CHAPTER: IV

BIOLOGICAL STUDIES OF AZADIRACHTIN AND IT'S DERIVATIVES AGAINST POLYPHAGOUS PEST *SPODOPTERA LITURA*

IV.1 ABSTRACT

In the present study, Azadirachtin-A (Aza-A) was subjected to a variety of synthetic transformations.

Thus, Aza-A was converted into a 22,23-dihydro-11-methoxy derivative **3** (Scheme: IV.1) via selective methylation (MeI-Ag_2O) followed by hydrogenation (H_2, Pd/C). This compound was then transformed into a decalin derivative **5** via a base-initiated fragmentation, which was subsequently converted into an α,β-enone decalin derivative **8** through a series of reactions. The structures of these compounds were confirmed by spectral as well as analytical data.

A probable mechanism for the base-initiated fragmentation of the diketocarbonate into a decalin fragment and a spiroketal moiety has been proposed.

The antifeedant activity and toxicity of the azadirachtin derivatives were assessed against the second instar larvae of polyphagous pest *Spodoptera litura* on castor leaves in comparison with crude material containing 9.14 % Aza-A as well as relatively pure 91 % Aza-A.

The present study indicates that the combination of toxicity and antifeedant activity of Aza-A provides good crop protection.

IV.2 INTRODUCTION AND OBJECTIVES

The army worm *Spodoptera litura*. Fabricius is one of the most devastating insect species, which attacks and damages almost all important crops leading to total crop loss.[1] Current control is achieved by spraying infested fields with synthetic insecticides, which has led to a number of problems such as toxicity to non target organisms, increased insect resistance, ecological imbalance, hazard

to pesticide appliers and environmental contamination with the potential of affecting the entire food chains.[2,3]

For these reasons, discovery of target specific methods of insect control based on plant source has been the major goal of research world wide in the last decade.[4]

Plants have evolved highly elaborate chemical defenses against insect attack and provide biologically active compounds based on which the search for an effective insect antifeedant can be focussed.[5,6]

Munakata [5] has defined 'an insect antifeedant as a substance which inhibits feeding but does not kill the insect directly, insect often remaining near to the treated plant and possibly dying through starvation'. This mode of action is in direct contrast to more traditional pesticides and insecticides, which display a rapid outright kill.[7]

Aza-A is one of the isomers forming a major constituent in the neem seed extracts showing high insecticidal activity.[8-10] It is shown to be insect antifeedant, antimalarial and insect growth inhibitor amongst many other biological activities.[11-15]

Due to these factors Aza-A is considered to be an ideal bio-pesticide and enjoys a prime place in integrated pest management programmes.[8]

Pradhan *et al* in 1962 first reported the antifeedant effect of neem seed extracts against locusts.[12] Since then, neem extracts have been found to affect nearly 200 insect species belonging to different orders.[16]

Butterworth and Morgan in 1971 showed that the azadirachtin was a very potent antifeedant against *Schistocerca gregaria*.[17] Its ability to act as a growth inhibitor was described by Schmutterer and

Rembold.[18] Following these studies different groups have reported the effects of azadirachtin and many of its structural analogues on various insect species belonging to different orders and the biological properties of the structurally simpler compounds have been far less systematically studied.[19-22,7]

Azadirachtin shows three specific modes of action in insects as reviewed by Mordue and Blackwell.[11] First, it has strong antifeedant activity due to its effects on chemoreceptors. Second, it affects ecdysteroid and juvenile hormone titers through a blockage of morphogenetic peptide hormone release, resulting in severe growth and molting aberrations. Third, it has direct detrimental and histo-pathological effects on most insect tissues, for example muscles, fat body and gut cuticular epithelial cells. Additionally, azadirachtin is systemic in plants through translocation to the leaves and growing parts thus providing an enhanced element of protection. The fact that azadirachtin shows a multitude of biological activity against insects suggests that resistance problems might also be reduced.[23]

Azadirachtin as an antifeedant has potential for pest management, however it is sensitive to sunlight. Therefore its full potential to be realized in the field, careful formulation is required. Incorporation into neem oil gives a degree of protection of the azadirachtin molecule.[24] Alternatively modifications of the azadirachtin molecule may significantly increase its stability. For example, hydrogenation of the C_{22}-C_{23} double bond of the dihydrofuran ring yields dihydro-azadirachtin, a more stable compound with greater potential for field use.[22] This compound is as active as azadirachtin in feeding trials with four economically important lepidopteran species[25] and with *Epilachna Varivestis* and *Locusta migratoria*.[26] It is however,

significantly less active than azadirachtin against the locust *Schistocerca gregaria*.[11]

Yamasaki and Klocke have prepared eight derivatives of azadirachtin and bioassayed them for their growth inhibitory and lethal activities against the agricultural pest *Heliothis vireseens*. The results indicated that the hydroxyl groups on azadirachtin are essential for the activity and that for maximum activity, the molecule must also have a lipophilic region possibly for transport phenomena.[22]

Blaney *et al*[13] assessed the antifeedant activity of azadirachtin, its derivatives and related limonoids in choice and no-choice bioassays against four species of lepidoptera namely *Spodoptera littoralis*, *Spodoptera frugiperda*, *Heliothis virescens* and *Heliothis armigera*. Azadirachtin and dihydroazadirachtin were the most potent of the forty compounds tested. The results showed that hydrogenation of the C_{22}-C_{23} double bond did not decrease the antifeedant activity and nature of the substituents at C_1, C_3 and C_{11} were important.[13] In addition, same group has also studied the effect of azadirachtin and twenty three other related compounds against *S. littoralis*, *S. frugiperda* and *H. virescens* in three different bioassay methods, such as oral cannulation, haemolymph injection and topical application. Overall, azadirachtin, 22,23-dihydroazadirachtin and 1-detigloyl-22,23-dihydroazadirachtin were the most active compounds. Generally, larvae were less sensitive to compounds when they were topically applied than when they were cannulated into the gut or injected into the haemolymph.[14]

