in DMSO solution strong hydrogen bonding to the solvent shifts hydroxyl proton signals downfield to τ6 or below, and reduces the rate of proton exchange sufficiently to sharpen the signal and permit the observation of hydroxyl proton splitting. (The rate of exchange is not so slow however as to prevent the disappearance of O<u>H</u> resonances soon after the addition of D_2O.) A primary hydroxyl group therefore appears as a triplet, a secondary group as a doublet, and a tertiary group as a singlet in the spectra of alcohols in DMSO solution. This technique has since been

used successfully by other workers[58] for the classification of alcohols though some difficulty has been reported[59] for compounds with strongly electron-withdrawing groups adjacent to the hydroxyl function.

The n.m.r. spectrum of azadirachtin in hexadeutero-dimethylsulphoxide (DMSO-d_6) is shown in Fig. 8. Three signals, integrating for one proton each and exchangeable with D_2O, at τ5.19 (doublet J$\sim$3Hz., coupled to τ5.75 H-C-OH), 4.82 (singlet) and 3.75 (singlet) indicated that azadirachtin contained one secondary and two tertiary hydroxyl groups. For convenience the tertiary groups were designated A (τ3.75) and B (τ4.82).

The n.m.r. spectrum of bis(trimethylsilyl)-azadirachtin in DMSO-d_6 solution (Fig. 9) showed an exchangeable signal integrating for one proton at τ5.08 (doublet J$\sim$3Hz.) indicating that a hydroxyl group remained unsilylated in the molecule, as was suspected from earlier evidence, and furthermore that this free group was secondary in nature. This fact gave rise to the possibility that the ion at $^{m}/e$ 864 in the mass spectrum of bis(trimethylsilyl)-azadirachtin was not the molecular ion as assumed by Butterworth but was an M-18 ion formed by loss of a molecule of water from the true molecular ion. Butterworth had calculated the molecular weight of 720 and formula $C_{35}H_{44}O_{16}$ for azadirachtin from the accurate mass of the ion at $^{m}/e$ 864 in the spectrum of the bis(trimethylsilyl)-ether, but if this was

an M-18 ion and not the molecular ion the true molecular weight of azadirachtin would be 738 with composition $C_{35}H_{46}O_{17}$.

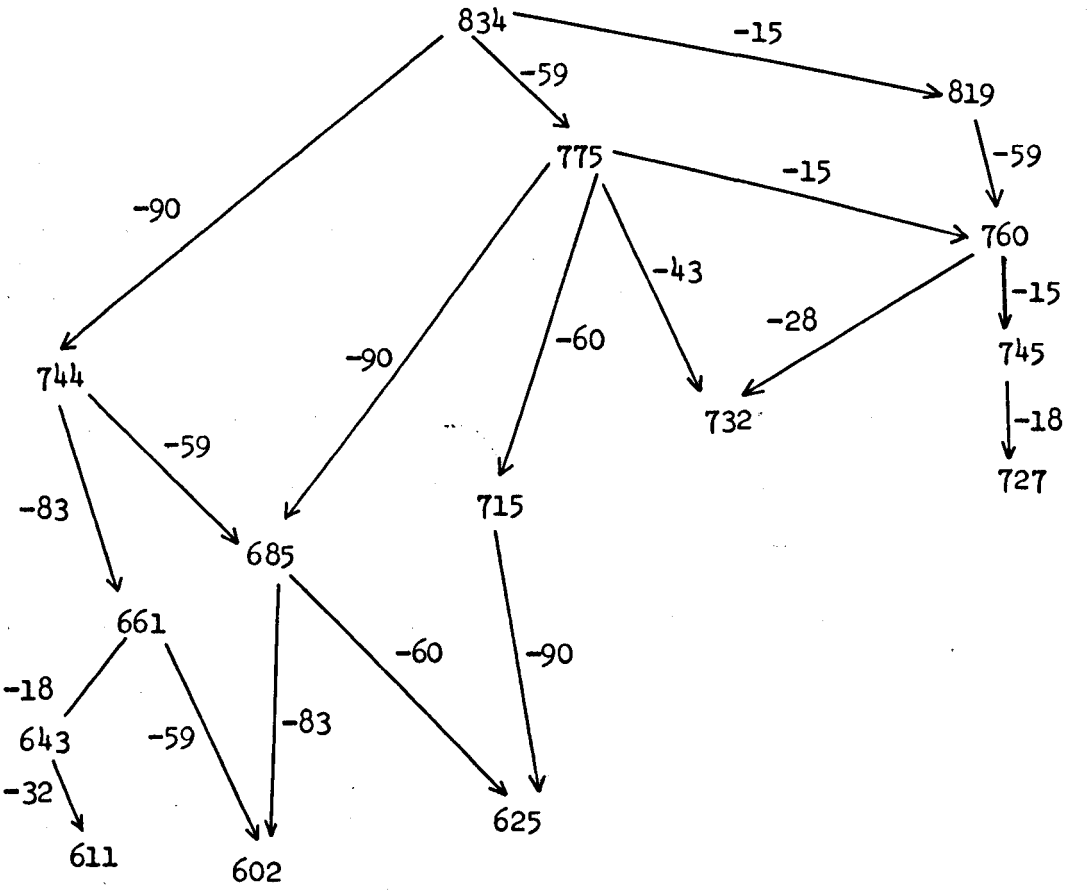
The suspicion that an M-18 ion was the highest ion seen in the mass spectrum of the bis(trimethylsilyl)-ether derivative was not out of context because none of the other derivatives prepared by Butterworth had shown a molecular ion in their mass spectra. If the molecular weight of azadirachtin was 720, the highest ion seen for these derivatives corresponded to M-18. If the true molecular weight of azadirachtin was 738 the highest ions seen for these compounds would have to be reassigned as M-36.

It was clearly of primary importance to prepare a derivative of azadirachtin with all three hydroxyl groups protected because the mass spectrum of such a compound would be expected to give an unequivocal molecular ion from which the molecular weight and formula of azadirachtin itself could be established. On the evidence described above of a shift of a one proton triplet (spectrum in $CDCl_3$) from τ5.3 (proposed H-C-OH) in azadirachtin to τ4.8 (proposed new H-C-OAc) in acetyl-azadirachtin, Butterworth had postulated that acetylation of azadirachtin occurred at a secondary hydroxyl group. The n.m.r. spectrum of bis(trimethylsilyl)-azadirachtin in DMSO-d_6 showed that silylation of azadirachtin occurred at the two tertiary hydroxyl groups, A and B. It seemed therefore that treatment of acetyl-azadirachtin with B.S.A., under similar conditions to those used by Butterworth on azadirachtin

itself, should produce an acetyl-bis(trimethylsilyl)-ether derivative with all three hydroxyl groups protected.

Acetyl-azadirachtin was therefore prepared by treating azadirachtin with boiling acetic anhydride[12] and purification of this compound followed by treatment with B.S.A. in chloroform solution at room temperature gave acetyl-trimethylsilyl-azadirachtin. The presence of only one trimethylsilyl-ether grouping was shown by one singlet integrating for nine protons at τ9.8 in the n.m.r. spectrum in $CDCl_3$(Fig. 10). The spectrum in DMSO-d_6 (Fig. 11) still showed the presence of an exchangeable one proton doublet at τ5.10 and the I.R. spectrum showed a broad absorption at 3500 cm^{-1}. It was clear that acetylation had taken place on one tertiary hydroxyl group and silylation had taken place on the other, leaving the secondary hydroxyl group unaffected throughout.

The mass spectrum of acetyl-trimethylsilyl-azadirachtin showed a peak at highest $^m/_e$ with accurate mass 834.3134 which was within 3 p.p.m. of 834.3114, the calculated mass for $C_{40}H_{54}O_{17}Si$. Because of the remaining free secondary hydroxyl group it was not certain if this was the molecular ion of the compound. If it was, then the molecular weight and formula for azadirachtin followed as 720 and $C_{35}H_{44}O_{16}$ as postulated by Butterworth. The prominent ions observed in the spectrum above $^m/_e$ 600 could be explained by losses of fragments known to be present in the molecule.



The evidence that both acetylation and silylation at room temperature left the secondary hydroxyl group unaffected prompted a further investigation of acetyl-azadirachtin in which Butterworth thought the secondary group was acetylated. The n.m.r. spectrum of acetyl-azadirachtin in DMSO-d_6 (Fig. 12) showed exchangeable one proton signals at τ5.20 (doublet) and τ4.53 (singlet) indicating that acetylation had occurred at the tertiary hydroxyl group A. It therefore became necessary to re-investigate the triplet resonance which moved from τ5.3 in the spectrum ($CDCl_3$) of azadirachtin to τ4.8 in acetyl-azadirachtin because Butterworth's assignment as a proton attached to the carbinol carbon atom susceptible to acetylation was clearly incorrect.

Butterworth had shown[60] that one proton triplets at τ5.3

and τ4.5 in the spectrum of azadirachtin in $CDCl_3$ solution both collapsed to a singlet on irradiation of the complex signal at τ7.7. The latter signal he assigned to a methylene group joined on one side to a carbon atom to which one proton (τ5.3) and a tiglate ester group were attached, and on the other side to a carbon atom to which one proton (τ4.5) and an acetate ester group were attached. The presence of such a structural entity was confirmed by chemical degradation[12] and led to part structure (15). In the n.m.r. spectrum ($CDCl_3$) of acetyl-azadirachtin (Fig. 7), irradiation at τ7.7 caused both the triplet at τ4.5 (H-C-OAc) and the triplet at τ4.8 to collapse to a singlet. This proved that the triplet at τ4.8 was the proton attached to the carbon atom bearing the tiglate ester grouping. It had presumably been shifted from its position at τ5.3 in azadirachtin by the anisotropic effect of a nearby group, possibly the new acetate residue in acetyl-azadirachtin.

As described earlier, Butterworth had proposed that the oxygen function attached at the 2-position of the dihydrofuran ring was not a hydroxyl group because the proton also attached at this position did not move on acetylation of azadirachtin. However, the new evidence that a secondary hydroxyl group remained unaffected in acetyl-azadirachtin made this evidence invalid and re-opened the possibility that there was a hemi-acetal structure based on the 2-position of the dihydrofuran ring. The spectrum of azadirachtin

in DMSO-d_6 solution (Fig. 8) displayed an absorption due to the proton at the 2-position of this ring as a singlet at τ4.42. If a hydroxyl group had been attached at the same carbon atom this signal would have appeared as a doublet by virtue of the coupling between hydroxyl and carbinol protons which occurs in DMSO solution. So on this evidence it can be stated that the oxygen function at the 2-position of the dihydrofuran ring is not a hydroxyl group.

Attempts were now made to protect all three hydroxyl groups in azadirachtin by forming a tris(trimethylsilyl)-ether derivative of the molecule. Butterworth had prepared the bis(trimethylsilyl)-ether derivative by treating azadirachtin with B.S.A. in chloroform solution at room temperature for 10 minutes. It was thought possible that exposure of azadirachtin to this reagent for a longer period of time might furnish a tris(trimethylsilyl)-ether derivative. Therefore azadirachtin was treated with B.S.A. in chloroform solution at room temperature for 19 hours. After removal of the volatile materials the crude product was investigated by t.l.c. eluting with ether–acetone (9:1). Two minor components were seen at R_F 0.26 and 0.69 corresponding to azadirachtin and its bis(trimethylsilyl)-ether derivative, but the two major products gave spots at R_F 0.41 and 0.51. The mixture was subjected to P.L.C. and the n.m.r. spectrum ($CDCl_3$, Fig. 13) of the product from the upper major band clearly indicated that it was a mono(trimethylsilyl)-ether derivative of azadirachtin. A singlet integrating for nine protons was visible at τ9.89 (-$SiMe_3$ group).

The n.m.r. spectrum of this compound in DMSO-d_6 (Fig. 14) showed two protons, exchangeable with D_2O, resonating at τ3.70 (singlet) and τ5.03 (doublet) indicating that the tertiary hydroxyl group B was protected. The compound was therefore designated B-trimethylsilyl-azadirachtin. The mass spectrum of this material showed only weak ions above $^m/_e$ 400 but a significant peak was observed for the molecular ion at $^m/_e$ 792. Other significant peaks observed above $^m/_e$ 650 were related to the molecular ion by successive losses of acetic acid, methanol, and a methoxy radical as shown below.

$$792 \longrightarrow 732 \longrightarrow 700 \longrightarrow 669$$

The material from the lower major band was further purified by preparative t.l.c. to give A-trimethylsilyl-azadirachtin. The n.m.r. spectrum of this compound in $CDCl_3$ solution is shown in Fig. 15. The spectrum in DMSO-d_6 (Fig. 16) showed exchangeable one proton signals at τ4.64 (singlet) and τ5.25 (doublet) indicating that tertiary hydroxyl group A was protected. The mass spectrum of this compound did not show peaks of significant intensity above $^m/_e$ 400 due to the low volatility of the substance.

Butterworth had reported[12] the formation of bis(trimethylsilyl)-azadirachtin on treating azadirachtin with B.S.A. in chloroform solution at room temperature for just 10 minutes and so the formation of the two mono(trimethylsilyl)-ether derivatives after 19 hours seemed anomalous. However it was found that the formation of the bis-derivative in such a short time as that reported by Butterworth

was only possible if a very pure sample of azadirachtin was used as starting material. If slightly impure azadirachtin was used, as in this present experiment, silylation progressed very slowly using B.S.A. in chloroform solution. However if dimethylformamide was used as solvent, silylation proceeded relatively rapidly even if the starting material was not of high purity. Indeed treatment of either azadirachtin or the two mono(trimethylsilyl)-ether derivatives with B.S.A. in dimethylformamide for 3 hours at room temperature invariably brought about complete conversion to bis(trimethylsilyl)-azadirachtin.

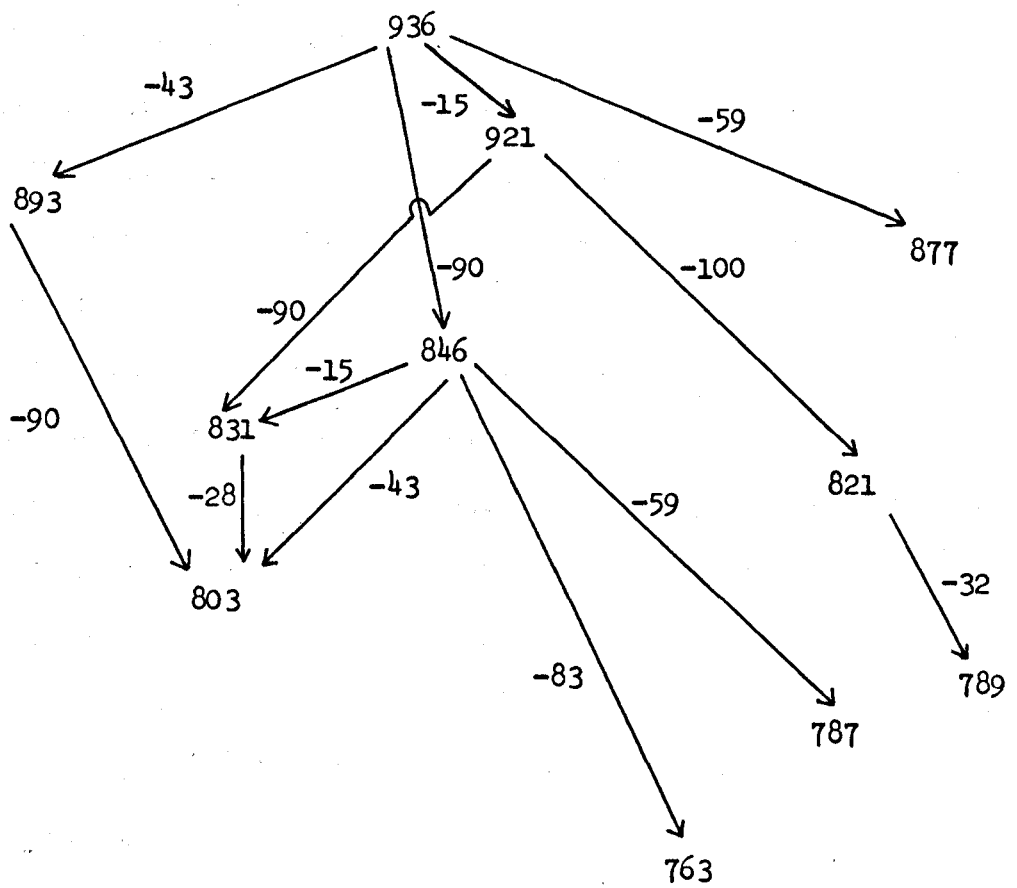
A number of attempts were now made to prepare a fully silylated derivative of azadirachtin by using more vigorous conditions. Early experiments involving various reagents, longer reaction times, different solvents, and elevated temperatures yielded only bis-(trimethylsilyl)-azadirachtin (see experimental section for summary). Chambaz and Horning[61] in 1967 reported that a mixture of B.S.A., trimethylsilyl-imidazole, and trimethylchlorosilane (3:3:2) in pyridine at 60° for 60 hours completely silylated 5β-pregnan-3α, 11β, 17α, 20α, 21-pentol (cortol) despite the high degree of steric hindrance to the 11β- and 17α- positions. Treatment of azadirachtin with the above mixture of three silylating reagents in pyridine at 60° for 90 hours yielded a mixture of tris(trimethylsilyl)-azadirachtin and some of the bis-derivative showing that the steric hindrance to to the secondary hydroxyl group in azadirachtin must be considerable.

The n.m.r. spectrum of the tris(trimethylsilyl)-ether in $CDCl_3$ (Fig. 17) showed three singlets, each integrating for nine protons, at τ9.76, τ9.83, and τ9.88 ($-SiMe_3$ groups), and a peak at τ5.75 ($\underline{H}$-C-$OSiMe_3$) instead of the peak at ∿τ5.4 ($\underline{H}$-C-OH) in azadirachtin itself. None of the resonances in this spectrum were exchangeable with D_2O and the I.R. spectrum of the tris(trimethylsilyl)-ether showed the complete absence of absorptions attributable to hydroxyl functions. In this compound the secondary hydroxyl group was clearly protected and yet no significant movement in the singlet in the n.m.r. spectrum attributable to the proton at the 2-position of the dihydrofuran ring was observed. This confirmed that the secondary hydroxyl group was not attached at this position.

The ion at highest $^m/_e$ in the mass spectrum of tris(trimethylsilyl)-azadirachtin was assumed to be the molecular ion as the protection of all three hydroxyl groups precluded the simple loss of water to give an M-18 peak. Accurate mass determination on this ion gave 936.3798, 2 p.p.m. error from the calculated mass 936.3816 for $C_{44}H_{68}O_{16}Si_3$. This confirmed that the molecular weight 720 and formula $C_{35}H_{44}O_{16}$ postulated by Butterworth for azadirachtin itself were correct and the possibility of the compound having molecular weight 738 and formula $C_{35}H_{46}O_{17}$ was eliminated.

Accurate mass determination on the ion at $^m/_e$ 877 in the mass spectrum of the tris(trimethylsilyl)-derivative gave 877.3688, $C_{42}H_{65}O_{14}Si_3$ requires 877.3682 (error 1 p.p.m.). This inferred

that this ion was formed by loss of a carboxymethyl radical from the molecular ion at $^{m}/e936$. All other prominent ions observed above $^{m}/e$ 750 were explained by loss of fragments already identified in the molecule.



The success of using DMSO-d_6 as a solvent for n.m.r. analysis of the derivatives already mentioned, prompted investigation of some other compounds by this technique. Butterworth had observed[62] that the one proton triplet at τ4.51 (H-C-OAc) in the spectrum of azadirachtin in $CDCl_3$ was moved upfield to above τ5 in the spectrum of deacetyl-azadirachtin. He therefore stated that a secondary

acetate group had been removed from the molecule. Although this conclusion is not in doubt, it has proved impossible to confirm by reference to the spectrum of deacetyl-azadirachtin in DMSO-d_6 (Fig. 18) because the multiplicity of the new OH resonance at τ5.35 (coupled to $\sim\tau$5.84, new H-C-OH) is hidden by partial overlap with another signal.

The n.m.r. spectrum in DMSO-d_6 solution of the lactone from the alkaline hydrolysis of dihydro-azadirachtin is shown in Fig. 19. Exchangeable signals integrating for one proton each were seen at τ2.71 (doublet J$\sim$3Hz), 4.68 (singlet) and 4.73 (singlet). The chemical shifts of these protons were so different from those observed for the OH protons in azadirachtin and its other derivatives examined, that it was not possible to decide whether one of the hydroxyl groups originally present in the dihydro-azadirachtin molecule was involved in the production of the lactone or if the new hydroxyl generated by loss of an acetate group was involved as suggested by Butterworth.

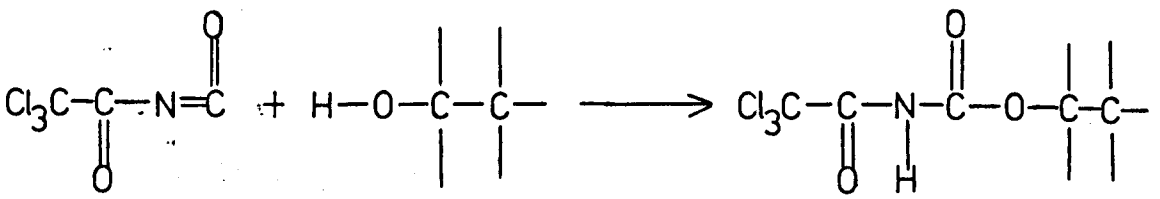
After the n.m.r. spectrum in DMSO-d_6 had demonstrated the presence of a third hydroxyl group in azadirachtin, special attention was paid to spectra in $CDCl_3$. Using pure samples, the OH resonance not previously observed has been recognised in the spectra of most of the derivatives mentioned. A full list of chemical shifts (τ values) for OH protons is shown below:

Chemical Shifts of OH protons (τ values) for Azadirachtin and some derivatives

	$CDCl_3$				$DMSO-d_6$			
	A	B	2°		A	B	2°	
Azadirachtin	~4.9	~6.9	~7.1	-	3.75(s)	4.82(s)	5.19(d)	-
Acetyl-azadirachtin	-	~6.7	~6.8	-	-	4.53(s)	5.20(d)	-
A-trimethylsilyl-azadirachtin	-	?	?	-	-	4.64(s)	5.25(d)	-
B-trimethylsilyl-azadiractin	~4.9	-	?	-	3.70(s)	-	5.03(d)	-
Bis(trimethylsilyl)-azadirachtin	-	-	~7.2	-	-	-	5.08(d)	-
Acetyl-trimethylsilyl-azadirachtin	-	-	~7.4	-	-	-	5.10(d)	-
Deactyl-azadirachtin	~4.9	~6.7	~6.9	~7.6	3.82(s)	4.85(s)	5.14(d)	5.35(?)
The lactone	~5.4, ~7.1, ~7.2				2.71(d), 4.68(s), 4.73(s)			

Angyal, Pickles and Rajendra[58] reported in 1964 that in DMSO solution equatorial hydroxyl groups display an OH doublet resonance with a coupling constant of approximately 8Hz, whereas theequivalent figure for axial hydroxyl groups is approximately 4Hz. The coupling constant observed for the secondary hydroxyl group resonance in azadirachtin and derivatives is approximately 3Hz which suggests that this group is axial in configuration if it is attached to a ring system.

The use of trichloroacetyl isocyanate for *in situ* reactions with alcohols for n.m.r. studies was first reported in 1965[63]. Trehan, Monder and Bose[64] in 1968 described its use with steroid alcohols of various types and claimed it had advantages over the DMSO method for determining the nature of hydroxyl groups in a compound. The reagent forms a carbamate with alcohols as shown below.



The NH signals of the carbamate appear in the relatively free area of the spectrum below τ2, and integration of these signals indicates the number of hydroxyl groups present in the

alcohol. Furthermore, a characteristic downfield shift (0.5 to 0.9 p.p.m. for primary alcohols, 1.0 to 1.5 p.p.m. for secondary alcohols) in the position of any carbinol protons on carbamate formation enables the nature of hydroxyl groups to be assigned. Even relatively hindered hydroxyl groups are claimed to react with trichloroacetyl isocyanate and the reagent may be used in excess as it has no protons to obscure the spectrum.

Addition of a slight excess of trichloroacetyl isocyanate to a deuterochloroform solution of azadirachtin in an n.m.r. tube caused an immediate brown discolouration of the solution and loss of definition in the spectrum. After 15 minutes a broad signal was apparent in the region τ1.1 but the spectrum was so ill-defined that no conclusions could be drawn from it. After 45 minutes the mixture was pumped at 0.1 mm. and the residue was subjected to P.L.C. eluting three times with ether–acetone (85:15). Materials were isolated from the five major bands visualised but in each case the material was inhomogeneous and gave an ill-defined n.m.r. spectrum. The azadirachtin molecule, and the 2,3-dihydrofuran ring system in particular, is known to be highly acid sensitive. It is possible that traces of acidic impurities in the trichloroacetyl isocyanate were responsible for the failure of this technique.

On the evidence of acetylation previously described, Butterworth proposed that azadirachtin contained a secondary hydroxyl group. Attempts were therefore made to confirm the

presence of such a group by its oxidation to a ketone. This work was undertaken before n.m.r. studies in DMSO-d_6 solution had confirmed the presence of a secondary hydroxyl group and shown its resistance to acetylation, and before silylation experiments had confirmed the high degree of steric hindrance to this group. The overall problems of this attempted oxidation were firstly to find a reagent which attacked the secondary hydroxyl group selectively, and secondly to avoid any acidic conditions which were vigorous enough to affect other sites in the molecule, for example the dihydrofuran ring.

Butterworth had already undertaken two separate oxidation procedures involving the use of potassium permanganate. He had found that treatment of azadirachtin with potassium permanganate in acetone, followed by esterification with diazo-methane of any carboxylic acid groups formed, produced an inseparable mixture of a large number of products. However, Butterworth did isolate and characterise a distinct compound from the oxidation of azadirachtin with slightly alkaline potassium permanganate and sodium metaperiodate in tertiary butanol and water (Lemieux Reagent [65-68]). This product was formed by oxidative cleavage of the carbon-carbon double bonds in the molecule. It was apparent therefore that any oxidising reagent containing potassium permanganate would not be suitable for the specific oxidation of the secondary hydroxyl group in azadirachtin.

Mild conditions for the oxidation of secondary hydroxyl groups to ketones using oxygen gas over finely divided platinum metal have been described[69, 70]. Experiments with steroid alcohols have shown however that hydroxyl groups which are sterically hindered to even a relatively slight degree are not oxidised under these conditions[71, 72]. Azadirachtin was recovered unchanged after treatment with oxygen over platinum metal in both acetone and ethyl acetate solution at elevated temperatures for considerable periods of time. This result is explained by the high degree of steric hindrance to the secondary alcohol group in the molecule highlighted by later silylation experiments.

Attention was then given to the use of chromium^{VI} reagents to oxidise the secondary hydroxyl group of azadirachtin to a ketone. Butterworth had already shown[73] that azadirachtin was not affected by Cornforth's Reagent (chromium trioxide-pyridine-water) over a period of two days and so the use of this conveniently mild reagent was precluded. An oxidation involving the commonly used Jones' Reagent[74] (chromium trioxide-sulphuric acid-water-acetone) was not attempted as it was thought that the azadirachtin molecule would be subject to drastic acid hydrolysis under such conditions. It was estimated that conditions more vigorous than in the case of Cornforth's Reagent but less acidic than in Jones' Reagent would be required to bring about the desired oxidation.

In 1953 Fieser published a description of several Cr^{VI}

oxidising reagents of increasing severity[75]. The mildest of these was a solution of potassium dichromate in glacial acetic acid and benzene. Accordingly a benzene solution of azadirachtin was treated with a slurry of potassium dichromate in glacial acetic acid at 8°. After 20 hours some starting material was still present and that which had reacted had given a complex mixture of products from which no pure component was isolated. The continued presence of starting material after 20 hours at 8° prompted a further attempt at oxidation with the same reagent at room temperature. However after 200 minutes at room temperature all the azadirachtin had reacted to give a complex mixture of materials in which no clear cut product was apparent.

A slightly different approach to the oxidation of azadirachtin with Cr^{VI} was then tried. A glacial acetic acid solution of chromium trioxide (Fieser's Reagent[76]) was added drop-wise to a solution of azadirachtin also in glacial acetic acid. Aliquots of the reaction mixture were removed after the addition of known amounts of oxidising reagent and worked up separately. Each aliquot was investigated by t.l.c. The patterns obtained as more oxidising agent was added showed the disappearance of starting material to give a complex mixture of a large number of products. No final product or simpler pattern of products was seen on addition of excess of this reagent.

Dihydro-azadirachtin, which does not contain the labile 2,3-dihydrofuran ring system, was then used as the starting material for oxidation by Fieser's Reagent. The reaction was monitored as

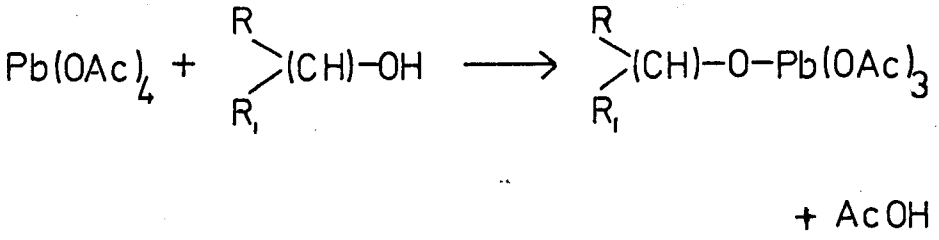
described above but the result obtained was just as unsatisfactory as that when azadirachtin itself was used.

A two-phase system for the oxidation of secondary hydroxyl groups in sensitive molecules has been described[77, 78]. A solution of the alcohol in ether or benzene is treated with a solution of sodium dichromate in sulphuric acid, acetic acid and water. The ketone formed on oxidation of the alcohol is distributed mainly in the organic solvent layer where it is relatively protected from further attack at any other sensitive sites such as carbon-carbon double bonds. Such an oxidation of azadirachtin was attempted using benzene as the solvent in the organic layer. The reaction was monitored by t.l.c. which showed that the disappearance of starting material was slow. However, after 29 hours at -10° and a further 17 hours at room temperature, the crude product obtained from the organic layer showed three major components on t.l.c. It is to be noted that the yield of material from the organic layer amounted to only about 30% of the starting quantity and no further material was obtained from the aqueous acidic layer by chloroform or ethyl acetate extraction. When the crude product was subjected to P.L.C., seven bands were visualised by U.V. light although only three were of significant intensity. The materials were isolated from all seven bands but in each case the yield was very low ($< 3\%$ of starting quantity). Investigation by t.l.c. showed that each of these materials was inhomogeneous and their n.m.r. spectra were all ill-defined.

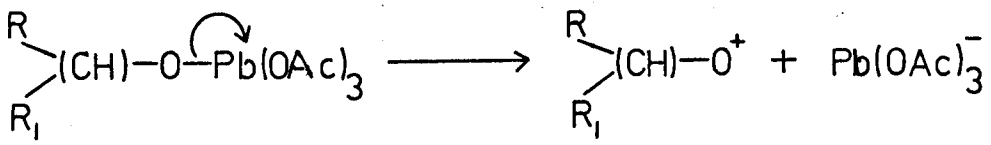
In summary, none of the Cr^{VI} reagents employed attacked the secondary hydroxyl group of azadirachtin specifically. Presumably the presence of double bonds in the molecule, and its general acid sensitivity, enabled other reactions to occur at least as readily as the oxidation of a highly sterically hindered hydroxyl group.

An unreported attempt at Oppenauer oxidation of azadirachtin was undertaken by Butterworth employing benzene as solvent and acetone as "hydrogen acceptor" in the presence of aluminium iso-propoxide. Azadirachtin was recovered unchanged despite treatment for several hours in this reaction mixture at reflux. It was thought that slightly more forcing conditions might bring about Oppenauer oxidation of the secondary hydroxyl group in azadirachtin. A number of steroid alcohols have been successfully oxidised using toluene as solvent with cyclohexanone as "hydrogen acceptor" in the presence of aluminium iso-propoxide[80]. Accordingly azadirachtin was treated with aluminium iso-propoxide in a dry mixture of toluene and cyclohexanone boiling under reflux for one hour. The product however was a complex mixture of a large number of materials running as a streak on a t.l.c. plate.

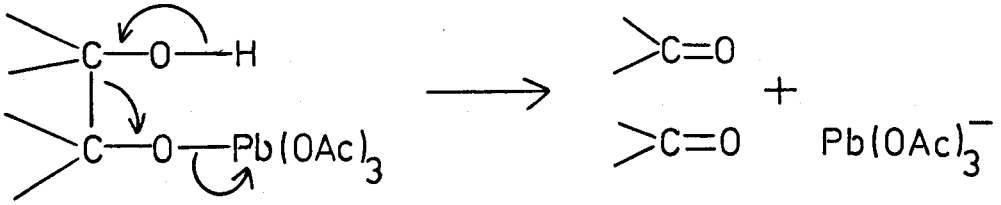
Lead tetraacetate has been widely used as an oxidising reagent and several reviews on its reactions with organic compounds of various classes have been published[81-83]. In benzene solution lead tetraacetate can oxidise secondary alcohols to ketones[81]. The first step in this reaction is thought to be alcoholysis of lead tetraacetate.



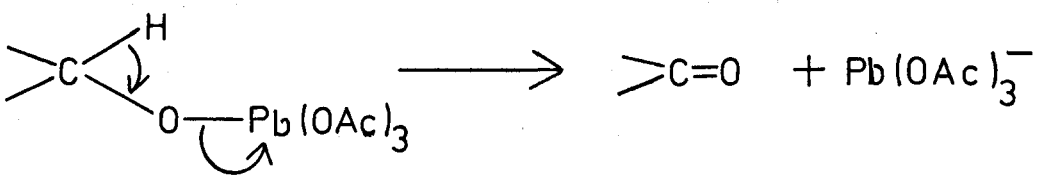
The oxidation step involves acceptance of the Pb-O electron pair by the strongly electronegative tetravalent lead to give a lead triacetate anion which then breaks down to give lead diacetate and an acetate anion.



The shift of the Pb-O electron pair in this reaction is possible only if the electron octet of the alcohol oxygen atom can be completed at the same time. In the case of α-glycols for example this is achieved by a shift of the electron pair of the second O-H bond and C-C bond fission results.



In the case of monohydric alcohols however the electron pair of a C-H bond must shift.

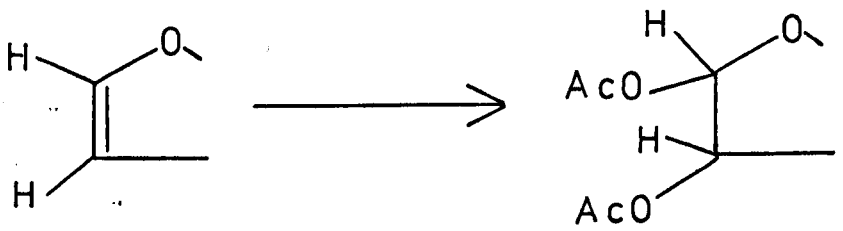


Although this is a less favoured shift than that which occurs in α-glycols, some secondary alcohols have been successfully oxidised to ketones by lead tetraacetate[81].

In an attempt to oxidise the secondary hydroxyl group of azadirachtin a benzene solution of the compound was treated with excess lead tetraacetate for 16 hours. The reaction was carried out at room temperature in darkness to minimise free radical reactions which can occur with this reagent. The doublets at τ3.6 and τ4.95 (J∿3Hz), assigned by Butterworth to the vinyl protons of the

2,3-dihydrofuran ring in azadirachtin, were not present in the n.m.r. spectrum ($CDCl_3$, Fig. 20) of the product I obtained from this reaction. The singlet assigned to the proton at the 2- position in the 2,3-dihydrofuran ring had also moved from its position at τ4.4 in azadirachtin. Clearly the 2,3-dihydrofuran ring had been modified in this reaction and the recovery of dihydro-azadirachtin unchanged following an analogous treatment with lead tetraacetate showed that this was the only site susceptible to attack. The original objective of oxidising the secondary hydroxyl group had therefore not been achieved, though the interesting product I had been obtained.

Lead tetraacetate is known to react with carbon-carbon double bonds to give diacetate adducts and when an electron donating group is present, as in a vinyl ether, the reaction is known to proceed in good yield under mild conditions[81] as illustrated below.



It was thought possible that material I was formed by such simple addition at the 2,3-dihydrofuran site of azadirachtin. The n.m.r. spectrum of I showed an additional singlet at τ7.9 but the large number of resonances in the vicinity made it impossible to

confirm by integration how many new acetate ester groups were present in the molecule. The peak at highest $^{m}/e$ in the mass spectrum of I was at $^{m}/e$ 720. It was possible that this represented an ion formed by loss of two acetoxy radicals from an unseen molecular ion at $^{m}/e$ 838 corresponding to a diacetate adduct ($C_{39}H_{50}O_{20}$). However the n.m.r. and mass spectral evidence was not sufficient to allow a definite diacetate adduct structure to be assigned to the material I.

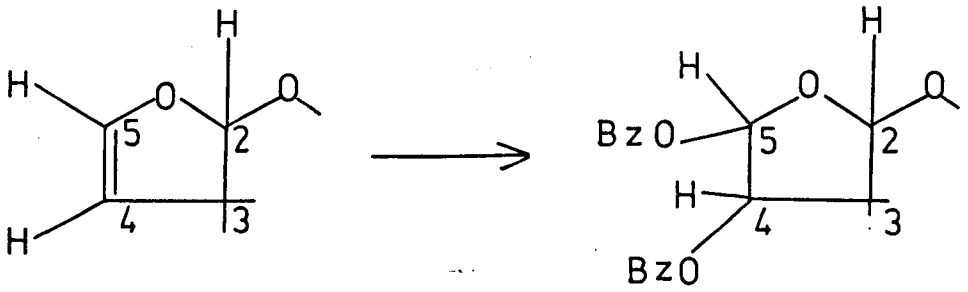
Material I was therefore treated with B.S.A. in dimethylformamide solution at room temperature to obtain a more volatile trimethylsilyl-ether derivative which might give a molecular ion in the mass spectrometer. The material II obtained as a product of this reaction gave an n.m.r. spectrum ($CDCl_3$, Fig. 21) with two singlets of different intensities attributable to the protons of two trimethylsilyl groups at τ9.89 and τ9.99. It appeared therefore that II was a mixture of mono and bis(trimethylsilyl)-ether derivatives of substance I, though t.l.c. in five different solvent systems failed to achieve separation. Furthermore the mass spectrum of the mixture II showed no significant peaks above $^{m}/e$ 864 and, since the mono and bis(trimethylsilyl)-ether derivatives of a possible diacetate adduct of azadirachtin would have molecular weights of 910 and 982 respectively, the ion at $^{m}/e$ 864 was probably not a molecular ion and the spectrum was of little help.

The failure to assign a definite structure to material I, either from its own spectra or by formation of a trimethylsilyl-ether

derivative, led to the adoption of a completely different approach to ascertain how many ester groups had been introduced into the azadirachtin molecule on attack by lead tetraacetate. It was decided to treat azadirachtin with lead tetrabenzoate which is reported[83] to act in an analogous way to lead tetraacetate. However the number of ester groups introduced into the azadirachtin molecule would be easier to ascertain by integration in the n.m.r. spectrum of the product, since the protons of benzoate groups resonate in the otherwise unoccupied region below τ3.

The lead tetrabenzoate required for this reaction was prepared from lead tetraacetate and benzoic acid by the method of Criegee et al[84]. Recrystallisation from methylene chloride gave a sample of lead tetrabenzoate with melting point 169°. Reported melting points for this compound are 176°[85] and 164°[84]. The variance observed is probably due to difficulties in removing methylene chloride from the sample[86].

Azadirachtin was treated with lead tetrabenzoate under the same conditions as those used in the reaction with lead tetraacetate. The material III obtained as product showed no I.R. absorption at 1625 $cm.^{-1}$ and the τ3.5 to τ5.0 region of its n.m.r. spectrum ($CDCl_3$, Fig. 22) confirmed that the 2,3-dihydrofuran system had undergone modification. The integration below τ3 in this spectrum showed that two benzoate ester groups had been introduced into the molecule.

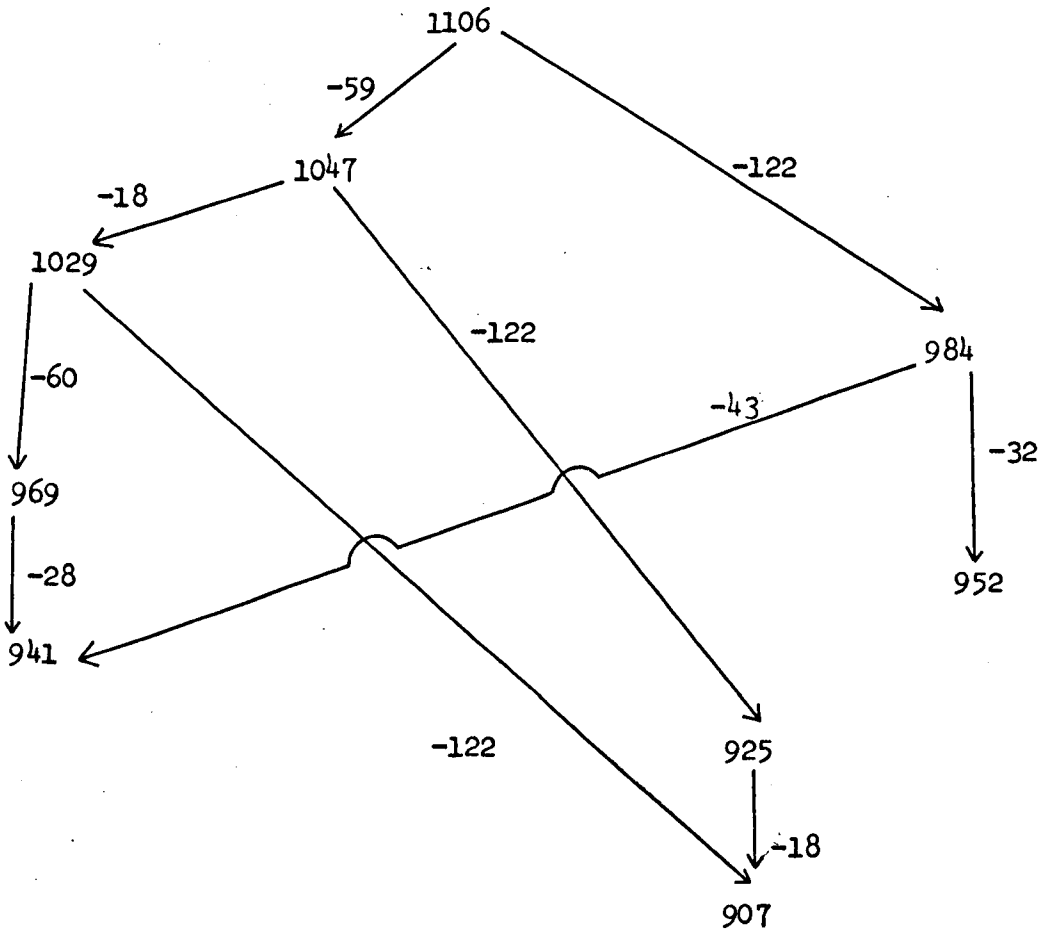


Spin decoupling experiments on material III revealed coupling between the signal at τ3.5 and that at τ4.38. The former signal is assigned to H5 and the latter to H4. The singlet for H2 is superimposed on the resonance for H4 at τ4.38. However the signals for H4 and H5 are not clean doublets. It is known[81] that the formation of adducts using lead tetracarboxylates may yield both cis and trans diesters and it is proposed that material III is a mixture of stereoisomers. The signals for H4 and H5 therefore consist of overlapping doublets of very similar chemical shifts attributable to the various stereoisomers present. Such overlap makes the H4-H5 coupling constant difficult to measure but it appears to be about 1 to 2 Hz.

Of a number of solvent systems tried, only ether-acetonitrile (97:3) separated the stereoisomers of material III on t.l.c. Using this system two separate components could be seen but their R_F values were so similar that a practical separation of large amounts of III into individual isomers was not attempted.

The molecular formula of the dibenzoate adduct III could not be confirmed from its mass spectrum as no peaks above $^{m}/e$ 200 were seen, presumably due to the low volatility of the material. However intense ions at $^{m}/e$ 122($C_6H_5COOH^+$), 105($C_6H_5CO^+$) and 77($C_6H_5^+$) confirmed the presence of benzoate ester groups in the molecule. Treatment of III with B.S.A. in dimethylformamide at room temperature in an attempt to obtain a derivative more amenable to mass spectrometry yielded as major product a material IV whose n.m.r. spectrum($CDCl_3$, Fig. 23) showed that it was probably a mixture of the mono and bis(trimethylsilyl)-ether derivatives of III.