Jacobson studied the importance of tiglic ester group in azadirachtin and other steroids and found that tiglic esters of cholesteroles/sitosterols are inactive antifeedant.[21] Jones *et al* reported that the azadirachtin and its derivatives, block the development of the

male malarial gamete *in vitro* in *Plasmodium berghei*. These findings suggest the possibility of developing an azadirachtin-based compound as an antimalarial.[27]

Over the past few years an extensive study has been carried out by Ley and his group towards the total synthesis of azadirachtin and preparation of various derivatives in order to find simple analogues that display similar biological activity.[7] In addition, a number of authors have reported different derivatives of azadirachtin and studied their structure activity relationships against different pests.[14,22,25]

Thus, Butterworth *et al* and other groups of worker have reported the formation of tertiary acetate on treatment of azadirachtin with acetic anhydride.[28] They have also prepared both bis and tris-trimethyl silylethers of azadirachtin for molecular weight determination.

Azadirachtin has been successfully alkylated using iodomethane, initially reported by Yamasaki and Klocke[22] as low yielding and difficult reaction. However, Ley *et al*[29] have slightly modified this procedure to allow the efficient C_{11}-OH methylation. In parallel with acylation and silylation, prolonged treatment affords 11,20-dimethoxyazadirachtin.[30]

Morgan and Butterworth[28] found azadirachtin to be resistant to hydrogenation under a variety of conditions. Successful conversion was accomplished with Adam's catalyst (PtO_2) at 50 lb in^{-2} by Yamasaki and Klocke.[22] Ley *et al* have found that azadirachtin is selectively reduced using palladium on charcoal to either 22,23-dihydroazadirachtin or 2',3', 22,23-tetrahydroazadirachtin depending on the duration of the reactions.[30,31]

Morgan and Butterworth[28] failed to oxidize azadirachtin using Cornforth's reagent. Ley *et al*[30] suggested that the resistance of the C_7-OH group of azadirachtin towards oxidation might be attributed to shielding of this functional group by the right hand side of the molecule, which is held in place by a network of hydrogen bonds.

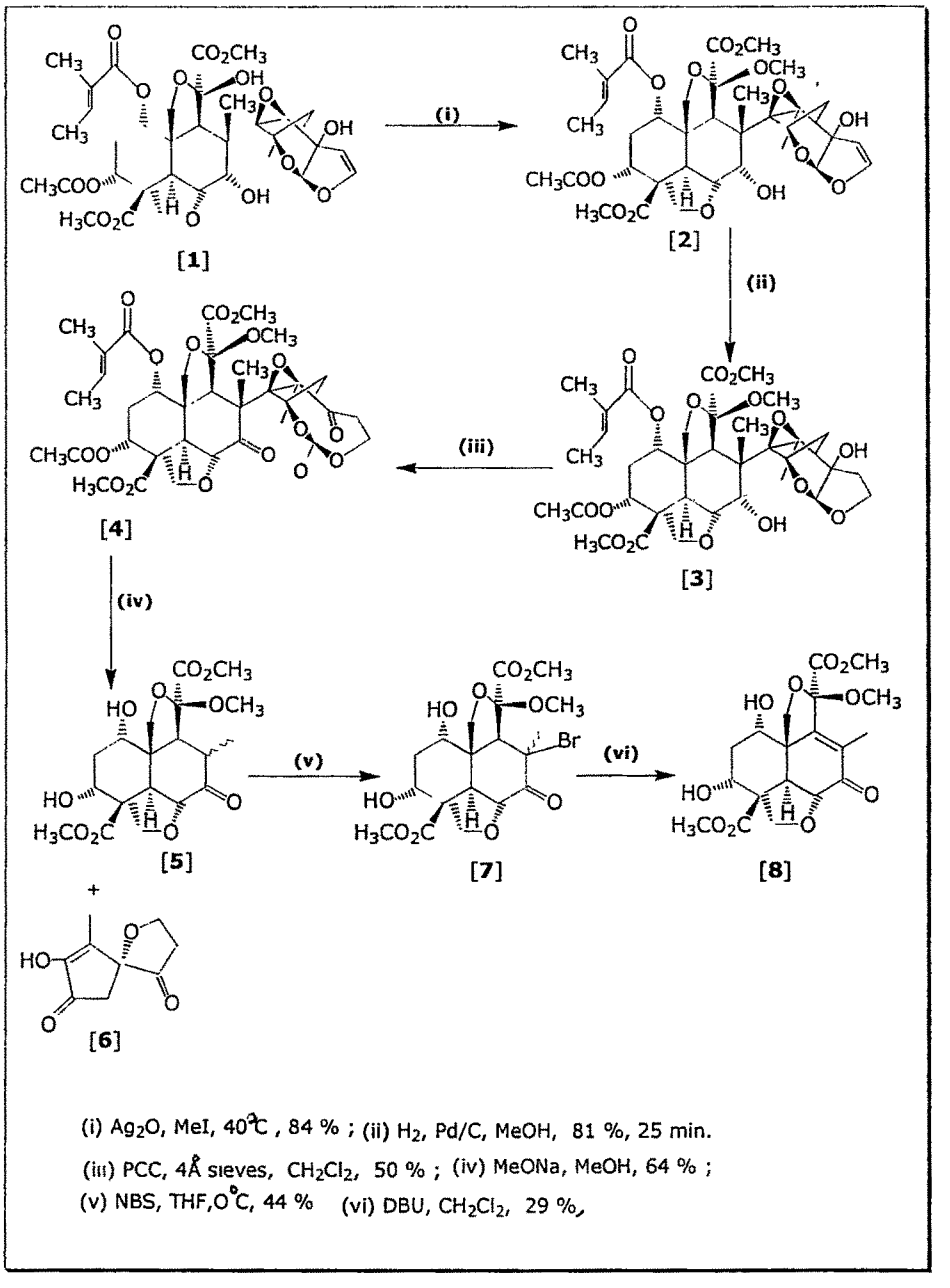
These groups have reported a pyridinium chlorochromate mediated oxidation of 22,23-dihydro product giving an unusual cyclic diketocarbonate.[29,30] Further treatment of the diketocarbonate with sodium methoxide in methanol caused a retro-aldol reaction to take place, giving the decalin **5** and an unusual spiro cyclic compound.[32]

The structure activity studies showed that the decalin and dihydrofuran acetal fragment of azadirachtin individually have insect antiifeedant property.[33]

Blaney *et al*[33] found that decalin and dihydrofuran acetal fragments exhibited antifeedant activity against larvae of *S. llittrolis*. These results indicated that the compounds were more active when tested in combinations than when tested singly. Although some of these combinations did show significant levels of antifeedant activity, some times coupled with a synergistic effect, they were not as active as either azadirachtin or dihydroazadirachtin.[33]

It is known that, the decalin portion of the azadirachtin itself has some antifeedant effect and changes to the functional groups present on the decalin fragment of the azadirachtin molecule, can alter the antifeedant activity of the molecule.[33-35]

In view of these observations, we focused our attention on functional group modification of decalin fragment and study the bioactivity of the various products. Our studies in this area are discussed in the following sections.



Scheme: IV.1 Preparation of Derivatives of Aza-A

IV.3 RESULTS AND DISCUSSION

In view of the above discussion Aza-A was converted into a decalin fragment **5** and spiroketal derivative **6** (Scheme: IV: 1) following the reported method of Ley *et al.*[32] The decalin fragment was then converted into α-bromodecalin derivative **7**, which was dehydro-brominated using 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) to give the corresponding α,β- enone derivative **8.**

We then examined the feeding response to crude azadirachtin containing material as well as pure materials and its derivatives of the polyphagous pest *Spodoptera litura*.

We also propose herein a probable mechanism of the base-initiated fragmentation of the diketocarbonate **4** into the decalin fragment **5** and spiroketal derivative **6.**

Thus, we have methylated Aza-A **1** (500 mg, 0.69 mmol) using methyl iodide (32 mL, excess) in presence of silver oxide (800 mg, 3.46 mmol, 5 equiv. excess), which after usual work up and chromatography gave 11-methoxyazadirachtin **2** in 84 % yield. All the physical and spectral data of the compound **2** were in excellent agreement with those reported in the literature.[30,32]

The IR spectrum of the compound **2** showed an absorption band at 1741 cm^{-1} diagnostic of a carbonyl group along with a strong band at 2854 cm^{-1} for the presence of methoxy group. Among the main features observed in the PMR spectrum of the compound **2** (Figure: IV.1) were the signals at δ_H 6.93 (dq, 1H at C_3'), 5.67 (s, 1H at C_{21}) 4.75 (dd, 1H at C_1), whereas the signal 3.79 (s, 3H) and 3.68 (s, 3H) for acetate methyls. The peak due to methoxy protons was observed at 3.32 (s, 3H at C_{11}).

11-Methoxyazadirachtin **2** (400 mg, 0.542 mmol) was selectively hydrogenated in methanol over palladium on charcoal (Pd/C, H_2) for 30 minutes, furnishing 22,23-dihydro-11-methoxyazadirachtin **3** in 81 % yield. The physical and analytical data of the compound **3** were also in good agreement with those reported in the literature.[30,32]

The IR spectrum of the compound **3** showed a band at 3444 cm^{-1} for the presence of intramolecular hydrogen bond and 1648 cm^{-1} for the presence of olefinic linkage. It also exhibited the band at 1738 cm^{-1} for the ester group and 1438 cm^{-1} for the $COCH_3$ group.

The PMR spectrum of the compound **3** (Figure: IV.2) displayed signals at δ_H 6.94 (dq, 1H at C_3'), 5.5 (s, 1H, at C_{21}) 3.91 (m, 1H at C_{23}) and 2.10 (m, 1H at C_{22}).

The hydrogenated product **3** (300 mg, 0.407 mmol) was oxidized at C_7 and C_{20} position using pyridinium chlorochromatic[36] (PCC) (876 mg, 0.40 mmol, 10 equiv.) in dichloromethane containing molecular sieves (4 A°) (1g) for 48 hours at room temperature, to obtain the corresponding diketocarbonate **4** in 55 % yield after usual work up and chromatography.

The compound **4** also gave matching spectral and analytical characteristics with those reported in the literature.[30,32] It showed a strong band at 2856 cm^{-1} for the presence of methoxy group in addition to the characteristic carbonyl doublets at 1620 and 1570 cm^{-1} in the IR spectrum. Its PMR (Figure:IV.3) displayed signals at δ_H 6.67 (dq, 1H, at C_3'), 5.50 (d, 1H, at C_3) 5.20 (d, 1H, at C_6), 4.80 (t, 1H, at C_1) and 4.52 (ddd, 1H, at C_{23}). It further gave signal at 3.80 (s, 3H) and 3.72 (s, 3H) for acetate methyls, in addition 1.92 (s, 3H) for the acetyl methyls.