Preparative t.l.c. eluting with chloroform-light petroleum-acetonitrile (1:3:1) separated IV into three components designated IVa (R_F 0.49), IVb (R_F 0.42) and IVc (R_F 0.36). The mass spectrum of IVa showed a molecular ion at $^{m}/e$ 1106 which was as expected for a bis(trimethylsilyl)-ether derivative of a dibenzoate adduct of azadirachtin ($C_{55}H_{70}O_{20}Si_2$), though the peak was not intense enough for an accurate mass determination. However the intense M-59 peak gave $^{m}/e$ 1047.3895 which was within 3 p.p.m. of 1047.3865, the calculated mass for $C_{53}H_{67}O_{18}Si_2$. This ion was formed by loss of a carboxymethyl radical from the molecular ion. All the significant ions above $^{m}/e$ 900 were accounted for by losses of fragments known to be present in the molecule as shown below.



The mass spectra of IVb and IVc did not show molecular ions but they were consistent with each of these compounds being a mono(trimethylsilyl)-ether derivative of a dibenzoate adduct of azadirachtin.

So by use of a trimethylsilyl-ether derivative it was confirmed that III was a dibenzoate adduct of azadirachtin, and the n.m.r. of III suggested that it was a mixture of stereoisomers. It is suggested by analogy that material I was probably a mixture of stereo-isomeric diacetate adducts of azadirachtin. However

uncertainty in the interpretation of its n.m.r. spectrum and the lack of a molecular ion in the mass spectrum of its trimethylsilyl-ether derivative makes this a tentative assignment.

No further attempts were made to oxidise the secondary hydroxyl group in azadirachtin. The failure to oxidise this group by any of the methods described above is explained in part at least by the high degree of steric hindrance to this part of the molecule indicated by later silylation experiments.

Evidence described earlier had shown that azadirachtin contained three hydroxyl groups and not two as Butterworth had suggested. Therefore only two oxygen atoms remained to be assigned in the molecule. It was thought possible that at least one of these might exist in a ketone function. Previously Butterworth had found that azadirachtin was resistant to reduction with sodium borohydride for a short time at room temperature but prolonged treatment had given a complex mixture of products probably due to alkaline hydrolysis. Attempts to form a 2,4-dinitrophenylhydrazone of azadirachtin had failed, due to the high sensitivity of the molecule to the acidic conditions employed in the preparation of such a derivative. Attempts to form a semicarbazone derivative had led only to the recovery of starting material and Butterworth had concluded therefore that if azadirachtin contained a ketone group it was in a hindered position.

The Optical Rotatory Dispersion (O.R.D.) and Circular Dichroism (C.D.) curves of azadirachtin were kindly determined by

Dr. Ryback on a Polarmatic 62 instrument at the Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent. The O.R.D. curve of azadirachtin was determined at a concentration of 1 mg./ml. with path length 10 mm. at 20° in ethanol and tetrahydrofuran solution (Figs. 24 and 25 respectively). Down to 270 nm. the specific rotation, $[\alpha]$ is moderate and negative. The curve is wavy indicating some weak Cotton effects which could not be analysed. Below 270 nm. the curve plunges downwards and measurements at wavelengths down to 217 nm. using a sample of unknown concentration did not detect a trough.

The C.D. curve above 250 nm. for an ethanol solution of azadirachtin at a concentration of 1 mg./ml. with path length 10 mm. at 20° is shown in Fig. 26. A curve (Fig. 27) showing a wider wavelength range was constructed using a solution of unknown concentration and the approximate values for the differential dichroic absorption, $\Delta\varepsilon$, were obtained by comparison with the accurate curve where overlap occurred. The C.D. shows a weak negative maximum at about 308 nm. where $\Delta\varepsilon$ is -0.14. A weak positive maximum, $\Delta\varepsilon$ + 1.1, is seen at 244 nm. and the curve then plunges downwards with $\Delta\varepsilon$ showing a value of -5 at 217 nm. without reaching an extremum. As neither the O.R.D. nor the C.D. curve has reached a negative maximum at 217 nm., this probably occurs below 210 nm. and is associated with the $n \rightarrow \pi^*$ transitions of the ester carbonyl chromophores in the molecule.

The C.D. negative maximum at 308 nm. is thought to be associated with the $n \rightarrow \pi^*$ transitions of a ketone carbonyl chromophore in the molecule. The negative maximum probably appears in this position due to overlap with the 244 nm. maximum Cotton effect without which it would probably appear somewhat below 308 nm. The 244 nm. positive maximum itself overlaps with a big negative maximum at lower wavelengths and its true peak probably occurs at a shorter wavelength.

The three observed Cotton effects overlap to give a surprisingly flat curve. An upward bulge might have been expected around 270-280 nm. where the peak of the negative 308 nm. Cotton effect should reinforce the peak of the positive 244 nm. Cotton effect. It is possible therefore that further unrecognised Cotton effects are present.

If a large fragment of the azadirachtin molecule containing fewer chromophores can be obtained, the simpler pattern of Cotton effects in its C.D. and O.R.D. curves should be of interest. Evidence from the C.D. curve of the whole molecule however does suggest the presence of a ketone. The unreactive nature of this group is presumably accounted for by steric hinderance as suggested by Butterworth.

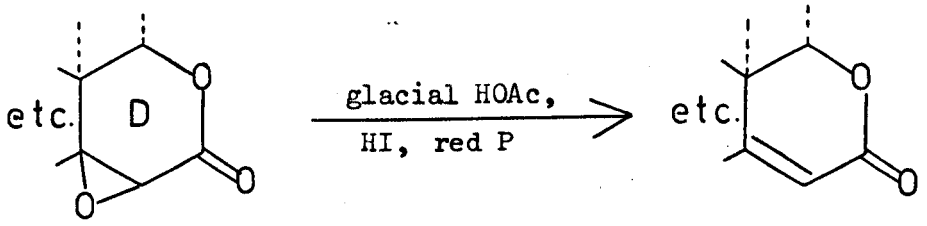
The evidence for a ketone described above left only one of the sixteen oxygen atoms in the azadirachtin molecule unassigned. Epoxide groups are well known in many naturally occurring compounds

and in limonoids isolated from the Meliaceae epoxide groups are common at the C14β - C15β position [eg: gedunin[26](7), epoxy-azadiradione[24](5)] and at the C8α - C30α position in the case of the bicyclononanolides [eg: Xylocarpin[28](9)]. In the compounds which still retain a full eight carbon side-chain (proto-limonoids) epoxidation at the C24-C25 position is known [eg: melianone[56]]. Also it has been suggested[14,15] that a C7α - C8α epoxy-compound may be an important intermediate in the <u>in vivo</u> shift of a methyl group from C14 in the tirucallane based compounds to the C8 position in the apo-euphol based compounds. This wide occurrence of epoxide groups in compounds from the Meliaceae prompted investigations to ascertain whether azadirachtin contained such an entity.

Protons attached to an oxirane ring usually absorb in the region τ6 to τ7 in the n.m.r. spectrometer[87]. This region is occupied by several groups of signals in the spectrum of azadirachtin and so n.m.r. spectroscopy was not of immediate use for detecting a possible epoxide group in the molecule. Similarly in the infra-red, absorptions associated with an epoxide group (ν3050 $cm.^{-1}$ C-H; ν1250, ν900, ν800 $cm.^{-1}$ C-O;[88]) would not be particularly outstanding in the spectrum of azadirachtin. Therefore attempts were made to recognise the presence of an epoxide by chemical means.

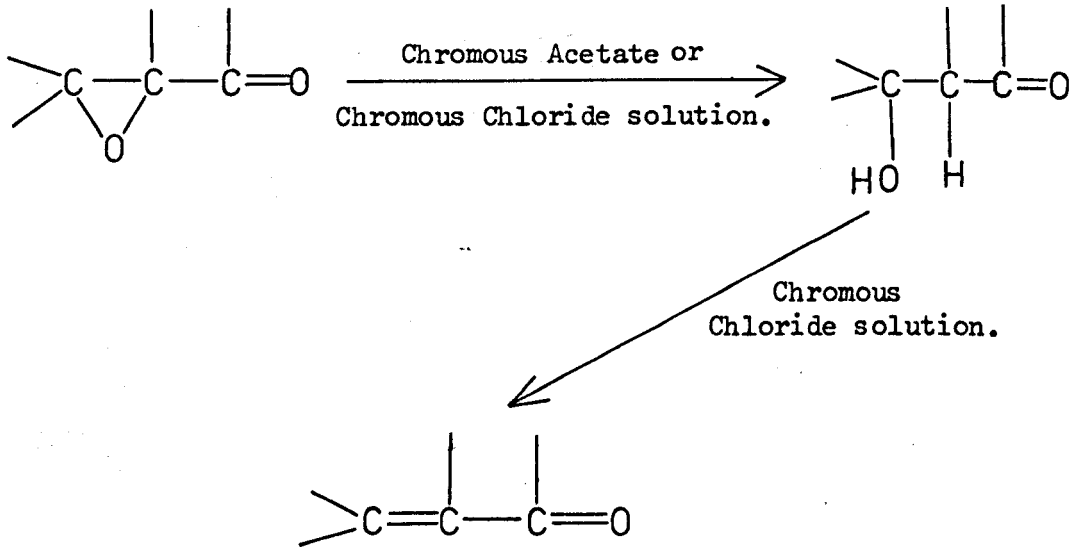
Before the full extent of the acid sensitivity of azadirachtin was appreciated, an attempt was made to detect a possible epoxide in the molecule by its reduction to an olefin using a mixture of

glacial acetic acid and hydriodic acid in the presence of red phosphorous. Such a reagent had been used by Barton et al[89] to reduce limonin to deoxylimonin.

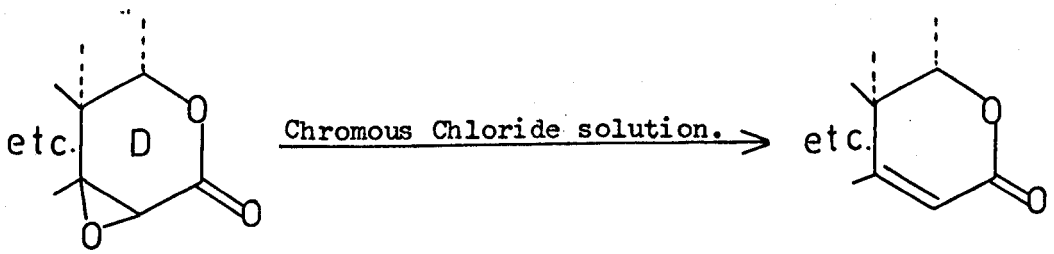


However azadirachtin was degraded to a complex mixture of acid hydrolysis products by this reagent. Milder conditions for the reduction of epoxides by hydriodic acid in chloroform solution have been reported[90] but these were not used on azadirachtin because it was feared that any further conditions involving this acid would produce similarly drastic degradation of the molecule.

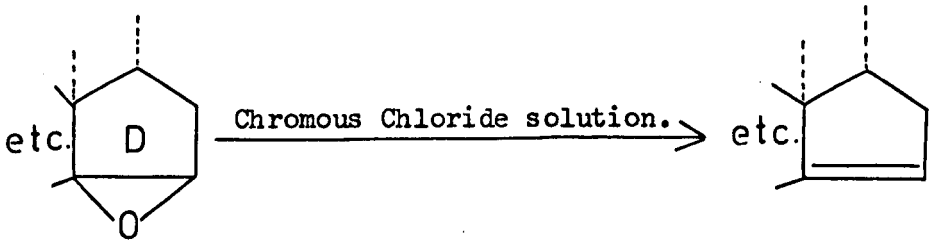
In 1954 Cole and Julian[91] described the reduction of epoxy-ketones with chromous salts. They showed that reduction with chromous acetate produced mainly a hydroxy-ketone but when chromous chloride solution was used the major product was the corresponding α,β-unsaturated ketone. This is shown schematically below.



This reaction has since been used successfully in the limonoid series. For example in 1962 Taylor et al[92] reduced khivorin to deoxy-khivorin using chromous chloride solution and Arigoni et al[20] have reported the reduction of limonin to deoxy-limonin by a similar method. Both these reactions are represented by the scheme below.



In 1963 Taylor et al[93] used chromous chloride solution to reduce an anthothecol derivative to the corresponding deoxy-compound, so demonstrating that the reductive elimination of an epoxide grouping is possible in the absence of an α-carbonyl function. The reaction is represented below.



Similarly xylocarpin (9) does not possess a ketone adjacent to the epoxide group but Okorie and Taylor[28] have reported that chromous chloride solution brings about reductive elimination to give the corresponding olefin.

The chromous chloride solution used in all such reactions is prepared by the treatment of chromic chloride with zinc metal and hydrochloric acid. The resulting solution which is then used to reduce the epoxy-compound is acidic. Indeed Cole and Julian reported that it is this acidity which is responsible for the second step in the reduction, namely dehydration of the initially produced alcohol to an olefin.

When azadirachtin was treated with the chromous chloride reagent at room temperature overnight in an effort to detect a possible epoxide group in the molecule, the product obtained was

a complex mixture of materials presumably due to acid hydrolysis. Dihydro-azadirachtin is more stable to acid than azadirachtin itself due to saturation of the labile 2,3-dihydrofuran double bond. An attempt was made therefore to detect a possible epoxide group in dihydro-azadirachtin by its reduction with chromous chloride solution. However a complex mixture of products more polar than the starting material were obtained, again as a result of acid hydrolysis.

Chromous acetate is an insoluble salt prepared by the addition of a solution of sodium acetate in water to a chromous chloride solution. The precipitate can be washed free of acid and so it was thought that this compound could be used to reduce a possible epoxide group in azadirachtin without incurring the problem of acid hydrolysis. Because of its non-acidic nature, chromous acetate reduces epoxides only as far as the corresponding alcohol and does not bring about dehydration.

When a solution of azadirachtin in acetone and acetic acid was shaken overnight with excess chromous acetate at room temperature the starting material was recovered unchanged. A similar result was obtained with dihydro-azadirachtin as starting material. These results were taken as strong evidence that azadirachtin does not contain an epoxide group.

In 1959 Cornforth et al[94] reported the use of a mixture of sodium iodide, zinc metal and sodium acetate in acetic acid to reduce epoxides to olefins. They proposed that the reaction proceeds by protonation of the epoxide and subsequent nucleophilic attack by an

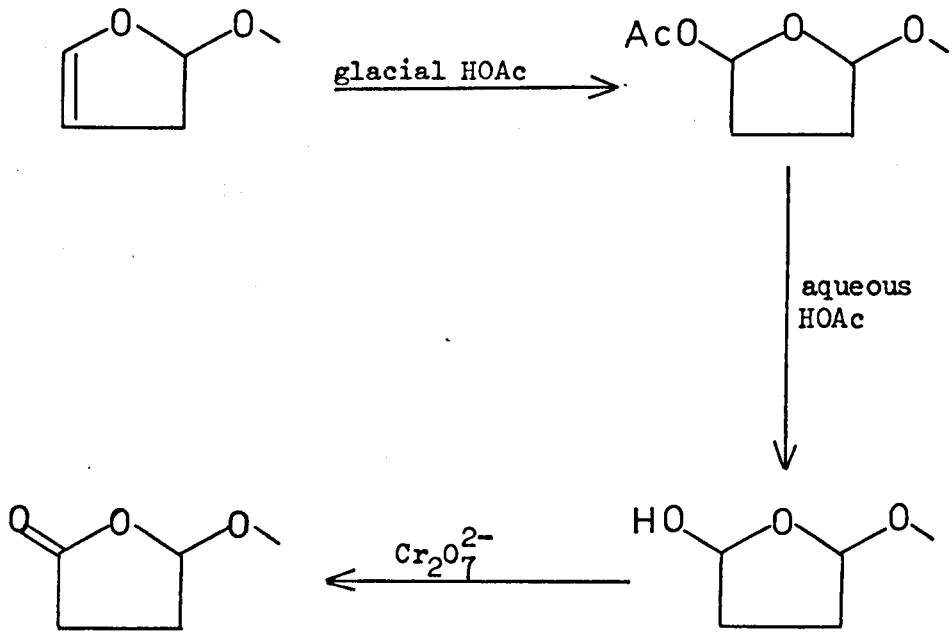
iodide ion to give an iodohydrin. The iodohydrin is then reduced by zinc and acetic acid to give the olefin. The sodium acetate present acts as a buffer. Such a reagent has been successfully used by Berkoz et al[95] to reduce 17β-acetoxy-5α,6α-epoxy-pregna-2-ene-20-one to the corresponding Δ5-6 compound.

Dihydro-azadirachtin was recovered unchanged after treatment with sodium iodide, zinc metal and sodium acetate in acetic acid overnight at room temperature. This was taken as confirmation that azadirachtin does not contain an epoxide function.

In the absence of evidence to the contrary it is proposed that the remaining oxygen atom in the azadirachtin molecule is present as an ether linkage. Butterworth postulated that an oxygen function was attached at the 2-position of the 2,3-dihydrofuran ring (part structure 14) and, as described earlier, n.m.r. evidence involving the signal due to the proton attached at the 2-position ruled out the possibility of a hemi-acetal structure. It is now proposed that an ether group is attached at this position. Such a structural entity is present in several naturally occurring compounds including clerodin[35] (20) isolated from Clerodendron infortunatum, clerodendrin A[47] (19) from Clerodendron tricotomum Thumb, and aflatoxins B1[36], G1[36] and M1[98] isolated from Aspergillus flavus. It should be noted that whereas azadirachtin is fully substituted at the 3-position of the 2,3-dihydrofuran ring, all the compounds mentioned above have a proton at the 3-position except aflatoxin M1 which is hydroxylated at this site.

The 2,3-dihydrofuran ring and the tiglate ester function are the two main sites at which the azadirachtin molecule is vunerable to chemical attack. By saturating the double bond of the 2,3-dihydrofuran ring Butterworth was able to undertake a series of reactions, commencing with specific attack at the tiglate ester double bond, which eventually led to the important conclusion that the tiglate ester entity was situated β to an acetate ester group on a six-membered ring in the azadirachtin molecule (part structure 15). It was thought that it might be profitable to attempt a degradation of azadirachtin by initial attack at the other reactive site in the molecule, namely the 2,3-dihydrofuran ring. Butterworth had shown that reagents that cleaved double bonds (e.g. ozonised air, Lemieux Reagent) attacked both such entities in the azadirachtin molecule. It was therefore necessary to find some other way of attacking the dihydrofuran ring which would leave the tiglate ester group intact.

Barton et al[35] found that treatment of clerodin (20) with glacial acetic acid at room temperature for three days brought about attack at the 2,3-dihydrofuran double bond and converted the compound into the corresponding hemi-acetal acetate. Hydrolysis of this ester using aqueous acetic acid gave the free hemi-acetal which was readily oxidised to a γ-lactone so proving the presence of a five-membered vinyl ether ring in the original compound. This reaction scheme is summarised below.



It was thought that a reaction scheme based on that described above would provide a method of specifically attacking azadirachtin at the dihydrofuran ring. It might provide a method of confirming that azadirachtin contained a five-membered ring vinyl ether system and would perhaps provide further knowledge of the chemistry of the molecule. Azadirachtin was therefore treated with glacial acetic acid but was recovered unchanged after one hour at room temperature. Treatment under the same conditions for prolonged periods of time did not produce any distinct new components and after six days the product was a mixture of a large number of materials which ran as a streak from the origin on t.l.c. eluting with ether-acetone (4:1). Treatment of azadirachtin with boiling glacial acetic acid for 25 minutes also

produced a complex mixture of products in which no distinct component was visualised on t.l.c.

Formic acid was then tried as an alternative to acetic acid in the attempt to make a hemi-acetal ester derivative of azadirachtin. Azadirachtin was treated with formic acid as a 10% solution in chloroform at room temperature and the reaction was monitored by t.l.c. After 13 hours some starting material remained but that which had reacted had given a complex mixture in which no distinct components were recognised.

Azadirachtin contains a substituent at the 3-position of the 2,3-dihydrofuran ring which clerodin does not. This may explain why clerodin gave a clean hemi-acetal ester with organic acids whereas azadirachtin gave a complex product mixture. Any hemi-acetal ester formed by treatment of azadirachtin with the organic acid may have been rendered unstable by the 3-substituent. The ring hemi-acetal structure may therefore have been opened and this would give a new hemi-acetal based at what was the 2-position in the dihydrofuran ring of the starting material. The new hemi-acetal itself may then have undergone cleavage in the acidic conditions. Thus with all the possible interactions which could have occurred with any nearby functional groups it is possible to rationalise the observed production of a complex mixture of products. Another possible explanation of the result obtained may be that some other part or parts of the molecule may have been susceptible to acid attack under the conditions used.

At a late stage in this work it was decided to investigate briefly the possibility of forming a hemi-acetal propionate by attacking the 2,3-dihydrofuran system of azadirachtin with propionic acid. Barton et al[35] had found that treatment of clerodin with propionic acid at room temperature for three days yielded clerodin hemi-acetal propionate. (They did not however report the successful hydrolysis of this compound to the free hemi-acetal.) Accordingly a small quantity of azadirachtin was treated with propionic acid at room temperature for six days. The material recovered showed two equally intense spots on t.l.c. eluting with chloroform-acetone (7:3) with minor stains in between. The lower spot corresponded to azadirachtin. The material from the upper spot was isolated by preparative t.l.c. Its mass spectrum did not show a molecular ion and the peak observed at highest $^{m}/e$ was at $^{m}/e$ 620. However an intense peak at $^{m}/e$ 57, possibly due to $CH_3CH_2-C\equiv O+$, suggested that a propionate ester group may have been introduced into the molecule. This possible propionic acid adduct of azadirachtin was not pursued due to shortage of time.

With fourteen of the oxygen atoms of azadirachtin assigned to one tiglate and two acetate esters, two carboxymethyl groups, three hydroxyl groups and a dihydrofuran ring and with the remaining two oxygen atoms tentatively assigned to a ketone group and an ether linkage, investigations on the carbon skeleton of the molecule were undertaken. From the number of double bond equivalents still

unaccounted for in the molecule it was clear that the compound contained six ring junctions including that of the 2,3-dihydrofuran ring. Dehydrogenation to form poly-cyclic aromatic compounds by pyrolysis with sulphur or selenium has found wide application[99] in the assignment of ring structures to natural products and it was decided to apply this technique to azadirachtin.

Sulphur has been known to become incorporated into organic molecules under pyrolysis conditions and this can lead to complications in the characterisation of products. It was decided therefore to pyrolyse azadirachtin with selenium which in general does not give organo-selenium compounds when a pyrolysis temperature above 300° is used. (It must be noted that the temperature required for selenium dehydrogenation is higher than that usually required for sulphur dehydrogenation and consequently more deep-seated changes in the organic molecule may occur.) It was also decided to carry out a preliminary vigorous reduction of azadirachtin with lithium aluminium hydride to give a poly-hydroxy compound which would probably have a greater tendency than the azadirachtin molecule itself to lose all its oxygen functions on pyrolysis[99]. Such a preliminary reduction of nimbin (10) was carried out by Sengupta, Sengupta and Khastgir[100] who then obtained 1,2,5-trimethylnaphthalene on dehydrogenation of the product.

Azadirachtin was treated with excess lithium aluminium hydride in tetrahydrofuran solution boiling at reflux for four hours and

stirring at room temperature overnight. Water was cautiously added and an attempt was made to extract the product with ethyl acetate. However an emulsion formed which did not separate on addition of sodium chloride. So the solvent was evaporated in vacuo and the solid residue was extracted several times with ethyl acetate to yield a viscous yellow oil. Investigation by t.l.c. showed that this was a mixture containing three major products, none of which absorbed ultra-violet light. All the material in the mixture was considerably more polar than azadirachtin itself as was expected for poly-hydroxy compounds formed by the reduction of carbonyl groups in the molecule. The formation of multiple products may be accounted for in two ways. First the reaction conditions may not have been vigorous enough to fully reduce all the azadirachtin present. Secondly epimeric products were probably formed by reduction of the ketone group thought to be present in the molecule, and this again may have increased the number of possible products. Nevertheless it was assumed that selenium dehydrogenation would be more successful on this crude product mixture than on azadirachtin itself.

The total product from the lithium aluminium hydride reduction of azadirachtin was thoroughly mixed with selenium powder before pyrolysis in an evacuated Carius tube at 320° for 66 hours. The black residue obtained was extracted with boiling absolute ethanol for 24 hours. The mixture of products isolated after filtration and evaporation of the solvent was investigated by gas liquid chromatography (g.l.c.) and mass spectrometry by means of a direct-link from a Pye Model No. 64

gas chromatograph to a RMU-6 mass spectrometer through a Watson-Biemann Separator. The chromatogram shown in Fig. 28 was obtained when a 9 ft. long $\frac{1}{8}$" diameter column of 5% S.E. 30 on Chromosorb G was used with a helium flow rate of 17 $^{ml.}$/min. at 152°.

The peaks designated A, B, C and D in the chromatogram had corrected retention times of less than 5 minutes and they represented materials with mass spectra which showed none of the characteristics of aromatic compounds. The peak designated E had a corrected retention time (6.2 minutes) close to that of an authentic dimethylnaphthalene (2,3-dimethylnaphthalene, 6.6 minutes)and the mass spectrum (Fig. 29) of the material from this peak led to its assignment as a dimethylnaphthalene. The major ions observed in this spectrum were attributed to the molecular ion (M^+ 156), a methyl-benztropylium ion (^{m}/e 155), the benztropylium ion (^{m}/e 141), the indenylium ion (^{m}/e 115), a methyl-tropylium ion (^{m}/e 105) and the tropylium ion (^{m}/e 91).

The mass spectrum (Fig. 30) of the material represented by peak F on the chromatogram (corrected retention time 11.0 minutes) led to its assignment as a trimethylnaphthalene. Prominent ions observed in this spectrum were the molecular ion (M^+ 170), M^+-methyl radical (^{m}/e 155), the benztropylium ion (^{m}/e 141), the indenylium ion (^{m}/e 115), a methyl-tropylium ion (^{m}/e 105) and the tropylium ion (^{m}/e 91).

The mass spectrum (Fig. 31) of the material from peak G (corrected retention time 14.6 minutes) led to its tentative

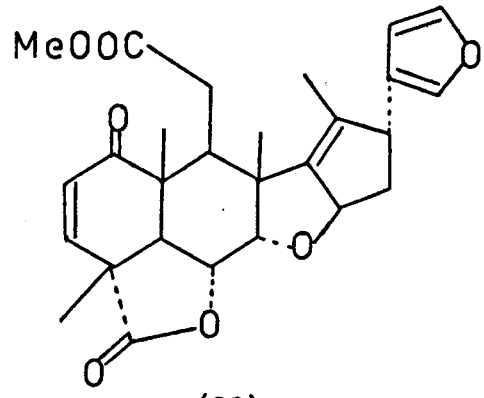
assignment as a tetramethylnaphthalene. The major ions in the spectrum were provisionally assigned as the molecular ion (M^+ 184), M^+-methyl radical ($^m/e$ 169), a dimethyl-indenylium ion ($^m/e$ 143), the indenylium ion ($^m/e$ 115), a methyl-tropylium ion ($^m/e$ 105) and the tropylium ion ($^m/e$ 91). It is thought that such a tetra-substituted naphthalene may have been produced by a methyl shift during pyrolysis. The shift of various groups under such conditions has been reported[99] and the phenomenon may be explained by a proposed mechanism of dehydrogenation which has hydride ion or hydrogen atom abstraction by selenium as its first step. Rearrangements and shifts in the remaining carbonium ion or free radical may then occur before aromatisation is completed[99].

The crude mixture of products from the selenium dehydrogenation was also investigated by g.l.c. at a variety of other temperatures. However no peak corresponding in retention time to an authentic sample of phenanthrene was seen.

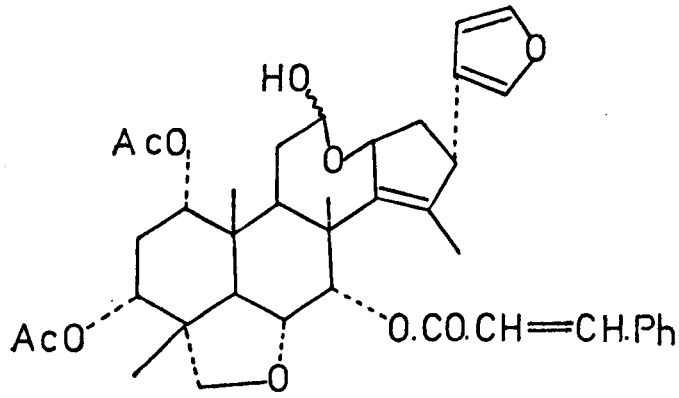
The total weight of the product mixture obtained by ethanol extraction of the pyrolysis residue was 11 mg. of which a considerable proportion appeared to be red selenium metal. Although only seven major peaks were seen in the chromatogram of this material, it was apparent from its overall appearance that other materials were being eluted from the column as "background" to these major peaks. In order to determine the positions of methyl group substitution in the naphthalene derivatives it would have been necessary to isolate

each of these materials and investigate them by I.R. and U.V. spectroscopy. Preparative g.l.c. was the only separation method applicable for these small quantities of material and this may not have yielded pure components because of the "background" materials. Therefore no attempt to isolate each individual naphthalene derivative was made. Nevertheless the evidence from mass spectrometry already offered was considered sufficient to establish that naphthalenes were present in this dehydrogenation product. This in turn implied that the azadirachtin molecule contained two fused six-membered carbocylic rings.

The assignment of such a fused ring system to the azadirachtin molecule suggested that it was not related to the B-seco-limonoid series as these compounds do not contain two intact six-membered carbocyclic rings fused together. However the C-seco-limonoids which have been isolated from neem do possess two intact fused six-membered carbocyclic rings, namely rings A and B. It is conceivable therefore that azadirachtin might constitute a highly oxidised member of this series. C-seco-limonoids so far isolated from neem include nimbin[29] (10) and its deacetyl-derivative[101], salannin[30] (11), nimbolide[102] (23) and the more recently reported nimbolin B[103] (24).



(23)



(24)

A considerable proportion of the present work on azadirachtin has involved n.m.r. spectrometry. These n.m.r. studies have proceeded mainly through efforts to compare and contrast the chemical shifts, and splitting patterns where applicable, of given signals in the spectrum of the parent compound with the appearance of the corresponding resonances in the spectra of a number of derivatives. All the derivatives involved in this work have been described earlier in an appropriate context. Most of the information to be discussed has come from 100 MHz. spectra, but in the case of azadirachtin itself a spectrum recorded at 220 MHz. was available and this proved to be extremely useful. The following discussion generally refers to spectra recorded in $CDCl_3$ solution. The only

advantage in using DMSO-d_6 as solvent came in the characterisation of OH resonances. Other details from spectra determined in this solvent will only be given where a definite correlation has been made with the corresponding feature in a spectrum recorded in $CDCl_3$ solution.

The n.m.r. spectrum of azadirachtin at 100 MHz. and 220 MHz. is shown in Figs. 1 and 32 respectively. The α-protons of a furan ring, a characteristic feature of the limonoids, absorb between τ2 and τ3. The spectrum of azadirachtin shows no signals in this area, thereby demonstrating the absence of such a grouping in the molecule. The signal at lowest field in the azadirachtin spectrum occurs at τ3.10 as a one proton multiplet. This resonance was assigned by Butterworth to the β-vinyl proton of the tiglate ester function. Spin decoupling experiments indicated that the multiplet possesses major coupling J∿10 Hz. with the protons of the β-methyl group which themselves give rise to a doublet at τ8.25. The vinyl proton also shows minor coupling J∿1 to 2 Hz. with the protons of the α- methyl group which appear as a broad singlet at τ8.2. As might be expected the chemical shifts of the resonances associated with the tiglate function are not altered to any significant degree in the derivatives of azadirachtin investigated except when the tiglate group itself has undergone attack. In the spectrum of azadirachtin in DMSO-d_6 solution the resonances attributed to the tiglate ester function were recognised at τ3.03 (β-H), τ8.19 (α-CH_3) and τ8.21 (β-CH_3).

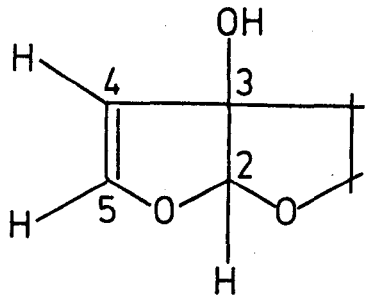
In the spectrum of azadirachtin the doublet at τ3.58 (J∿3 Hz.) integrating for one proton is coupled to a similar signal at τ4.95. These signals do not appear in the spectrum of dihydroazadirachtin (Fig. 2). Instead this derivative displays multiplets integrating for two protons each at τ5.9 and τ7.8. Butterworth therefore assigned the doublets in the azadirachtin spectrum to the vinyl protons at the 5- and 4-positions respectively on the 2,3-dihydrofuran ring (part structure 14). Either of these doublets collapses to a clean singlet on irradiation of the other. The absence of further coupling led Butterworth to propose that the 3-position must be fully substituted.

On the evidence of its movement upfield to τ4.72 on hydrogenation of azadirachtin, Butterworth assigned the one proton singlet resonance at τ4.36 to a proton at the 2-position of the 2,3-dihydrofuran ring which is further deshielded by an adjacent oxygen atom. It has since been proposed that this oxygen is ethereal in nature. The singlet character of the resonance for the proton at the 2-position is further evidence that the 3-position is fully substituted.

The multiplicities of the resonances for H2, H4 and H5 in azadirachtin clearly contrast with those of the corresponding signals in compounds possessing a proton attached at C3. In clerodin[35] (20) for example the presence of such a proton means that H2 is split to a doublet (J∿6Hz.) while H4 and H5 appear as multiplets (numbering as in part structure 14).

The nature of the substituent at the 3-position of the

dihydrofuran ring in azadirachtin is as yet unknown. Aflatoxin M1[98] contains the structural unit shown below (25, numbering in accord with part structure 14) and it was considered possible that azadirachtin could similarly possess a hydroxyl group attached at C3.



(25)

A direct comparison between the chemical shifts of the H2, H4 and H5 resonances of azadirachtin and aflatoxin M1 was considered to be of little help because the anisotropy of the neighbouring aromatic system in the latter affects the shielding of nearby protons. However Holzapfel et al[98] successfully acetylated the hydroxyl group of aflatoxin M1 and information as to the resulting changes of position of the H2, H4 and H5 resonances was sought in order that a comparison might be made with the appropriate signals in derivatives of azadirachtin in which hydroxyl groups were protected. Unfortunately details of the n.m.r. spectrum of acetyl-

aflatoxin M1 were not available as only small quantities of the substance had been obtained[104].

Nevertheless tables were assembled showing the chemical shifts of H2, H4 and H5 for derivatives of azadirachtin in which hydroxyl groups were protected to ascertain if informative variations occurred in the position of these resonances. The table for spectra determined in $CDCl_3$ solution is shown below; τ values are quoted and changes in p.p.m. from the figures for azadirachtin itself are given in parentheses.

	H2	H4	H5
Azadirachtin	4.36	4.95	3.58
Acetyl-azadirachtin	4.38(+.02)	4.95 (0)	3.55(-.03)
A-trimethylsilyl-azadirachtin	4.41(+.05)	5.00(+.05)	3.57(-.01)
B-trimethylsilyl-azadirachtin	4.42(+.06)	4.98(+.03)	3.58 (0)
Bis(trimethylsilyl)-azadirachtin	4.46(+.10)	4.97(+.02)	3.58 (0)
Acetyl-trimethylsilyl-azadirachtin	4.45(+.09)	4.95 (0)	3.58 (0)

The corresponding figures for spectra determined in DMSO-d_6 solution are shown below.

	H2	H4	H5
Azadirachtin	4.42	4.99	3.53
Acetyl-azadirachtin	4.43(+.01)	4.95(-.04)	3.52(-.01)
A-trimethylsilyl-azadirachtin	4.48(+.06)	4.97(-.02)	3.50(-.03)
B-trimethylsilyl-azadirachtin	4.34(-.08)	4.85(-.14)	3.41(-.12)
Bis(trimethylsilyl)-azadirachtin	4.38(-.04)	4.81(-.18)	3.51(-.02)
Acetyl-trimethylsilyl-azadirachtin	4.30(-.12)	4.79(-.20)	3.40(-.13)

The changes summarised above are clearly too small to enable a hydroxyl group to be assigned to the 3-position of the dihydrofuran ring in azadirachtin. However it does seem that protection of the A hydroxyl group, as in acetyl-azadirachtin and A-trimethylsilyl-azadirachtin, causes less of a change in the chemical shifts of the protons under scrutiny than does protection of hydroxyl group B. Therefore, in the uncertain event of there being a hydroxyl group at the 3-position of the dihydrofuran ring in azadirachtin, that group is more likely to be the one designated B.

On the basis of evidence obtained by chemical degradation, Butterworth proposed the part structure 15 for azadirachtin. From spin decoupling experiments he was able to assign the triplet at τ4.51 in the spectrum of azadirachtin to the proton H-C-OAc shown. He recognised that the methylene protons of this ring system give rise to part of the complex absorption at τ7.7 and that the signal for the H-C-OTig proton occurs in the group of resonances at about τ5.3.

Later spin decoupling experiments have shown that the latter proton appears as a triplet at τ4.83 in the spectrum of acetyl-azadirachtin (Fig. 7). At first sight there would appear to be three possible explanations for the downfield shift of the H-C-OTig proton on acetylation of azadirachtin, namely an additional inductive effect, a Van der Waal's deshielding, or a new anisotropic effect. The known substitution on the H-C-OTig carbon atom is such that introduction of the new acetate group in the formation of acetyl-azadirachtin must occur at least four bonds away from the H-C-OTig proton. This is too great a distance for an increased inductive effect to account for the magnitude of the downfield shift observed. Bis(trimethylsilyl)-azadirachtin and A-trimethylsilyl-azadirachtin both have a silyl-ether group protecting the same hydroxyl site that is protected in acetyl-azadirachtin yet in the spectra of both of these derivatives (Figs. 6 and 15) the H-C-OTig signal is not moved downfield from its position in azadirachtin. Therefore the possibility that the extra deshielding of the H-C-OTig proton in acetyl-azadirachtin is due to a Van der Waal's interaction can probably be discounted. It seems most likely that the downfield shift is brought about by the anisotropic effect of the carbonyl group of the new acetate ester. However it must also be noted that the H-C-OTig signal is moved down to τ4.80 in the spectrum of tris(trimethylsilyl)-azadirachtin (Fig. 17), though here the introduction of three new bulky groups into the molecule may well have brought a new Van der Waal's interaction into play.

In its position at τ4.83 in acetyl-azadirachtin, the H-C-OTig resonance (which Butterworth wrongly assigned to a new H-C-OAc proton) does not overlap with any other signal and is clearly seen to be a triplet. Irradiation at the τ7.7 methylene resonance causes the triplets at τ4.51 (H-C-OAc) and τ4.83 (H-C-OTig) to collapse to clean signlets proving conclusively that no other protons are involved in this spin-spin system. Such a closed system exists in the A ring of salannin[30] (11) and it appears that azadirachtin contains such a structural unit. Further evidence for an intact A ring in azadirachtin came from selenium dehydrogenation of the compound which suggested that both ring A and ring B might still be intact. Furthermore all the limonoids except methyl ivorensate[31] (12) so far isolated from the Meliaceae contain an intact A ring[22] so if azadirachtin is related in some way to these compounds it too might be expected to contain such an entity.

The H-C-OAc and H-C-OTig triplet resonances both show a coupling of about 3 Hz. and this is characteristic of protons in an equatorial attitude[105]. Therefore the tiglate and acetate ester groups are arranged 1,3-diaxial on the six-membered ring. The two esters in the A ring of salannin are also 1,3-diaxial and α in configuration and this is the stereochemistry of all limonoids with a 1,3 relationship of free or esterified hydroxyl groups in this ring[21]. This is further evidence that azadirachtin contains a structural unit corresponding to an intact A ring.

In the 220 MHz. spectrum of azadirachtin (Fig. 32) the signals between τ7.6 and τ7.9 are much better resolved than at 100 MHz. (Fig. 1). At 220 MHz. a broadened doublet integrating for two protons is seen at τ7.72 distinctly separated from nearby signals. This resonance is assigned to the protons of the methylene group in the proposed A ring system. The major coupling of this signal is gem-coupling of 11 Hz; minor coupling of 3Hz. with the H-C-OAc and H-C-OTig protons is also observed.

The n.m.r. spectrum of deacetyl-azadirachtin (Fig. 5), prepared by mild alkaline hydrolysis of the parent compound, does not show a triplet at τ4.51, indicating that the acetate of the proposed A ring system is no longer present. A new one proton signal (proposed new H-C-OH) appears at τ5.67 and the new H-C-OH signal is tentatively assigned to the region around τ7.7. The H-C-OTig signal appears at τ5.19, as a distinct triplet which does not overlap the other signal in the τ5.3 region.

The preferential removal of the acetate group from the proposed A ring on alkaline hydrolysis of azadirachtin suggests that this ester is in an exposed position in the molecule, possibly corresponding to the C3 position in salannin (11). Similar evidence of preferential removal of the acetate function on alkaline hydrolysis of salannin led Overton *et al*[30] to assign the acetate ester to the C3 position and the tiglate ester to the relatively more hindered C1 position. A similar arrangement may well exist in azadirachtin.

Overton et al[30] also reported a diol formed as a minor product of the alkaline hydrolysis of salannin by loss of both the acetate and the tiglate ester group of the A ring. No corresponding derivative has been isolated from the alkaline hydrolysis of azadirachtin. (Indeed no other distinct component beside deacetyl-azadirachtin has been observed in the crude product from such a reaction.) This may be because approach to the tiglate function is more hindered by other groups than is the case in salannin, or perhaps other areas of the azadirachtin molecule are at least as vunerable to attack by alkali and so the resulting formation of multiple products disguises the possible presence of a deacetyl-detigloyl-derivative.

In the spectrum of azadirachtin in DMSO-d_6 solution (Fig. 8) the protons of the proposed A ring system are recognised at τ4.71 (H-C-OAc), τ5.48 (H-C-OTig) and τ7.8 (methylene). As previously described, the multiplicity of the new OH resonance (τ5.35) in the spectrum of deacetyl-azadirachtin in DMSO-d_6 (Fig. 18) was uncertain due to partial overlap with nearby signals. It was therefore not possible to confirm by this evidence that the new OH generated by alkaline hydrolysis of azadirachtin was secondary in character. It could be seen that this new OH signal was coupled to a signal at τ5.84 (proposed new H-C-OH) but again the multiplicity of this signal was disguised by overlap with other resonances.

In the 100 MHz. spectrum of azadirachtin shown (Fig. 1) a broad one proton singlet, which disappears on addition of D_2O, occurs

at τ4.95 where it overlaps with the doublet resonance assigned to the β-vinyl proton of the dihydrofuran ring. This OH resonance, as described earlier, has been observed as far downfield as τ4.59 in a concentrated sample (560 mg./ml.) and as far upfield as τ5.05 in a dilute sample (40 mg./ml.). Such movement is characteristic of an OH proton susceptible to inter-molecular hydrogen bonding. Butterworth had suspected that an OH resonance was present in this region of the spectrum and he suggested that it might represent a secondary hydroxyl group. However the absence of this signal in the spectra of acetyl-azadirachtin (Fig. 7) and A-trimethylsilyl-azadirachtin (Fig. 15) indicated that it was in fact due to the tertiary hydroxyl group designated A. In the spectrum of azadirachtin in DMSO-d_6 solution (Fig. 8) this OH signal appears as a singlet at τ3.75.

In the spectrum of azadirachtin at 100 MHz. (Fig. 1) resonances due to a total of four protons overlap to give a complex absorption in the area between τ5.22 and τ5.56. As described previously, the low field section of this complex signal is assigned to the proton H-C-OTig of the proposed A ring system. This signal is probably centred at about τ5.23 and its triplet nature is only revealed when it moves downfield, as for example in acetyl-azadirachtin.

With one of the resonances in this complex group definitely assigned to the proton H-C-OTig, the nature of the other signals in this area of the azadirachtin spectrum will be considered. Butterworth

reported[106] that irradiation on a doublet (J~10Hz.) of variable position at about τ6.7 caused the extreme highfield portion of the complex signal around τ5.5 to coalesce with the rest of the grouping. The present work has indicated that the apparent variation from one sample of azadirachtin to another in the position of the doublet around τ6.7 is due to coincidental overlap with two OH resonances in this region of the spectrum. Removal of these OH resonances by D_2O exchange reveals that the doublet is centred at τ6.65 and integrates for one proton. It displays a coupling of 12 Hz. with a doublet centred at τ5.4. The latter signal appears as a split doublet due to further coupling of 2 Hz. with a signal at about τ8.3 which will be discussed later. The downfield portion of this split doublet at τ5.4 overlaps with other signals in the area but the upfield portion stands out distinctly both at 100 MHz. and 220 MHz. It is this upfield portion which coalesces with the rest of the resonances in the region when the coupling of 12 Hz. is destroyed by irradiation at τ6.65.

The split doublet signal at τ5.4 is not moved significantly in the spectra of derivatives of azadirachtin which have various hydroxyl groups protected. The signal is recognised at τ5.62 in the spectrum of azadirachtin in DMSO-d_6 solution. The chemical environment of the proton represented by this resonance is not yet known. The relatively high deshielding it experiences could be caused by a combination of several effects including perhaps the aniostropic effect of one or more of the six carbonyl functions in the molecule.

Butterworth assigned the H-C-OH proton of azadirachtin to the group of resonances between τ5.22 and τ5.56 because a one proton signal was moved downfield from this area to τ4.83 on acetylation of azadirachtin and this he proposed was the new H-C-OAc resonance. However it has since been proved that acetylation of azadirachtin occurs at a tertiary hydroxyl group and it is the H-C-OTig signal which moves downfield. Nevertheless the H-C-OH proton of azadirachtin is now assigned to the area τ5.22 to τ5.56 on evidence gained from tris(trimethylsilyl)-azadirachtin which is the only derivative so far prepared in which the secondary hydroxyl group is protected. In the spectrum of this compound (Fig. 17) a signal has been moved from about τ5.4 in azadirachtin to τ5.75. It is proposed that this is the H-C-OH signal in azadirachtin and the H-C-OSiMe$_3$ signal in the tris(trimethylsilyl)-ether. The H-C-OSiMe$_3$ signal at τ5.75 overlaps with no other signals and appears as a broad or slightly split singlet, but nothing is known of its possible coupling.

In the spectrum of azadirachtin in DMSO-d$_6$ solution (Fig. 8) either irradiation of the exchangeable doublet at τ5.19 (J∿3Hz., H-C-OH) or addition of D$_2$O sharpens a signal at τ5.74. The multiplicity of this signal is obscured by overlap with other absorptions but it is confidently assigned to the H-C-OH proton in the molecule.

The final proton involved in the complex pattern between τ5.22 and τ5.56 in the spectrum of azadirachtin appears to be centred at about τ5.3. The spectrum of tris(trimethylsilyl)-azadirachtin

(Fig. 17) provides a unique view of this resonance because in this compound the H-C-OTig and H-C-OH signals of azadirachtin itself are moved away from the area. This leaves only the split doublet (J∿12Hz. and 2Hz.) which has already been discussed, and the one proton signal at present under consideration. In the spectrum of the tris(trimethylsilyl)-ether at 100 MHz. this signal appears between the two major portions of the split doublet. It seems to be either a broad, or a slightly split singlet, but nothing is known about the possible coupling of this proton. It probably resonates at this relatively low field position due to a combination of deshielding effects in this highly oxygenated molecule.

In summary the complex pattern in the area τ5.22 to τ5.56 in the spectrum of azadirachtin has been assigned as follows:

One proton	triplet	τ5.23 (J∿3Hz.)	H-C-OTig
One proton	broad/split singlet	∿τ5.3	unknown
One proton	broad/split singlet	∿τ5.4	H-C-OH
One proton	split doublet	τ5.4 (J∿12 and 2Hz.)	unknown

Resonances attributable to a total of ten protons occur in the region τ5.8 to τ6.2 in the spectrum of azadirachtin. There is such a high density of overlapping signals in this area that all assignments made here must retain a certain degree of uncertainty.

Between τ5.8 and τ6.1 in the spectrum at 220 MHz. (Fig. 32) there are clearly two doublet resonances though only three peaks are observed at 100 MHz. (Fig. 1) due to overlap. The lower of these doublets is centred at τ5.88 and the upper at τ5.97. Both doublets integrate for one proton and both display a coupling of 8 Hz. but they are not coupled to each other. Spin decoupling has shown that the doublet at τ5.88 is coupled ($J \sim 8$Hz.) to a one proton doublet resonance at τ6.38, the downfield arm of which is partially obscured in the 220 MHz. spectrum (Fig. 32), and totally obscured in the 100 MHz. spectrum (Fig. 1) by the three proton singlet at τ6.33 attributed to a carboxymethyl group. It is suspected that the doublet at τ5.97 is coupled ($J \sim 8$Hz.) to a one proton doublet centred at τ6.25 and almost totally obscured at 100 MHz., and partially obscured at 220 MHz., by the carboxymethyl singlet at τ6.22. However practical difficulties due to the close proximity of these signals have precluded spin decoupling experiments which might have confirmed this assignment.

All ten protons in this area are accounted for by the complex pattern described above. Unfortunately little is known about the chemical environment of the protons represented by the series of doublets but they probably represent two independent AB systems. A chemical shift of about τ6 is often observed for the α-protons of an ether linkage and one or more of the protons represented by these doublets may exist in such an environment. A summary of this region of the spectrum appears below.

One proton	doublet	τ5.88 (J∿8Hz., to τ6.38)	unknown
One proton	doublet	τ5.97 (J∿8Hz., to τ6.25?)	unknown
Three protons	singlet	τ6.22	carboxymethyl
One proton	doublet	τ6.25 (J∿8Hz., to τ5.97?)	unknown
Three protons	singlet	τ6.33	carboxymethyl
One proton	doublet	τ6.38 (J∿8Hz., to τ5.88)	unknown

Difficulties in interpreting the area of the spectrum from τ5.8 to 6.5 both in azadirachtin and its derivatives has mainly been due to the presence of the two carboxymethyl singlets which tend to obscure the small, split signals in the vicinity. It has been widely reported[87] that addition of benzene to n.m.r. samples in for example $CDCl_3$ or CCl_4 solutions can cause shifts of carboxymethyl resonances to the extent of 1 p.p.m. in some cases. Addition of deutero-benzene (C_6D_6) to a solution of azadirachtin in $CDCl_3$ moved the lower-field carboxymethyl singlet upfield by a small amount, with a maximum shift of 0.15 p.p.m. being obtained in a solvent mixture containing 15% or more of C_6D_6. A maximum upfield shift of 0.05 p.p.m. was observed for the upper carboxymethyl resonance. Clearly these solvent dependent shifts were too small to leave an unobstructed view of the other signals in the area. Furthermore

an additional difficulty arose through loss of resolution in the spectrum when the proportion of benzene in the solvent exceeded about 10%.

A possible alternative technique for clearing the carboxymethyl signals from this crowded area would have been to remove the methyl esters by alkaline hydrolysis and then to re-esterify using deutero-diazomethane[54]. This was not pursued because it was suspected that residual multiplet signals due to traces of material containing partially deuterated carboxymethyl groups would confuse the relevant area of the spectrum. Furthermore the poor yields previously obtained in the alkaline hydrolysis of azadirachtin suggested that the process would entail the loss of a considerable amount of material.

Variations in the pattern in the region τ5.8 to τ6.5 in the derivatives of azadirachtin are apparent but it has proved impossible to follow what happens in detail due to the presence of the large carboxymethyl resonances. However spin decoupling experiments on this area of the acetyl-azadirachtin spectrum have proved interesting. In the spectrum of this compound (Fig. 7) the lowest field doublet in this area occurs at τ5.82. It is thought to correspond to the doublet at τ5.88 in azadirachtin itself but is coupled to a doublet at τ6.19 which lies beneath the lower carboxymethyl resonance. In azadirachtin itself the doublet at τ5.88 is coupled to a doublet at τ6.38, that is underneath the upper carboxymethyl singlet. It appears

therefore that acetylation of azadirachtin causes a one proton doublet to move downfield from τ6.38 to τ6.19. Although the chemical environment of this proton is not known it must be in such a position that it is further deshielded in acetyl-azadirachtin, possibly due to the anisotropic effect of the new acetate ester function.

Between τ6.6 and τ6.8 in the spectrum of azadirachtin at 100 MHz. (Fig. 1) three peaks are seen. Two of these are the arms of a one proton doublet centred at τ6.65 coupled ($J \sim 12$Hz.) to the one proton split doublet at τ5.4 as previously described. The relatively large coupling constant indicates that if the protons concerned are positioned on a ring system, they are probably both axial in attitude[105]. The other signal in this area is a one proton singlet at τ6.67 which at 100 MHz. lies between the two arms of the above mentioned doublet and at 220 MHz. overlaps with the upfield arm of this resonance.

Variations in the position of the singlet and doublet signals under consideration throughout a series of azadirachtin derivatives are summarised below. (τ values are quoted.)

In some cases the signals are not recognised (n.r.) because they are shifted downfield to below about τ6.45 and obscured or disguised by the complex group of signals in the vicinity of the carboxymethyl peaks. The factors that cause these variations in chemical shifts are not understood as the chemical environments of the protons involved have not been elucidated.

	Doublet (J~12Hz.)	Singlet
Azadirachtin	6.65	6.67
Acetyl-azadirachtin	6.63	6.83
A-trimethylsilyl-azadirachtin	n.r.	6.70
B-trimethylsilyl-azadirachtin	n.r.	6.68
Acetyl-trimethylsilyl-azadirachtin	6.51	6.82
Bis(trimethylsilyl)-azadirachtin	n.r.	6.71
Tris(trimethylsilyl)-azadirachtin	n.r.	n.r.
Deacetyl-azadirachtin	6.74	6.72

In the spectrum of some samples of azadirachtin the region around τ6.7 is further complicated by the presence of one, or sometimes two OH resonances. Generally however these two resonances are observed at higher fields, commonly at about τ6.9 in one case and about τ7.1 in the other. The resonance at about τ6.9 corresponds to the exchangeable singlet seen at τ4.82 in the spectrum in DMSO-d_6 solution and is assigned to the tertiary hydroxyl group B. This group has not been successfully acetylated but is silylated in B-trimethylsilyl-azadirachtin, bis(trimethylsilyl)-azadirachtin, acetyl-trimethylsilyl-azadirachtin and tris(trimethylsilyl)-azadirachtin. The absorption at about τ7.1 in the spectrum of azadirachtin in $CDCl_3$ corresponds to the exchangeable doublet at τ5.19 in the spectrum determined in DMSO-d_6 solution and is therefore assigned to the secondary hydroxyl group in the molecule. This group is

resistant to acetylation and oxidation and is only silylated under very vigorous conditions. On these grounds it is thought to exist at a highly sterically hindered site on the molecule. It remains as a free hydroxyl group in all the derivatives of azadirachtin so far prepared except tris(trimethylsilyl)-azadirachtin.

As previously described, determination of the spectrum of azadirachtin through a range of concentrations in $CDCl_3$ enabled an OH resonance to be assigned to the τ4.9 region. During these experiments it was difficult to follow the movements of the two OH resonances which at the concentrations routinely used absorb in the τ6.9 and τ7.1 regions. However it is certain that at the highest concentration employed (560 mg./ml.), both these OH protons absorbed below τ6.6.

Between τ7.6 and τ7.9 in the spectrum of azadirachtin a complex group of signals representing four protons is observed. The overlap of signals is too great in the 100 MHz. spectra of azadirachtin and its derivatives for conclusions to be drawn about the individual resonances involved. However in the 220 MHz. spectrum of azadirachtin (Fig. 32) this area contains three separate resonances. The central resonance appears as a broadened doublet integrating for two protons and centred at τ7.72. This resonance is assigned to the methylene protons of the proposed A ring system as previously described. It is proposed that the major coupling of 11 Hz. observed for this methylene resonance is due to gem-interaction. There

is also vicinal coupling of 3 Hz. with the H-C-OTig (τ5.23) and H-C-OAc (τ4.51) protons of the proposed A ring system.

The lowfield resonance in the area τ7.6 to τ7.9 appears at 220 MHz. as a doublet (J∿5Hz.) centred at τ7.64 and integrating for one proton. Spin decoupling experiments have failed to demonstrate the source of the observed coupling. The high field resonance in this area appears as a broad doublet centred at τ7.86 and integrating for one proton. The major coupling for this signal appears to be about 6 Hz. but from its overall appearance it is clear that there is further splitting which gives a half-line width of about 20 Hz. Therefore the proton concerned is probably in an axial configuration if it is attached to a ring system[105].

Nothing definite is known about the source of the coupling observed for the signals at τ7.64 and τ7.86. However in acetylazadirachtin irradiation at about τ5.4 causes some sharpening in the complex absorption between τ7.6 and τ7.9. This effect is not due to the known spin-spin coupling between the H-C-OTig proton and the methylene group in the proposed A ring because in this compound the H-C-OTig proton absorbs downfield at τ4.83. There are broad or split singlets at ∿τ5.4 (H-C-OH) and ∿τ5.3 (unknown) with unassigned coupling and so one or both of these may be coupled to either the signal at τ7.64, or to that at τ7.86, or to both in some way. It is not possible to distinguish between these possibilities in the spectrum at 100 MHz. due to the overlap of signals but future spin decoupling

experiments at 220 MHz. may prove fruitful. Such experiments might most profitably be carried out on tris(trimethylsilyl)-azadirachtin because in the spectrum of this derivative the H-C-OH proton of the parent compound is moved upfield to τ5.75 (H-C-OSiMe$_3$) and so is quite distinct from the unknown signal at about τ5.3. It should therefore be possible to tell which, if any, of these signals is coupled to the unknown resonances at τ7.64 and τ7.86.

A summary of the area between τ7.6 and τ7.9 in the spectrum of azadirachtin is given below.

One proton	doublet	τ7.64 (J∿5Hz.)	unknown
Two protons	broad doublet	τ7.72 (J∿11Hz. and 3Hz.)	A ring methylene
One proton	broad doublet	τ7.86 (J∿6Hz.)	unknown

The chemical shifts of the two unknown protons in this group suggest that either, or both of them may be attached to a carbon atom which is situated α to the ketonic function thought to be present in the molecule. However no definite assignment can be made because such chemical shifts may well arise from a combination of other factors. If a derivative of the proposed ketone function is made at some time in the future it will be interesting to see if some change occurs in the chemical shifts of these protons.

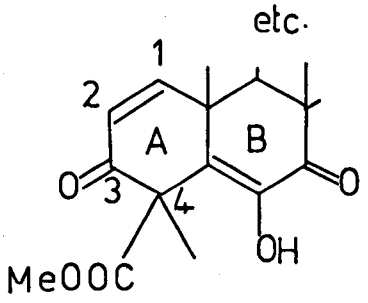
The region τ7.9 to 8.6 in the n.m.r. spectrum of azadirachtin integrates for sixteen protons and is dominated by a series of methyl group absorptions. Two singlets integrating for three protons each are normally seen at τ8.00 and τ8.08, though in samples at high concentration these may overlap at about τ8.08. These singlets are each assigned to the methyl group of an acetate ester. The slight variation in position which these signals can show makes it impossible to state which of them is lost when the acetate group of the proposed A ring system is removed by alkaline hydrolysis.

Upfield of these acetate peaks are seen the methyl group resonances of the tiglate ester function. A broad singlet assigned to the α-methyl group is seen at τ8.2 and a doublet (J∿10 Hz.) assigned to the β-methyl group is centred at τ8.25. Irradiation at this point causes collapse of the one proton multiplet at τ3.10 assigned to the β-H of the tiglate function.

The methyl groups of the acetate and tiglate ester functions account for twelve of the sixteen protons resonating in this area of the spectrum. Butterworth recognised an additional broad singlet at τ8.3, but due to the large number of signals in the vicinity he was unsure if it integrated for two or three protons. Butterworth stated that if this was a three proton signal then the methyl group it represented must be tertiary as no coupling is apparent. He put forward two possible explanations for the lowfield position of this suggested methyl resonance[107]. Either it was a vinyl methyl group

situated on a fully substituted double bond to account for the lack of coupling, or it was a tertiary methyl group highly deshielded by a number of oxygenated functions or anisotropic effects.

The uncertainty about this signal at τ8.3 in the azadirachtin spectrum is still not absolutely resolved. In the spectrum of tris(trimethylsilyl)-azadirachtin in $CDCl_3$ solution, and in the spectra of several compounds in DMSO-d_6 solution, this resonance stands apart from nearby signals and appears in the region τ8.5 to 8.6 as a broad singlet integrating for three protons. It is therefore definitely assigned to a methyl group. However the environment of this group is still uncertain. Possibly it is a vinyl methyl group analogous to the C26 methyl groups in nimbin[29] (10) and salannin[30] (11) which show slight broadening due to coupling of 1 to 2 Hz. with H15 in these molecules. However a source of coupling to the τ8.3 signal in azadirachtin has not yet been found, and furthermore there is no chemical or spectral evidence for the existence of a further double bond in this molecule. More probably this signal is due to a methyl group highly deshielded by a combination of several factors. The limonoid hirtin[108] possesses the part structure shown below (26) and the methyl group attached at C4 absorbs as low as τ8.17 due to the combination of deshielding effects it experiences from the various nearby functions.



(26)

It is conceivable that a methyl group attached at an analogous site on the proposed A ring of azadirachtin might similarly be highly deshielded because acetate ester and tiglate ester functions are thought to be attached at C3 and C1 respectively (numbering as in part structure 26) and possibly a carboxymethyl group may be attached at C4 also. The presence of oxygenated functions in the proposed B ring might then further deshield the methyl group sufficiently for it to resonate as low as τ8.3. These tentative proposals do not however account for the broadened nature of the signal and further work will be required before a definite environment can be assigned to the methyl group concerned.

The final one proton signal to be considered in the τ7.9 to 8.6 region of the azadirachtin spectrum is completely obscured by methyl group resonances in all spectra except that of tris(trimethylsilyl)-azadirachtin. In this case the tiglate methyl signals are further downfield than normal, and the unknown methyl group resonance described above appears further upfield than normal. A distinct

one proton doublet (J∿2 Hz.) is therefore seen at τ8.23 in this spectrum. This signal collapses to a singlet on irradiation of the split doublet (J∿12 Hz. and 2 Hz.) at τ5.4 due to destruction of the smaller coupling. Little is known of the chemical environment of the proton which resonates at τ8.23 but its low coupling constant of 2 Hz. suggests that if it is situated on a ring system it is probably equatorial in attitude[105].

In summary the resonances in the azadirachtin spectrum between τ7.9 and 8.6 have been assigned as shown below.

Three protons	singlet	τ8.0	$CH_3CO.O-$
Three protons	singlet	τ8.08	$CH_3CO.O-$
Three protons	broad singlet	τ8.2	tiglate, α-methyl
Three protons	doublet (J∿10 Hz.)	τ8.25	tiglate, β-methyl
One proton	doublet (J∿2 Hz.)	∿τ8.2	unknown
Three protons	broad singlet	∿τ8.3	unknown methyl group

The n.m.r. spectra of most samples of azadirachtin, and many samples of its derivatives, show signals of some description above τ8.6. These signals are usually weak, variable in appearance and position from one sample to another, and are not coupled to any of the signals known to be associated with protons of the azadirachtin molecule. Furthermore all 44 protons in the molecule are accounted for by the survey given above of the region from τ3 to τ8.6. Any signals which occurred above τ8.6 were therefore dismissed as being

due to small amounts of impurities in the sample concerned. These impurities were possibly derived from the silica gel used in making the P.L.C. and t.l.c. plates by which azadirachtin and its derivatives were purified.

In the azadirachtin spectrum the absence of the characteristic tertiary methyl group signals in the τ9 region associated with the limonoids shows that the substance is not a simple member of this class of compounds. However it is possible that azadirachtin is derived from this class, perhaps by processes of oxidation and decarboxylation in which several angular methyl groups have been modified. In several limonoids including nimbin[29] (10), salannin[30] (11), nimbolide[102] (23) and nimbolin A and B[103] (24) isolated from neem, the α-methyl group attached at the C4 position has undergone oxidation of some kind. In nimbin oxidation has proceded as far as the carboxylic acid which appears as its methyl ester. Such a process may well have occurred at the analogous position in azadirachtin and further oxidation and decarboxylation may have occurred at other sites during the biogenisis of the molecule to leave only one unchanged tertiary methyl group: that which resonates at τ8.3. However the possible link between azadirachtin and the limonoids is still a tentative one.

From all the n.m.r. studies described above, signals for every one of the 44 protons of the azadirachtin molecule have been recognised between τ3 and τ8.6. The environment of 33 of these protons has been definitely established leaving a further 11 protons

to be assigned.

In the last few years interest has grown in the use of paramagnetic metal complexes as shift reagents to simplify complicated n.m.r. spectra, and it was thought that such a reagent might be of use in clarifying the azadirachtin spectrum. Early shift reagents reported include nickel dibromide trihydrate used by Zaev et al.[109] in their study of certain pyrazoles, and cobalt acetylacetonate used by Szarek and Baird[110] during work on a number of alcohols. These reagents produced small shifts and caused serious line broadening. Recently, more efficient shift reagents have been based on complexes of lanthanide metals with organic ligands. Hinckley has reported the use of the dipyridine adduct of tris(dipivalomethanato)europium, $Eu(DPM)_3.2py$, to simplify the n.m.r. spectrum of cholesterol[111] and testosterone[112]. However it is $Eu(DPM)_3$ without associated pyridine which has found the widest application as an n.m.r. shift reagent. It was first used by Sanders and Williams[113,114] on relatively simple alcohols, amines, ketones, esters, ethers and nitro-compounds but has since found widespread use in the simplification of the n.m.r. spectra of more complicated compounds including steroids and terpenoids[115,116]. The tris(dipivalomethanato) complexes of praseodymium[117] and ytterbium[118-120] have also been widely used. Recently work on the effect of certain shift reagents on ^{14}N and ^{31}P n.m.r. spectra has been reported[120,121].

A shift reagent operates by reversible complexation with the lone pair electrons of functional groups in a substrate molecule. A given proton in the substrate then experiences a shielding or deshielding effect to a degree which depends upon its distance from the metal, and on the nature of the functional group involved. The effect is thought to operate through bonds and through space[122]. Although the original spectrum may have contained a number of overlapping resonances, addition of a shift reagent may result in signals becoming sufficiently separated from each other for first order coupling patterns to be observed. Of the lanthanide complexes which might be used as shift reagents, $Eu(DPM)_3$ and $Pr(DPM)_3$ have been preferred because they are reasonably soluble in the solvents commonly used in n.m.r. work and they produce shifts of a convenient magnitude while causing a relatively slight degree of line broadening. Recently complexes of praseodymium and europium with a partially flourinated organic ligand have been reported to possess even more convenient properties in these respects[123].

It was decided to record the n.m.r. spectrum of azadirachtin in the presence of $Eu(DPM)_3$, which in general causes a downfield shift of protons in a substrate, and separately in the presence of $Pr(DPM)_3$ which in general causes upfield shifts. It was hoped that in the presence of one or other of these reagents, most of the overlapping signals in the azadirachtin spectrum could be separated

from each other. At the time this work was carried out $Eu(DPM)_3$ and $Pr(DPM)_3$ were not commercially available. Both complexes were therefore prepared from the corresponding metal nitrate and 2,2,5,5-tetramethyl-3,5-heptanedione(dipivaloylmethane). The basic procedure adopted was that of Eisenkraut and Sievers[124] but following the instructions of Sanders and Williams[113] precautions to exclude oxygen during the isolation of the products were not undertaken. Both complexes were purified by recrystallisation and sublimation and gave satisfactory melting points.

The deutero-chloroform used as solvent in these spectroscopy experiments was first allowed to stand for a minimum of 48 hours over molecular seive (4A, preheated at 120°) in an attempt to remove traces of water and acid which can cause decomposition of the complex. Each experiment was performed by adding solid praseodymium or europium complex stepwise to a solution of azadirachtin in purified $CDCl_3$ in an n.m.r. tube. However it was found that addition of each portion of complex led to the formation of a fine precipitate which destroyed the definition of the spectrum. The cause of this problem was not discovered and it was only overcome by allowing the precipitate to settle for a few hours and then decanting the supernatant liquid into another n.m.r. tube in which the spectrum could then be recorded. In this way the n.m.r. spectrum of azadirachtin was recorded in the presence of various amounts of added shift reagent. However it was not possible to

know accurately the weights of azadirachtin and complex present in each sample because certain quantities of both substances always remained in the tube from which the solution had been decanted.

The spectra obtained by this method were not of great value. As the amount of complex present was gradually increased, virtually all the signals in the spectrum were seen to move relative to an internal tetramethylsilane standard, but overlapping signals did not move apart from each other to any significant degree. The movement of signals en bloc, and a certain amount of line broadening, produced spectra in which the overlap of signals was at least as great as in the spectrum before the addition of shift reagent. Therefore very little information was gained from these experiments. The only signals to move at a significant rate relative to the other resonances were the three already assigned to the OH protons in the molecule. This was expected because association of a metal to the lone pair electrons of a hydroxyl group is reported[114] to be stronger than to the other functions such as esters and ethers known to be present in azadirachtin. The movement of virtually all the signals in the spectrum was probably caused by the presence of so many oxygen functions at which reversible complexation could occur, that all the protons in the molecule were near enough to a metal atom, either through bonds or space, to be affected to some extent.

The azadirachtin used in the experiments with shift reagents

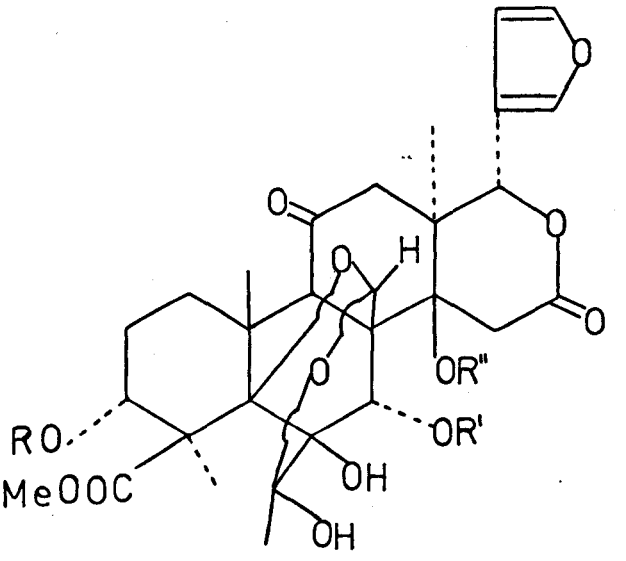
was conveniently recovered by P.L.C.

Chemical studies, first undertaken by Butterworth and continued in this present work, had identified all the functional groups of azadirachtin and established that the molecule contained two fused six-membered carbocyclic rings and a dihydrofuran ring. Attention was therefore turned to preparing a crystalline derivative for use in X-ray diffraction studies which, it was hoped, would elucidate the complete structure and stereochemistry of the molecule. This new approach was adopted because studies on azadirachtin through its chemical reactions had been slow, tedious, and often unfruitful. For example it had generally been difficult to find suitable reagents and conditions to carry out reactions specifically at a required site on the molecule. Large quantities of the starting material were not available and yields in most reactions, especially those of a degradative nature, had been very poor. The molecule had already been attacked at the two most reactive sites, namely the tiglate ester function and the 2,3-dihydrofuran ring, and any further degradative studies starting at these points would have involved several known steps before any new information could be gained. Therefore the most convenient way of elucidating this structure seemed to be by X-ray crystallography.

With the modern computer techniques available, X-ray crystallography is a powerful tool for the structural analysis of organic compounds[125]. In 1960 the study of the limonoids received

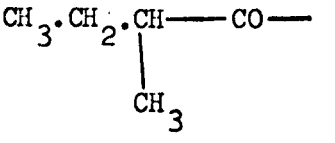
a tremendous boost from the elucidation by X-ray crystallography of the structure of limonin[126], the compound from which this class derived its name. The structure of this compound was solved by the heavy-atom method using the convenient derivative 7-epilimonol iodoacetate. Other pertinent examples of structure elucidation by X-ray studies on specially prepared heavy-atom derivatives are the limonoids mexicanolide[127,128] and swietenine[129,130] and the diterpenoid clerodin[35] (20). Recently the power of X-ray crystallographic methods not requiring a heavy atom was demonstrated when the structure of the limonoid utilin (13) was elucidated by diffraction studies on a crystal of the compound itself[33]. This compound displays two structural features not previously recognised in the limonoids. One is the methylene bridge between C4 and C1, and the other is the ortho-acetate function. Neither of these novel features appears in the structure (27) originally proposed for utilin in 1967 by Taylor and Wragg[131] on evidence gained from degradative and spectral studies on this compound and the closely related entandrophragmin.

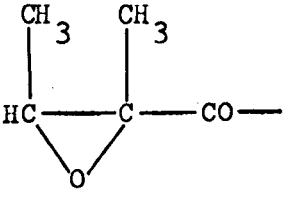
Crystals of azadirachtin had never been prepared, though Butterworth had shown that the compound could be precipitated from carbon tetrachloride as an amorphous powder. Therefore modifications to the structure of azadirachtin were sought which might give a derivative, suitable for crystallisation, containing a heavy atom as a marker for X-ray studies.



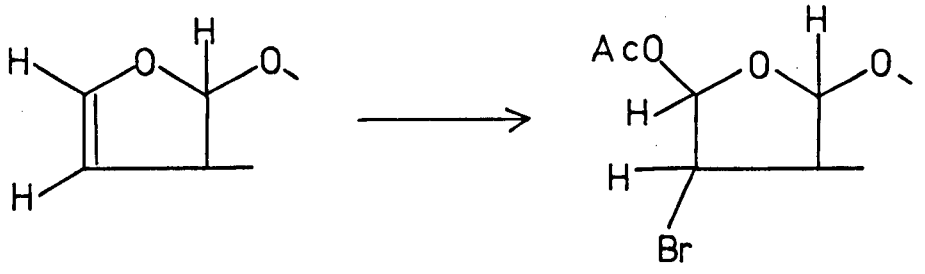
(27) R, R^1, R^{11} =

$CH_3.CO.$—





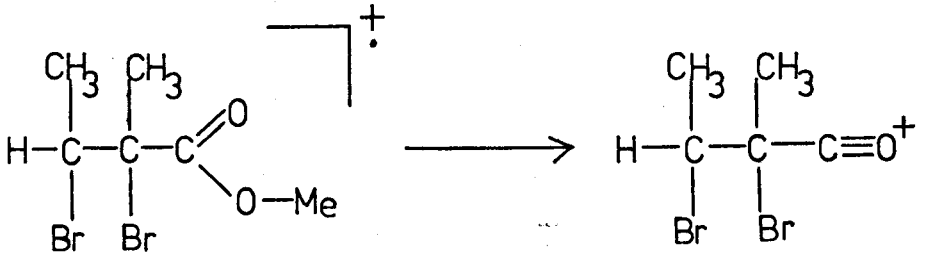
Barton et al had reported[35] that treatment of clerodin (20) with bromine and sodium acetate in glacial acetic acid for 90 minutes at room temperature produced a bromohydrin acetate derivative as illustrated below.



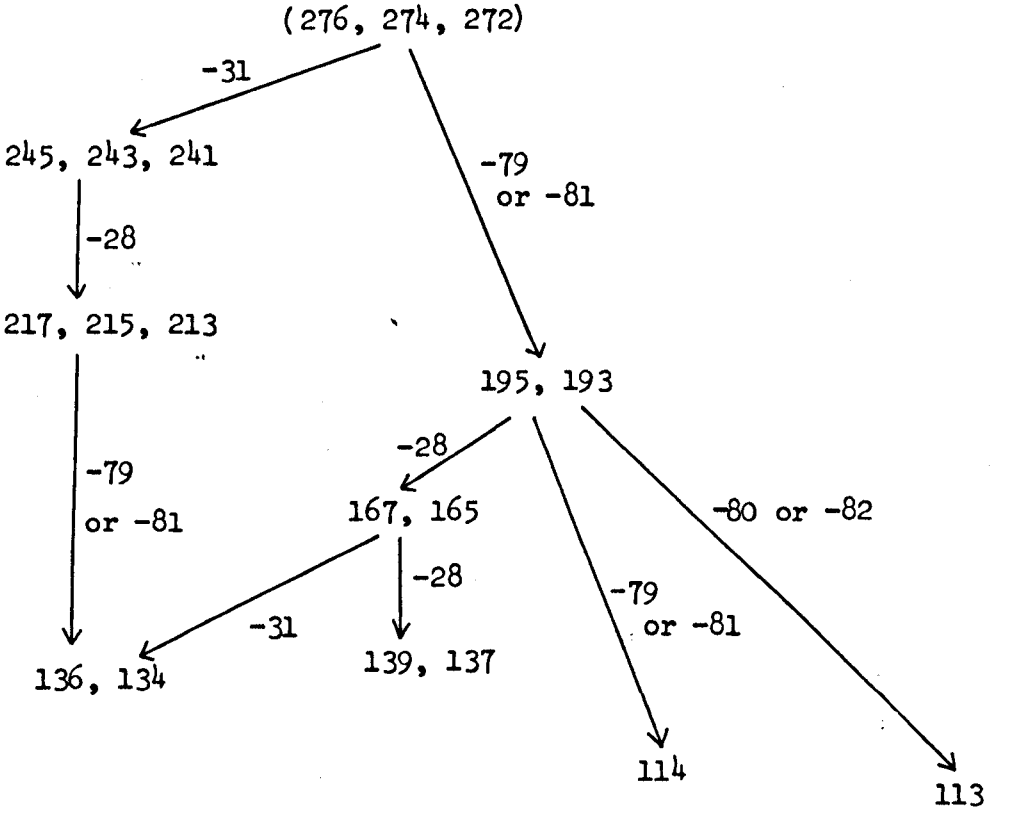
Treatment of azadirachtin under exactly the same conditions in the hope of producing a crystalline bromohydrin acetate gave in reasonable yield a new compound with R_F 0.56 on t.l.c. eluting with chloroform-acetone (7:3). The n.m.r. spectrum of this derivative ($CDCl_3$, Fig. 33) showed no vinyl proton doublet resonances at τ3.58 and τ4.95, and the absence of an I.R. absorption at 1625 $cm.^{-1}$ confirmed that the 2,3-dihydrofuran ring had undergone modification. The I.R., U.V. and n.m.r. spectra showed that the tiglate ester function had also undergone attack. The compound was tentatively assigned as acetoxy-tribromo-azadirachtin with a bromohydrin acetate structure modifying the dihydrofuran site (by analogy with clerodin) and the tiglate ester double bond now saturated by two bromine atoms.

The presence of one new acetate group in the molecule was suggested by integration of the τ8 region in the n.m.r. spectrum,

but the presence of nearby signals from the now modified tiglate ester function made complete interpretation of this region difficult. Modification of the tiglate ester residue of azadirachtin by addition of bromine had not previously been reported, indeed Butterworth had found that only the 2,3-dihydrofuran ring was attacked on treatment of a chloroform solution of azadirachtin with bromine in the presence of suspended calcium carbonate. In order to confirm that such a simple addition to the tiglate ester had occurred in this case, methyl tiglate was treated with bromine and sodium acetate in glacial acetic acid at room temperature for 90 minutes. The product of this model reaction was assigned as the simple dibromo-adduct (methyl 2,3-dibromo-2-methylbutyrate) on the following spectral evidence. Resonances were observed in the n.m.r. spectrum at τ5.2 (one proton quartet, C3-$\underline{H}$), τ6.2 (three proton singlet, O-$C\underline{H}_3$), τ8.05 (three proton singlet, C2-$C\underline{H}_3$) and τ8.12 (three proton doublet, C4 methyl group). The mass spectrum did not show the expected molecular ion triplet centred at $^m/e$ 274, but a triplet centred at $^m/e$ 243 was assigned to an ion formed by expulsion of a methoxyl radical from the unobserved molecular ion. (Such fragmentation was also observed for methyl tiglate itself.)



All the major peaks above $^{m}/e$ 100 could be explained by losses of fragments known to be present in the molecule.



On the evidence of this model reaction it seemed certain that the tiglate function in azadirachtin itself had undergone simple modification to the dibromo-adduct.

The expected molecular weight of the proposed acetoxy-tribromo-azadirachtin was 1022 but the compound was so involatile in the mass spectrometer that no ions were seen above $^{m}/e$ 370. In an attempt to make a more volatile trimethylsilyl-derivative the proposed acetoxy-tribromo-azadirachtin was treated with B.S.A. in chloroform solution for two hours at room temperature, but no reaction occurred. However treatment with B.S.A. in dimethyl-formamide solution at room temperature for five hours produced two products, both less polar than the starting compound. The less polar of these, which was assumed to be silylated to the greater extent and therefore more volatile, was isolated by preparative t.l.c. In the mass spectrometer it showed a molecular ion as a multiplet between $^{m}/e$ 1160 and 1166 which was as expected for a bis(trimethylsilyl)-ether derivative of acetoxy-tribromo-azadirachtin.

Later confirmation that three bromine atoms had been introduced into the azadirachtin molecule on treatment with bromine and sodium acetate came when micro-analysis gave bromine 22.22% for the product. Acetoxy-tribromo-azadirachtin $C_{37}H_{47}O_{18}Br_3$ requires bromine 23.54%. The degree of inaccuracy suggested the presence of impurities in the sample but was not great enough to

suggest that any more or less than three bromine atoms were present in the molecule. The sum total of evidence available suggested that the original tentative assignment of this derivative as acetoxy-tribromo-azadirachtin was correct.

The solubility of acetoxy-tribromo-azadirachtin was similar to that of azadirachtin and so carbon tetrachloride was used as the solvent in attempts to crystallise this derivative. However only an amorphous powder was obtained on all occasions, whether precipitation was brought about by rapid or slow cooling of a concentrated solution, or if the solvent was allowed to evaporate slowly from a solution of this compound.

Attempts were made to produce a derivative of azadirachtin in which both tertiary hydroxyl groups, and possibly even the hindered secondary hydroxyl group, were protected by acetylation in the hope that such a compound would be crystalline. Butterworth had shown that treatment of azadirachtin with boiling acetic anhydride for 10 minutes produced a non-crystalline mono-acetyl derivative, and later evidence indicated that the tertiary hydroxyl group A was protected in this compound. Azadirachtin was therefore treated with boiling acetic anhydride for periods of time in excess of 10 minutes to ascertain whether further acetylation could be accomplished. The reaction was carried out on a small scale and aliquots were removed at various times for investigation by t.l.c. It was found that after about 15 minutes a large number of compounds

was produced which gave an ill-defined streak on t.l.c. If acetylation of the tertiary hydroxyl group B or the secondary hydroxyl group had occurred, the product was not visible on t.l.c. due to the presence of many other materials. These had presumably been produced on attack by traces of free acid present in the acetic anhydride either as an initial impurity or as a result of the acetylation reaction. Removal of the initial acetic acid impurity by using freshly distilled acetic anhydride did not give more favourable results.

Butterworth had shown that treatment of azadirachtin with acetic anhydride in the presence of pyridine left the starting material unchanged and that the use of boron triflouride ethereate instead of pyridine led to the production of multiple products. Therefore methods involving acetic anhydride were abandoned and attempts were made to form a di- or tri-acetyl derivative of azadirachtin by treatments using acetyl chloride.

A chloroform solution of azadirachtin was treated with acetyl chloride in the presence of a catalytic amount of pyridine for 110 minutes at room temperature. Investigation of the product by t.l.c. revealed one major component with R_F 0.54 eluting with chloroform-acetone (7:3), and R_F 0.45 eluting with ether-acetone (4:1). The material was subjected to P.L.C. eluting twice with chloroform-acetone (4:1) and the solid from the main band was further purified by preparative t.l.c. eluting twice with ether-

acetone (4:1) to give the major product which was designated V. The n.m.r. spectrum of material V (Fig. 34) indicated that it was not a simple acetyl derivative of azadirachtin and indeed the complex group of signals between τ7.5 and τ8.9 was first taken to indicate that the sample had retained some impurities despite the two chromatographic procedures used in its isolation. However V gave a single spot on t.l.c. using four different eluants which suggested that it was homogeneous. The absence of doublet resonances at τ3.58 and τ4.95 in the n.m.r. spectrum of V indicated that the 2,3-dihydrofuran ring of azadirachtin had undergone attack in the reaction, and this was confirmed by the absence of an I.R. absorption at 1625 $cm.^{-1}$ However the characteristic absorptions of the tiglate ester function were seen in the I.R. and U.V. spectra of substance V. The mass spectrum showed ions at $^{m}/e$ 83 and 55 confirming the presence of the tiglate function but the high mass region was not helpful. The ion at highest $^{m}/e$ occurred at $^{m}/e$ 734 but this was not considered to be the molecular ion, because firstly it was an inexplicable 14 mass units above the molecular weight of azadirachtin, and secondly several of the ions below could not have been derived from this ion by losses of fragments normally associated with azadirachtin derivatives. Substance V could not be fully correlated with azadirachtin and it is possible that it was a mixture which was not separated by any of the solvent systems used during t.l.c. investigations. However

it may have been a material produced by some form of rearrangement of the azadirachtin molecule initiated by acidity in the acetyl chloride.

An attempted acetylation of azadirachtin with acetyl chloride in excess pyridine at 0° was monitored by removal of aliquots at various time intervals for investigation by t.l.c. After 5 hours the azadirachtin remained unchanged but a brown stain was seen at the origin of the t.l.c. plate. A control experiment, in which no azadirachtin was present, proved that this brown polar material was formed by interaction between the acetyl chloride and the pyridine. This interaction occurred even though the acetyl chloride was freshly obtained and the pyridine had been dried over barium oxide and distilled.

As azadirachtin had not been affected at 0°, the treatment with acetyl chloride in pyridine solution was repeated at 10° and again monitored by t.l.c. Azadirachtin was seen to be unchanged after 4 hours, but after 6 hours the chromatogram was dominated by heavy streaking over most of the plate, which control experiments showed was due to materials produced by interaction between the pyridine and acetyl chloride. When the reaction was repeated at room temperature an equally unsatisfactory result was obtained. It was clear therefore that any genuine acetyl derivatives of azadirachtin produced by reaction with acetyl chloride and pyridine could not be successfully detected or isolated by chromatography

due to the presence of unwanted materials derived from the reagents. Further attempts to form a di- or tri-acetyl derivative of azadirachtin were not made.

The structures of limonin[20,126] and mexicanolide[127,128] were solved by X-ray diffraction studies on specially prepared iodoacetyl derivatives, and several attempts were made to prepare such a derivative of azadirachtin in the hope that it might be crystalline. Iodoacetyl derivatives are generally prepared by chloroacetylation of a hydroxyl group, followed by treatment with sodium iodide in boiling acetone to bring about halogen exchange. Attempts were therefore first made to chloroacetylate azadirachtin. It was known that an acetyl derivative of hydroxyl group A in the azadirachtin molecule was formed on treatment of the compound with boiling acetic anhydride for 10 minutes, and it was thought that a similar treatment using chloroacetic anhydride might chloracetylate the same group. Accordingly azadirachtin was treated with chloroacetic anhydride at the temperature of boiling acetic anhydride (140°) for 5 minutes, but the product appeared on t.l.c. as an inseparable mixture of a large number of materials. Such a mixture was probably produced by attack on the azadirachtin molecule at this high temperature by traces of chloroacetic acid which is a considerably stronger acid than acetic acid. It was necessary therefore to treat azadirachtin with chloroacetic anhydride under milder conditions.

Some starting material was recovered when azadirachtin was

treated with chloroacetic anhydride at 60° for 2 hours but that which had reacted had produced a complex mixture of products, again presumably due to acid attack. An analagous result was obtained when azadirachtin was treated with chloroacetic anhydride in chloroform solution at room temperature for 62 hours. The addition of 2 drops of pyridine to such a reaction mixture brought about the production of multiple products within 20 hours at room temperature.

From all these results it appeared that the specific chloroacetylation of a hydroxyl group in the azadirachtin molecule was not a practical possibility because the presence of the required product, if formed, would be disguised by an ill-defined mixture of other materials produced by acid attack.

Dihydro-azadirachtin is more stable in acidic conditions than the parent molecule due to saturation of the acid labile 2,3-dihydrofuran system, so several attempts at chloroacetylation were made using this derivative as starting material. Dihydro-azadirachtin was recovered unchanged from treatment with chloroacetic anhydride at 140° for 15 minutes, but when the reaction was prolonged to 90 minutes a complex mixture of products showing no distinct components on t.l.c. was obtained.

The chloroacetylation stage in the preparation of the iodoacetyl derivatives of both limonin and mexicanolide was achieved by methods involving chloroacetyl chloride, and several

attempts to chloroacetylate dihydro-azadirachtin were made using this reagent. Jacobs and Heidelberger[132] had reported the chloroacetylation of aniline by chloroacetyl chloride at 0^{o} in the presence of sodium acetate and acetic acid to remove hydrochloric acid as it was formed. Such conditions seemed conveniently mild for the first attempt to chloroacetylate dihydro-azadirachtin with the acid chloride. However the starting material was recovered unchanged from the appropriate reaction mixture after 90 minutes.

There have been several reports of the successful use of a pyridine-chloroacetyl chloride mixture as a chloroacetylating agent. However it was found that pyridine and the acid chloride interacted to give products which obscured the chromatographic region occupied by dihydro-azadirachtin on t.l.c. and P.L.C. plates, and the use of this reagent mixture was therefore precluded in this case.