When compound **4** (100 mg, 0.012 mmol) was treated with sodium methoxide (120 µL, 0.680 mmol, 30 % w/w, 5 equiv.) in methanol (5

mL) at room temperature for 24 hours, to give the decalin fragment **5** (64 %) along with the spiroketal derivative **6** (50.82 %) after usual work-up and purification by column chromatography and preparative HPLC.

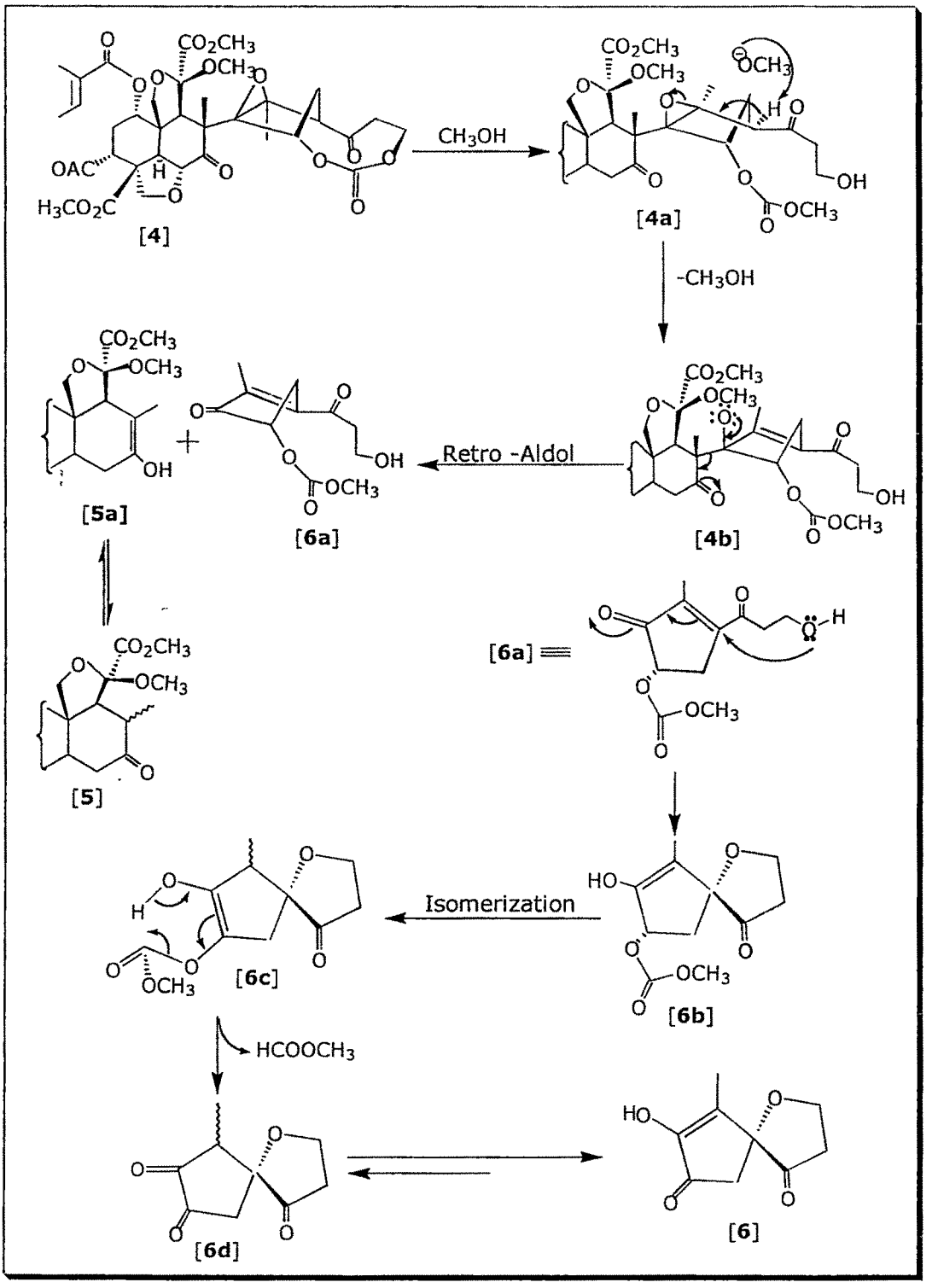
The elemental as well as spectral characteristics of the compounds **5** and **6** were in good agreement with those reported in the literature.[29,30,32]

The IR spectrum of the compound **5** showed a band at 3446 cm^{-1} diagnostic of a hydroxyl group and 1733 cm^{-1} for the presence of an ester as well as 1716 cm^{-1} for the presence of a carbonyl group. It also displayed a band at 1114 cm^{-1} for the 2° alcohol group. Its PMR spectrum (Figure:IV.4) gave signals at δ_H 4.87 (dd, 1H, at C_6), 4.40-4.46 (m, 1H, at C_3), 4.04-4.14 (m, 4H, at C_1, C_{19} and C_{28}), 3.80 (s, 3H) and 3.60 (s, 3H) for acetate methyls. It further gave signal 3.49 (s, 3H) for methoxy proton and 3.30 (d, 1H) for hydroxyl group.