Kamiya et al[133] had reported the protection of a hydroxyl group in a tetracyclic triterpenoid by bromoacetylation with bromoacetyl chloride in dimethyformamide solution, so an attempt to chloroacetylate dihydro-azadirachtin in this mildly basic solvent was made. Azadirachtin was recovered unchanged after 4 hours at room temperature in a dimethylformamide solution of chloroacetyl chloride. However when a similar reaction mixture was heated on a water bath for 10 minutes a mixture of many materials running as a streak on t.l.c. was obtained. Presumably the reaction mixture

was sufficiently acidic to cause degradation of dihydro-azadirachtin at the higher temperature.

An attempt was made to chloroacetylate dihydro-azadirachtin by treatment with undiluted chloroacetyl chloride at 9°, and the progress of the reaction was monitored by t.l.c. eluting twice with ethyl acetate-benzene (9:1). After 17 hours no starting material remained at R_F 0.27 but two major products were seen at R_F 0.39 and R_F 0.49, with a minor component at R_F 0.19. The product mixture was subjected to P.L.C. eluting with the above mentioned solvent system to give three bands designated A, B and C in order of increasing R_F value. Very little material was isolated from band A (which corresponded to the minor component) and when t.l.c. investigation showed that this material was inhomogeneous it was discarded. The material from band B was further purified by preparative t.l.c. eluting with chloroform-acetone (7:3) to give a material designated VI. The n.m.r. spectrum of substance VI ($CDCl_3$, Fig. 35) could not be correlated with that of dihydro-azadirachtin itself. The region around τ6.4 suggested that VI was a mixture of products yet it gave a single spot on t.l.c. with four different solvent systems. The region between τ7.6 and τ8.8 was also puzzling. An I.R. absorption at 1650 $cm.^{-1}$ and peaks at $^m/e$ 83 and 55 in the mass spectrum indicated that the tiglate group was intact in the molecule. This function, and the acetate groups presumably present, accounted for some of the n.m.r.

resonances around τ8 but the origin of the peaks above τ8.3 was unknown. The high mass region in the mass spectrum of VI was generally uninformative with no ions visible above $^{m}/e$ 702.

The material isolated from band C was further purified by preparative t.l.c. eluting with ether–acetonitrile (4:1) to give a substance VII. The n.m.r. spectrum ($CDCl_3$, Fig. 36) of this substance also defied complete interpretation. It seemed to indicate that VII was a mixture, yet the substance gave only one spot on t.l.c. with three different solvent systems. The appearance of peaks above τ8.3, as in the case of VI, was particularly puzzling. The mass spectrum of VII was uninformative in the high $^{m}/e$ regions, but significant ions at $^{m}/e$ 83 and 55 showed that the tiglate ester function was still present and this was confirmed by an I.R. absorption at 1650 $cm.^{-1}$

Although substances VI and VII were not identified, it was clear that neither of them was the simple chloroacetyl derivative of dihydro-azadirachtin which was sought. It is thought that VI and VII were either inhomogeneous materials which were not separated by the solvent systems used in t.l.c. investigations, or they may have been produced by some form of rearrangement of the dihydro-azadirachtin molecule in the acidic reaction conditions.

Treatment of dihydro-azadirachtin with undiluted chloroacetyl chloride at room temperature for 5 hours also yielded substances VI and VII, but a reaction at 100° for 25 minutes produced an

ill-defined mixture of materials, presumably due to acid attack.

The structure of the limonoid swietenine was elucidated by X-ray analysis of its 3-detigloyl-3-p-iodobenzoyl derivative[129,130] and it was thought that a detigloyl-iodoacetyl or a detigloyl-p-iodobenzoyl derivative of azadirachtin might be similarly convenient. Butterworth had reported[12] a method for the selective removal of the tiglate function of dihydro-azadirachtin. The two stage procedure involved oxidative cleavage of the tiglate double bond with Lemieux Reagent[65-68] (sodium metaperiodate-potassium permanganate) to give detigloyl-pyruvyl-dihydro-azadirachtin, followed by selective removal of the pyruvate ester group by hydrolysis with sodium bicarbonate solution to give detigloyl-dihydro-azadirachtin. However the overall yield reported for these two stages was only about 7% and it was realised that attempts to chloroacetylate detigloyl-dihydro-azadirachtin would only be practicable if this material could first be prepared in reasonable quantity. In both stages of the preparation Butterworth had reported the recovery of some starting material and the route was therefore re-investigated to ascertain whether increased reaction times would bring about complete conversion of starting material at each stage, so improving the overall yield.

The oxidation of dihydro-azadirachtin was carried out by Butterworth for 75 minutes, and it was found in this present work that after 100 minutes all the starting material had undergone

reaction. However the recovery of material after this extended period was only 52% as against 92% reported by Butterworth, but there was the compensation that the sample of detigloyl-pyruvyl-dihydro-azadirachtin obtained was completely free of dihydro-azadirachtin and could be used as starting material for the second stage without intervening chromatography and the losses that entails.

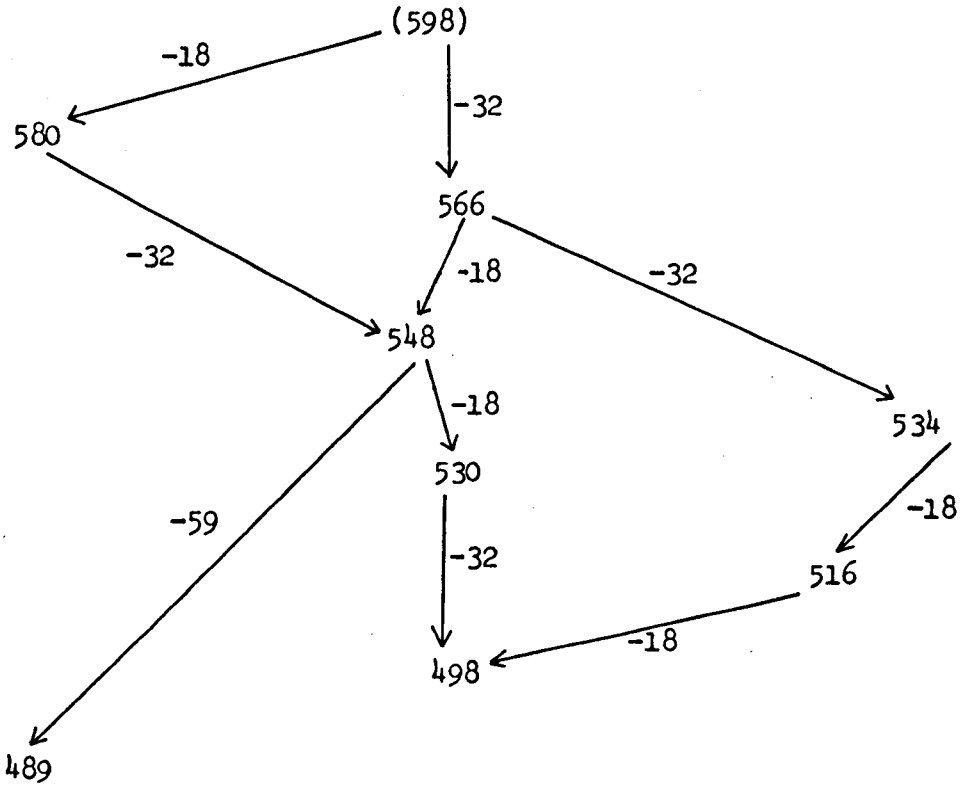
Butterworth had allowed the hydrolysis of detigloyl-pyruvyl-dihydro-azadirachtin with sodium bicarbonate solution to proceed for 70 minutes. In this present work it was found that some starting material still remained after 100 minutes and so the hydrolysis was continued for a further 3 hours. The amount of material recovered from this extended procedure was rather low and on t.l.c. eluting twice with chloroform-acetone (7:3) it showed a faint spot at R_F 0.40 corresponding to the starting compound detigloyl-pyruvyl-dihydro-azadirachtin, a more intense spot at R_F 0.25 corresponding to detigloyl-dihydro-azadirachtin, and a new major spot at R_F 0.19. Attempts to improve the yield of detigloyl-azadirachtin by extending the hydrolysis reaction time had therefore failed because a new more polar product had been produced before all the starting material had reacted.

The new product was isolated by two successive applications of the preparative t.l.c. technique, each time eluting twice with chloroform-acetone (7:3). Only 2 mg. of the product was obtained

and it was assigned as deacetyl-detigloyl-dihydro-azadirachtin from the features of its accumulated n.m.r. spectrum in $CDCl_3$ solution which is shown in Fig. 37. (The spectrum of Butterworth's detigloyl-dihydro-azadirachtin is shown in Fig. 38 for comparison.) The integration around $\tau 8$ in the spectrum of this new compound showed that only one acetate group remained in the molecule, and the absence of an H-C-OAc resonance at $\tau 4.5$ indicated that the acetate ester in the proposed A ring system had been removed in the alkaline conditions. The signal for the new H-C-OH proton appeared above $\tau 5$. The ill-defined absorption above $\tau 8.6$ was assigned to impurities in the sample, probably eluted from the silica used on the chromatographic plates.

The mass spectrum of deacetyl-detigloyl-dihydro-azadirachtin did not show the expected molecular ion at $^m/e$ 598. However a weak M-18 ion was seen at $^m/e$ 580, and other significant ions above $^m/e$ 500 could be accounted for by losses of methanol or water from this penta-hydroxy compound as shown below.

The yield of deacetyl-detigloyl-dihydro-azadirachtin was poor in this case because the conditions used were not originally aimed at its production. However if this compound is prepared in sufficient quantity in the future, n.m.r. spin decoupling studies on it may be fruitful because there are few resonances due to ester functions to obscure the smaller signals. The compound contains a β-diol system in the proposed A ring and the possible

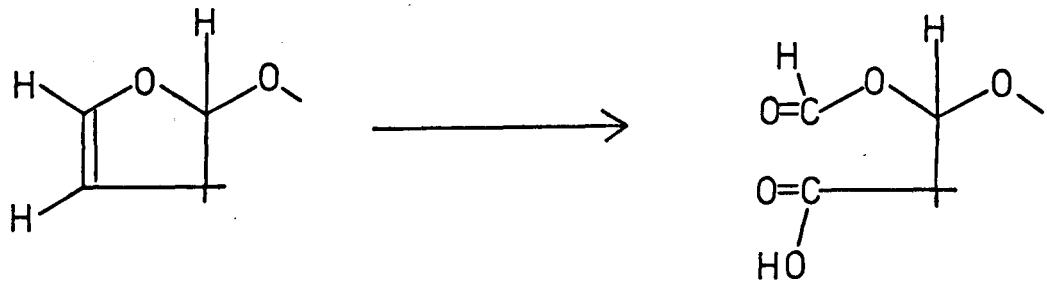


oxidation of this to a dione might also be of interest.

The scheme to produce an iodoacetyl or iodobenzoyl derivative of detigloyl-dihydro-azadirachtin was abandoned because it was considered impracticable to undertake a scheme using the detigloyl derivative as starting material when the above investigations had shown that the preparation of this compound was costly in terms of the amount of azadirachtin consumed. Future efforts might be made to iodoacetylate the new hydroxyl group in deacetyl-azadirachtin because it is thought that this is in a more exposed position on the proposed A ring than the hydroxyl group generated when the tiglate ester is removed. However this scheme too would be costly because deacetyl-azadirachtin can only be prepared from azadirachtin

in about 15% yield. Also the molecule contains the 2,3-dihydrofuran system which may have to be saturated before exposure to an acidic reagent in the chloroacetylation step.

Butterworth found that the treatment of azadirachtin with Lemieux Reagent[65-68] (sodium metaperiodate-potassium permanganate) converted the tiglate group to a pyruvate ester and split the 2,3-dihydrofuran ring double bond to give a formate ester and a carboxylic acid function. The latter process is illustrated below.



Attempts were made to form the caesium salt of the carboxylic acid function of this azadirachtin derivative in the hope that such a compound might be crystalline and amenable to X-ray studies. The Lemieux Oxidation of azadirachtin was carried out following Butterworth's precedure and on t.l.c. eluting with ether–acetone (4:1) the product showed an elongated spot at the origin characteristic of a carboxylic acid, with a faint, diffuse stain at about R_F 0.63. Purification of the carboxylic acid by chromatography

was impracticable because of its high polarity, and attempts to form a caesium salt were therefore performed using the crude Lemieux Oxidation product. This material in hot 95% ethanol was treated with a 95% ethanol solution containing the amount of caesium carbonate calculated to be required for salt formation. Attempts to bring about precipitation of a possible caesium salt of the azadirachtin derivative by rapid cooling of the solution failed. The solution was therefore stored in the dark and the solvent was allowed to evaporate slowly at room temperature. However the precipitate obtained by this method was a yellow glassy material which displayed no crystalline chracteristics. A further attempt to crystallise a possible caesium salt from aqueous acetone (1:34) was also unsuccessful.

Several possible approaches towards a crystalline derivative of azadirachtin might be exploited in the future. For example, attempts to form a crystalline metal salt of the Lemieux Oxidation product of azadirachtin might be performed using other alkali metals, or perhaps silver. X-ray analysis of metal salts of organic acids is well known, and it is interesting to note that the structure of the antibiotic polyetherin A, whose molecular weight is within four mass units of that of azadirachtin, was readily solved by X-ray studies on its silver salt[134]. There would seem therefore to be no immediate reason why the structure of azadirachtin could not be solved in this way if a crystalline salt could be obtained.

Alternatively attempts to prepare an iodobenzoyl derivative of one of the hydroxyl groups in azadirachtin or dihydro-azadirachtin might lead to a crystalline derivative. Attempts to form the iodobenzoyl derivative of the new hydroxyl group in deacetyl-azadirachtin might also be undertaken.

A small scale reaction described earlier suggested that it might be possible to form a hemi-acetal propionate by attack of propionic acid at the 2,3-dihydrofuran ring site in the azadirachtin molecule. If this could be confirmed, an analogous reaction employing β-bromopropionic acid might give a heavy-atom crystalline derivative. If β-bromopropionic acid were to prove too strongly acidic, an acid such as ɣ-bromobutyric acid in which the halogen is positioned further from the carboxyl function might prove more convenient.

To summarise, the groupings shown below have been definitely assigned to the azadirachtin molecule.

	Oxygen atoms	Double bond equivalents
One tiglate ester	2	2
Two acetate esters	4	2
Two carboxymethyl groups	4	2
One 2,3-dihydrofuran ring	1	2
Three hydroxyl groups	3	-
	14	8

In addition the following entities are tentatively assigned to the molecule.

	Oxygen atoms	Double bond equivalents
One ether linkage (probably at the 2-position of the dihydrofuran ring)	1	-
One ketone carbonyl	1	1
	2	1

In this way all sixteen oxygen atoms in the azadirachtin molecule are accounted for. From the molecular formula $C_{35}H_{44}O_{16}$ the azadirachtin molecule contains fourteen double bond equivalents and nine of these are assigned as shown above. It appears therefore that azadirachtin contains five rings in addition to the dihydrofuran ring. The results from selenium dehydrogenation suggest the presence of two fused six-membered carbocyclic rings, and various pieces of evidence described previously suggest that a structural unit equivalent to the A and B rings of the limonoids remains intact in the azadirachtin molecule.

There are eleven carbon atoms which cannot be part of the azadirachtin skeleton. This total is made up of five in the tiglate function, four in the two acetate esters and two in the carboxymethyl groups. The skeleton of azadirachtin therefore contains twenty-four carbon atoms which is two less than the

skeleton of a tetranor-triterpenoid.

Simple tetranor-triterpenoids possess five tertiary methyl groups. Presuming that azadirachtin is derived from this class of compounds, two of these methyl groups may have been oxidised to carboxylic acids which appear as their methyl esters, and a further two have probably been completely removed by oxidation and subsequent decarboxylation to leave the novel twenty-four carbon atom skeleton. The remaining methyl group in azadirachtin presumably gives rise to the signal at τ8.3 in the n.m.r. spectrum of the compound. Overall twenty-one carbon atoms, as well as two oxygen atoms incorporated in the dihydrofuran ring with the adjoining ether linkage, remain to construct a total of six rings.

Of the forty-four protons in the azadirachtin molecule the environment of thirty-three is known from the n.m.r. studies described earlier. The chemical shift and appearance of the signals for the remaining eleven protons are also known, but no definite assignments as to the environments of these protons can yet be made. Further work on the n.m.r. spectrum of azadirachtin and derivatives will be facilitated when spin-decoupling at 220 MHz. becomes routinely available. One of the most interesting derivatives which should be investigated by this technique is tris(trimethylsilyl)-azadirachtin because, as has already been described, the spectrum of this compound gives a unique opportunity for close investigation of the τ5.2 to τ5.5 area, and the one proton

doublet at τ8.23.

Spin-decoupling experiments at 220 MHz. may also, in the future, help to eliminate some of the other uncertainties left in this work. For example such a study on the lactone from the alkaline hydrolysis of dihydro-azadirachtin might elucidate the relationship between this derivative and azadirachtin itself, thereby yielding further important information on the relative positions of various functions in the parent molecule. As suggested earlier this particular task might be further simplified by the introduction of deuterium atoms into the lactone at known positions.

However in the long term the complete elucidation of the structure and stereochemistry of azadirachtin seems more likely to be achieved by X-ray diffraction studies rather than by future n.m.r. work. The difficulty of finding a suitable crystalline derivative of azadirachtin remains, but several possible approaches have been discussed earlier. Of these the most promising alternatives would seem to be the formation of a hemi-acetal β-bromopropionate at the 2,3-dihydrofuran site, the formation of an iodobenzoate derivative of an alcohol function in the molecule, or the production of a convenient metal salt of the Lemieux Oxidation product of azadirachtin.

The Anti-feeding Activity of Azadirachtin and some Derivatives

The feeding test procedure used in these studies is described in the experimental section. The method was based on the bioassay used by Butterworth[12] in the isolation of azadirachtin from neem seeds, though several changes were incorporated in an attempt to ensure a sufficiently high consumption of control papers for the tests to be considered valid. For example tests were only performed during the summer months when the insects had been receiving a regular diet of fresh grass because it was found that locusts reared on the senescent grass of the winter generally consumed only small amounts of control paper during feeding experiments. Groups of five rather than four locusts were preferred, and the jars employed were of a more convenient neck-width and overall size than those used by Butterworth. Haskell and Mordue had reported[39] that locusts consumed a maximum amount of sucrose impregnated filter paper after 18 hours without food, and consequently this starvation period was employed in the present experiments rather than the 24 hours used by Butterworth. Before presentation to the insects all the papers were dampened with water in order that they might be as palatable as possible, though the amount of liquid used was not sufficient to wash any of the test substance or the sucrose from the paper. A test period of 24 hours was preferred to the 8 hours used by Butterworth in the hope that the extended time

would lead to the maximum possible consumption of paper. The final modification made to Butterworth's procedure was that the results for each test substance at each concentration were expressed as a percentage anti-feeding activity relative to the control and not as a percentage eaten relative to the control.

Tests carried out by Butterworth had shown[11] that azadirachtin possessed 100% anti-feeding activity at test solution concentrations down to about 0.04 mg./l., and the present experiments confirmed that the limit of 100% activity for this compound lay at a concentration between 0.05 mg./l. and 0.005 mg./l. Further feeding tests were then undertaken using various known derivatives of azadirachtin in an attempt to identify the site or sites in the molecule responsible for the high level of biological activity. All the compounds were carefully purified by chromatography immediately before impregnation of the papers because it was realised that the presence of a small quantity of a highly active compound as an impurity in a relatively inactive compound could give misleading results in a bioassay. The results obtained for a number of azadirachtin derivatives are given in full in the experimental section and are discussed below.

Dihydro-azadirachtin showed 100% anti-feeding activity at a test solution concentration of 0.5 mg./l. and an activity of 99% at 0.05 mg./l. For this derivative therefore, the borderline for 100% activity occurred at a slightly higher concentration than

for the parent compound but the difference was not outstanding. Deacetyl-azadirachtin also was virtually as efficient an anti-feedant as the parent compound, showing a limit of 100% activity just above 0.05 mg./l.

The limit of 100% activity for acetyl-azadirachtin was at a concentration between 5 and 0.5 mg./l. which was approximately one hundred times higher than that at which the limit for azadirachtin occurred. This suggested that the tertiary hydroxyl group A in azadirachtin makes an important contribution to the physiological activity of the molecule.

In the compounds bis(trimethylsilyl)-azadirachtin and acetyl-trimethylsilyl-azadirachtin the tertiary hydroxyl groups A and B are both protected. These materials each showed a limit of 100% activity around 500 mg./l., about ten thousand times the concentration required for azadirachtin and approximately one hundred times that required for acetyl-azadirachtin. These results indicated that both the tertiary hydroxyl groups in the azadirachtin molecule make important contributions to its physiological activity.

In an effort to ascertain if one tertiary hydroxyl group was significantly more important than the other to the anti-feeding activity of the molecule, tests were performed using the two known mono-trimethylsilyl-ether derivatives of azadirachtin. Both A-trimethylsilyl-azadirachtin and the B-trimethylsilyl-ether

derivative showed a limit of 100% activity between 50 and 5 mg./l. It seems therefore that groups A and B make roughly equal contributions to the physiological activity of azadirachtin.

All the tests on the locust anti-feeding activity of azadirachtin carried out by Butterworth at Keele, and by Haskell and Mordue at the A.L.R.C. in London, had employed the desert locust Schistocerca gregaria Forsk. Tests were now carried out to ascertain if azadirachtin showed comparable activity against the african migratory locust Locusta migratoria migratorioides Reiche and Fairmaire. The procedure employed was identical to the routine test discussed above except that nymphs of the migratory locust on the fourth day of the fifth instar were used. The results from these experiments (see experimental section) indicated that azadirachtin possessed only 74% anti-feeding activity against this species at a test solution concentration as high as 500 mg./l. Additionally it showed zero activity against the migratory locust at 0.05 mg./l., a concentration at which the compound is 100% active against S. gregaria.

The work of Sinha and Gulati[8] had shown that neem seeds possess anti-feeding activity against both the desert locust and the african migratory locust. It may be that azadirachtin is the constituent in the seeds which is responsible for the activity against both insects, though the latter species is clearly less sensitive to the compound. Alternatively there may be another

constituent or combination of constituents in neem seeds which possesses higher anti-feeding activity than azadirachtin against the migratory locust but a relatively low activity against S. gregaria. In order to distinguish between these two possibilities an extraction and fractionation of neem seeds monitored by feeding tests employing the migratory locust would need to be undertaken.

A study of the interaction of azadirachtin with the mouth-parts of the migratory locust has yet to be undertaken. If pursued however the results may make an interesting comparison with those obtained by Haskell and Mordue[39] from such experiments with the desert locust.

A number of workers have studied the biological effect of azadirachtin on a variety of insect species. The results so far available are described in the introduction to this thesis. Work of this nature is continuing.

EXPERIMENTAL

General Procedures

Dimethylformamide (D.M.F.) and pyridine were purified by distillation from barium oxide. Dimethylsulphoxide and hexadeuterodimethylsulphoxide were dried over molecular sieves type 4A.

Melting points were determined on a Kofler hot stage apparatus and are uncorrected.

Infrared absorption spectra were recorded on a Perkin Elmer 257 grating spectrophotometer in chloroform or carbon tetrachloride solution using 1 mm. cells. Ultraviolet absorption spectra were recorded on a Unicam S.P. 800 spectrophotometer using 1 cm. cells and commercial 95% ethanol as solvent.

^{1}H Nuclear magnetic resonance (n.m.r.) spectra were routinely recorded on a Perkin Elmer R10 60 MHz. instrument using deuterochloroform as solvent and tetramethylsilane as internal standard. 100 MHz. spectra were recorded on a Varian HA-100D instrument at the Physico-Chemical Measurements Unit (P.C.M.U.) Harwell, or on a Varian HA-100 instrument at Manchester University.

Unless otherwise stated mass spectra were recorded on a Hitachi Perkin Elmer RMU-6 instrument. Accurate mass determinations were carried out on an A.E.I. MS 902 instrument at the P.C.M.U., Harwell.

The optical rotatory dispersion (O.R.D.) and circular dichroism (C.D.) curves of azadirachtin were determined on a Polarmatic 62 instrument at the Milstead Laboratory of Chemical Enzymology, Sittingbourne.

B.S.A. refers to the silylation reagent bis(trimethylsilyl)-acetamide.

Light petroleum refers to the light petroleum fraction, b.p. 60-80°.

Floridin Earth refers to Floridin Earth XXS supplied by B.D.H. Ltd.

Chromatography

Chloroform, toluene, and ether used in column chromatography were dried over calcium chloride. Acetone used for column chromatography was dried over calcium sulphate.

Analytical thin layer chromatography (t.l.c.) was carried out on 5 x 20 cm. and 20 x 20 cm. glass plates coated with a 0.25 mm. layer of Kieselgel PF_{254} (Merck). The solvent was allowed to run 10 cm. or 15 cm. up the plate. Components were visualised under ultraviolet light and by exposure to iodine vapour.

Preparative thin layer chromatography was carried out on 20 x 20 cm. glass plates coated with a 0.25 mm. layer of Kieselgel PF_{254} (Merck). The separated components, visualised by ultraviolet light or by exposure of a thin strip of the plate to iodine vapour, were isolated by scraping off the silica and eluting in a column

with redistilled acetone. The acetone was removed and the residue, which contained some silica, was dissolved in chloroform, filtered and the solvent evaporated.

Preparative thick layer chromatography (P.L.C.) was carried out on 20 x 20 cm. and 40 x 20 cm. glass plates coated with a 2.5 m.m. layer of Kieselgel PF_{254} (Merck). The separated components were visualised and isolated as above.

Feeding Tests

Tests for anti-feeding activity were routinely performed using nymphs of the desert locust Schistocerca gregaria Forsk. on the fifth day of the fifth instar. The hoppers were housed at a temperature of 25-30° and were normally provided with a diet of fresh grass and bran. However all food was removed from the cages for 18 hours before the start of a feeding test.

Feeding test papers (Whatman No. 1, 5.5 cm.) were prepared by immersing in a chloroform solution of the substance under test, drying, spraying with 10% aqueous sucrose solution, and drying again. The papers were weighed, and then dampened with water (approximately 0.15 ml.) just before presentation to the insects.

The nymphs were separated into groups of five in 2 lb. confectionary jars, each of which contained a metal climbing frame. The jars were arranged around a 100 watt electric bulb which was lit for 15 continuous hours per day. A pair of test papers, fixed

upright in a cork by means of paper clips, was placed in each jar. One pair of papers which had been impregnated with sucrose solution only was presented to one of the groups of nymphs as a control. After 24 hours all the papers were removed and weighed.

The test was considered valid if 100 mg. or more of the pair of control papers had been eaten. The weight eaten from a pair of test papers was expressed as a percentage of the weight of control papers consumed. The figure so calculated was subtracted from one hundred to give the percentage anti-feeding activity of the test substance at the particular concentration concerned.

Tests for Anti-feeding Activity on Azadirachtin and some Derivatives

(i) Azadirachtin

Concentration of test solution (mg./l.)	Activity
5	100%
0.5	100%
0.05	100%
0.005	95%

(ii) Dihydro-azadirachtin

Dihydro-azadirachtin was prepared[12] by hydrogenation of azadirachtin over Adam's catalyst.

Concentration of test solution (mg./l.)	Activity
5	100%
0.5	100%
0.05	99%
0.005	75%

(iii) Deacetyl -azadirachtin

Deacetyl-azadirachtin was prepared[12] by mild alkaline hydrolysis of azadirachtin.

Concentration of test solution (mg./l.)	Activity
5	100%
0.5	100%
0.05	98%
0.005	40%

(iv) Acetyl-azadirachtin

Acetyl-azadirachtin was prepared[12] by the treatment of azadirachtin with boiling acetic anhydride.

Concentration of test solution (mg./l.)	Activity
50	100%
5	100%
0.5	75%
0.05	97%
0.005	64%

(v) Bis(trimethylsilyl)-azadirachtin

Bis(trimethylsilyl)-azadirachtin[12] was prepared by the treatment of azadirachtin in dimethylformamide with B.S.A. for two hours at room temperature.

Concentration of test solution (mg./l.)	Activity
500	98%
50	63%
5	64%
0.5	60%
0.05	18%
0.005	11%

(vi) Acetyl-trimethylsilyl-azadirachtin

The procedure for the preparation of acetyl-trimethylsilyl-azadirachtin by the treatment of acetyl-azadirachtin with B.S.A. is described later in the Experimental section.

Concentration of test solution (mg./l.)	Activity
500	100%
50	92%
5	59%
0.5	20%
0.05	zero

(vii) A-trimethylsilyl-azadirachtin

A-trimethylsilyl-azadirachtin was prepared as described later in the Experimental section.

Concentration of test solution (mg./l.)	Activity
500	100%
50	100%
5	97%
0.5	45%
0.05	zero
0.005	25%

(viii) B-trimethylsilyl-azadirachtin

B-trimethylsilyl-azadirachtin was prepared as described later in the Experimental section.

Concentation of test solution (mg./l.)	Activity
500	100%
50	100%
5	97%
0.5	66%
0.05	25%
0.005	zero

A Test for the Possible Anti-feeding Activity of Azadirachtin on the African Migratory Locust

A feeding test was carried out using groups of nymphs of the african migratory locust Locusta migratoria migratorioides on the fourth day of the fifth instar under conditions otherwise identical to those described previously. The following results were obtained for azadirachtin:

Concentration of test solution (mg./l.)	Activity
500	74%
50	32%
5	95%
0.5	41%
0.05	zero
0.005	zero
0.0005	zero

Revised Procedure for the Isolation of Azadirachtin

Indian neem seeds (2 Kg.) (T.P.I., 1970, 71), were ground with 95% ethanol (3 l.) in a food mixer. The mascerate was filtered, the solid material was returned to the mixer, ground again with 95% ethanol (2 l.) and the mascerate filtered. The ethanol was evaporated from the combined extracts to leave a dark brown oily residue (254 g.) which was dissolved in aqueous methanol (5:95, 1300 ml.) and washed twice with light petroleum (1300 ml., 200 ml.).

The light petroleum extracts were combined and washed with aqueous methanol (5:95, 150 ml.) and the combined aqueous methanol extracts were evaporated to leave a brown sticky gum (142 g.).

The gum was dissolved in hot methanol (150 ml.) and Floridin Earth (270 g.) was added with shaking. The solvent was removed from the slurry under reduced pressure and the resulting free-flowing powder was added to the top of a column of Floridin Earth (1 Kg.) made up in toluene. The column was eluted successively with toluene (1800 ml.), toluene-ether (1:1, 1 l.), toluene-ether (1:3, 1 l.), ether (2 l.) and ether containing the following percentages by volume of acetone: 1% (1 l.), 1.5% (1 l.), 2.5% (1 l.), 3.5% (1 l.) and 4% (2.5 l.). The fractions (250 ml. each) eluted by the ether-acetone mixtures were investigated by n.m.r. spectroscopy, and those which contained azadirachtin were combined to give a residue (3.2 g.) which was purified by P.L.C. eluting twice with ether-acetone (85:15) to give azadirachtin (1.2 g.) m.p. 151-156°, running as a single spot R_F 0.50 on t.l.c. eluting with ether-acetone (4:1).

Preliminary Extraction of Neem Leaves

Dry Neem Leaves (5 g.) were crushed under 95% ethanol (100 ml.) in a mortar. After filtration the solid was returned to the mortar, crushed with a further quantity of ethanol (50 ml.), and again filtered. The combined filtrates were evaporated under vacuum to

leave a dark green oil (592 mg.) which was subjected to a routine feeding test.

The following results were obtained:

Concentration of test solution (g./l.)	Activity
10.0	100%
1.0	84%
0.1	68%

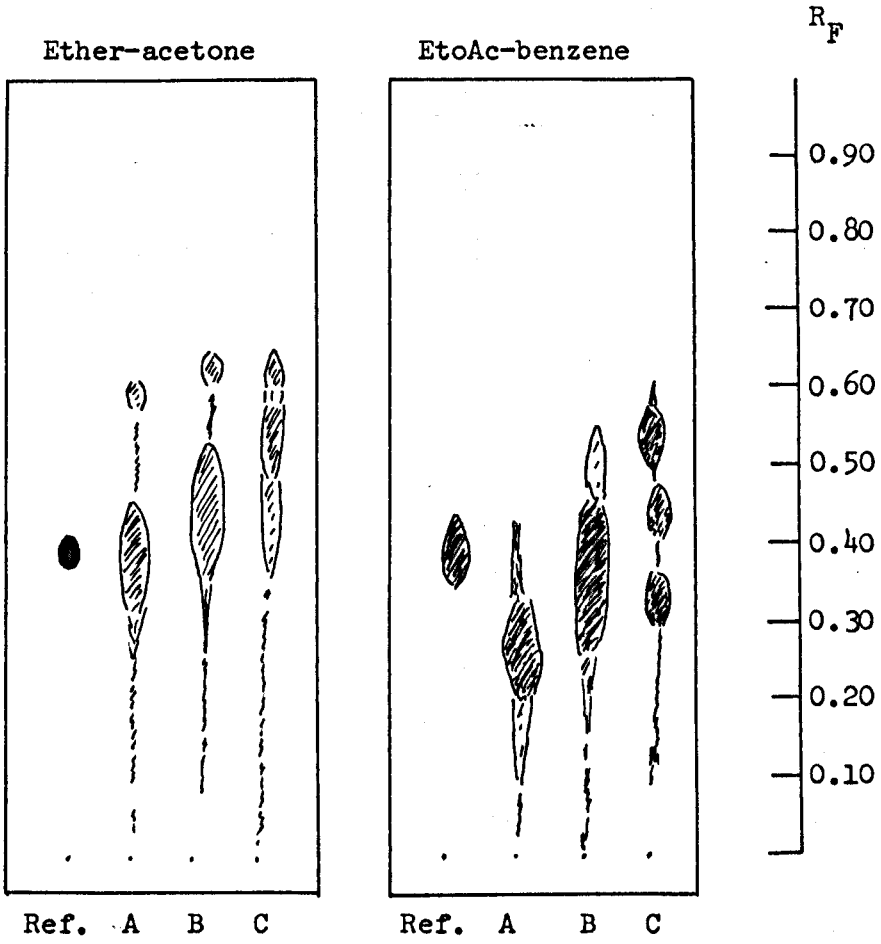
An Investigation of Neem Leaves as a Possible Source of Azadirachtin

Dry leaves (1 Kg.) of neem (Azadirachta indica) obtained from Pakistan were broken up by hand and ground in a Waring blender with 95% ethanol (2 l.). After filtration the remaining solid was returned to the blender and ground again with more ethanol (2 l.). The ethanol was evaporated from the combined filtered extracts to leave a dark green sticky gum (114 g.) which was dissolved in methanol (350 ml.). Floridin Earth (200 g.) was added with shaking and the solvent was removed from the resulting slurry by pumping at reduced pressure (0.1 mm.). The free-flowing powder obtained was added to the top of a column of Floridin Earth (1 Kg.) made up in toluene and the column was eluted successively with toluene (1 l.), toluene-ether mixtures (1:1, 1 l. and 1:3, 1.5 l.), ether (3 l.) and ether-acetone mixtures containing the following percentages by volume of acetone: 1% (0.5 l.), 2% (0.5 l.), 5% (4.5 l.), 8% (1 l.), 10% (0.5 l.) and 12% (0.5 l.).

Each fraction (250 ml.) was examined on t.l.c. eluting with ether–acetone (85:15) or chloroform–acetone (7:3). The fractions eluted from the column by ether containing 2% and 5% acetone were shown to include material which flowed alongside a reference spot of azadirachtin. These fractions were combined to give a green amorphous powder (1.54 g.) which was subjected to P.L.C. eluting twice with ether–acetone (92:8). Seven separate bands were visualised and the materials isolated from the three bands with R_F close to that of azadirachtin were designated A (93 mg., R_F 0.38), B (111 mg., R_F 0.47), and C (79 mg. R_F 0.53).

Further investigation on t.l.c. eluting with ether–acetone (85:15) or ethyl acetate–benzene (4:1) showed that A, B and C were all inhomogeneous as shown in the diagram below.

The n.m.r. spectrum of each of these substances indicated that azadirachtin was not present as a major component.



In each case the reference material was authentic azadirachtin.

The three materials were subjected to a feeding test and the following results were obtained:

Sample	Concentration of test solution (mg./l.)	Activity
	50	100%
A	5	99%
	0.5	zero
	50	100%
B	5	81%
	0.5	63%
	50	100%
C	5	81%
	0.5	zero

Attempted Isolation of Meliantriol[9] from Neem Seeds

Neem seeds (1 Kg.) were ground with chloroform (2 l.) in a Waring blender. After filtration the remaining solid was returned to the blender and reground with a further quantity of chloroform (1 l.). After standing at room temperature for 24 hours the mixture was filtered and the solid again returned to the blender, ground with chloroform, and allowed to stand for a further two days. After filtration all the extracts were combined and the chloroform evaporated to leave a greenish-brown oil (266 g.), which was taken up in light petroleum (400 ml.) and the solution was extracted four times with nitromethane (200 ml., 2 x 100 ml., 50 ml.). Evaporation of the combined nitromethane extracts left a brown residue (32 g.). A portion (16 g.) of this residue was dissolved in a small volume of benzene and applied to a column of Florisil (400 g.) made up in hexane.

The column was eluted successively with benzene (500 ml.), hexane-ether mixtures (9:1, 500 ml.; 3:1, 500 ml.; 3:2, 500 ml. and 1:3, 500 ml.), ether (2 l.) and chloroform-acetone mixtures containing the following percentages by volume of acetone: 10% (2.75 l.), 15% (500 ml.), 20% (250 ml.), 30% (250 ml.), 40% (500 ml.), 60% (500 ml.), 80% (250 ml.) and 90% (500 ml.).

The fractions (250 ml. each) eluted from the column by chloroform-acetone mixtures containing 10% to 60% acetone all contained material which ran on t.l.c. in the R_F region quoted[9]

for meliantriol (R_F 0.33 in chloroform-acetone 7:3). These fractions were combined to give a residue (2.5 g.) which was dissolved in a small volume of chloroform and applied to a column of Kieselgel H (85 g.) made up in chloroform. The column was eluted with chloroform (2.5 l.) and chloroform-acetone mixtures containing amounts of acetone increasing steadily from 2% to 55% by volume (total amount 1.5 l.).

All the fractions (100 ml. each) were examined by t.l.c. but none contained a material with all the running properties quoted[52] for meliantriol: R_F 0.33 in chloroform acetone (7:3), 0.47 in ethyl acetate-benzene (4:1), and 0.62 in ethyl acetate. Attempts to recognise meliantriol in any of the fractions by using n.m.r. spectroscopy were unsuccessful, though azadirachtin (total weight approx. 0.6 g.) was present in several of the fractions which were eluted from the column by chloroform.

Selected fractions were further purified by P.L.C. eluting with chloroform-acetone (7:3). The materials from all the bands isolated were screened by t.l.c. and n.m.r. spectroscopy but meliantriol was not found.

Several selected fractions and materials from P.L.C. bands were dissolved in acetone, and pentane was added to turbidity. In no case was meliantriol obtained as a precipitate.

Preparation of the Lactone from the Alkaline Hydrolysis of Dihydro-azadirachtin

The hydrolysis of dihydro-azadirachtin was carried out with 2½% aqueous potassium hydroxide as described by Butterworth[12]. The ethyl acetate solution obtained after acidification and extraction, was left to stand overnight at 0° before removal of solvent and re-esterification with ethereal diazomethane. This change in Butterworth's basic procedure did not result in an increased yield of the lactone.

As described by Butterworth[79], a pure sample of the lactone was not obtained when the crude product from the alkaline hydrolysis of dihydro-azadirachtin was subjected to P.L.C. eluting twice with ether-acetone (4:1). A further purification by P.L.C. eluting twice with chloroform-acetone (7:3) resulted in a homogeneous sample of the lactone, R_F 0.30 in ether-acetone (4:1) and 0.41 in chloroform-acetone (7:3). ν_{max} ($CHCl_3$) 3400 (broad), 1725 (strong) and 1650 $cm.^{-1}$ (weak). The n.m.r. spectrum is shown in Fig. 4. The mass spectrum showed prominent ions above $^m/e$ 550 at $^m/e$ 648 (16), 630 (100), 616 (50), 605 (83), 598 (33), 589 (34), 588 (70), 587 (70), 573 (33), 571 (32), and 553 (40). (% abundance relative to $^m/e$ 630.)

Preparation of the Bis(trimethylsilyl)-ether Derivative of the Lactone from the Alkaline Hydrolysis of Dihydro-azadirachtin

The lactone (3 mg.) in dimethylformamide (1 ml.) was treated with B.S.A. (1 ml.) for seventy-six hours at room temperature.

Removal of volatile components at reduced pressure (0.1 mm.) left a glassy residue which on t.l.c. eluting with ether–acetone (4:1) showed two spots at R_F 0.49 and 0.75.

The residue was subjected to preparative thin-layer chromatography eluting twice with ether–acetone (92:8). The two resulting bands were scraped off, the silica extracted with dry chloroform, and the chloroform evaporated. The upper band gave the bis(trimethylsilyl)-ether derivative of the lactone (<1 mg.), M^+ 792.3192, $C_{38}H_{56}O_{14}Si_2$ requires 792.3206 (error 2 p.p.m.). The mass spectrum showed other prominent ions above $^m/_e$ 600 at 777 (89), 749 (25), 733 (100), 702 (7), 689 (23), 677 (9), 659 (9), 649 (14), 633 (14), 631 (14), 615 (20) and 602 (10). (% abundance relative to $^m/_e$ 733.)

Attempted Oxidation of Deacetyl-azadirachtin with Cornforth's Reagent[55] (Chromium Trioxide–Pyridine–Water)

Cornforth's reagent was prepared by adding chromium trioxide (1 g.) in water (1 ml.) to pyridine (10 ml.) cooled in ice.

Deacetyl-azadirachtin[12] (65 mg.) was treated with Cornforth's reagent (3 ml.) for 71 hours at room temperature. The solution was extracted with chloroform and the chloroform was washed with water and evaporated to leave a dark brown residue (35 mg.) which on t.l.c. eluting with chloroform–acetone (7:3) showed elongated spots R_F 0.37 and 0.51, and a minor spot corresponding to deacetyl-

azadirachtin at R_F 0.35. The residue was subjected to P.L.C. eluting three times with chloroform-acetone (85:15) but the materials isolated were found to be inhomogeneous.

Preparation of Acetyl-trimethylsilyl-azadirachtin

Acetyl-azadirachtin[12] (140 mg.) in dry chloroform (5 ml.) was treated with B.S.A. (1 ml.) for one hour at room temperature. Removal of volatile components under reduced pressure (0.1 mm.) left a yellow solid (250 mg.) which on t.l.c. eluting with ether-acetone (4:1) showed a major spot R_F 0.55 with a weak spot R_F 0.28 corresponding to acetyl-azadirachtin.

The residue was subjected to P.L.C. eluting twice with ether-acetone (91:9). The major band was scraped off and the silica extracted with dry chloroform. Evaporation of the chloroform left a colourless amorphous powder acetyl-trimethylsilyl-azadirachtin (73 mg.), m.p. 131-135°, R_F 0.55 in ether-acetone (4:1). ν_{max} (CCl_4) 3500 (broad), 1745 (strong), 1652 (weak) and 1615 $cm.^{-1}$ (weak). The n.m.r. spectrum is shown in Fig. 10. The mass spectrum showed prominent ions above $^m/e$ 600 at $^m/e$ 834 (4), 775 (4), 760 (2), 745 (3), 744 (2), 727 (3), 715 (5), 685 (27), 661 (100), 647 (6), 643 (6), 633 (3), 625 (14) and 602 (42). (% abundance relative to $^m/e$ 661.)

An accurate mass measurement on the molecular ion gave 834.3134, $C_{40}H_{54}O_{17}Si$ requires 834.3114 (error 2.5 p.p.m.).

Preparation of Two Mono(trimethylsilyl)-ether Derivatives of Azadirachtin

Azadirachtin (270 mg.) in chloroform (1.5 ml.) was treated with B.S.A. (1.5 ml.) for 19 hours at room temperature. Removal of the volatile components at reduced pressure left a yellow solid (280 mg.) which on t.l.c. eluting with ether-acetone (9:1) showed major spots R_F 0.41 and 0.51, with minor components at R_F 0.26 corresponding to azadirachtin and 0.69 corresponding to bis(trimethylsilyl)-azadirachtin[12].

The residue was subjected to P.L.C. eluting twice with ether-acetone (49:1). The two major bands were scraped off, the silica was extracted with chloroform, and the chloroform evaporated.

From the upper major band was isolated B-trimethylsilyl-azadirachtin as a white solid (63 mg.), m.p. 135-138°, R_F 0.51 in ether-acetone (9:1). ν_{max} 3560 (sharp), 3470 (broad), 1730 (strong), 1710 (shoulder), 1650 (weak) and 1610 $cm.^{-1}$ (weak). The n.m.r. spectrum is shown in Fig. 13. The mass spectrum showed significant peaks above $^m/e$ 650 at $^m/e$ 792 (25), 732 (75), 700 (81), and 669 (100). (% abundance relative to $^m/e$ 669)

From the lower major band was isolated a solid (43 mg.) which was further purified by preparative t.l.c. eluting twice with ether-acetone (9:1) to give A-trimethylsilyl-azadirachtin (36 mg.), m.p. 141-145°, R_F 0.41 in ether-acetone (9:1). ν_{max} 3520 (shoulder), 3440 (broad), 1740 (strong), 1710 (strong),

1650 (weak) and 1610 cm.$^{-1}$ (weak). The n.m.r. spectrum is shown in Fig. 15. The mass spectrum did not show peaks above $^{m}/e$ 400.

Attempted Preparation of Completely Silylated Azadirachtin

Azadirachtin was treated under the conditions summarised in the table below. In each case, when the stated reaction time had elapsed, the volatile components were removed under reduced pressure (0.1 mm.) to leave bis(trimethylsilyl)-azadirachtin.

	Reagent	Solvent	Temperature	Time
(i)	B.S.A.	D.M.F.	20°	3 hours
(ii)	B.S.A.	D.M.F.	77°	23 hours
(iii)	Trimethylsilylimidazole	D.M.F.	20°	20 hours
(iv)	B.S.A. + Trimethylsilylimidazole + Trimethylchlorosilane, (3:3:2)	Pyridine	20°	47 hours

Preparation of Tris(trimethylsilyl)-azadirachtin

A mixture of azadirachtin (205 mg.), pyridine (1.2 ml.), B.S.A. (1 ml.), trimethylsilylimidazole (1 ml.), and trimethylchlorosilane (0.66 ml.) was heated at 60° for 90 hours. The volatile components were removed by pumping at 0.1 mm. for one hour at 100° to leave a dark brown oily residue (425 mg.) which on t.l.c. eluting with benzene-ethyl acetate (4:1) showed spots at R_F 0.37 and 0.17.

The residue was subjected to P.L.C. eluting twice with benzene-ethyl acetate (4:1) and the two resulting bands were scraped off. The silica was extracted with chloroform, and the chloroform evaporated. The material (60 mg.) from the lower band was shown to be slightly impure bis(trimethylsilyl)-azadirachtin by n.m.r. spectroscopy and comparative t.l.c.

The material (51 mg.) from the upper band was further purified by preparative t.l.c. eluting twice with benzene-acetonitrile (92:8). The major band was worked up as above to give a white amorphous solid tris(trimethylsilyl)-azadirachtin (33 mg.), m.p. 151-153°, R_F 0.37 in benzene-ethyl acetate (4:1), 0.38 in benzene-acetonitrile (92:8), 0.73 in ether-acetone (99:1) and 0.50 in chloroform-light petroleum-acetonitrile (1:3:1). ν_{max} (CCl_4) 1745 (strong), 1710 (strong), 1650 (weak) and 1615 $cm.^{-1}$ (weak). The n.m.r. spectrum is shown in Fig. 17. The mass spectrum showed prominent ions above $^m/e$ 700 at $^m/e$ 936 (7), 921 (2), 893 (2), 877 (50), 846 (2), 831 (1), 821 (1), 803 (4), 789 (8), 787 (3), 763 (75), 749 (9), 721 (6) and 705 (100). (% abundance relative to $^m/e$ 705.)

An accurate mass measurement on the molecular ion gave 936.3798, $C_{44}H_{68}O_{16}Si_3$ requires 936.3816 (error 2 p.p.m.). An accurate mass measurement on the ion at $^m/e$ 877 gave 877.3688, $C_{42}H_{65}O_{14}Si_3$ requires 877.3682 (error 1 p.p.m.).

Attempted Oxidation of Azadirachtin with Oxygen over Platinum

(i) In ethyl acetate:

Adam's catalyst (13 mg.) suspended in ethyl acetate (180 ml.) was shaken with hydrogen at 30 p.s.i. for 30 minutes. The suspension was flushed with nitrogen and azadirachtin (29 mg.) in ethyl acetate (20 ml.) was added. The mixture was shaken with oxygen at 20 p.s.i. for 5 hours at 40° and for a further 20 hours at room temperature. Filtration, and evaporation of the solvent yielded unchanged azadirachtin (28 mg.).

(ii) In acetone:

Hydrogen was bubbled through a suspension of Adam's catalyst (58 mg.) in freshly distilled acetone (50 ml.) for one hour. The suspension was flushed with nitrogen and azadirachtin (19 mg.) was added. Oxygen was continuously bubbled through the reaction mixture which was stirred and heated at reflux for 12 hours and stirred at room temperature for a further 34 hours. Filtration, and evaporation of the solvent left unchanged azadirachtin (18 mg.).

Attempted Oxidation of Azadirachtin with Chromium^{VI} Reagents

(i) A cooled slurry of potassium dichromate (360 mg.) in glacial acetic acid (20 ml.) was added to a cooled solution of azadirachtin (350 mg.) in dry benzene (40 ml.) and the resulting mixture was kept at 8° for 20 hours. The mixture was diluted with water and extracted with chloroform. The chloroform was washed with 5%

aqueous sodium carbonate solution, twice washed with water, and evaporated to leave a residue (241 mg.) which on t.l.c. eluting with ether–acetone (95:15) showed a major spot at R_F 0.43 corresponding to starting material, with minor staining above and below this position.

The residue was subjected to P.L.C. using the same eluant and azadirachtin (70 mg.) was recovered from the major band. The materials isolated from the minor bands all proved to be inhomogeneous.

When a similar reaction was carried out at room temperature for 200 minutes and the product was worked up as before, a residue was obtained which contained no azadirachtin. The material proved to be a complex mixture of a large number of components which could not be separated on P.L.C. eluting with ether–acetone (95:15).

(ii)[76] To a solution of azadirachtin (19 mg.) in glacial acetic acid (2 ml.) was added drop-wise with stirring a solution of chromium trioxide in glacial acetic acid at a concentration of 5 mg./ml. Aliquots of the reaction mixture were removed in turn after the addition of 1 ml., 3 ml., 4 ml., 5 ml. and 7 ml. of the reagent. Each aliquot was diluted with water, extracted with chloroform, and the chloroform was washed with water and evaporated. Each sample was investigated by t.l.c. eluting with ether–acetone (85:15) or chloroform–acetone (7:3). It was found that the starting material had all reacted before removal of the third aliquot

and the product was a complex mixture of inseparable materials.

A similar procedure using dihydro-azadirachtin as the starting material gave a parallel result.

(iii) Concentrated sulphuric acid (3.2 ml.) was added to a solution of sodium dichromate (2.38 g.) in glacial acetic acid (1 ml.) and water (10 ml.). A portion (2 ml.) of this oxidising reagent was carefully added to a two-phase mixture of azadirachtin (332 mg.) in benzene (200 ml.) and water (40 ml.). The mixture was gently stirred at room temperature for 17 hours and left at -10° for a further 29 hours. The organic layer was isolated and chloroform (150 ml.) was added. The solution was washed with dilute aqueous sodium carbonate solution followed by water, and the solvent was evaporated to leave a yellow solid (100 mg.) which on t.l.c. eluting with chloroform-acetone (7:3) showed components at R_F 0.27, 0.42 and 0.55 with considerable streaking in between.

The residue was subjected to P.L.C. eluting with chloroform-acetone (4:1) and seven bands were visualised. Material was isolated from the seven bands but in no case was a pure product obtained.

Attempted Oppenhauer Oxidation of Azadirachtin

Cyclohexanone (2 ml., dried over calcium chloride) was added to toluene (30 ml., dried over calcium chloride and distilled) and a portion (5 ml.) of the mixture was distilled to remove traces of water. To the remainder was added azadirachtin (21 mg.) and

aluminium isopropoxide (76 mg.) and the reaction mixture was heated for one hour at reflux. Chloroform (50 ml.) was added and the solution was washed once with dilute aqueous acetic acid and several times with water. Evaporation of the solvent left a yellow gum (15 mg.) which on t.l.c. eluting with ether-acetone (85:15) or chloroform-acetone (7:3) proved to be a complex mixture of a large number of components running as a streak from the origin almost to the solvent front.

Treatment of Azadirachtin with Lead Tetraacetate

Lead tetraacetate (1 g.), dampened with acetic acid, was added to a solution of azadirachtin (208 mg.) in dry benzene (20 ml.) and the resulting slurry was stirred in the dark at room temperature for 16 hours. Chloroform (25 ml.) and water (30 ml.) were added and stirring was continued for a further 30 minutes. The mixture was filtered through Hyflo Supercel and the organic layer was washed with dilute aqueous sodium carbonate solution, water, and evaporated. The residue (150 mg.) on t.l.c. eluting with chloforom-acetone (7:3) showed a spot R_F 0.60 with slight streaking below.

P.L.C. of the residue eluting twice with chloroform-acetone (85:15) showed one major band from which was isolated a white solid I (46 mg.) m.p. 162-166°, R_F 0.60 in chloroform-acetone (7:3) and 0.42 in ether-acetone (85:15). ν_{max} (CCl_4) 3600 (sharp), 3480 (broad), 1740 (strong), 1720 (shoulder), and 1650 $cm.^{-1}$ (weak). The n.m.r.

spectrum is shown in Fig. 20. The mass spectrum showed weak ions above $^{m}/e$ 600 at 720 (12), 702 (12), 688 (32), 660 (100), 642 (34) and 632 (24). (% abundance relative to $^{m}/e$ 660.)

Treatment of Dihydro-azadirachtin with Lead Tetraacetate

Dihydro-azadirachtin (5 mg.) in dry benzene (1 ml.) was stirred in the dark with lead tetraacetate (100 mg., moist with acetic acid) for 17 hours at room temperature. Work up as above yielded unchanged dihydro-azadirachtin (3 mg.) as shown by comparative t.l.c. in three different solvent systems.

Silylation of the Material I

A sample (250 mg.) of crude material I from the treatment of azadirachtin with lead tetraacetate was dissolved in dimethylformamide (9 ml.), and B.S.A. (4 ml.) was added. After 20 hours at room temperature the volatile components were removed by pumping at 0.1 mm. to leave a residue (301 mg.) which on t.l.c. eluting with ether-acetone (99:1) showed a major component at R_F 0.48 and a minor component at R_F 0.67.

The crude product was subjected to P.L.C. eluting with ether-acetone (99:1) and from the major band was isolated the material II, as a white powder (131 mg.), m.p. 158-162°, which ran as a single spot on t.l.c. in ether-acetone (99:1, R_F 0.48), chloroform-acetone (24:1, R_F 0.56), chloroform-methanol (49:1,

R_F 0.66), ethyl acetate-benzene (7:3, R_F 0.56), and ether-acetonitrile (49:1, R_F 0.44). ν_{max} (CCl_4) 3600 (sharp), 1730 (strong), and 1650 $cm.^{-1}$ (weak). The n.m.r. spectrum is shown in Fig. 21. In the mass spectrum, which showed no molecular ion, prominent peaks above $^m/e$ 600 appeared at $^m/e$ 864 (1), 849 (1), 838 (1), 832 (1), 812 (2), 806 (1), 791 (100), 773 (4), 763 (7), 731 (50), 703 (10), 675 (6), 641 (8) and 601 (6). (% abundance relative to $^m/e$ 791.)

Treatment of Azadirachtin with Lead Tetrabenzoate

Lead tetrabenzoate was prepared from lead tetraacetate and benzoic acid by the method of R. Criegee et al.[84] m.p. 169°. (Lit., m.p. 176°[85] and 164°[84]).

Lead tetrabenzoate (1 g.) was added to azadirachtin (262 mg.) in dry benzene (15 ml.) and the resulting slurry was stirred for 20 hours in the dark at room temperature. Chloroform (20 ml.) and dilute aqueous sodium carbonate solution were added with stirring and after 5 minutes the mixture was filtered through Hyflo Supercel. The organic layer was washed with water and evaporated to leave a colourless powder (235 mg.) which on t.l.c. eluting with chloroform-acetone (85:15) showed major and minor components at R_F 0.60 and 0.25 respectively. A reference spot of azadirachtin ran to R_F 0.33.

The residue was subjected to P.L.C. eluting twice with chloroform-acetone (92:8) and from the major band was isolated the

material III, a dibenzoate adduct of azadirachtin as a colourless solid (103 mg.) m.p. 162-164°. ν_{max} (CCl_4) 3450 (broad), 1730 (strong), and 1650 $cm.^{-1}$ (weak). The n.m.r. spectrum is shown in Fig. 22. The mass spectrum showed no ions above $^{m}/e$ 186.

The product III ran as a single spot on t.l.c. in the following solvent systems: chloroform-ethyl acetate (3:2, R_F 0.39), ethyl acetate-benzene (7:3, R_F 0.47 and 85:15, R_F 0.70) and chloroform-methanol (19:1, R_F 0.78). However t.l.c. eluting twice with ether-acetonitrile (97:3) showed two components at R_F 0.52 and 0.56 but these could not be adequately separated on P.L.C. or preparative t.l.c.

Silylation of the Dibenzoate Adduct III

A sample (238 mg.) of crude dibenzoate adduct III was dissolved in dimethylformamide (8 ml.) and B.S.A. (4 ml.) was added. After 20 hours at room temperature the volatile components were removed under reduced pressure (0.1 mm.) to leave a yellow residue (257 mg.) which on t.l.c. eluting with chloroform-acetone (49:1) showed a single spot R_F 0.60 with slight streaking below. (Dibenzoate adduct III R_F 0.25)

The product was subjected to P.L.C. eluting three times with ethyl acetate-benzene (15:85) and from the major band was isolated a silylated material IV as a white solid (111 mg.), m.p. 151-154°, ν_{max} (CCl_4) 3600 (weak), 1730 (strong), and 1650 $cm.^{-1}$ (weak). The n.m.r. spectrum which is shown in Fig. 23 suggested

that IV was a mixture.

Material IV ran as a single spot on t.l.c. in the following solvent systems: ether–acetone (99:1, R_F 0.57), chloroform–acetonitrile (49:1, R_F 0.40), ether–acetonitrile–benzene (11:1:8, R_F 0.48) and benzene–acetonitrile (9:1, R_F 0.55 after two elutions). However two elutions with chloroform–light petroleum–acetonitrile (1:3:1) showed two major components IVa (R_F 0.49) and IVb (R_F 0.42), and a minor component IVc (R_F 0.36).

Separation of such close running components was not practicable by P.L.C. but when a sample (13 mg.) of material IV was subjected to preparative t.l.c. the three fully separated components were isolated.

The mass spectrum of material IVa (4 mg.), the bis(trimethylsilyl)-ether derivative of the dibenzoate adduct III showed prominent ions above $^m/_e$ 900 at $^m/_e$ 1106 (1), 1047 (4), 1029 (2), 984 (6), 969 (2), 952 (4), 941 (2), 925 (100) and 907 (4). (% abundance relative to $^m/_e$ 925.) Accurate mass determination was not possible on the weak molecular ion at $^m/_e$ 1106 but the peak at $^m/_e$ 1047, derived by loss of a carboxymethyl radical from the molecular ion, gave 1047.3895, $C_{53}H_{67}O_{18}Si_2$ requires 1047.3865 (error 3 p.p.m.).

In the mass spectrum of material IVb (3 mg.), a mono(trimethylsilyl)-ether derivative of material III, the peak at highest $^m/_e$ was at $^m/_e$ 853. The accurate mass of this ion was 853.3103, corresponding to the composition $C_{43}H_{53}O_{16}Si$ (calculated mass

853.3102) which suggested that it was formed by loss of one molecule of benzoic acid and a carboxymethyl radical from the unseen molecular ion.

Accurate mass determination on the peak at highest $^{m}/e$ in the mass spectrum of IVc (2 mg.) gave 853.3103 which indicated that this material was isomeric with IVb.

Attempted Reduction of Azadirachtin with Hydriodic Acid

Azadirachtin (100 mg.) in glacial acetic acid (8 ml.) was heated at reflux for 2 hours with hydriodic acid (55%, specific gravity 1.7, 5 ml.) in the presence of red phosphorous (50 mg.). The reaction mixture was filtered into boiling water (60 ml.) containing sodium bisulphite. The resulting yellow suspension was filtered through Hyflo Supercel in a buchner funnel and the filtrate was extracted four times with chloroform (40 ml., 30 ml., 20 ml. and 10 ml.). The chloroform was washed with water and evaporated to leave a brown residue (49 mg.) which was only slightly soluble in a small volume of chloroform, insoluble in carbon tetrachloride, but readily soluble in acetone. On t.l.c. eluting with ether–acetone (1:4) the material showed a diffuse stain in the region R_F 0.24 only when the plate was exposed to iodine vapour. The residue was subjected to P.L.C. eluting with ether–acetone (1:4) but proved to be a complex mixture of materials appearing in the region R_F 0.15 to R_F 0.62.

Attempted Reduction of Azadirachtin with Chromous Salts

All experiments with chromous salts were carried out in a glove-box under an atmosphere of nitrogen.

(i) With chromous chloride reagent:-

Excess zinc metal (approx. 8 g.) was added to a solution of chromic chloride (4 g.) in water (5 ml.) and concentrated hydrochloric acid (9.5 ml.). When reduction of the green chromic salt to the blue chromous salt was complete and the evolution of hydrogen had ceased, the mixture was filtered and an aliquot (5 ml.) of the filtrate was added to a solution of azadirachtin (290 mg.) in acetone (5 ml.) and glacial acetic acid (5 ml.). The reaction mixture was shaken overnight at room temperature. The acetone was removed under reduced pressure, water (100 ml.) was added, and the mixture extracted four times with chloroform (2 x 50 ml., 40 ml., 20 ml.). The combined chloroform extracts were washed with 5% aqueous sodium bicarbonate solution and with water. Evaporation of the chloroform left a residue (163 mg.) which on t.l.c. eluting with ether-acetone (7:3) proved to be a mixture of a large number of inseparable components. The bulk of the material showed as a streak from R_F 0.15 to R_F 0.50 and there was no starting material evident at R_F 0.55. The crude mixture was subjected to P.L.C. eluting with ether-acetone (7:3) but no pure products were isolated.

(ii) With chromous acetate:

Excess zinc metal (approx. 8 g.) was added to a solution of

chromic chloride (4 g.) in water (5 ml.) and concentrated hydrochloric acid (9.2 ml.). When evolution of hydrogen had ceased the reaction mixture was filtered. To the filtrate was added a solution of sodium acetate (11 g.) in water (14.2 ml.) and the resulting mixture was stirred until a red precipitate of chromous acetate was formed. The mixture was filtered and the chromous acetate was washed with a little water, 95% ethanol, and ether and allowed to dry.

Chromous acetate (approx. 1 g.) was added to azadirachtin (20 mg.) in acetone (4 ml.) and glacial acetic acid (4 ml.) and the mixture was shaken overnight at room temperature. The acetone was removed under reduced pressure, water was added, and the insoluble chromous salt was allowed to undergo atmospheric oxidation to the soluble chromic salt. The solution was extracted with chloroform and the chloroform was washed with 5% aqueous sodium bicarbonate solution and with water.

The chloroform was evaporated and the residue (17 mg.) on t.l.c. eluting with ether-acetone (7:3) showed a spot at R_F 0.55 corresponding to starting material and a spot at R_F 0.78 corresponding to an impurity in the reagent.

Attempted Reduction of Dihydro-azadirachtin with Chromous Salts

The chromous salt reagents were prepared as described above.

(i) With chromous chloride reagent:

An aliquot (5 ml.) of chromous chloride reagent was added to a solution of dihydro-azadirachtin (180 mg.) in acetone (5 ml.) and glacial acetic acid (5 ml.) and the mixture was shaken overnight at room temperature. The acetone was removed under reduced pressure, the mixture was extracted with chloroform, and the chloroform was washed with 5% aqueous sodium bicarbonate solution and with water. The solvent was evaporated to leave a residue (100 mg.) which on t.l.c. eluting with ether–acetonitrile (85:15) proved to be a complex mixture of products running as a streak on the plate from the origin to R_F 0.40. The material was subjected to P.L.C. eluting with ether–acetonitrile (9:1) but no pure products were isolated.

(ii) With chromous acetate:

Chromous acetate (approx. 1 g.) was added to a solution of dihydro-azadirachtin (68 mg.) in acetone (5 ml.) and glacial acetic acid (5 ml.). The mixture was shaken overnight at room temperature. The acetone was removed under reduced pressure, water was added, and the insoluble chromous salt was allowed to undergo oxidation by the atmosphere to the soluble chromic salt. The solution was extracted with chloroform and the chloroform was washed with 5% aqueous sodium bicarbonate solution and with water. Evaporation of the solvent left a residue (51 mg.) which on t.l.c. eluting with ether–acetonitrile (85:15) showed spots at R_F 0.27 corresponding

to starting material and R_F 0.82 corresponding to an impurity in the reagent. The residue was subjected to P.L.C, eluting with ether-acetonitrile (85:15) and dihydro-azadirachtin (25 mg.) was recovered.

Attempted Reduction of Dihydro-azadirachtin via a Possible Iodohydrin

To a solution of dihydro-azadirachtin (100 mg.) in glacial acetic acid (15 ml.) was added sodium acetate (286 mg.), sodium iodide (752 mg.), and zinc dust (287 mg.). The reaction mixture was stirred overnight at room temperature. Water was added to the brown reaction mixture which was then extracted with chloroform. The chloroform was washed with aqueous sodium thiosulphate solution, with aqueous sodium carbonate solution, and with water. Evaporation of the chloroform left a residue (65 mg.) which on t.l.c. eluting with ether-acetone (4:1) showed a spot R_F 0.25 corresponding to dihydro-azadirachtin and a trace of material as a streak from R_F 0.30 to R_F 0.55.

Treatment of Azadirachtin with Glacial Acetic Acid

(i) Dissolution of azadirachtin (4 mg.) in glacial acetic acid (1 ml.) for one hour at room temperature, followed by removal of the solvent at reduced pressure, left azadirachtin (4 mg.) unchanged. However when azadirachtin (3 mg.) was treated with glacial acetic

acid (1 ml.) for 6 days at room temperature, removal of the solvent *in vacuo* left a residue (3 mg.) which on t.l.c. eluting with ether-acetone (4:1) showed heavy streaking from the origin to R_F 0.14 with a complex series of faint stains from R_F 0.23 to 0.40.

(ii) Azadirachtin (2 mg.) was treated with glacial acetic acid (2 ml.) boiling at reflux for 25 minutes. Removal of the solvent *in vacuo* left a residue (2 mg.) which on t.l.c. eluting with ether-acetone (4:1) proved to be a complex mixture of materials running as a streak from the origin to R_F 0.70.

Treatment of Azadirachtin with Formic Acid

(i) A mixture of formic acid (98-100%, 1 ml.) and chloroform (9 ml.) was prepared, and azadirachtin (5 mg.) was treated with a portion (2 ml.) of this mixture for 13 hours at room temperature. The solvent was removed *in vacuo* and the residue (5 mg.) on t.l.c. eluting with chloroform-acetone (7:3) showed a spot R_F 0.40 corresponding to azadirachtin, with a diffuse stain above and heavy streaking to the origin in which no distinct components were recognised.

(ii) Azadirachtin (3 mg.) was treated with formic acid (98-100%, 2 ml.) for 20 minutes at 0°. The solvent was removed under reduced pressure and the residue (3 mg.) on t.l.c. eluting with chloroform-acetone (7:3) showed an intense stain at the origin

and an inseparable mixture of materials running as a streak from the origin to R_F 0.75.

Treatment of Azadirachtin with Propionic Acid

Azadirachtin (2 mg.) was treated with propionic acid (0.5 ml.) for 6 days at room temperature. Evaporation of the solvent *in vacuo* left a residue (2 mg.) which on t.l.c. eluting with chloroform-acetone (7:3) showed spots of approximately equal intensity at R_F 0.52 and R_F 0.40, the latter corresponding to starting material.

The residue was subjected to preparative t.l.c. eluting twice with chloroform-acetone (4:1). The mass spectrum of the material (<1 mg.) isolated from the upper band showed no peaks above $^m/e$ 600. However a significant peak, possibly due to the ion $CH_3CH_2-C\equiv O^+$ from the fragmentation of a propionate ester function, was observed at $^m/e$ 57.

Reduction of Azadirachtin by Lithium Aluminium Hydride

The tetrahydrofuran used in this experiment was dried and purified by heating at reflux over lithium aluminium hydride for several hours followed by distillation.

Azadirachtin (151 mg.) was dissolved in tetrahydrofuran (10 ml.) and excess lithium aluminium hydride (approx. 1 g.) was added. The mixture was heated at reflux for four hours and

stirred overnight at room temperature. Water (25 ml.) was added slowly with stirring and when the excess lithium aluminium hydride had reacted, the mixture was shaken with ethyl acetate. An emulsion formed which did not separate into two layers on addition of sodium chloride. Excess ethanol was added and the resulting mixture was evaporated to dryness. The solid residue was broken up and stirred with hot ethyl acetate. The suspension was filtered and the solid was extracted with hot ethyl acetate a further three times in the same way. The solvent was removed from the combined filtrates to leave a yellow oil (89 mg.) which was sparingly soluble in a small volume of ethyl acetate and readily soluble in ethanol. The material on t.l.c. eluting with ether-acetone (1:3) showed spots on exposure to iodine vapour at R_F 0.15, 0.21 and 0.36 with considerable streaking in between. A reference spot of azadirachtin ran to R_F 0.63.

Selenium Dehydrogenation of the Product from Lithium Aluminium Hydride Reduction of Azadirachtin

A sample (89 mg.) of the product from the reduction of azadirachtin with lithium aluminium hydride was dissolved in absolute ethanol (1 ml.) in a Carius tube and selenium powder (146 mg.) was added. The solvent was removed and the tube was evacuated (oil pump, 0.1 mm.), sealed, and heated at 320° for 66 hours. After cooling, the crushed tube and contents were extracted

with absolute ethanol boiling at reflux for 24 hours. Filtration and evaporation of the solvent left a residue (11 mg.) which was subjected to gas liquid chromatography (g.l.c.) on a Pye Model No. 64 Gas Chromatograph using a 9 ft. long, $\frac{1}{8}$" diameter column of 5% S.E. 30 on Chromosorb G. At 152^{o} with a helium flow rate of 17 ml./min. the following peaks were recorded:

Peak	Corrected Retention Time	Relative Peak Area
A	2.9 min.	19%
B	3.2 min.	3%
C	4.1 min.	6%
D	4.8 min.	29%
E	6.2 min.	21%
F	11.0 min.	100%
G	14.6 min.	61%

The chromatogram obtained is shown in Fig. 28. Several known materials were subjected to g.l.c. under the same conditions and the following results were obtained:

Sample	Corrected Retention Time
Naphthalene	2.2 min.
1-Methylnaphthalene	3.8 min.
2,3-Dimethylnaphthalene	6.6 min.
Phenanthrene	>25.0 min.

The selenium pyrolysis product was further investigated by means of a direct link from the gas chromatograph to a RMU-6 Mass Spectrometer via a Watson-Biemann Separator.

The mass spectra of the materials from peaks A, B, C and D did not yield any useful information. The mass spectrum (Fig. 29) of the material corresponding to peak E showed prominent ions at $^{m}/e$ 156 (100), 155 (30), 141 (64), 119 (16), 115 (18), 105 (42), 91 (13), 77 (16), and 65 (7). (% abundance relative to the molecular ion $^{m}/e$ 156.) The material was assigned a dimethylnaphthalene structure.

The mass spectrum (Fig. 30) of the material represented by g.l.c. peak F showed prominent ions at $^{m}/e$ 170 (100), 169 (11), 155 (78), 141 (8), 133 (8), 128 (8), 119 (7), 115 (32), 105 (9), 91 (10) and 77 (6). (% abundance relative to the molecular ion $^{m}/e$ 170.) The material was therefore identified as a trimethylnaphthalene.

The mass spectrum (Fig. 31) of the material from g.l.c. peak G showed prominent ions at $^{m}/e$ 184 (53), 169 (100), 143 (95), 115 (90), 105 (41), 93 (17), 91 (90) and 71 (24). (% abundance relative to $^{m}/e$ 169.) The substance was tentatively assigned a tetramethylnaphthalene structure.

Preparation of Acetoxy-tribromo-azadirachtin

Bromine (0.15 ml.) was added to azadirachtin (169 mg.) and

sodium acetate (1.17 g.) in glacial acetic acid (27.3 ml.), and the mixture was left at room temperature for 90 minutes. Water (75 ml.) was added and the mixture was extracted four time with chloroform (25 ml., 20 ml., 20 ml., and 10 ml.). The chloroform was washed several times with water and evaporated to leave a yellow solid residue (215 mg.) which on t.l.c. eluting with chloroform-acetone (7:3) showed a spot R_F 0.56 as the major component.

The residue was subjected to P.L.C. eluting twice with chloroform-acetone (4:1) and from the major band was isolated acetoxy-tribromo-azadirachtin (62 mg.) m.p. 179-182°, R_F 0.56 in chloroform-acetone (7:3), 0.51 in ether-acetone (4:1), 0.55 in ethyl acetate-benzene (85:15) and 0.37 in ether-acetonitrile (92:8). ν_{max} ($CHCl_3$) 3550 (broad), 1735 (strong) and 1603 $cm.^{-1}$ (weak). No U.V. absorption maximum was observed above 200 nm. The n.m.r. spectrum is shown in Fig. 33. No ions were observed above $^m/_e$ 370 in the mass spectrum.

Micro-analysis (F. B. Strauss) gave Br 22.22%, $C_{37}H_{47}O_{18}Br_3$ requires Br 23.54%.

Silylation of Acetoxy-tribromo-azadirachtin

Acetoxy-tribromo-azadirachtin (7 mg.) in dimethylformamide (0.5 ml.) was treated with B.S.A. (0.5 ml.) at room temperature for 5 hours. Pumping at 0.1 mm. left a solid (7 mg.) which on

t.l.c. eluting with chloroform-acetone (7:3) showed spots at R_F 0.60 and 0.68.

The residue was subjected to preparative t.l.c. eluting with the same solvent system and from the upper band was isolated the bis(trimethylsilyl)-ether derivative of acetoxy-tribromo-azadirachtin (0.5 mg.), which in the mass spectrometer showed a very weak molecular ion as a multiplet from $^{m}/e$ 1160 to 1166.

Treatment of Methyl Tiglate with Bromine and Sodium Acetate

Bromine (0.15 ml.) was added to methyl tiglate (65 mg.) and sodium acetate (1.17 g.) in glacial acetic acid (27.3 ml.). After 90 minutes at room temperature, water (75 ml.) was added and the mixture was extracted with chloroform. The chloroform was washed with water and evaporated to leave a colourless liquid (45 mg.) which on t.l.c. eluting with carbon tetrachloride showed a major component at R_F 0.20 and a very weak spot at R_F 0.07. A reference spot of starting material ran to R_F 0.13.

A portion (15 mg.) of this crude product was subjected to preparative t.l.c. eluting with carbon tetrachloride-chloroform (1:1) and from the major band was isolated methyl 2,3-dibromo-2-methyl butyrate as a colourless liquid (8 mg.), characterised by the following spectral evidence. The n.m.r. spectrum in carbon tetrachloride showed resonances at τ5.2 (one proton, quartet), τ6.2 (three protons, singlet), τ8.05 (three protons,

singlet) and τ8.12 (three protons, doublet). The mass spectrum did not show a molecular ion but prominent ions above $^{m}/e$ 100 were observed at $^{m}/e$ 245 (2), 243 (4), 241 (2), 217 (5), 215 (10), 213 (5), 195 (30), 193 (30), 167 (8), 165 (8), 139 (42), 137 (50), 136 (35), 114 (30) and 113 (100). (% abundance relative to $^{m}/e$ 113.)

Treatment of Azadirachtin with Acetyl Chloride-Pyridine

(i) Azadirachtin (191 mg.) in chloroform (10 ml.) containing pyridine (10 drops) was treated with acetyl chloride (1 ml.) for 110 minutes at room temperature. Chloroform (40 ml.) and water (40 ml.) were added with shaking and the organic layer was washed with 5% aqueous potassium bicarbonate solution and several times with water. Evaporation of the solvent left a yellow solid (184 mg.) which on t.l.c. eluting with chloroform-acetone (7:3) showed a major spot R_F 0.54 with traces of material above and below.

The substance was subjected to P.L.C. eluting twice with chloroform-acetone (4:1) and from the major band was isolated a residue (46 mg.) which was further purified by preparative t.l.c. eluting twice with ether-acetone (4:1) to give a colourless solid V (24 mg.), m.p. 175-178°, R_F 0.54 in chloroform-acetone, 0.45 in ether-acetone (4:1), 0.34 in ethyl acetate-benzene (85:15) and 0.24 after two elutions with chloroform-acetonitrile (88:12). ν_{max} ($CHCl_3$) 3380 (broad), 1735 (strong), 1705 (shoulder) and 1650 $cm.^{-1}$ (weak). λ_{max} 216 nm. $E^{1\%}_{1\ cm.}$ 170. The n.m.r. spectrum

is shown in Fig. 34. The mass spectrum, which showed no molecular ion, displayed prominent peaks above $^{m}/e$ 600 at $^{m}/e$ 734 (3), 716 (9), 702 (39), 689 (21), 669 (100), 658 (33), 646 (4), 631 (14), 620 (17) and 602 (25). (% abundance relative to $^{m}/e$ 669.) Ions characteristic of the fragmentation of the tiglate ester group were seen at $^{m}/e$ 83 and 55.

(ii) Azadirachtin (15 mg.) in pyridine (3 ml.) was treated with acetyl chloride (0.2 ml.) at 0°. Aliquots were removed from the reaction mixture after known intervals of time, worked up as described above, and investigated by t.l.c. eluting with ether-acetone (4:1) or chloroform-acetone (7:3). The results obtained showed that azadirachtin remained unchanged even after five hours. Highly polar material was visible at the origin but a control experiment not involving azadirachtin showed that this material was produced by the interaction of pyridine and acetyl chloride.

When similar reactions were performed at 10°, and room temperature, the azadirachtin remained unchanged after 4 hours and 15 minutes respectively. After these times the chromatograms obtained were confused by the presence of heavy stains caused by materials produced by interaction of acetyl chloride with pyridine.

Attempted Chloroacetylation of Azadirachtin

(i) Azadirachtin (4 mg.) was treated with a solution of chloroacetic anhydride (150 mg.) in chloroform (1.5 ml.) for

62 hours at room temperature. Chloroform (10 ml.) was added and the solution was washed with 5% aqueous potassium bicarbonate solution and several times with water. The chloroform was evaporated, a small volume of toluene was added, and the residue was pumped at 0.1 mm. and 100°. The remaining solid (2 mg.) on t.l.c. eluting with ether-acetone (4:1) showed a spot R_F 0.50 corresponding to starting material, with slight streaking above and below.

(ii) Pyridine (2 drops) was added to an ice-cooled solution of azadirachtin (4 mg.) and chloroacetic anhydride (150 mg.) in chloroform (1.5 ml., dried over calcium chloride). After 20 hours at room temperature chloroform (10 ml.) and water (10 ml.) were added. The organic layer was washed with water, 5% aqueous potassium bicarbonate solution, and again with water. The solvent was removed, a small volume of toluene was added, and the residue was pumped at 0.1 mm. and 100° to leave a solid (2 mg.) which on t.l.c. eluting with ether-acetone (4:1) proved to be a complex mixture running as a streak from the origin to R_F 0.70.

(iii) Azadirachtin (3 mg.) and chloroacetic anhydride (1 ml.) were heated together at 60° for 2 hours. Work up as before yielded a residue (1 mg.) which on t.l.c. eluting with ether-acetone (4:1) showed a spot corresponding to starting material, with heavy staining above and below in which no distinct components were apparent.

A similar result was obtained when the reaction was carried out at 140° for 5 minutes.

Attempted Chloroacetylation of Dihydro-azadirachtin

(i) Dihydro-azadirachtin (5 mg.) and chloroacetic anhydride (1 ml.) were heated together at 140° for 15 minutes. Chloroform (10 ml.) was added and the resulting solution was washed with 5% aqueous potassium bicarbonate solution and several times with water. The solvent was evaporated, a small volume of toluene was added, and the residue was pumped at 0.1 mm. and 100° to leave a solid (2 mg.) which on t.l.c. was shown to be unchanged starting material.

When a similar reaction was carried out for 90 minutes the product was a complex mixture in which no distinct component was apparent.

(ii) Saturated aqueous sodium acetate solution (1 ml.) was added to dihydro-azadirachtin (8 mg.) in glacial acetic acid (1 ml.). The resulting solution was treated with chloroacetyl chloride (14 drops) at 0° for 90 minutes. Work up as described above led to the recovery of unchanged starting material.

(iii) A solution of dihydro-azadirachtin (193 mg.) in chloroacetyl chloride (2 ml.) was kept at 9° for 17 hours. Work up in the usual manner gave a pale yellow solid (189 mg.) which on t.l.c.

eluting twice with ethyl acetate-benzene (9:1) showed major components at R_F 0.39 and 0.49 with a minor component at R_F 0.19. A reference spot of dihydro-azadirachtin ran to R_F 0.27. The material was subjected to P.L.C. eluting three times with ethyl acetate-benzene (9:1). Three bands were visualised and designated A, B and C in order of increasing R_F.

The material (8 mg.) isolated from band A was shown by further t.l.c. to be inhomogeneous and was discarded.

The yellow solid (58 mg.) isolated from band B was further purified by preparative t.l.c. eluting with chloroform-acetone (7:3) to give a colourless solid VI (33 mg.), m.p. 181-184°, R_F 0.39 after two elutions with ethyl acetate-benzene (9:1), 0.42 in chloroform-acetone (7:3), 0.56 after two elutions with ether-acetonitrile (4:1) and 0.19 after two elutions with ether-light petroleum-acetonitrile (2:1:2). ν_{max} (CCl_4) 3440 (broad), 1750 (strong), 1715 (strong), and 1650 $cm.^{-1}$ (weak). The n.m.r. spectrum is shown in Fig. 35. The mass spectrum, which did not show a molecular ion, displayed prominent peaks above $^m/e$ 600 at $^m/e$ 702 (5), 692 (6), 684 (3), 674 (5), 660 (40), 642 (100), 632 (21), 617 (20) and 603 (20). (% abundance relative to $^m/e$ 642.) Intense ions characteristic of the fragmentation of the tiglate ester group were seen at $^m/e$ 83 and 55.

The material (32 mg.) isolated from band C was further purified by preparative t.l.c. eluting twice with ether-acetonitrile

(4:1) to give a colourless solid VII (19 mg.), m.p. 163-166°, R_F 0.41 in ether-acetonitrile (4:1), 0.49 after two elutions with ethyl acetate-benzene (9:1) and 0.31 after two elutions with ether-light petroleum-acetonitrile (2:1:2). ν_{max} ($CHCl_3$) 3345 (broad, 1710 (strong) and 1650 $cm.^{-1}$ (shoulder). The n.m.r. spectrum is shown in Fig. 36. The mass spectrum did not contain a peak which could be readily assigned to a molecular ion. Prominent peaks above $^m/e$ 600 were observed at $^m/e$ 732 (3), 705 (34), 692 (100) and 610 (39). (% abundance relative to $^m/e$ 692.) Intense ions from the fragmentation of the tiglate ester function were seen at $^m/e$ 83 and 55.

(iv) A solution of dihydro-azadirachtin (5 mg.) in chloroacetyl chloride (1 ml.) was left at room temperature for 5 hours. Work up as before gave a residue which on t.l.c. eluting with ethyl acetate-benzene (9:1) showed components identical to materials VI and VII.

(v) Dihydro-azadirachtin (3 mg.) and chloroacetyl chloride (1 ml.) were heated together on a water bath for 25 minutes. Work up as before yielded a residue (1 mg.) which on t.l.c. eluting with ethyl acetate-benzene (9:1) proved to be a mixture of inseparable materials running as a streak from the origin almost to the solvent front.

(vi) Dihydro-azadirachtin (4 mg.) in dimethylformamide (0.3 ml.)

was treated with chloroacetyl chloride (10 drops) for 4 hours at room temperature. Work up as before gave unchanged starting material (3 mg.).

When a similar reaction mixture was heated on a water-bath for 10 minutes and worked up as before, a residue was obtained which consisted of an inseparable mixture of materials running as a streak on t.l.c. from the origin to the solvent front.

Oxidation of Dihydro-azadirachtin with Lemieux Reagent (Sodium Metaperiodate-Potassium Permanganate)

The Lemieux Reagent[65-68] was prepared as a solution of sodium metaperiodate (629 mg.) and potassium permanganate (11 mg.) in water (30 ml.).

The pH of a solution of dihydro-azadirachtin (69 mg.) in t-butanol (15 ml.) and water (25 ml.) was adjusted to about 7.7 by addition of sodium carbonate. A portion (22.5 ml.) of the Lemieux Reagent was then added and the resulting solution was left to stand at room temperature for 100 minutes. The reaction mixture was extracted with chloroform which was then washed with water and evaporated to leave a light yellow solid (36 mg.) which on t.l.c. eluting twice with chloroform-acetone (7:3) showed a spot at R_F 0.40 corresponding to an authentic sample of detigloyl-pyruvyl-dihydro-azadirachtin obtained from Butterworth.

Hydrolysis of Detigloyl-pyruvyl-dihydro-azadirachtin

The sample (36 mg.) of detigloyl-pyruvyl-dihydro-azadirachtin obtained directly from the Lemieux Oxidation of dihydro-azadirachtin described above, was dissolved in methanol (5 ml.) and treated with saturated aqueous sodium bicarbonate solution (3.5 ml.) for 100 minutes at room temperature. The reaction mixture was extracted with chloroform which was then washed with water and evaporated, to leave a solid (31 mg.) which on t.l.c. eluting twice with chloroform-acetone (7:3) showed a spot at R_F 0.25 corresponding to detigloyl-dihydro-azadirachtin and a slightly more intense spot at R_F 0.40 corresponding to the starting material.

The total material (31 mg.) in methanol (10 ml.) was treated with saturated aqueous sodium bicarbonate solution (5 ml.) for a further 3 hours at room temperature. Work up as before yielded a light yellow solid (19 mg.) which on t.l.c. eluting twice with chloroform-acetone (7:3) showed a faint spot at R_F 0.40 corresponding to detigloyl-pyruvyl-dihydro-azadirachtin, a spot at R_F 0.25 corresponding to detigloyl-dihydro-azadirachtin, and a major spot at R_F 0.19.

The material was subjected to preparative t.l.c. eluting twice with chloroform-acetone (7:3) and from the major band was isolated a solid (5 mg.) which after further purification by preparative t.l.c. eluting twice with chloroform-acetone (7:3) yielded deacetyl-detigloyl-dihydro-azadirachtin (2 mg.), R_F 0.19 after two elutions with chloroform-acetone (7:3), and 0.12 after

two elutions with ether-acetone (4:1). The n.m.r. spectrum (C.A.T., 109 scans) in $CDCl_3$ solution is shown in Fig. 37. The mass spectrum did not show the molecular ion at $^m/_e$ 598, but significant ions above $^m/_e$ 500 were observed at $^m/_e$ 580 (2), 566 (3), 548 (4), 534 (20), 530 (8), 516 (100) and 512 (5). (% abundance relative to $^m/_e$ 516.)

Attempted Preparation of the Caesium Salt of the Product from Lemieux Oxidation of Azadirachtin

The pH of a solution of azadirachtin (223 mg.) in t-butanol (80 ml.) and water (120 ml.) was adjusted to about 7.7 by addition of sodium carbonate. A solution of sodium metaperiodate (1.72 g.) and potassium permanganate (24 mg.) in water (80 ml.) was added, and the reaction mixture was stirred for 70 minutes at room temperature. The solution was then acidified with dilute hydrochloric acid and extracted with chloroform. The chloroform was washed with water and evaporated to give a yellow solid (117 mg.) which on t.l.c. eluting with ether-acetone (4:1) showed an elongated spot at the origin chracteristic of a carboxylic acid, and a faint diffuse stain at about R_F 0.63.

A portion (50 mg.) of the crude carboxylic acid in hot 95% ethanol (1 ml.) was treated with caesium carbonate (11 mg.) also in hot 95% ethanol (5 ml.). No precipitate was obtained on rapid cooling of the resulting solution and so the solvent was allowed

to evaporate slowly at room temperature in the dark. No crystalline material was obtained.

The glassy solid was redissolved in aqueous acetone (1:34, 17.5 ml.) and the resulting solution was kept at room temperature in the dark. The solvent evaporated slowly but no crystalline material was obtained.

Preparation of Lead Tetrabenzoate

Lead tetrabenzoate was prepared from lead tetraacetate and benzoic acid by the method of Criegee et al[84]. Recrystallisation from methylene chloride gave a pale yellow solid, m.p. 169° (lit. m.p. 164°[84]; 176°[85]).