The retro-aldol degradation sequence for the cleavage of C_8-C_{14} bond in Aza-A is shown in Scheme IV.2. This transformation involves C_{13}-C_{14} epoxide ring opening assisted by an abstraction of a proton on the bridgehead carbon C_{17} by the methoxide ion as shown in (**4a**, Scheme: IV.2). This is followed by the subsequent retro-aldol cleavage of C_8-C_{14} bond to furnish the cyclopentenone derivative [**6a**] along with the enol [**5a**], which tautomerizes to the decalin fragment [**5**].

The intra molecular Michael-type addition in [**6a**], then furnishes the spiro compound [**6b**], which after isomerization to [**6c**] followed by an intra molecular elimination perhaps via a six-membered transition state gives the spiro ketal [**6**], via [**6d**].

The decalin fragment **5** was initially brominated using pyridine hypo bromide per bromide[37,29] to give the α-bromodecalin derivative **7**.



Scheme: IV.2 Probable Mechanism for the Formation of 8-hydroxy-9-methyl-1-oxa-spiro [4,4]non-8-en-4,7-dione [6d]

However this reaction resulted in furnishing in low yield (19 %) of the compound **7**.

Hence, it was thought to employ N-bromo succinimide (NBS) for this purpose. Thus, the treatment of **5** (25 mg, 60.3 μmol) with NBS[38] (10.74 mg, 0.060 mmol, 1:1) in dry THF for 30 minutes (0-5°C) furnished the α-bromodecalin derivative **7** in (44 %) yield.

All the physical and spectral characteristics of the compound **7** were found in good agreement with those reported in literature.[32] The IR spectrum of the compound **7** showed a band at 3409, 2947, 1735, 1651, 1029 and 653 cm^{-1}. Its PMR spectrum (Figure: IV. 5) showed peaks at δ_H 4.96 (dd, 1H, at C_6), 4.76 (m, 1H, C_3), 4.15 (dd, 1H, C_1), 4.0 (d, 1H, CH_2 at C_{28}), 3.79 (d, 1H, CH_2 at C_{19}), 3.71 (s, 3H, $COOC\underline{H}_3$), 3.67 (d, 1H, CH_2 at C_{28}), 3.62 (s, 3H, $COOC\underline{H}_3$), 3.60 (d, 1H, CH_2 at C_{19}), 3.38 (br s, OH), 3.21 (s, 3H, $OC\underline{H}_3$ at C_{11}), 3.21 (s, 3H, $OC\underline{H}_3$ at C_{11}), 3.20 (s, 1H, C_9), 2.22 (m, 1H, CH_2 at C_2), 1.95 (s, 3H, $C\underline{H}_3$ at C_8) and 1.81 (dd, 1H, CH_2 at C_2).

The compound **7** (20 mg 0.04 mmol) was then treated with DBU[39](8 μL,0.048 mmol) in dichloromethane (10 mL) for 2 hours. The reaction mixture was washed with water to remove hypobromide salts and then combined organic extracts were washed with aqueous HCL (1 %, 5 mL) to remove the un-reacted DBU. The combined organic extracts were washed further with water and brine solution and dried over anhydrous sodium sulphate. The solvent was removed under vacuum and the residue was purified by the chromatography gave the α,β-enone decalin derivative **8** as white solid (29 %).

A stretch at 1683 cm^{-1} in IR (Figure: IV.6) and the disappearance of the signal due to C_9 proton in PMR spectrum (Figure: IV.7) indicated the formation of **8**.

PMR spectrum of the compound **8** displayed peaks at δ_H 5.01 (d, 1H, C_6), 4.72 (m, 1H, C_3), 4.59 (dd, 1H, C_1), 4.21 (d, 1H, CH_2 at C_{28}), 4.03 (d, 1H, CH_2 at C_{19}), 3.81 (s, 3H, $COOC\underline{H}_3$), 3.74 (d, 1H, CH_2 at C_{28}), 3.68 (s, 3H, $COOC\underline{H}_3$), 3.57 (d, 1H, CH_2 at C_{19}), 3.32 (br s, 1H, OH), 3.23 (s, 3H, $OC\underline{H}_3$ at C_{11}), 2.22 (m, 1H, CH_2 at C_2), 2.01 (ddd, 1H, CH_2 at C_2) and 1.98 (s, 3H, olefinic CH_3).

Results of the Biological Studies

Azadiradhtin is a classic example of a natural plant defense chemical affecting feeding, primarily through chemoreception, but also through a reduction in food intake, due to toxic effects if consumed. Attempts were now focused on checking the biological activities (in terms of antifeedant index and insect toxicity (LC_{50} value) of the crude material containing 9.14 % Aza-A, 91 % pure Aza-A and each of the derivatives (2-5, 7, 8, Scheme:IV.1) against polyphaous pest. For obtaining these data, second instar larvae of *Spodoptera litura* were used to assess the antifeedant activity and toxicity of our compounds.

The antifeedent activity of crude and pure materials and the derivatives (2-5, 7, 8) were evaluated by leaf discs bioassays. The data on antifeedent index at different concentrations (in ppm) of these materials are presented in Table:IV.1.

Azadirachtin and its derivatives generated a linear dose response curve (Figure: IV.8) with respect to percentage protection of leaves against feeding by second instar larvae of *S. litura*, with the increase in concentration of different materials under examination. There was a corresponding increase in antifeedent index up to 100 ppm, which remains almost constant thereafter.