Preparation of Tris(dipivalomethanato)europium

Tris(dipivalomethanato)europium, $Eu(DPM)_3$, was prepared from 2,2,6,6-tetramethyl-3,5-heptanedione (dipivaloylmethane) and europium nitrate by the method of Eisenkraut and Sievers[124]. Recrystallisation from hexane followed by sublimation gave a pale yellow solid, m.p. 190° (lit.[124] 187-189°).

Preparation of Tris(dipivalomethanato)praseodymium

Tris(dipivalomethano)praseodymium, $Pr(DPM)_3$, was prepared from 2,2,6,6-tetramethyl-3,5-heptanedione and praseodymium nitrate by the method of Eisenkraut and Sievers[124]. Recrystallisation (twice) from hexane followed by sublimation gave a pale green solid, m.p. 221° (lit.[124] m.p. 222-224°). (All m.p.'s in a sealed evacuated capilliary tube.)

100 MHz. N.M.R. Spectrum ($CDCl_3$) of

Azadirachtin

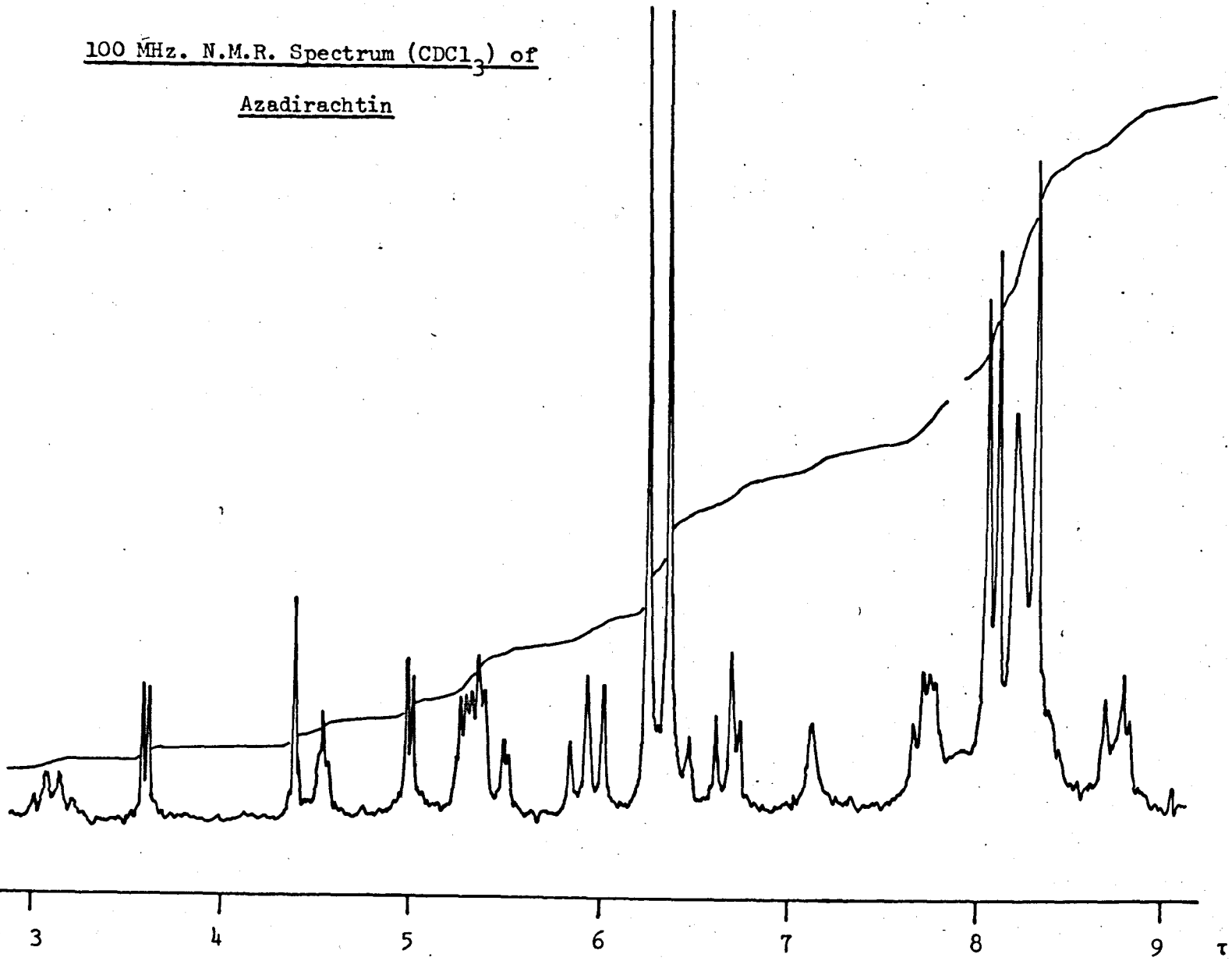


Fig. 1.

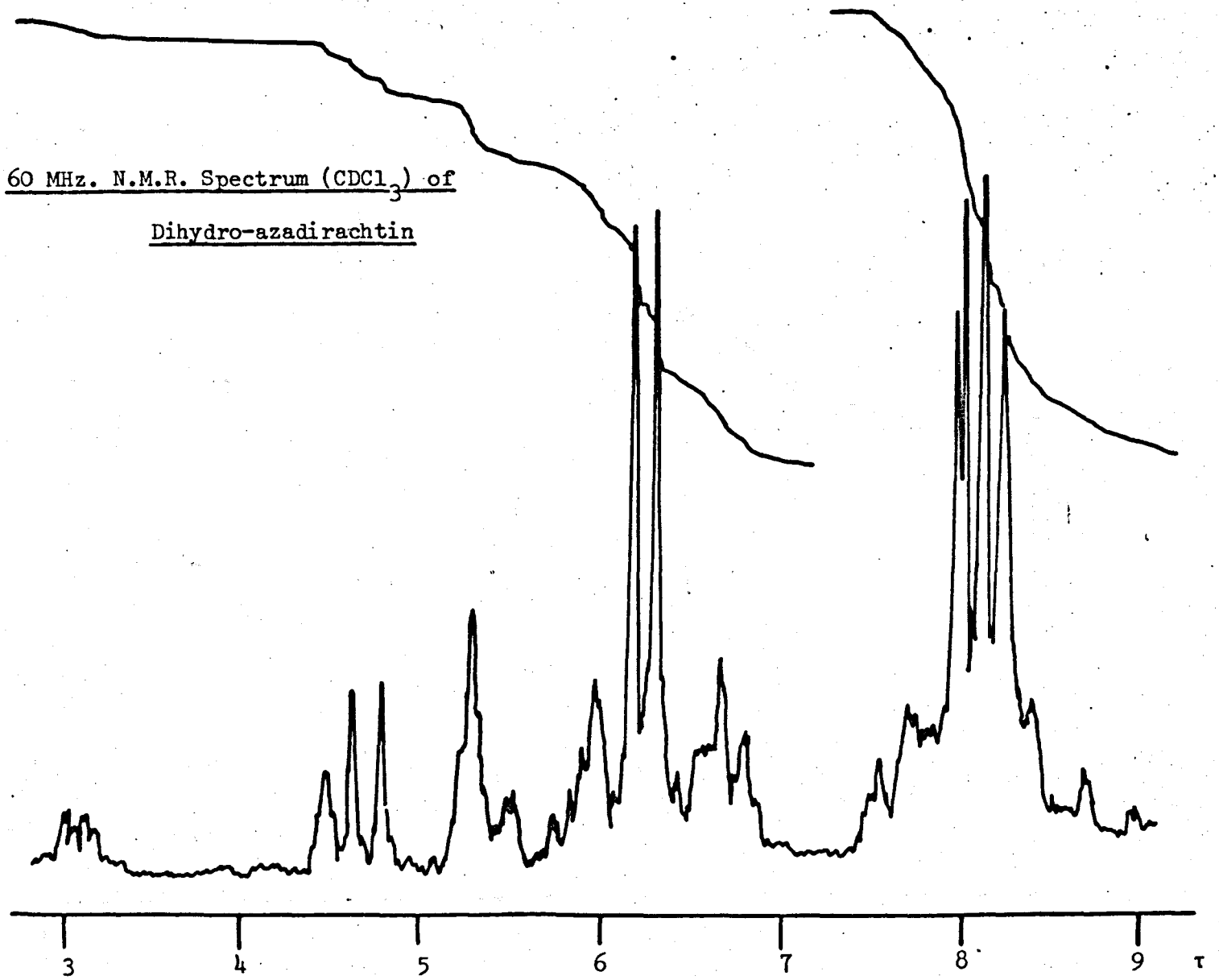

60 MHz. N.M.R. Spectrum ($CDCl_3$) of
Dihydro-azadirachtin
3
4
5
6
7
8
9
τ

Fig. 2.

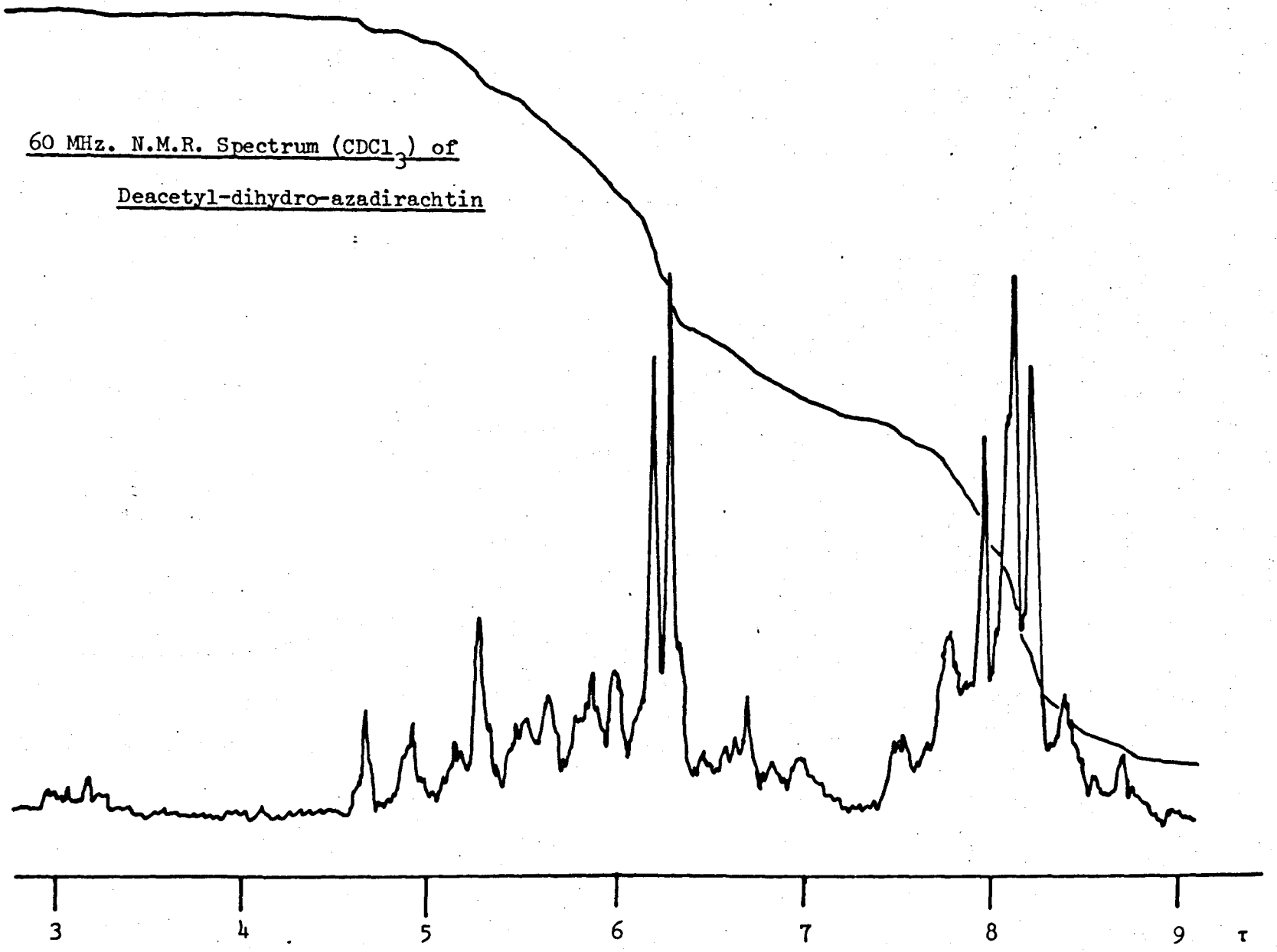

60 MHz. N.M.R. Spectrum ($CDCl_3$) of
Deacetyl-dihydro-azadirachtin
3
4
5
6
7
8
9
τ

Fig. 3.

100 MHz. N.M.R. Spectrum ($CDCl_3$) of

the Lactone from the Alkaline Hydrolysis of Dihydro-azadirachtin

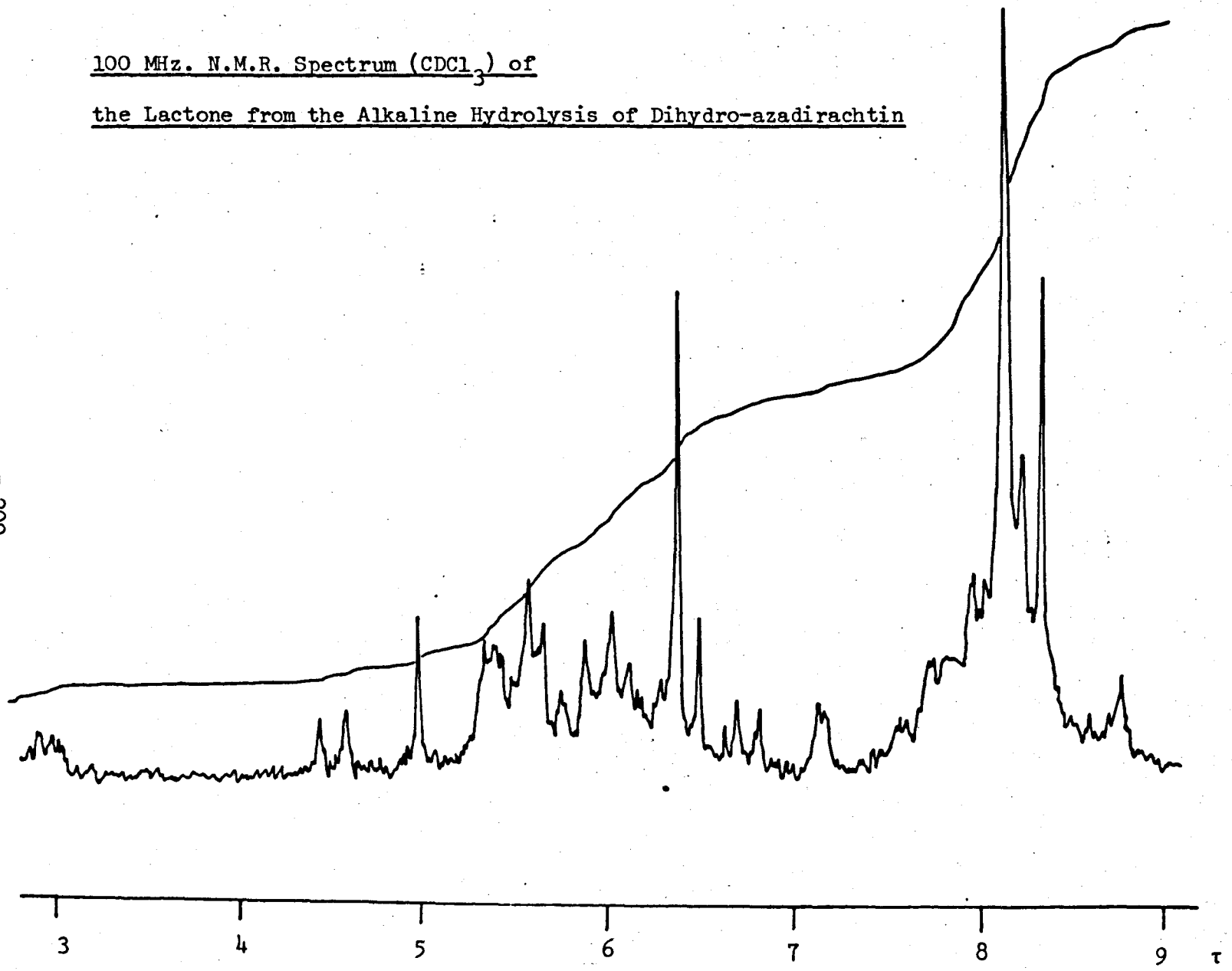


Fig. 4.

100 MHz. N.M.R. Spectrum ($CDCl_3$) of

Deacetyl-azadirachtin

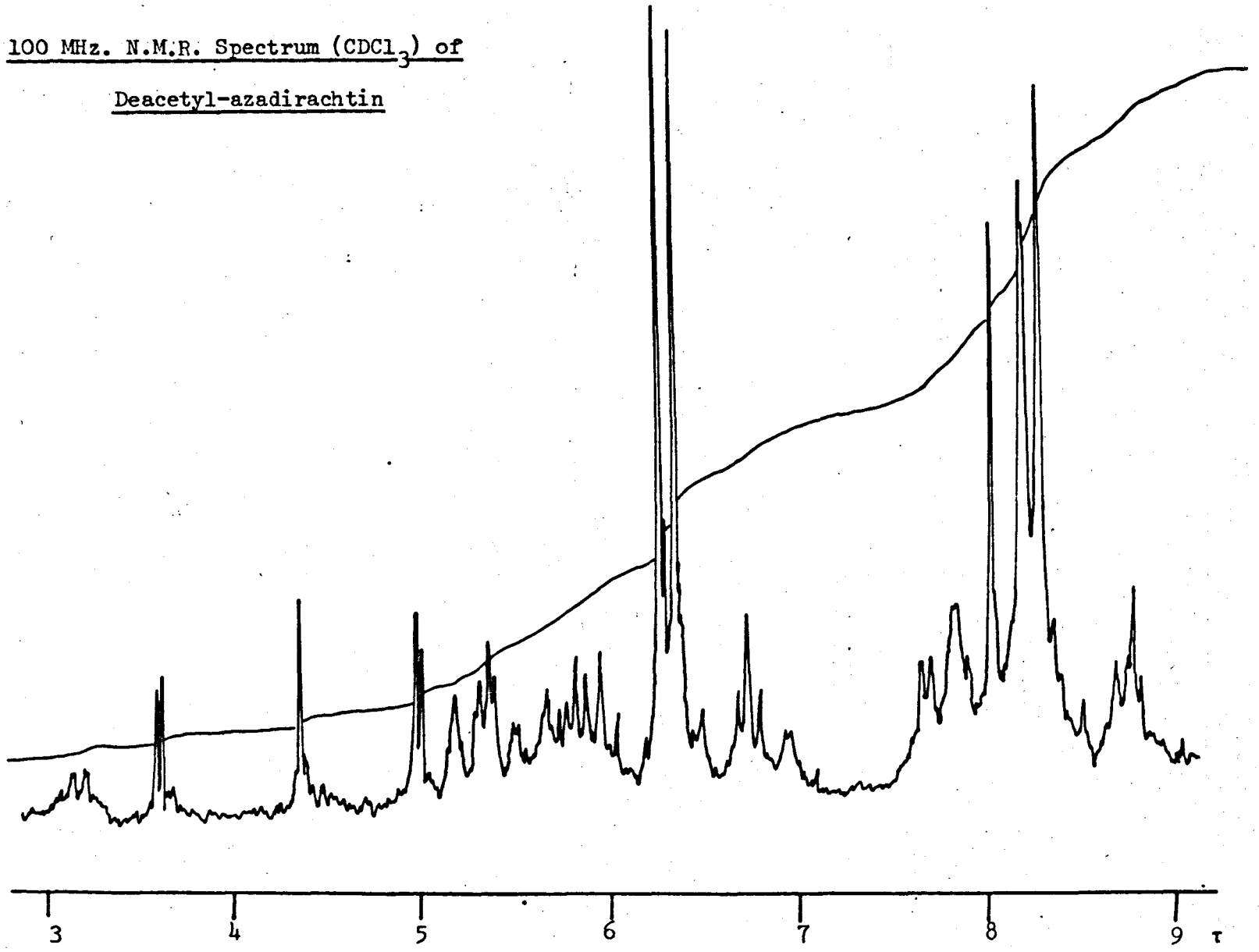


Fig. 5.

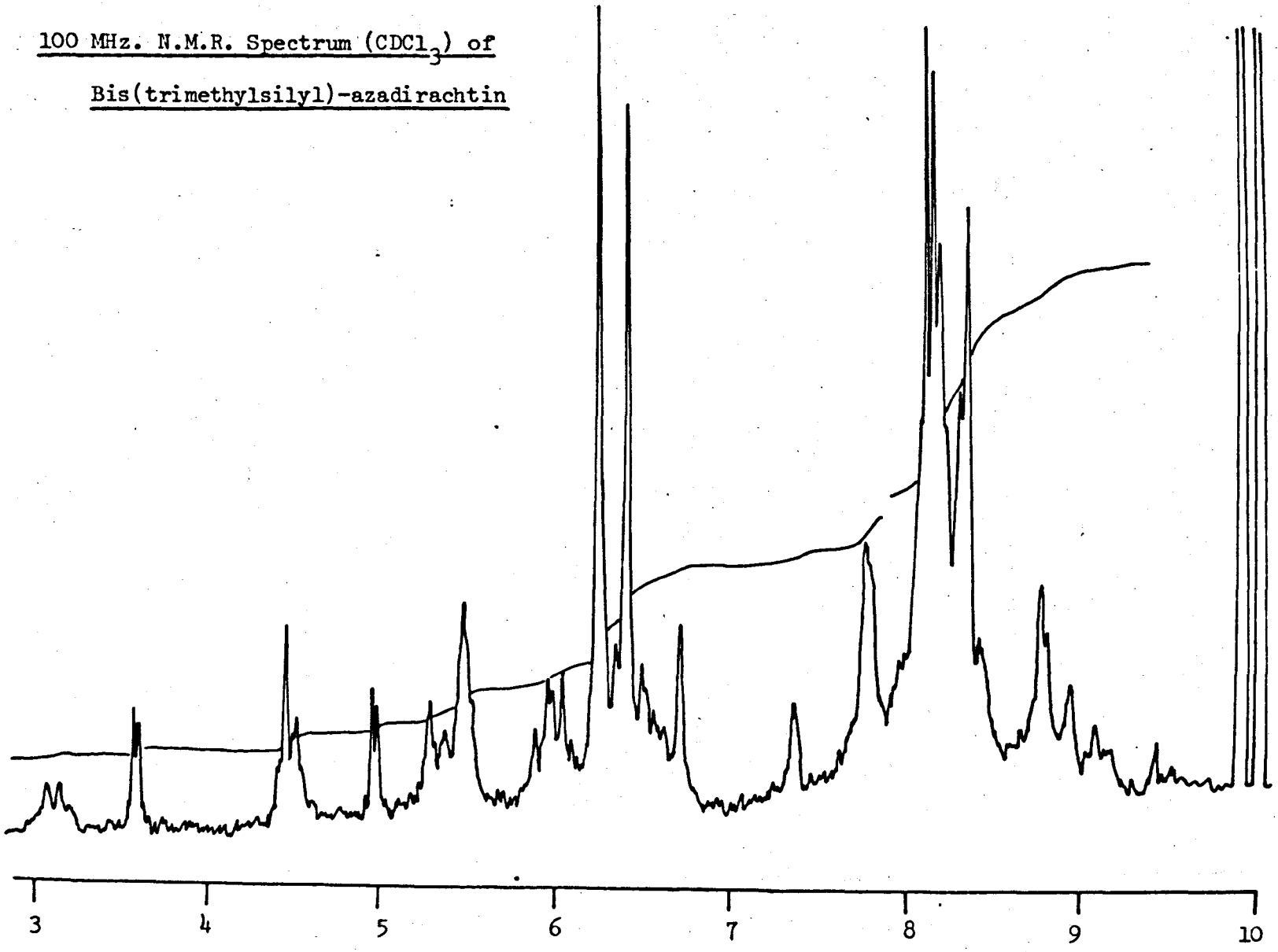

100 MHz. N.M.R. Spectrum ($CDCl_3$) of
Bis(trimethylsilyl)-azadirachtin
3
4
5
6
7
8
9
10
τ

Fig. 6.

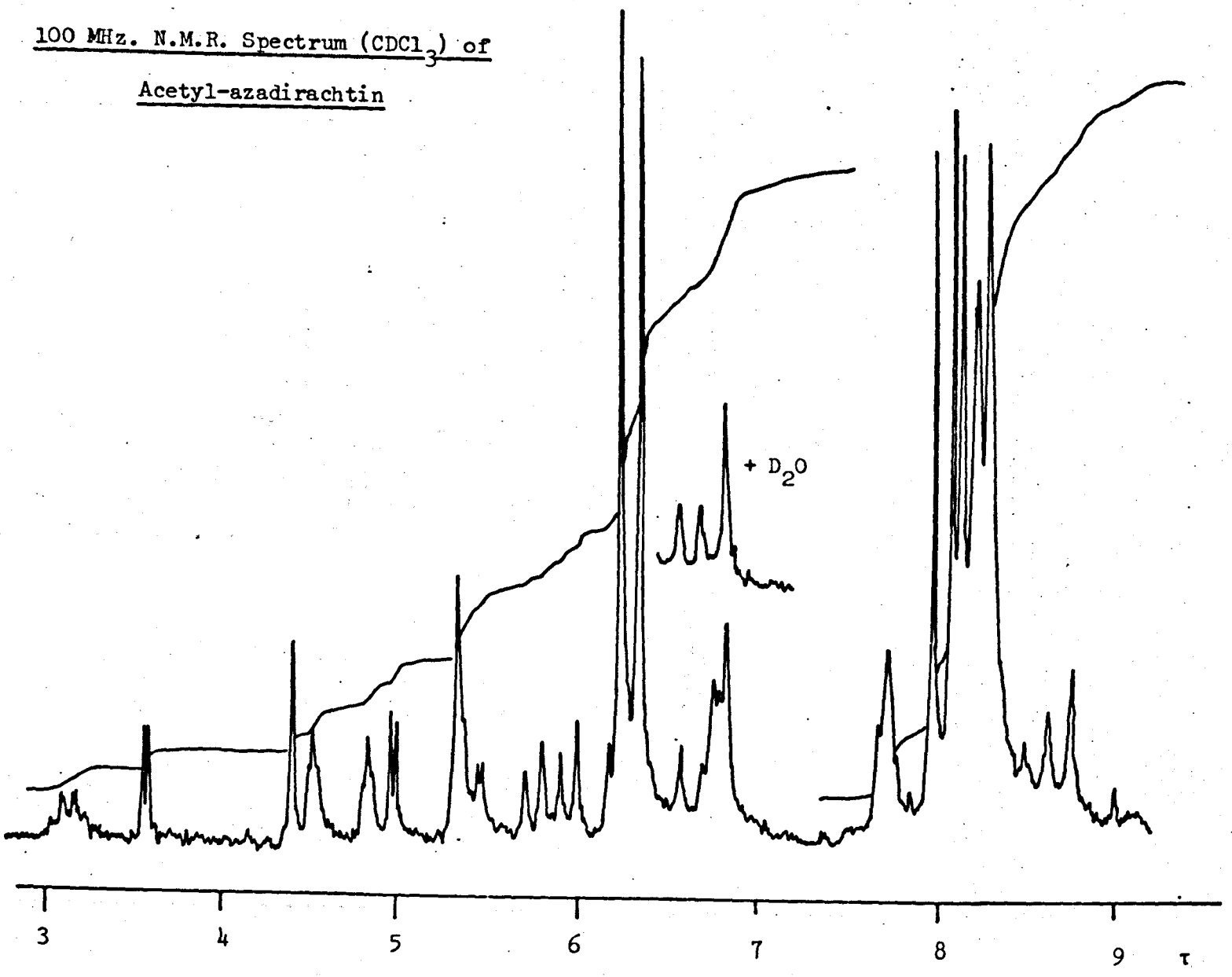

100 MHz. N.M.R. Spectrum ($CDCl_3$) of
Acetyl-azadirachtin
+ D_2O
3
4
5
6
7
8
9
τ

Fig. 7.

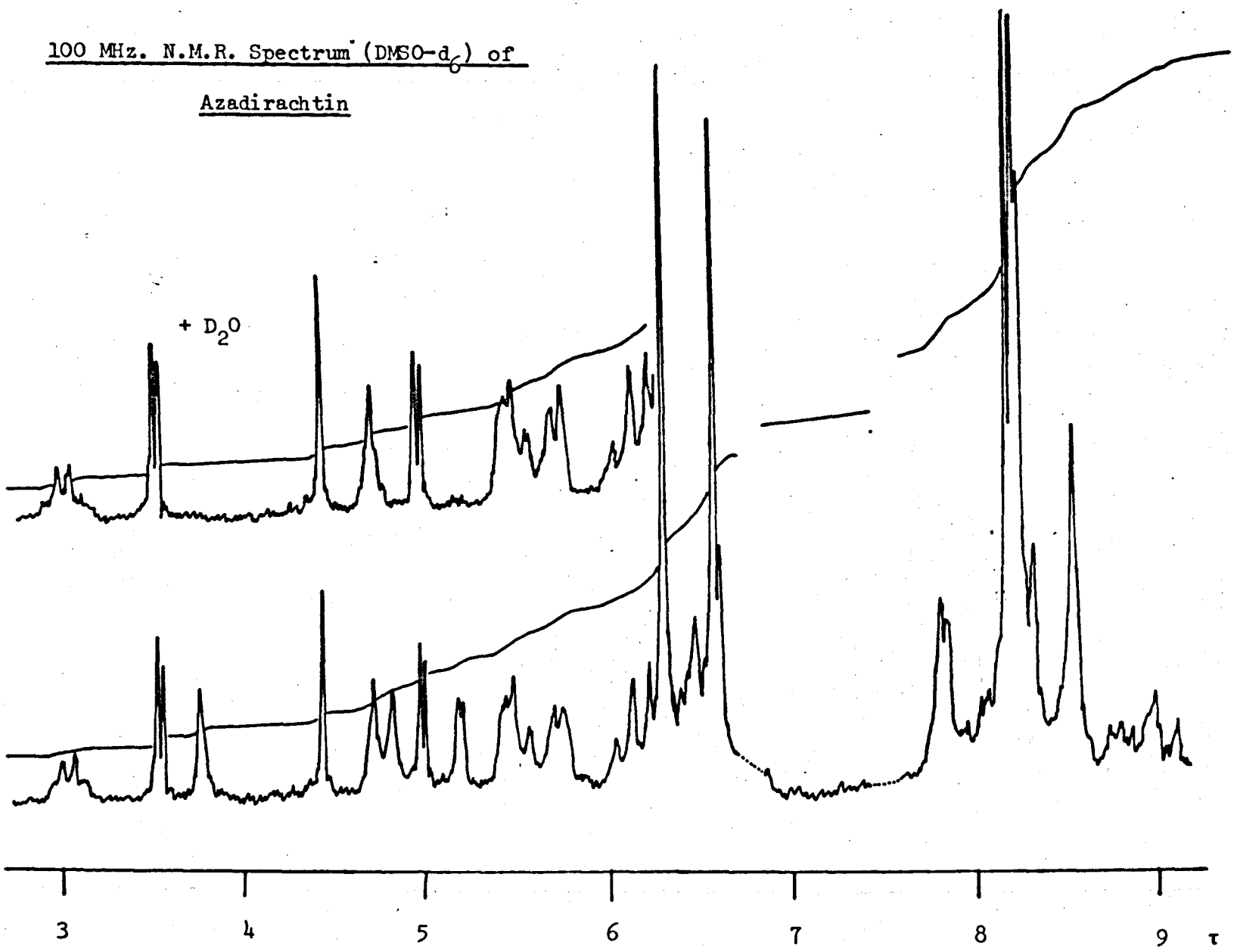


Fig. 8.

100 MHz. N.M.R. Spectrum (DMSO-d_6) of Bis(trimethylsilyl)-azadirachtin

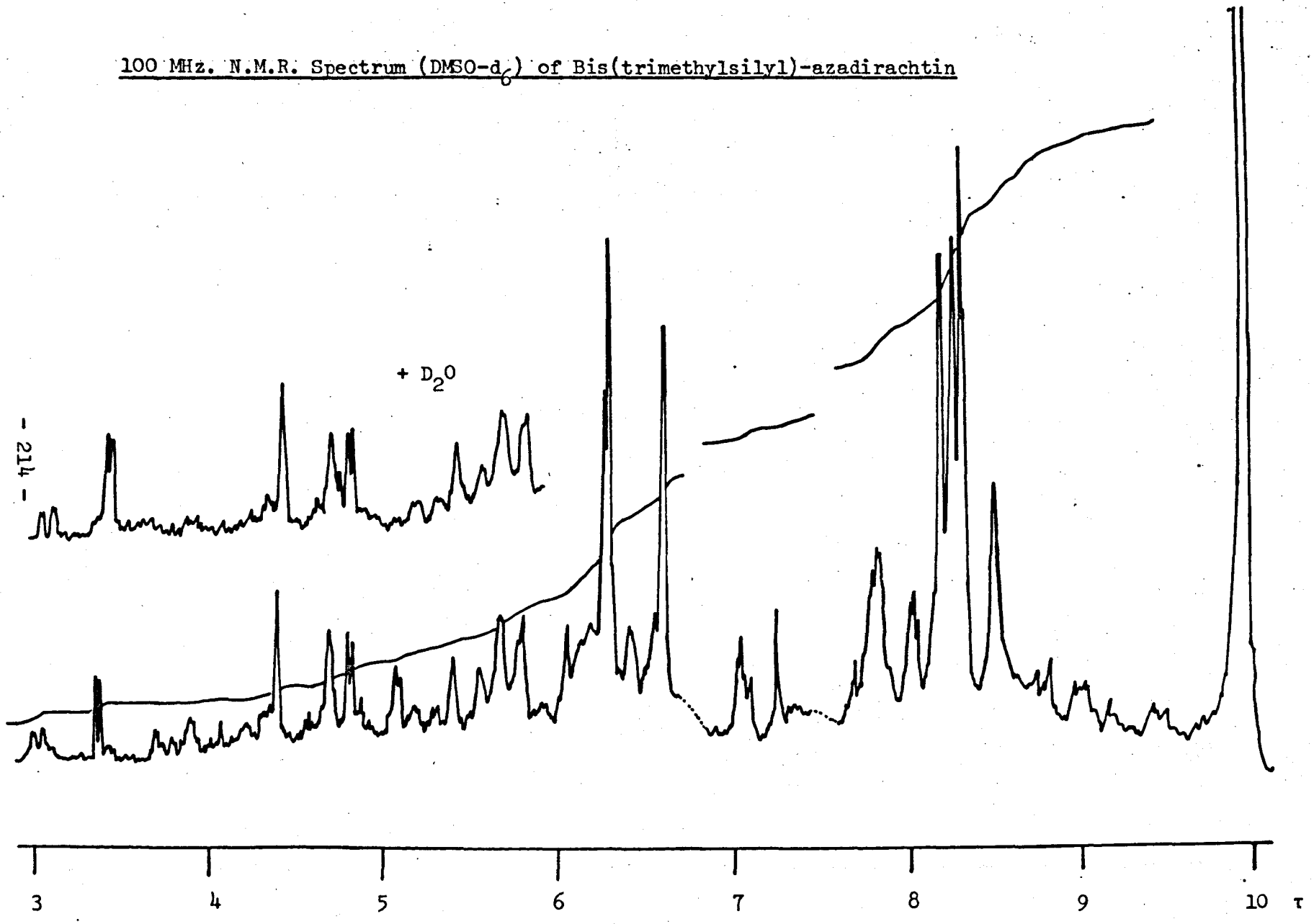


Fig. 9

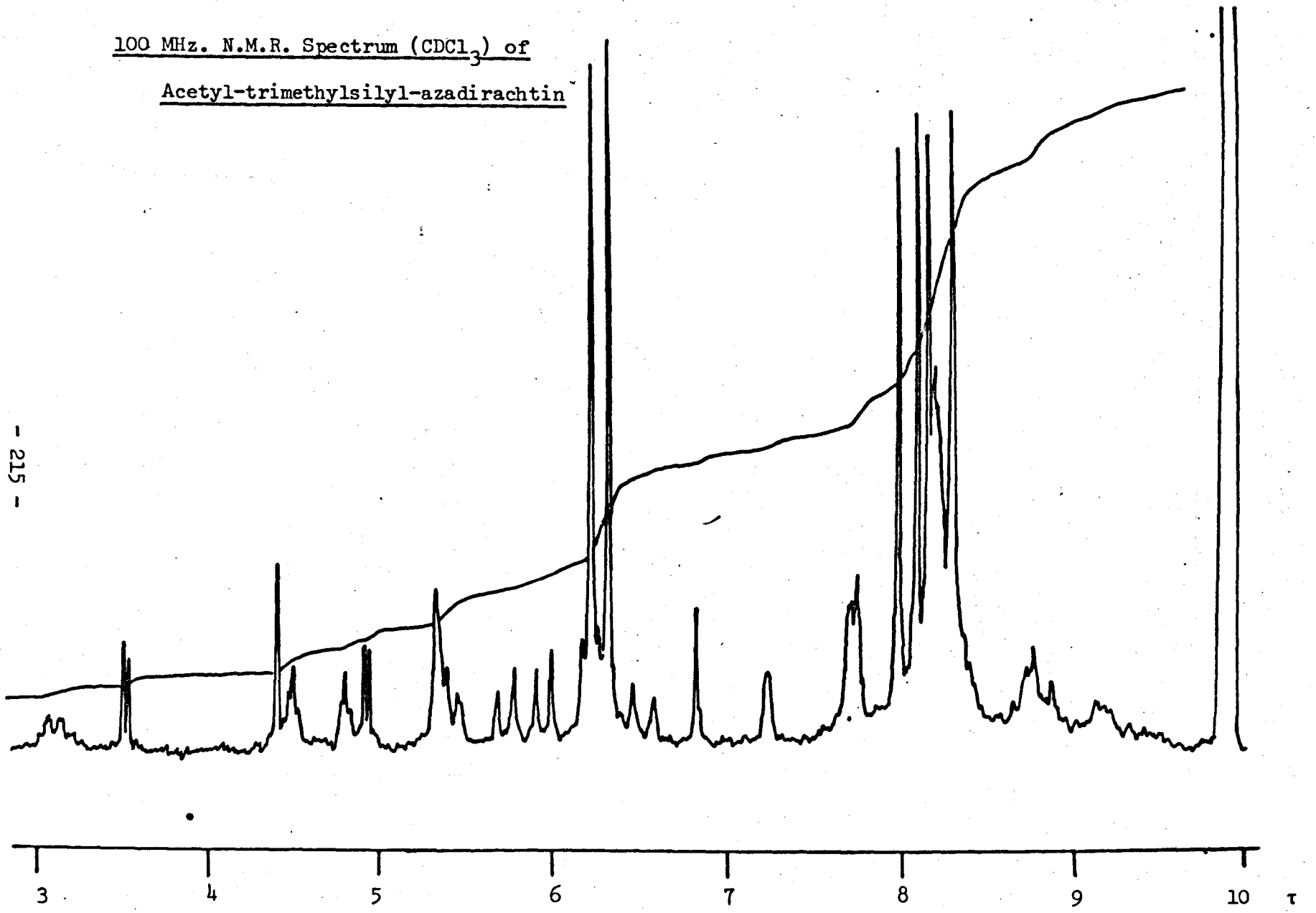

100 MHz. N.M.R. Spectrum ($CDCl_3$) of
Acetyl-trimethylsilyl-azadirachtin
3
4
5
6
7
8
9
10
τ

Fig. 10.

100 MHz. N.M.R. Spectrum (DMSO-d_6) of

Acetyl-trimethylsilyl-azadirachtin

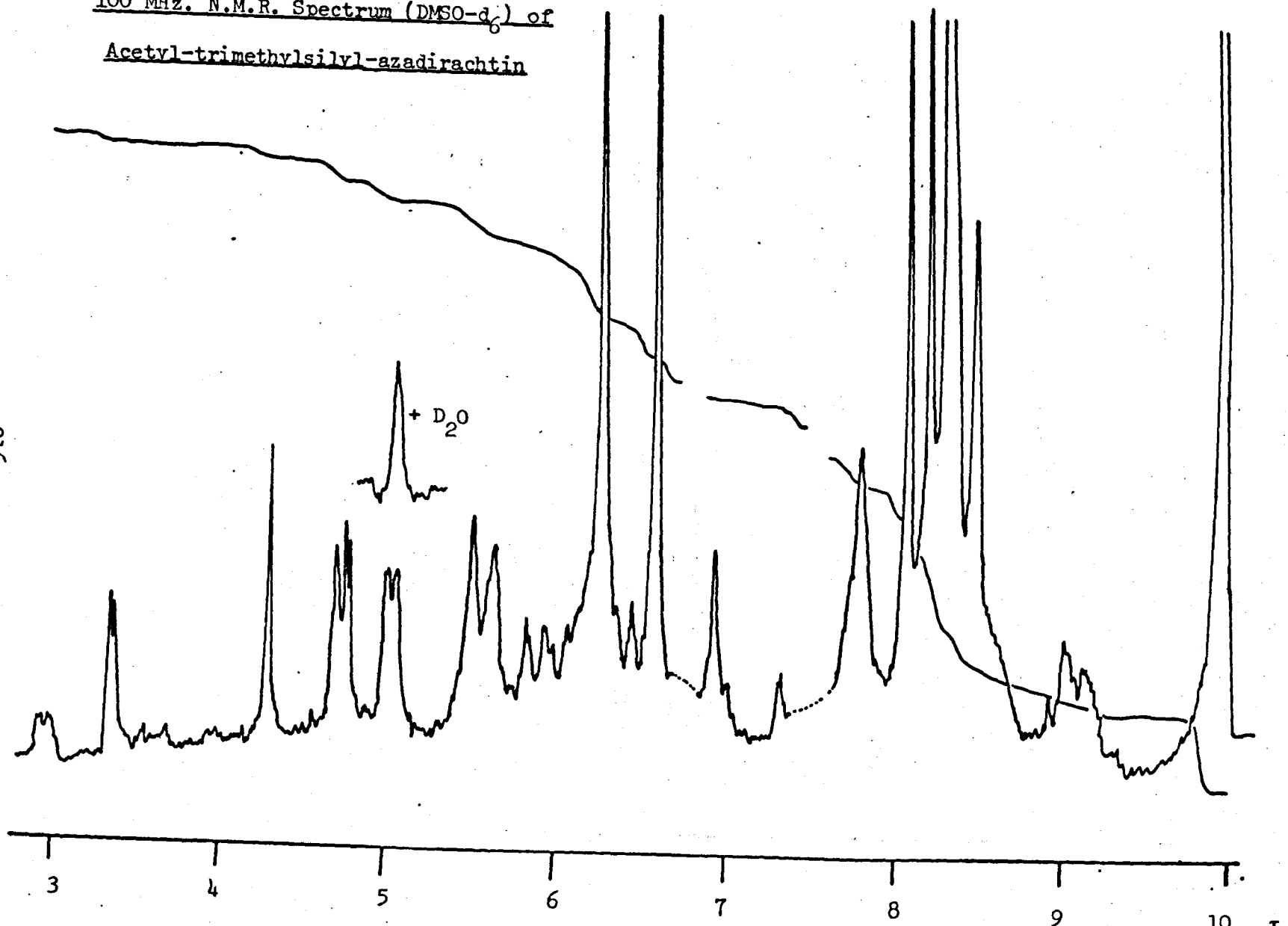


Fig. 11.

60 MHz. N.M.R. Spectrum (DMSO-d_6) of

Acetyl-azadirachtin

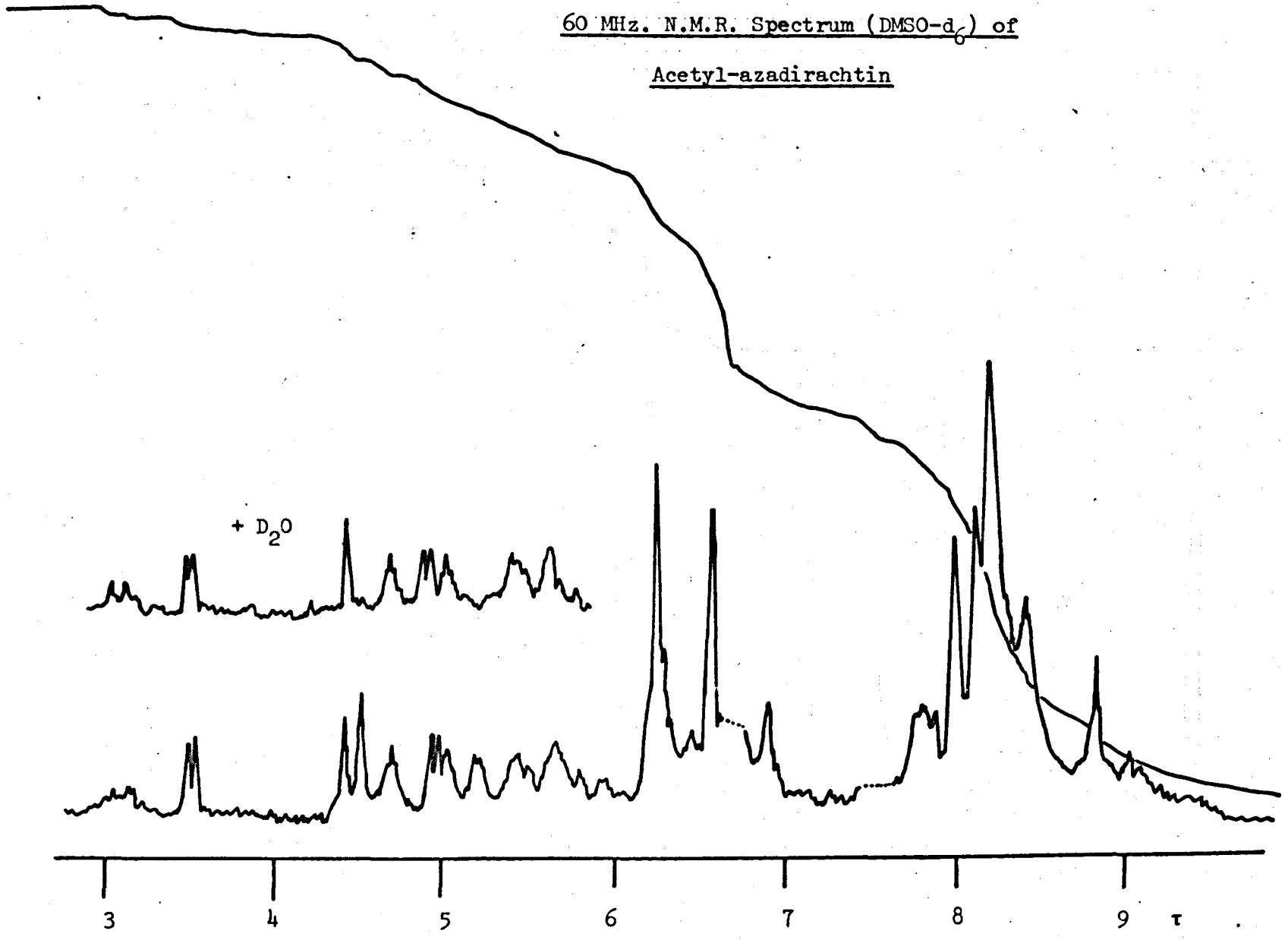


Fig. 12.

60 MHz. N.M.R. Spectrum ($CDCl_3$) of B-trimethylsilyl-azadirachtin

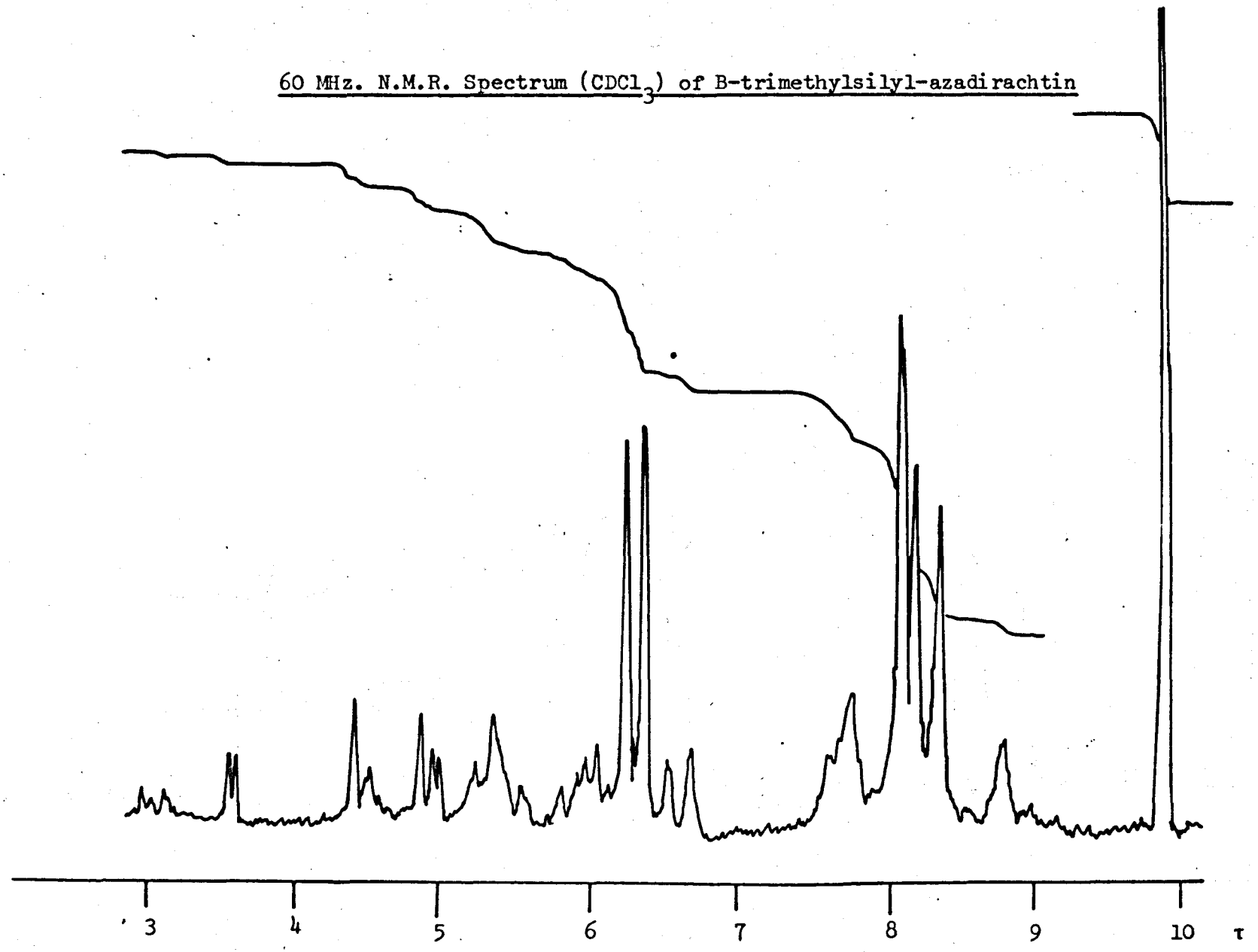


Fig. 13.

100 MHz. N.M.R. Spectrum (DMSO-d_6) of B-trimethylsilyl-azadirachtin

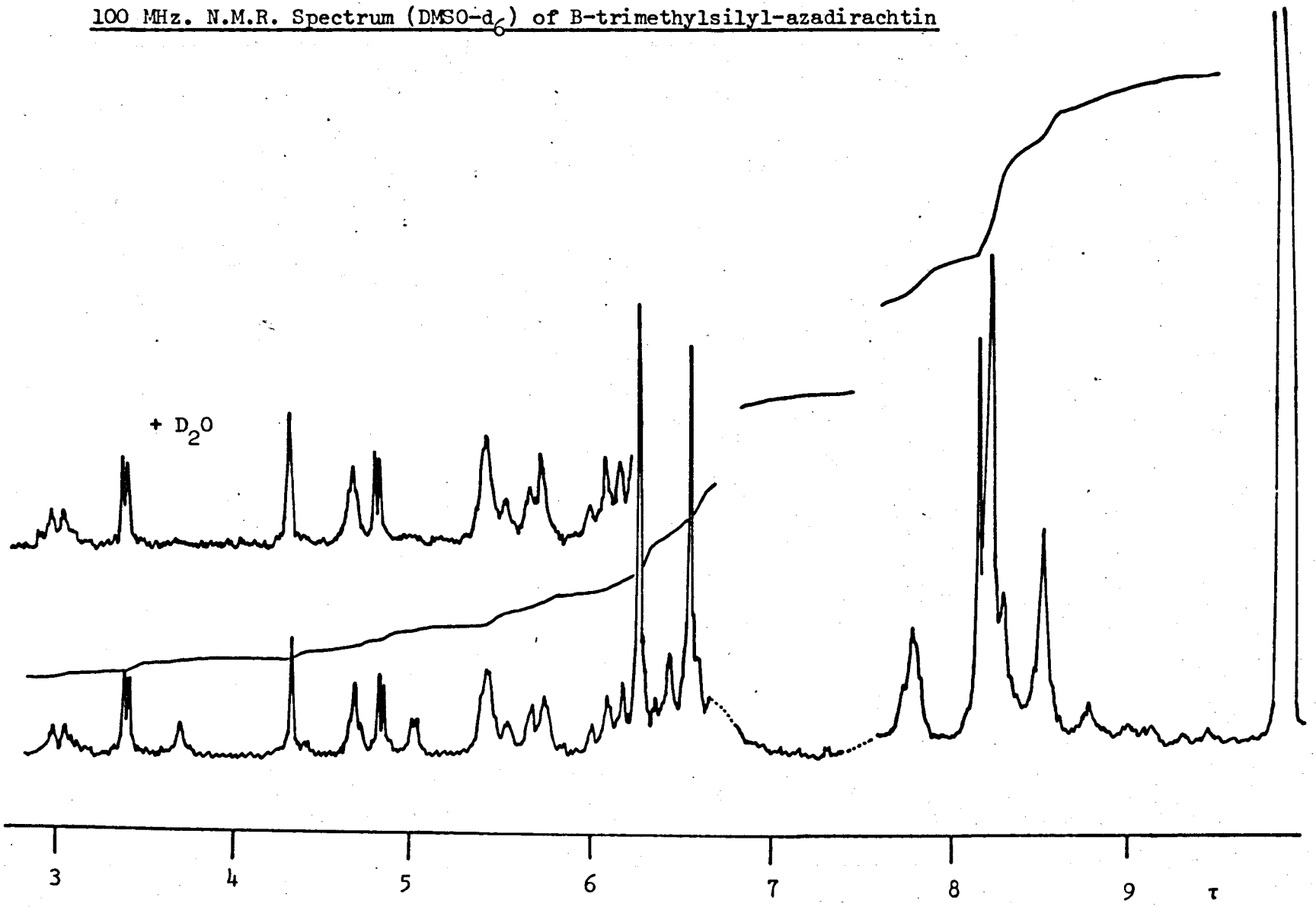


Fig. 14.

60 MHz. N.M.R. Spectrum ($CDCl_3$) of A-trimethylsilyl-azadirachtin

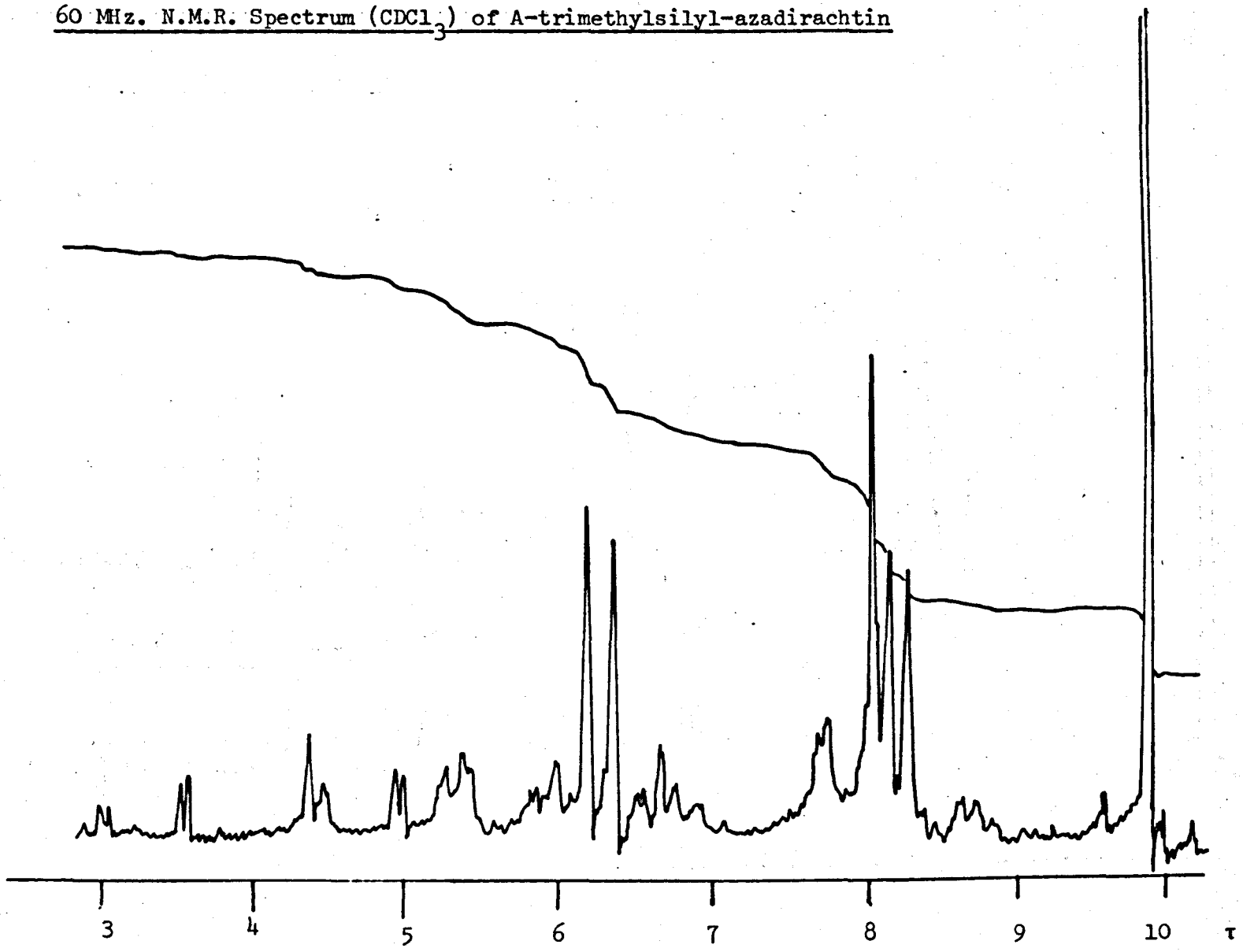
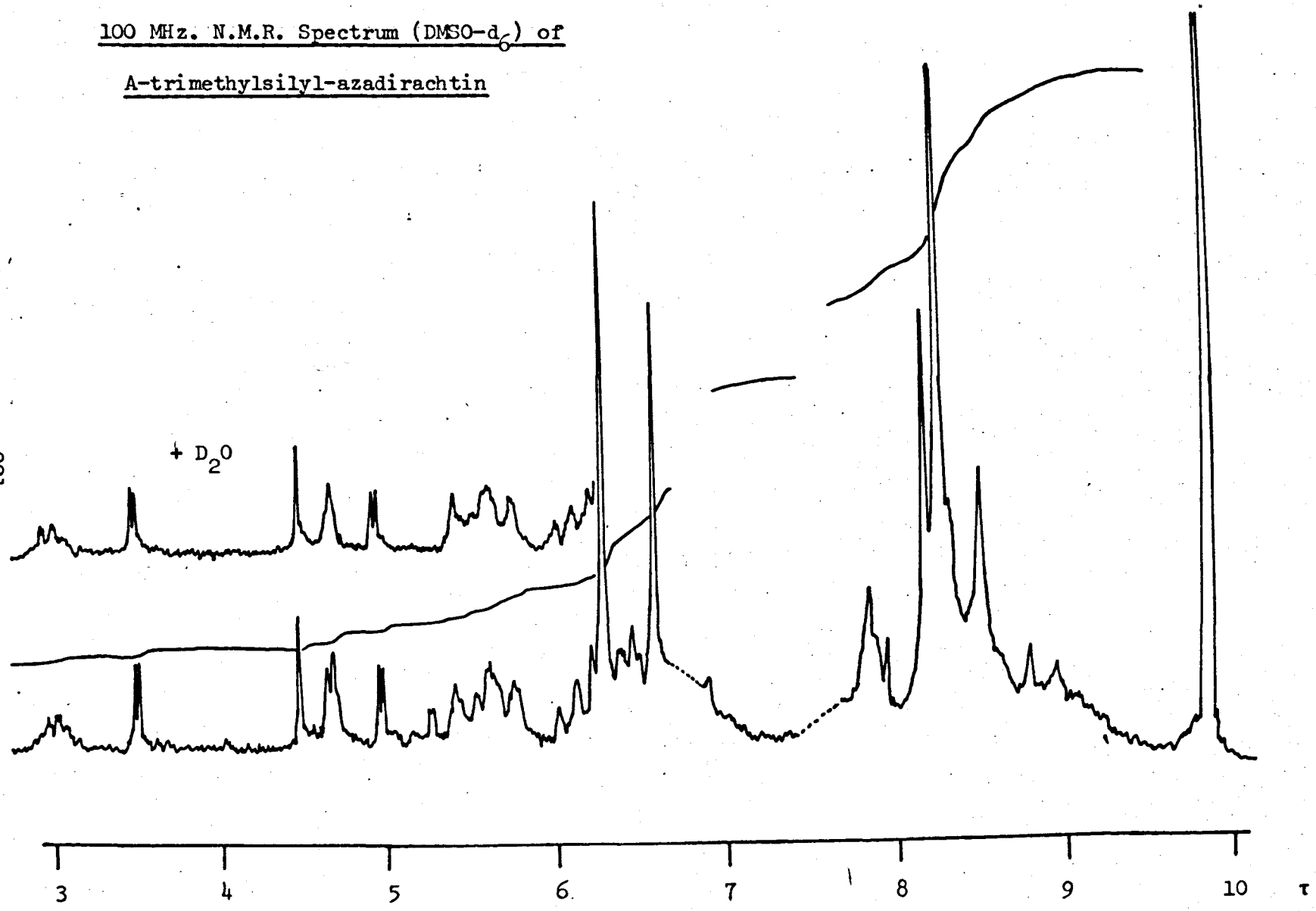


Fig. 15.


100 MHz. N.M.R. Spectrum (DMSO-d_6) of
A-trimethylsilyl-azadirachtin
+ D_2O
3
4
5
6
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8
9
10
τ

Fig. 16.

100 MHz. N.M.R. Spectrum ($CDCl_3$) of

Tris(trimethylsilyl)-azadirachtin

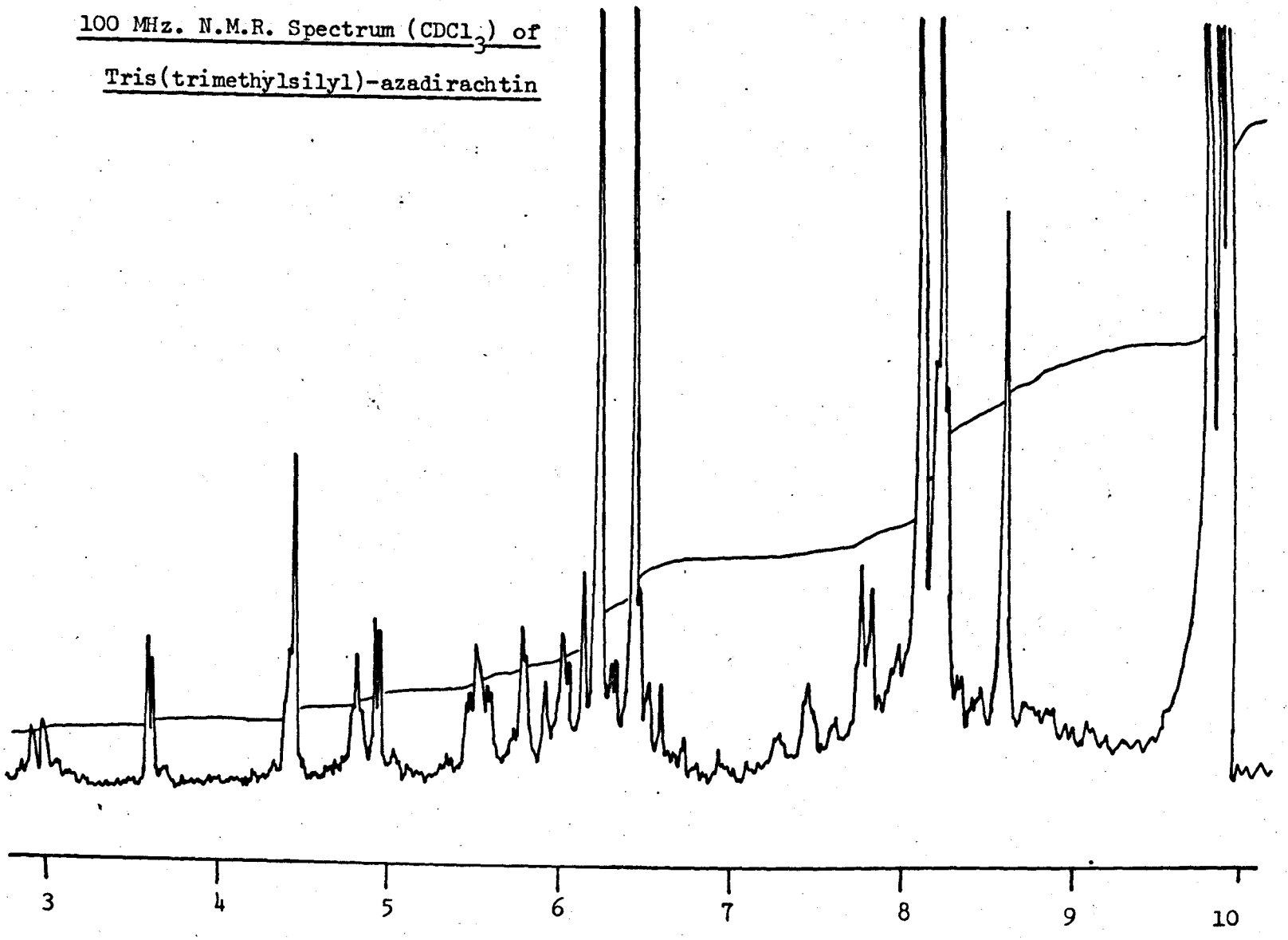


Fig. 17.

100 MHz. N.M.R. Spectrum (DMSO-d_6) of Deacetyl-azadirachtin

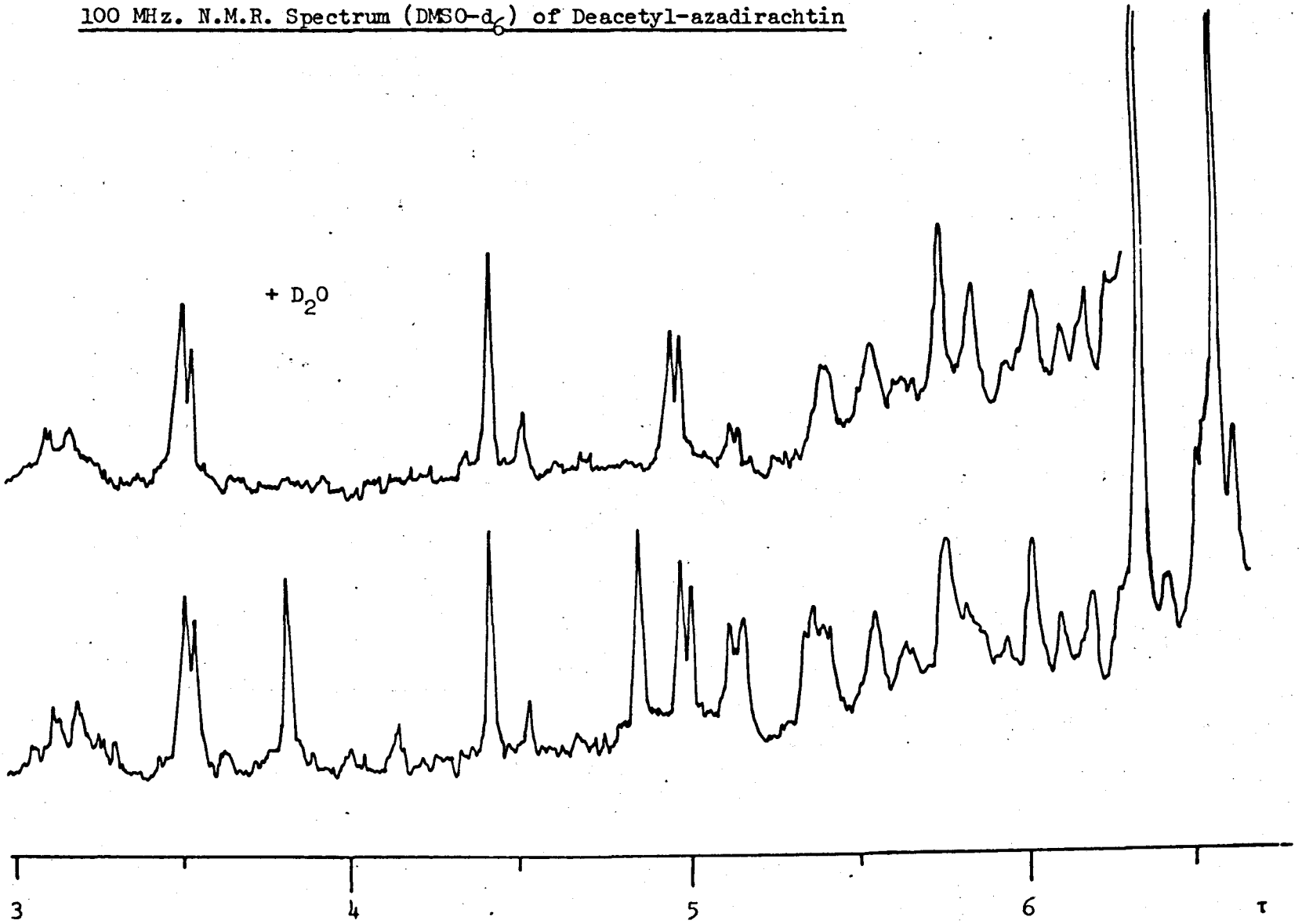


Fig. 18.

100 MHz. N.M.R. Spectrum (DMSO-d_6) of the Lactone from the Alkaline Hydrolysis of Dihydro-azadirachtin

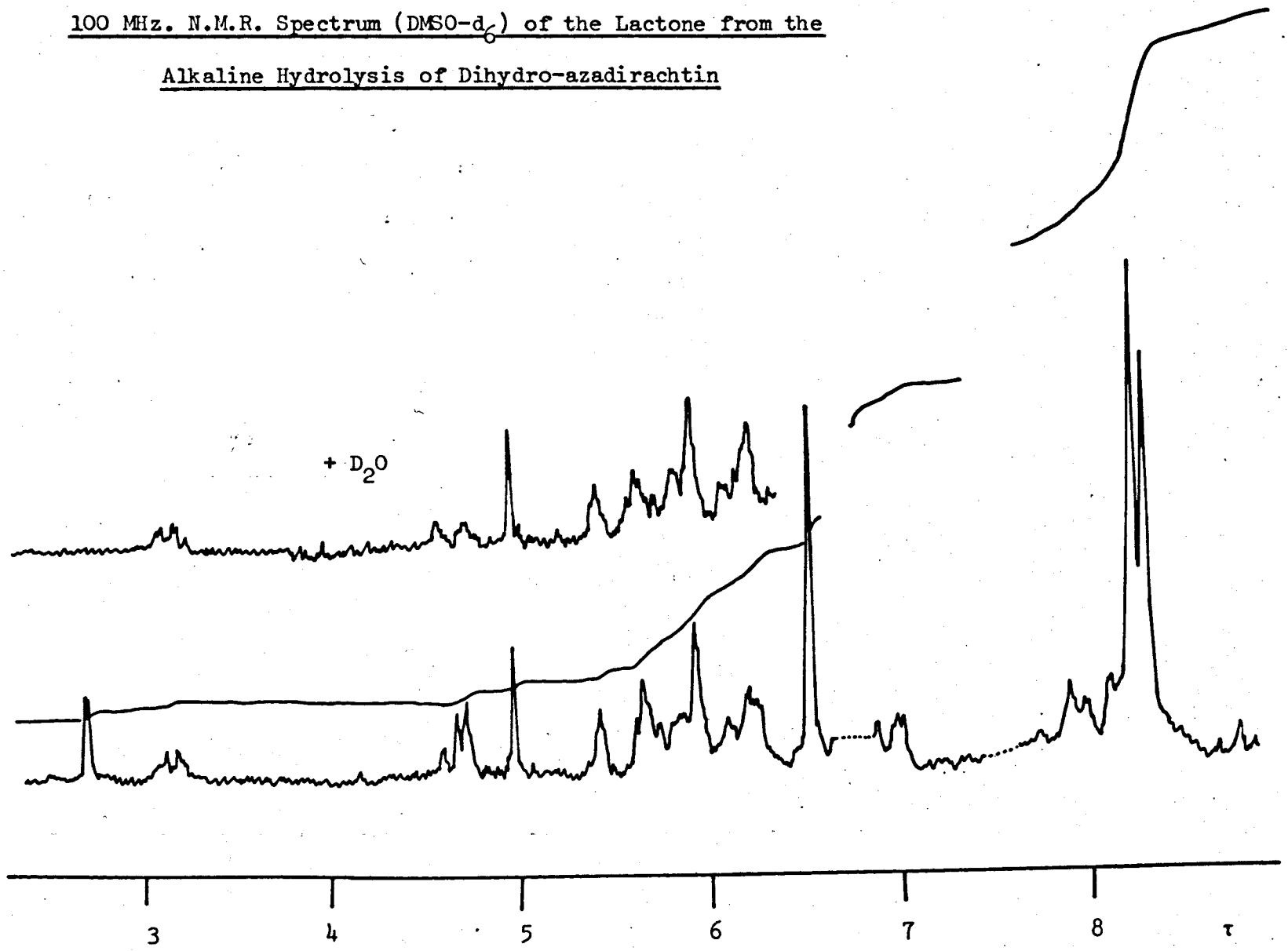


Fig. 19.

100 MHz. N.M.R. Spectrum ($CDCl_3$) of Material I

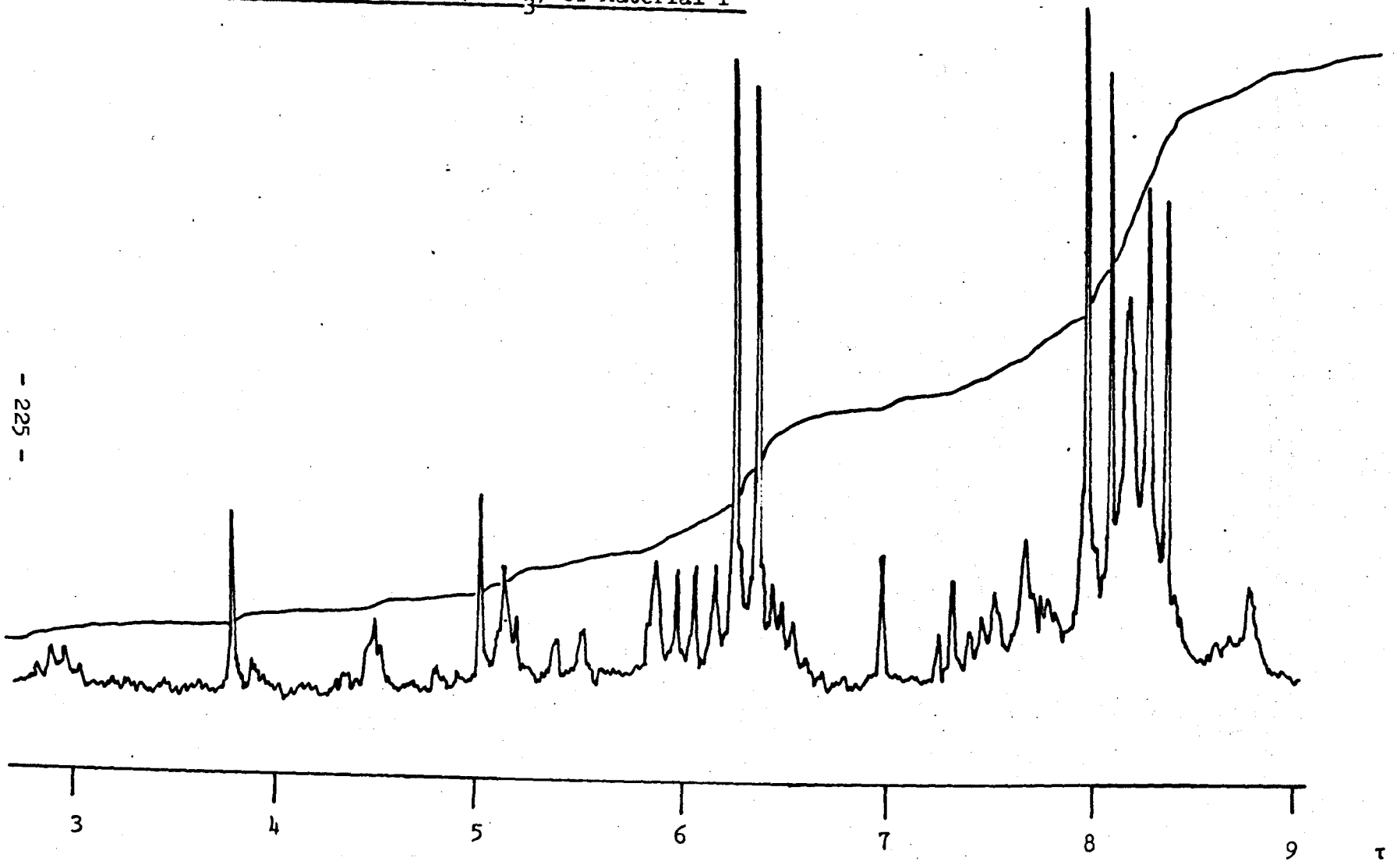


Fig. 20.

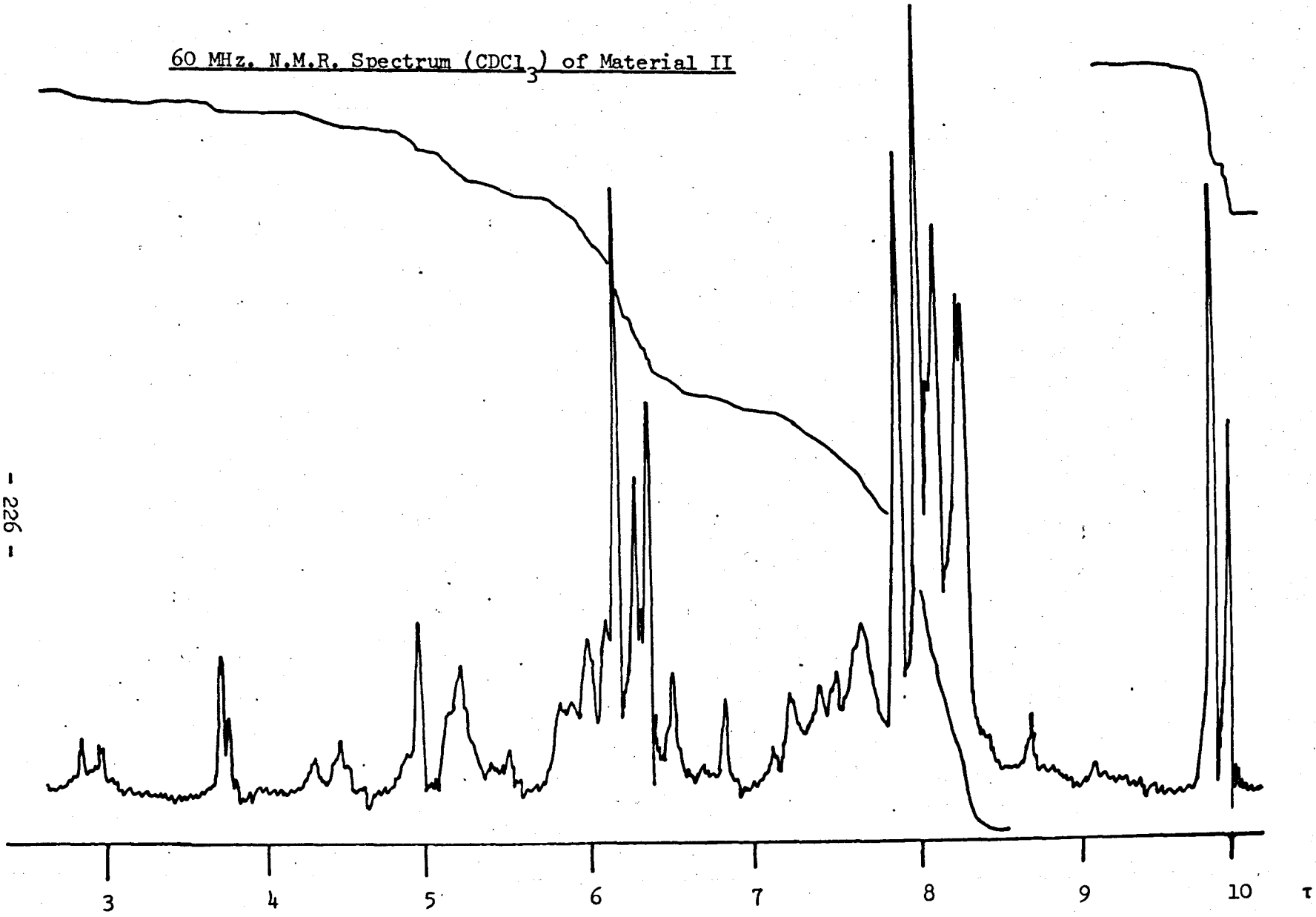


Fig. 21.

100 MHz. N.M.R. Spectrum ($CDCl_3$) of

Material III

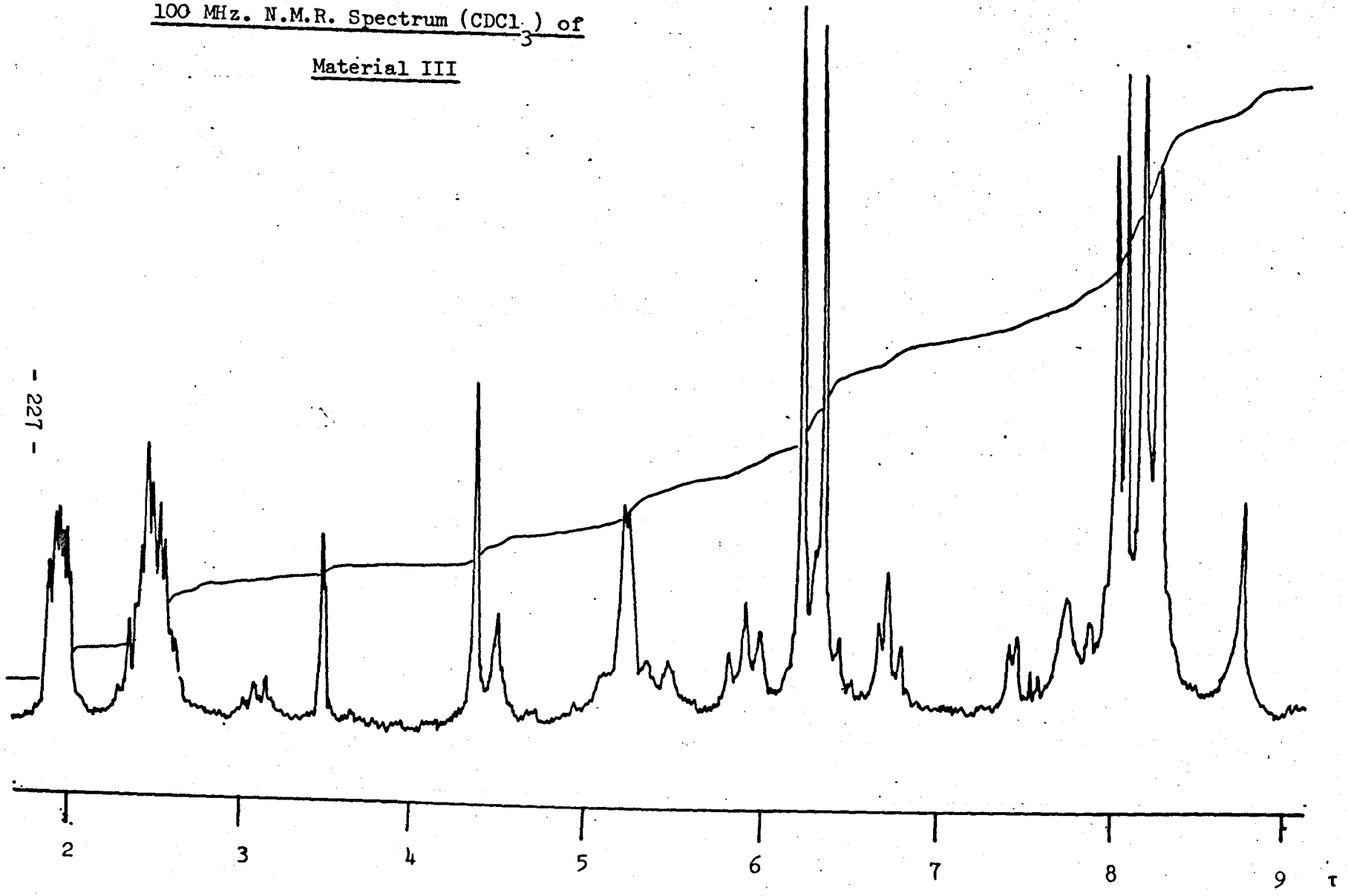


Fig. 22.

60 MHz. N.M.R. Spectrum ($CDCl_3$) of Material IV

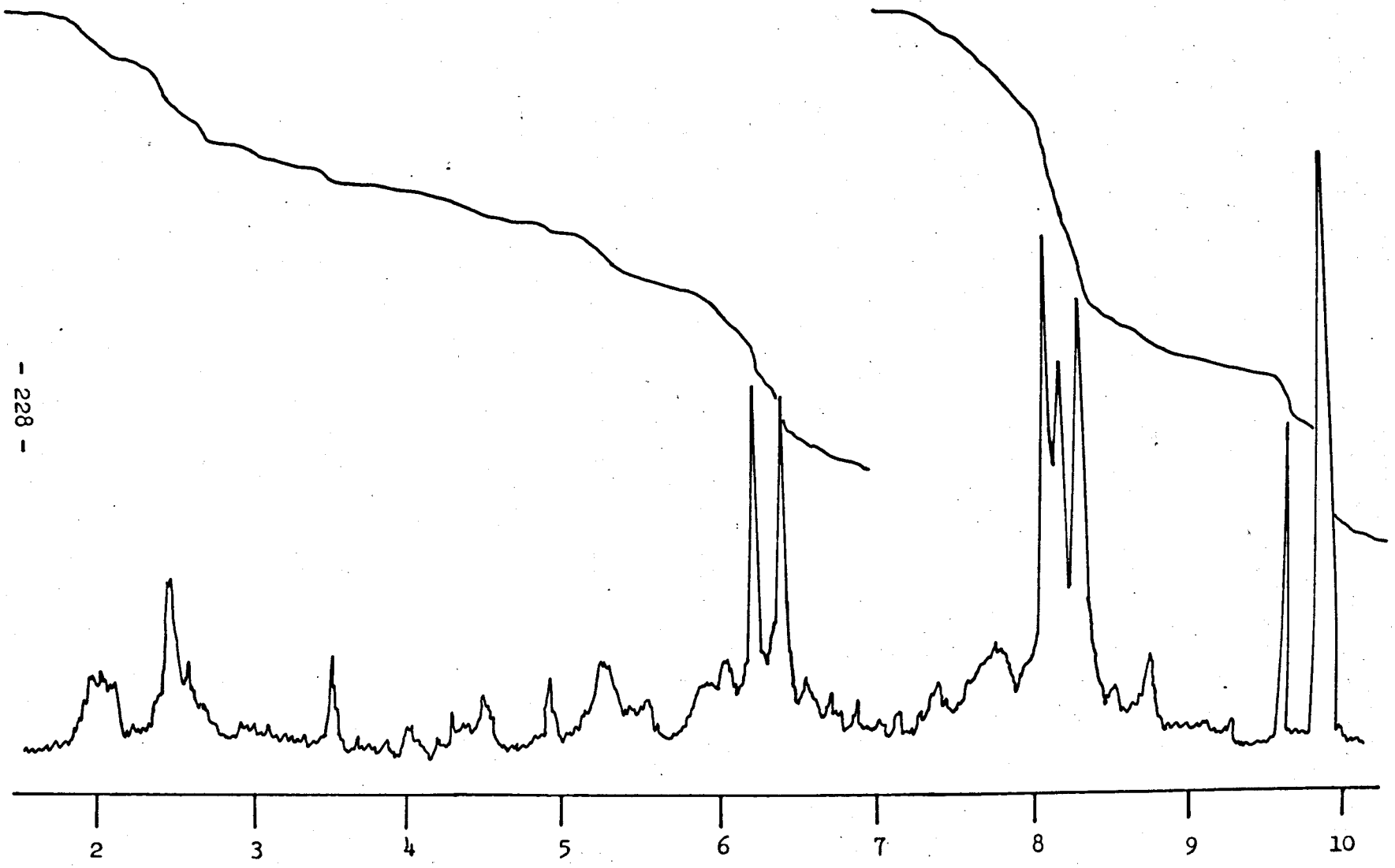


Fig. 23.