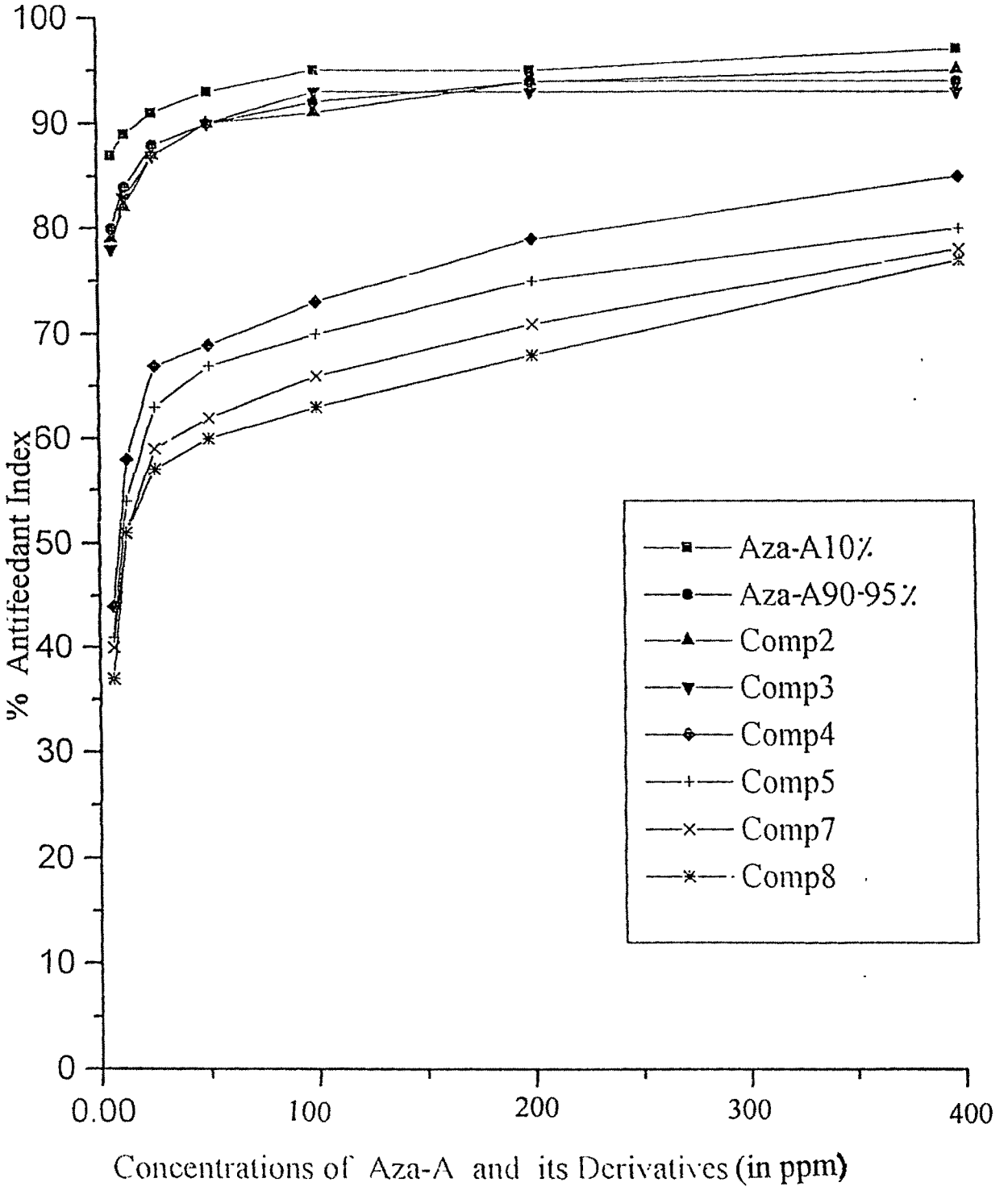


Figure: IV.8 Plot of antifeedant index versus concentrations of Aza-A its derivatives

From the antifeedent data obtained (Table:IV.1) it is possible to draw some general conclusions concerning the structure activity relationship in these materials.

The data revealed that crude material containing 9.14 % Aza-A was found to be more effective than 91 % pure Aza-A and other derivatives. This could be due to the synergistic effect of the other terpenoids present in the crude material.

The data further showed that methylation of the C_{11} hydroxy group in **1** and hydrogenation of the C_{22}-C_{23} enol-ether double bond in **2** did not significantly alter the activity of Aza-A. Our results would support those of Morgan's[28] results who showed that hydrogenation of the dihydrofuran ring did not decrease activity. The result showed that hydrogenation of the C_{22}-C_{23} double bond in azadirachtin does not significantly influence antifeedant activity. This is an important chemical feature as hydrogenation results in more stable compounds.

A comparison of the activities of the diketocarbonate **4**, the decalin fragment **5**, α-bromodecalin derivative **7** and α,β-enone derivative **8** with azadirachtin **1** and 22,23-dihydro-11-methoxy derivative **3** showed a significant decrease in the activity, which indicates that the hydroxyl groups at C_7 and C_{20} positions are important for maximum activity.

The insect toxicity of crude material containing 9.14 % Aza-A, 91 % Aza-A and its derivatives (**2-5**, **7**, **8**) against *S. litura* was determined by surface contamination bio assay method and the data thus obtained were subjected to probit analysis to work out the LC_{50} values, which are presented in Table IV.2.

The results indicated that crude material containing 9.14 % Aza-A showed strong toxicity effect in the polyphagous insect. Further observation indicated that compounds **4**, **5**, **7** and **8** have lower

insect toxicity, whereas compounds **1**, **2** and **3** have relatively higher insect toxicity against the armyworm. These results are in close agreement with those obtained for the antifeedant activity (*vide supra*).

These results and those of Blaney *et al*[25] and Yamasaki *et al*[22] suggested that the hydrogenation of C_{22}-C_{23} double bond did not decrease antifeedant activity and the hydroxyl groups on azadirachtin are essential for biological activity. In addition our results also indicated that the crude material containing 9.14 % azadirachtin shows more activities than the other compounds tested, which could be due to the synergistic effect of the other terpenoid compounds present in the crude material.

Observations described here also indicated that crude material containing 9.14 % Aza-A, 91 % Aza-A, 11-methoxyazadirachtin and 22,23-dihydro derivative possess a combination of antifeedant activity and insect toxic properties. Further it appears that antifeedancy may be insufficient for chemical defense of neem tree and the combination of insect toxicity and antifeedancy of Aza-A provides better crop protection.

IV.4 EXPERIMENTAL

Melting point were determined by the open capillary method and are uncorrected. IR spectra were recorded on a Perkin-Elmer PC-16-FTIR spectrophotometer. PMR spectra (200 MHz) were recorded on a Bruker 200-FT NMR using $CDCl_3$ as solvent containing tetramethyl silane as an internal standard and chemical shift values are reported in δ, ppm downfield with respect to TMS. Microanalyses were performed on a Perkin-Elmer 2400 series II instrument.

Thin layer chromatographic (TLC) analyses were accomplished on 0.25 mm thick (20 × 20 cm) pre-coated layer of silica gel 60 F-254 (Merck). The plates were developed in solvent system, as described in chapter II (section: II.6). The developed plates were air-dried, sprayed with 3 % (w/v) vanillin in absolute alcohol (100 mL) containing 1 % (v/v) conc. H_2SO_4 and gentle heating. Products were detected by characteristic green colour of the spot. The reactions were monitored using TLC with suitable solvent system for separation.

Aza-A was purified and isolated from neem seed kernels by preparative HPLC method and determination of its purity by analytical HPLC method, were done by following the procedure described in chapter II (section: II.6). Aza-A derivatives for example, 11-methoxyazadirachtin, dihydro derivative, diketocarbonate, decalin fragment and spiro ketal derivative were prepared by following reported method of Ley and his group.[32]

All solvents and reagents were purified by standard procedures as necessary. The experimental procedures are described in the order the compounds appear in scheme: IV.1.

Preparation of 11-methoxyazadirachtin (2)

To a stirred solution of Aza-A (**1**, 500 mg., 0.69 mmol) in iodomethane (32 mL, excess) was added silver oxide (800 mg, 3.46 mmol, 5 equiv.) in portions over a period of 1 hour in the dark. Stirring was continued for further 3 hours at 40-43°C temperature. After completion of the reaction (TLC), reaction mixture was cooled, filtered through Nylon-66 filter media (0.45 micron removal rating, 0.13 dia) and solvent was removed under reduced pressure. The residue was purified by flash chromatography (80 % ethyl acetate/ light petrol) to give 11-methoxy azadirachtin **2** as a colourless solid (430 mg, 84 %), m.p. 152-157°C.

IR ν_{max} : 3446, 2956, 2854, 1741, 1649, 1620, and 1045 cm^{-1}

PMR δ_H : 6.93 (dq, 1H at C3'), 6.43 (d, 1H at C_{23}), 5.67 (s, 1H at C_{21}), 5.05 (d, 1H at C_{22}), 4.60 (dd, 1H at C_6), 4.58 (br, s, 1H at C_7), 4.08 (d, 1H at C_{28}), 3.79 (s, 3H, at $CO_2C\underline{H}_3$), 3.72 (d, 1H at C_{28}), 3.68 (s, 3H at $CO_2C\underline{H}_3$), 3.32 (s, 3H $OC\underline{H}_3$ at C_{11}), 2.38 (dd, 1H at C_{17}), 2.33 (ddd, 1H at C_{22}) 1.96 (s, 3H, OAC at C_3) and 1.64 (s, 3H, CH_3 at C_{30})

Analysis : Found C; 58.87 %, H; 6.61 %

Requires C; 58.85, % H; 6.26 % for $C_{36}H_{46}O_{16}$.

Preparation of 22,23-dihydro-11-methoxyazadirachtin (3)

A stirred solution of 11-methoxyazadirachtin (**2**, 400 mg, 0.542 mmol) in anhydrous methanol (25 mL) containing 10 % palladium on charcoal (30 mg) was hydrogenated at 1 atm for 25 minutes at room temperature. The mixture was then filtered through Nylon-66 filter media and solvent was removed under reduced pressure. The residue was purified by flash column chromatography (gradient

elution 60-100 % ethyl acetate/petrol) to give 22,23-dihydro product **3** as a colour less solid (325.27 mg, 81.04 %), m.p. (157-160 C).

IR ν_{max} : 3444, 2926, 1738, 1648, 1438, 1044 and 734 cm^{-1}

PMR δ_H : 6.94 (dq, 1H at C3'), 5.51 (s, 1H at C_{21}), 3.91 (m, 1H at C_{23}), 3.31 (s, 3H $OC\underline{H}_3$ at C_{11}) and 2.10 (m, 1H at C_{22}).

Analysis : Found C; 58.54 %, H; 6.39 %

Requires C; 58.69, % H; 6.52 % for $C_{36}H_{48}O_{16}$.