O.R.D. of Azadirachtin in Ethanol
(1 mg./ml.)

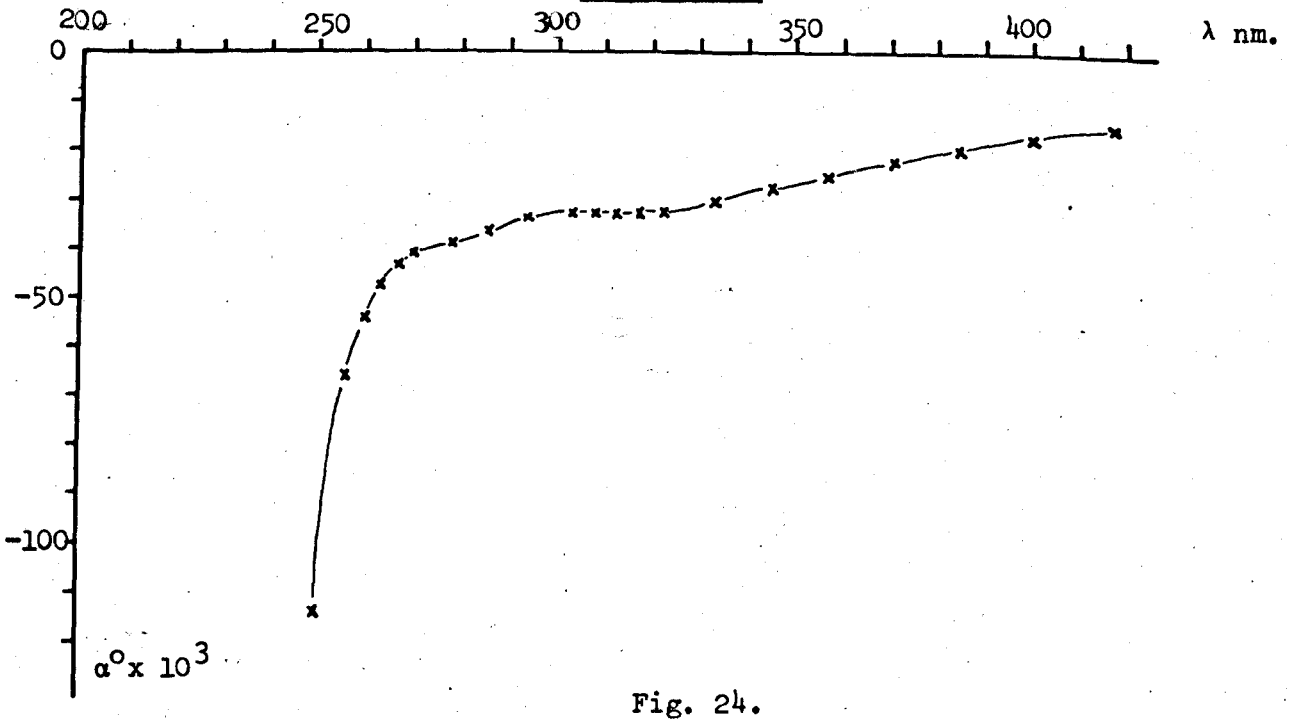


Fig. 24.

O.R.D. of Azadirachtin in Tetrahydrofuran
(1 mg./ml.)

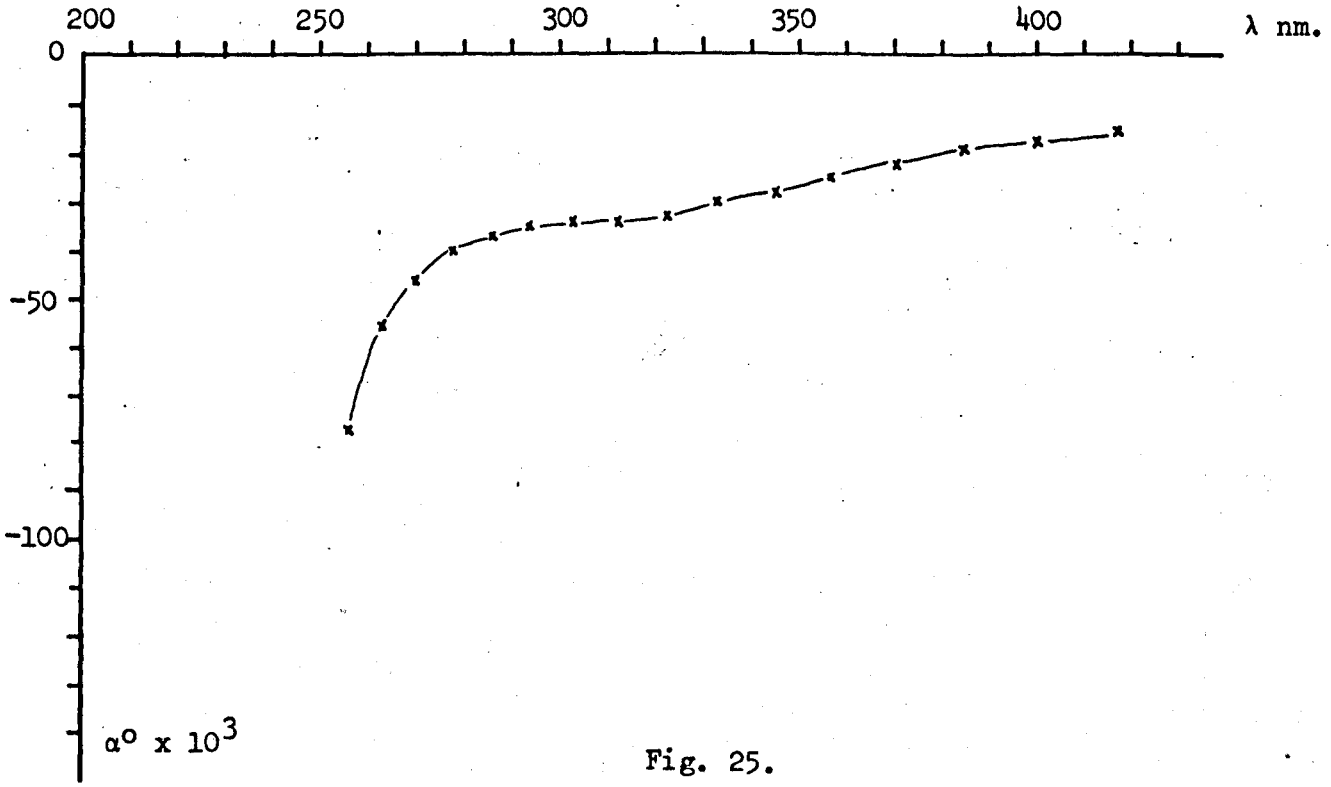


Fig. 25.

C.D. of Azadirachtin in Ethanol
(1 mg./ml.)

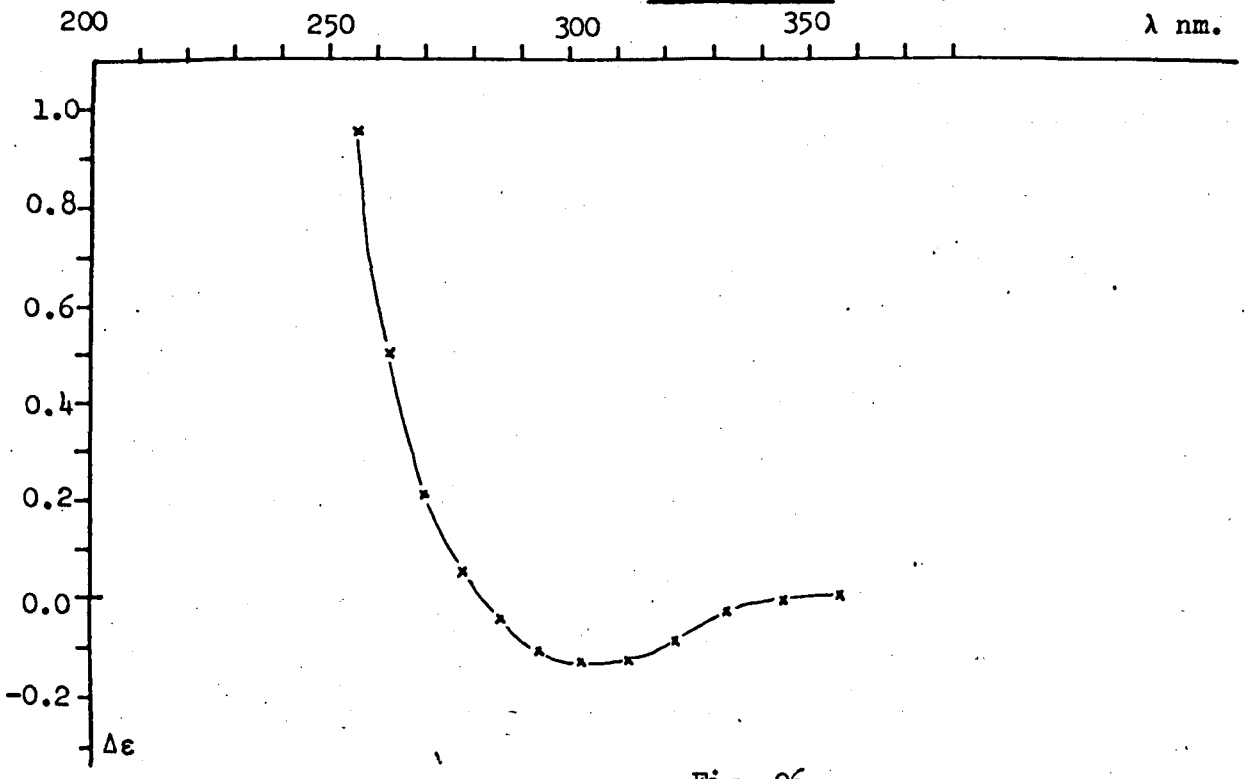


Fig. 26.

C.D. of Azadirachtin in Ethanol
(concentration unknown)

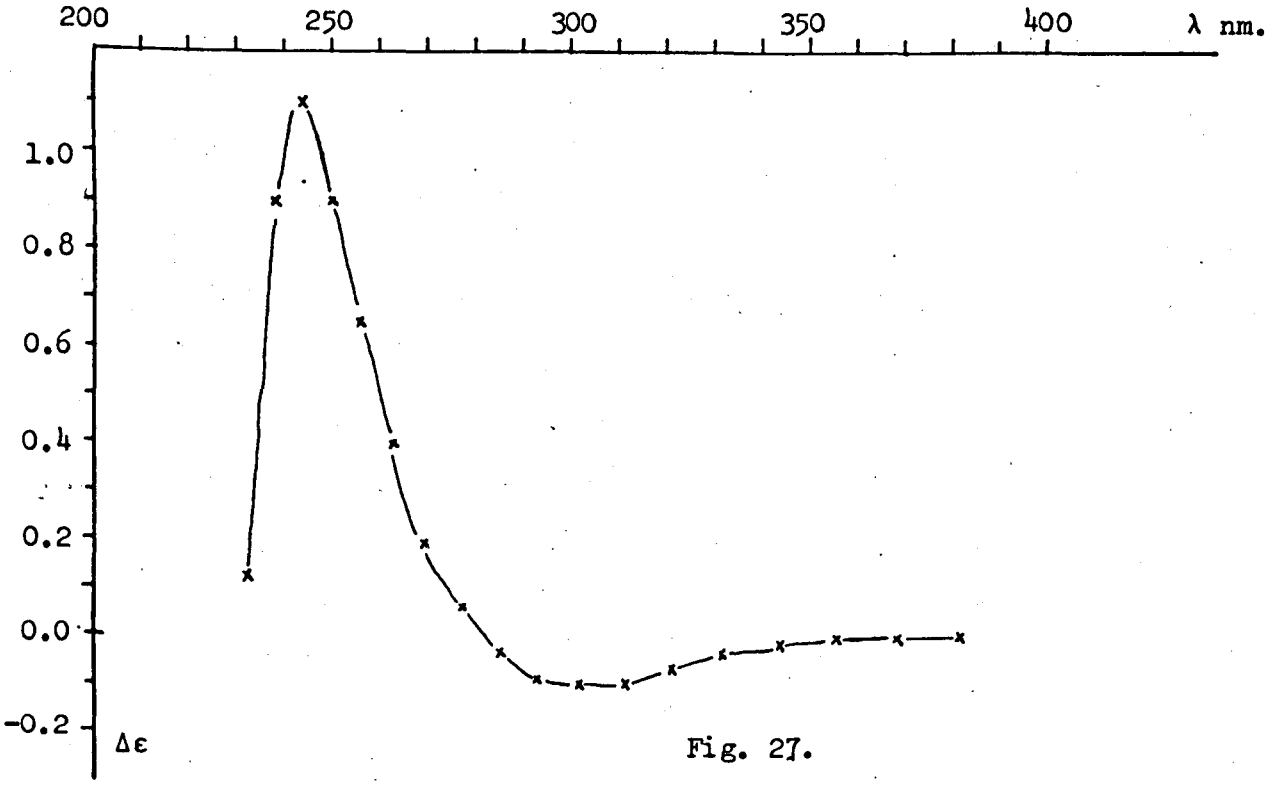


Fig. 27.

G.L.C. Trace for the Product from Selenium Dehydrogenation of L.A.H. Reduced Azadirachtin

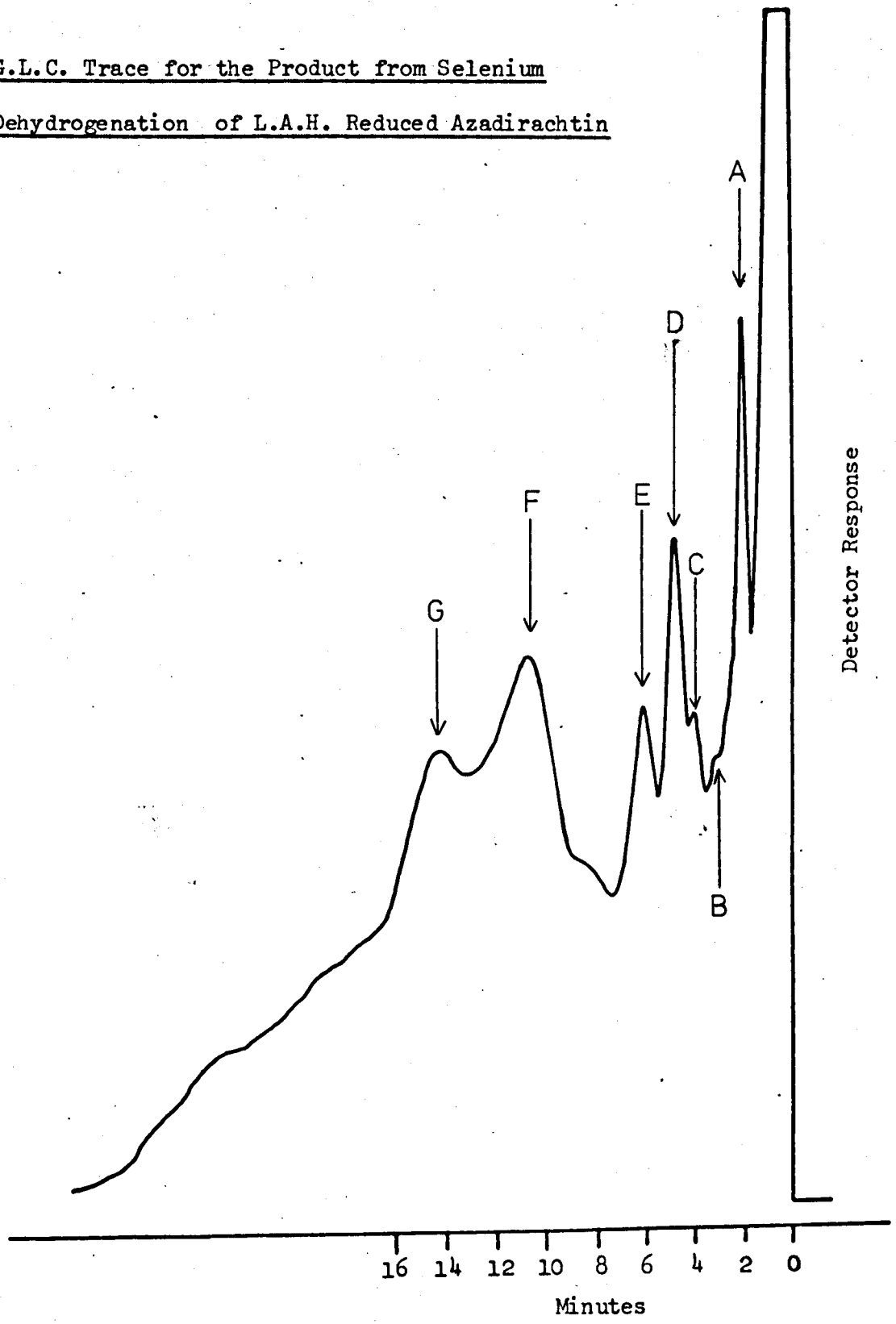


Fig. 28.

Mass Spectrum of Material from Peak E

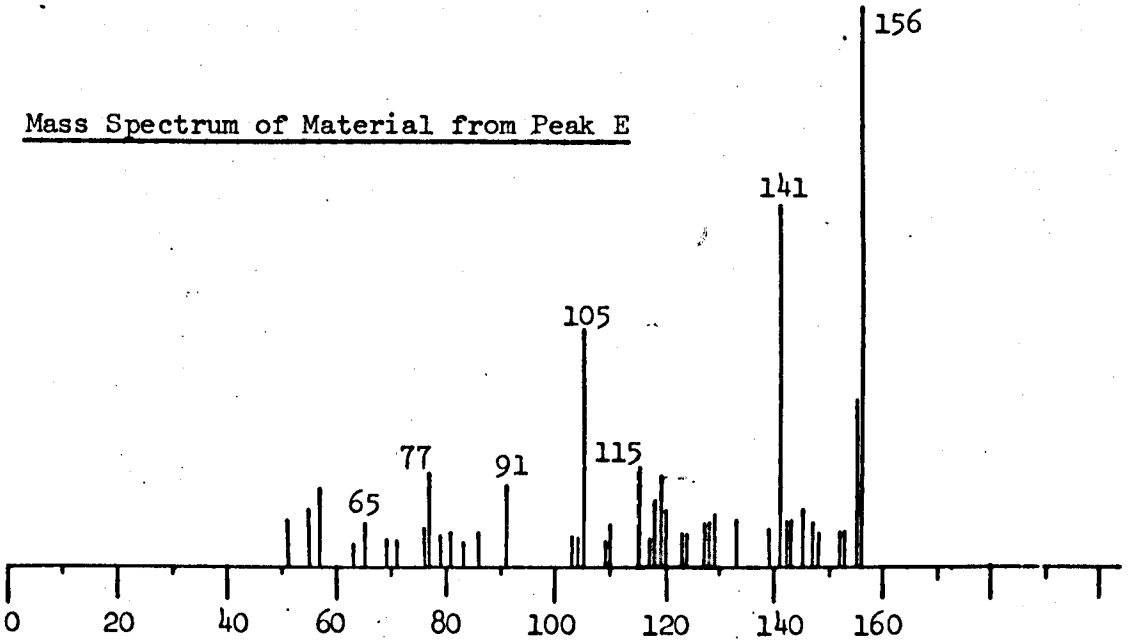


Fig. 29.

Mass Spectrum of Material from Peak F

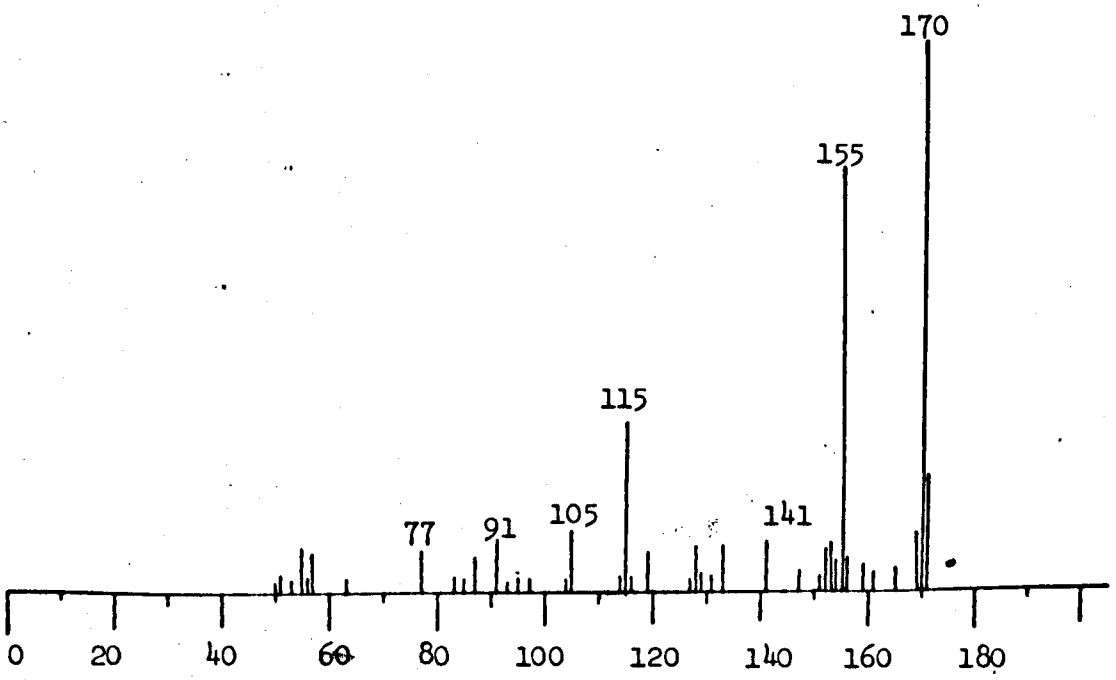


Fig. 30.

Mass Spectrum of Material from Peak G

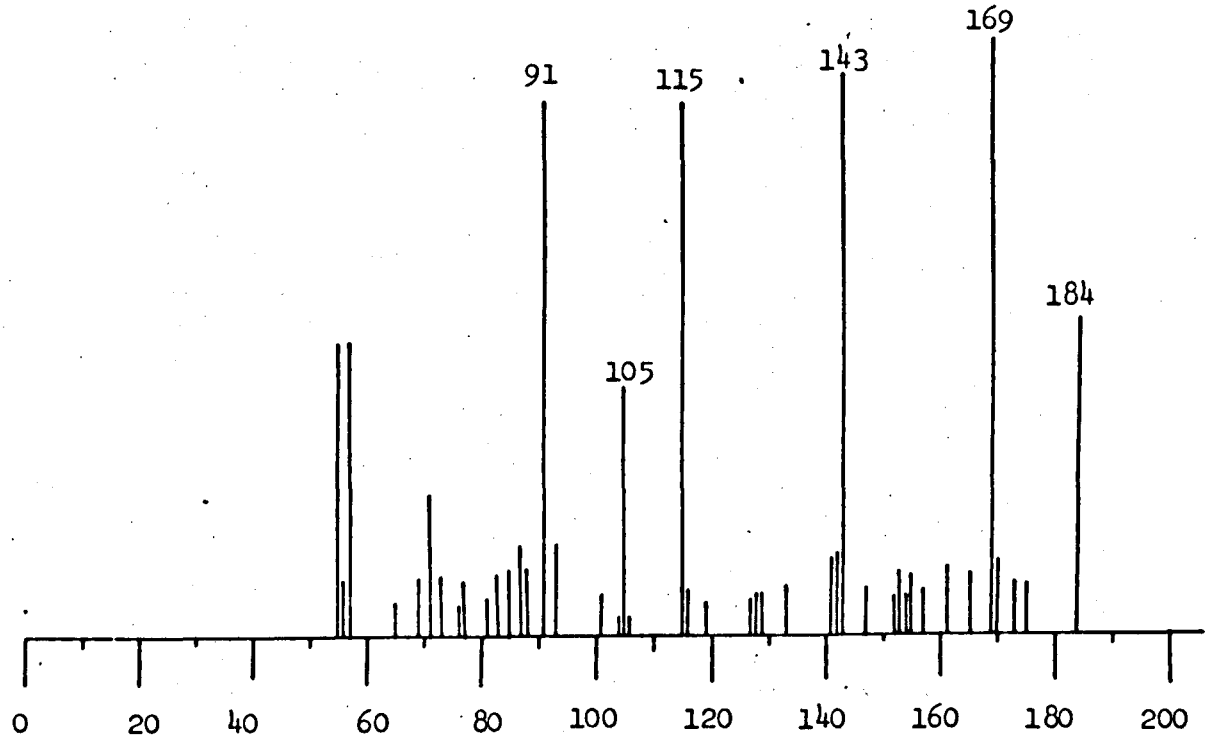


Fig. 31.

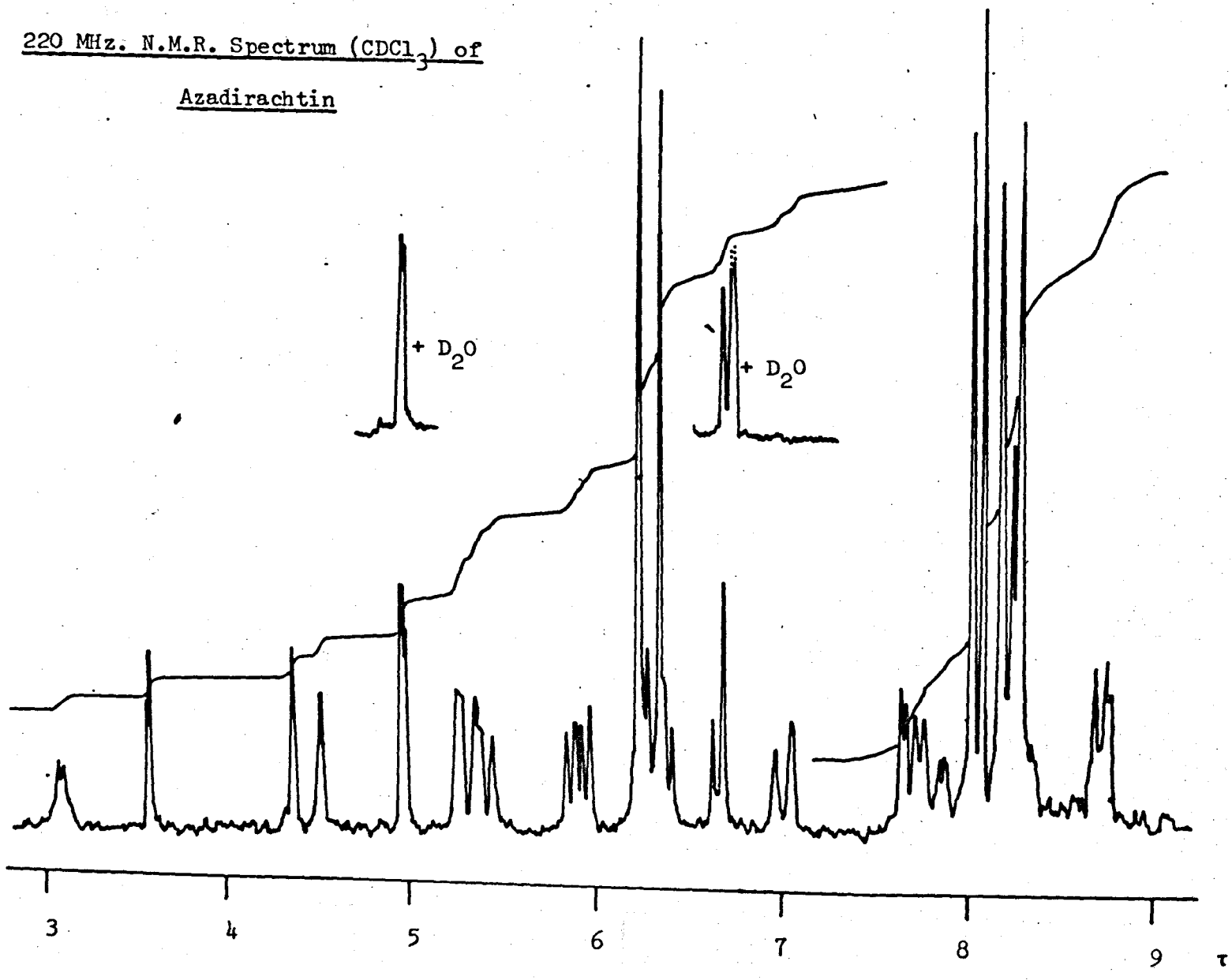

220 MHz. N.M.R. Spectrum ($CDCl_3$) of
Azadirachtin
+ D_2O
+ D_2O
3
4
5
6
7
8
9
τ

Fig. 32.

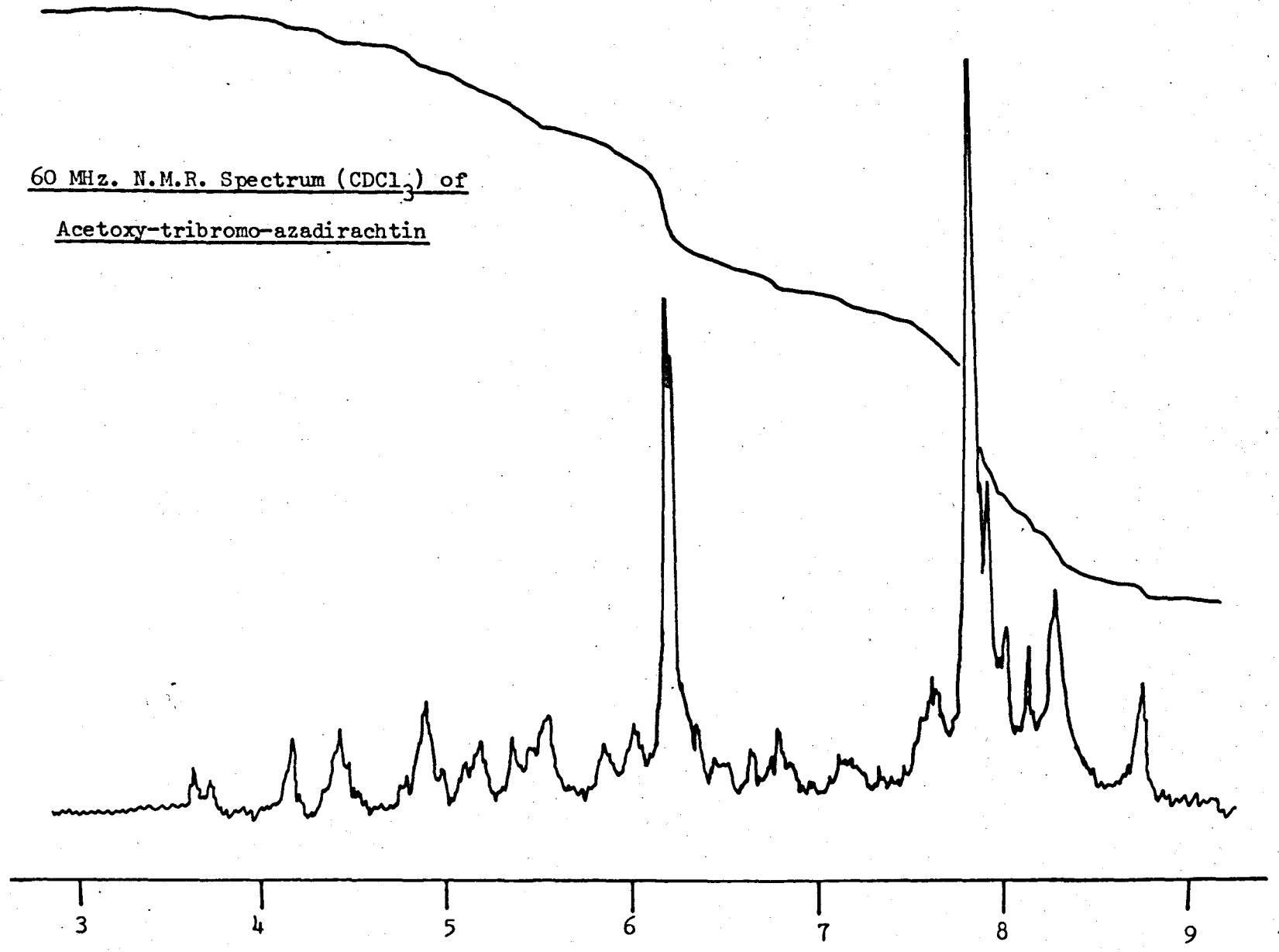

60 MHz. N.M.R. Spectrum ($CDCl_3$) of
Acetoxy-tribromo-azadirachtin
3
4
5
6
7
8
9
τ

Fig. 33.

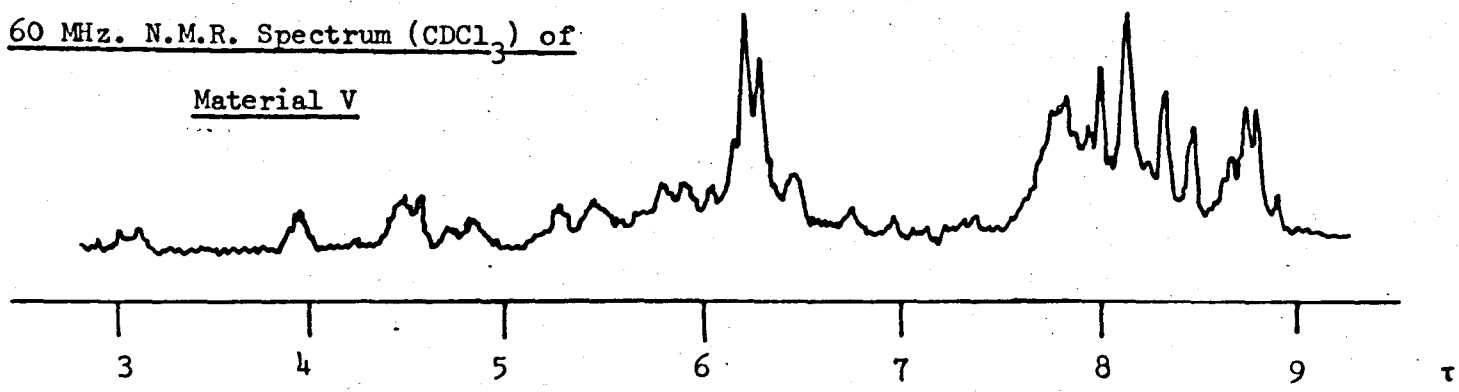

60 MHz. N.M.R. Spectrum ($CDCl_3$) of
Material V
3
4
5
6
7
8
9
τ

Fig. 34.

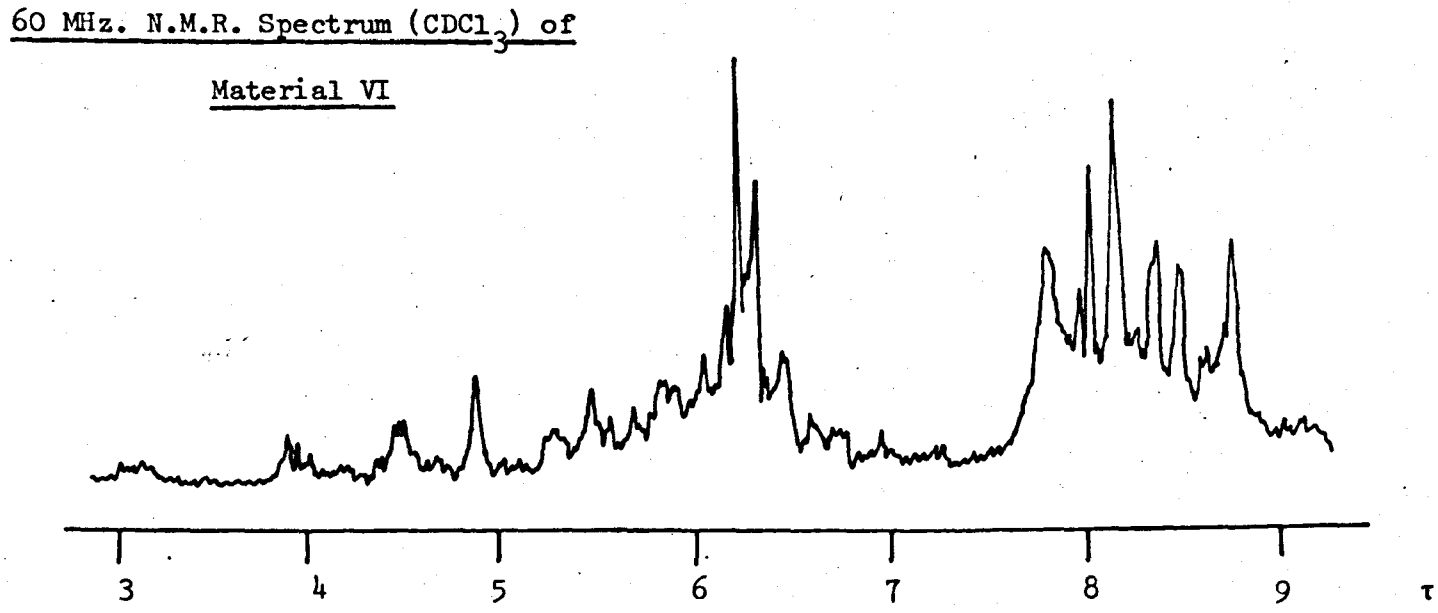

60 MHz. N.M.R. Spectrum ($CDCl_3$) of
Material VI
3
4
5
6
7
8
9
τ

Fig. 35.

60 MHz. N.M.R. Spectrum ($CDCl_3$) of Material VII

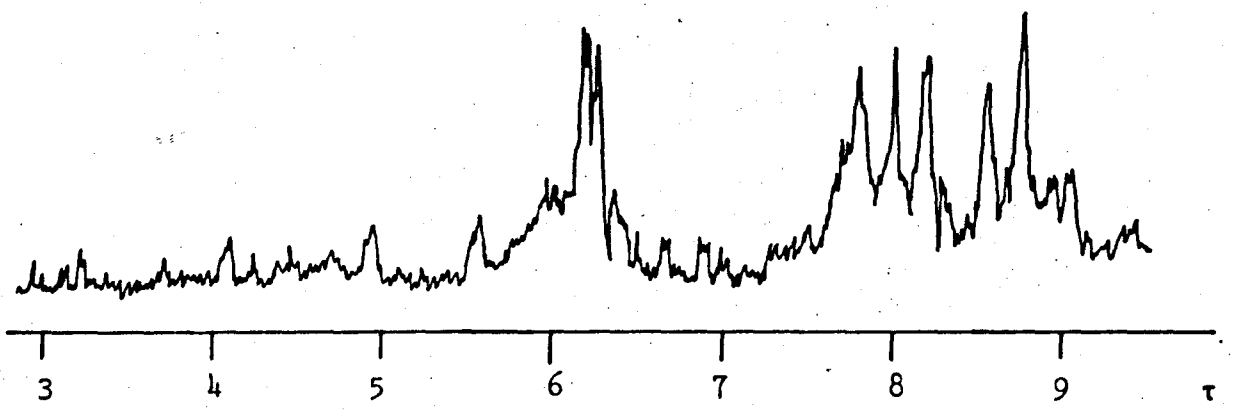
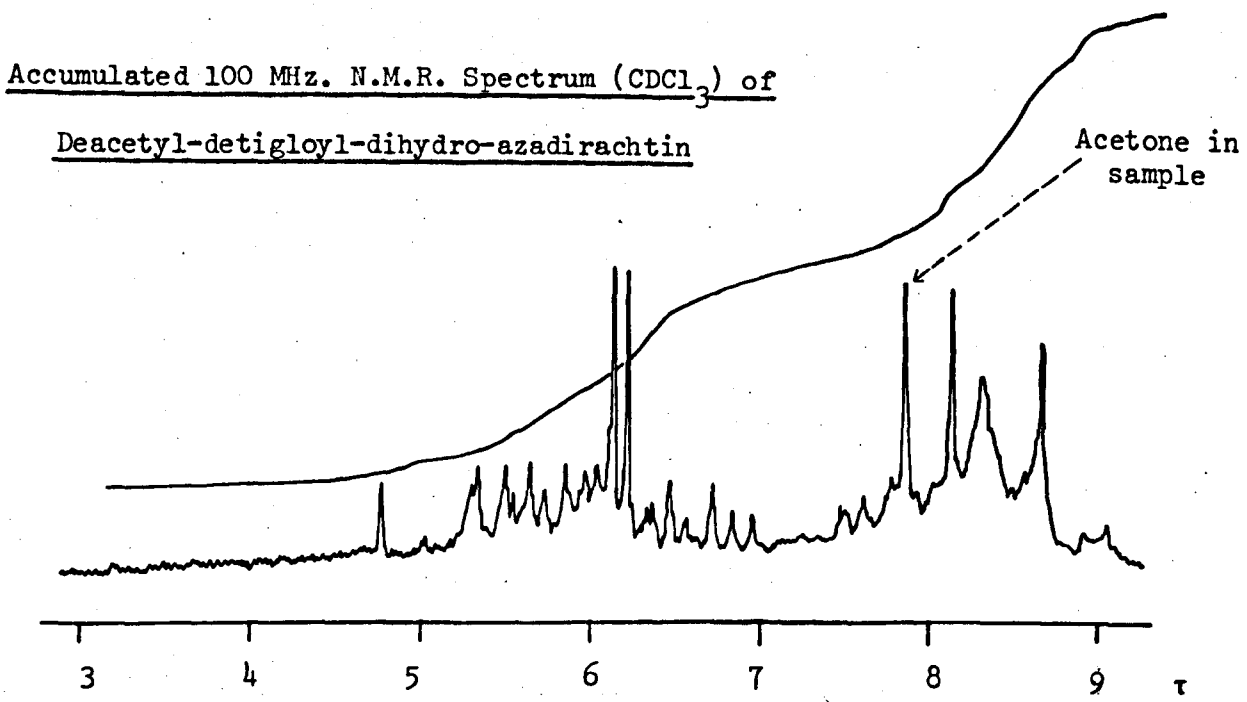
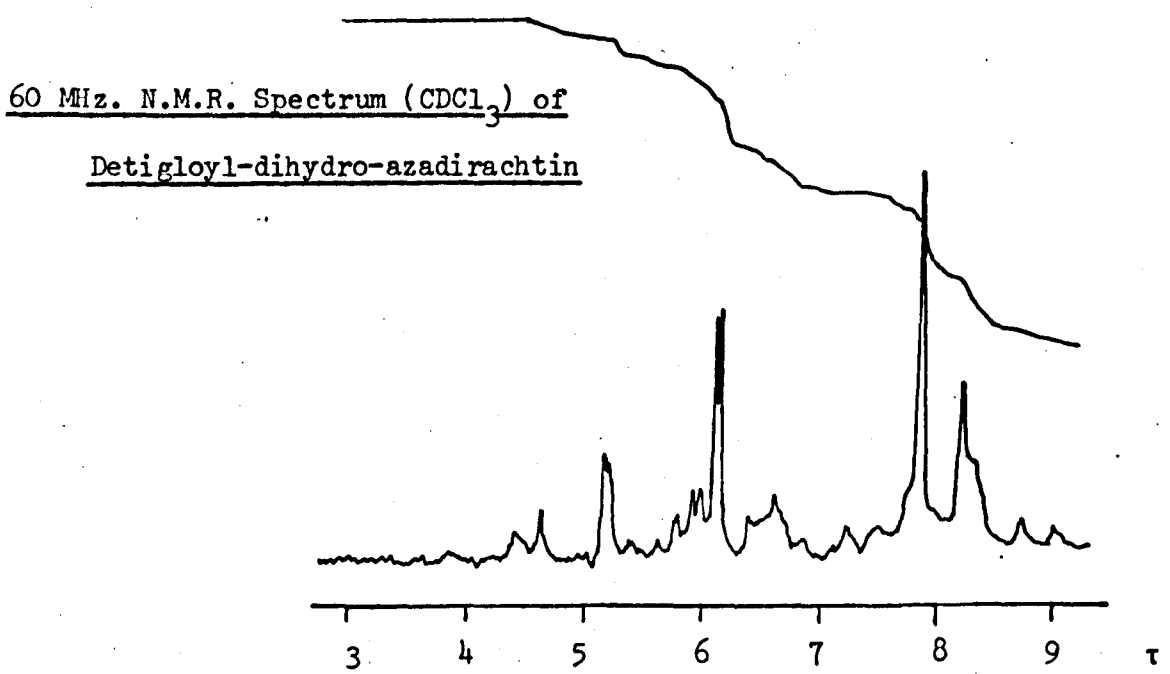


Fig. 36



Accumulated 100 MHz. N.M.R. Spectrum ($CDCl_3$) of Deacetyl-detigloyl-dihydro-azadirachtin

Fig. 37.



60 MHz. N.M.R. Spectrum ($CDCl_3$) of Detigloyl-dihydro-azadirachtin

Fig. 38.

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INDEX OF STRUCTURE DIAGRAMS