Preparation of Diketocarbonate (4)

To stirred solution of 22,23-dihydro-11-methoxy azadirachtin (**3**, 300 mg, 0.407 mmol) in dichloromethane (10 mL) was added PCC (876 mg, 0.40 mol, 10 equiv.) and activated 4 Å molecular sieves (1 g) portion wise. The reaction mixture was further stirred at room temperature for 48 hours, after which time ethyl acetate (10 mL) was added and stirred continued for another 10 minutes. The mixture was filtered through Nylon-66 filter media and the filtrate was evaporated in vacuum. Purification by flash chromatography (gradient elution 70-100 % ethyl acetate/petrol) gave the diketocarbonate as a solid **4** (170.54 mg, 55.93 %), m. p. 150-152 °C (dec.).

IR ν_{max} : 2958, 2856, 1738, 1610, 1570 and 1380 cm^{-1}

PMR δ_H : 6.67 (dq, 1H at C3'), 5.50 (d, 1H at C_3), 5.20 (d, 1H at C_6), 4.52 (ddd, 1H at C_{23}), 3.80 (s, 3H, at $CO_2C\underline{H}_3$), 3.72 (s, 3H at $CO_2C\underline{H}_3$), 3.31 (s, 3H, $OC\underline{H}_3$ at C_{11}), 1.92 (s, 3H, OAc at C_3) and 1.64 (s, 3H, CH_3 at C_{30})

Analysis : Found C; 57.10 %, H; 6.93 %

Requires C; 57.75 % H; 5.92 % for $C_{36}H_{44}O_{17}$.

Preparation of Decallin fragment (5) and Spiro ketal derivative (6)

A solution of diketocarbonate (**4**, 100 mg, 0.012 mmol) in anhydrous methanol (15 mL) was stirred at room temperature for 40 minutes. Afterwards solution was cooled to 0° C and sodium methoxide (120 µL, 0.680 mmol, 30 % w/w, 5 equiv.) was added via syringe. The solution turned pale yellow and stirred further for 24 hours. In this solution acetic acid (CH_3COOH) was added and some of the colour discharge. The solution was stirred for 5 minutes, poured into saturated solution of $NaHCO_3$ (30 mL) and extracted with dichloromethane (4 × 20 mL). The combined extracts were further washed with $NaHCO_3$ (10 mL) and water (10 mL). Organic layer was dried over sodium sulphate and solvent removed under reduced pressure to give crude residue, which was purified by chromatography (gradient elution of 70-100 % ethyl acetate /petrol) gave decalin fragment **5** (30.51 mg, 64.85 %).

IR ν_{max} : 3446, 2929, 1733, 1716, 1435, 1240 and 1111 cm^{-1}

PMR δ_H : 4.87 (dd, 1H at C_6), 4.40-4.46 (m, 1H at C_3), 4.04-4.14 (m, 4H at C_1, C_{19} or C_{28}), 3.80 (s, 3H, at $CO_2C\underline{H}_3$), 3.60 (s, 3H at $CO_2C\underline{H}_3$), 3.49 (s, 3H, $OC\underline{H}_3$ at C_{11}), 3.30 (d,1H, OH), 2.73 (d, 1H, C_5) and 2.20-2.31 (d, 1H, C_2).

Analysis : Found C; 58.54 %, H; 6.39 %

Requires C; 55.07 % H; 6.20 % for $C_{19}H_{26}O_{10}$.

Further elution of the column furnished compound 6 (10.51 mg., 50.82 %), m.p. 125-130°C

Analysis : Found C; 59.78 %, H; 5.83 %

Requires C; 59.34 % H; 5.49 % for $C_9H_{10}O_4$.

Preparation of α-Bromodecalin derivative (7)

To a stirred solution of NBS (10.74 mg, 0.060 mmol) in dry tetrahydrofuran (10 mL) was added the decalin compound (**5**, 25 mg, 0.0603 mmol) in two portions. The reaction mixture was stirred for 30 minutes while maintaining the temperature between 0–5°C. After which it was poured into a saturated solution of sodium bicarbonate (5 mL) followed by extraction of the aqueous layer with dichloromethane (3 × 10 mL). The combined organic extracts were washed with water (5 mL), brine (5 mL) and dried over anhydrous sodium sulphate. The solvent was removed under reduced pressure to furnish a residue, which was purified by flash column chromatography over silica gel (230-400 mesh size). Elution of the column with ethyl acetate and light petrol (40 : 60 v/v) gave the α-bromodecalin derivative **7** as a yellowish white solid (13.2 mg, 44 %) mp. 149-151°C (dec.)

IR ν_{max} : 3409, 2947, 1735, 1718, 1651, 1029, and 653 cm^{-1}

PMR δ_H : 4.96 (dd, 1H, C_6), 4.76 (m, 1H, C_3), 4.15 (dd, 1H, C_1), 4.0 (d, 1H, CH_2 at C_{28}), 3.79 (d, 1H, CH_2 at C_{19}), 3.71 (s, 3H, $COOC\underline{H}_3$), 3.67 (d, 1H, CH_2 at C_{28}), 3.62 (s, 3H, $COOC\underline{H}_3$), 3.60 (d, 1H, CH_2 at C_{19}), 3.38 (br s, OH), 3.21 (s, 3H, $OC\underline{H}_3$ at C_{11}), 3.20 (s, 1H, C_9), 2.22 (m, 1H, CH_2 at C_2), 1.95 (s, 3H, $C\underline{H}_3$ at C_8), 1.81 (dd, 1H, CH_2 at C_2),

Analysis : Found C; 44.99 %, H; 5.97 %

Requires C; 46.24 %, H; 5.07 %; for $C_{19}H_{25}O_{10}Br$.

Preparation of α,β-Enone decalin derivative (8)

To a solution of the α-bromodecalin derivative (**7**, 20 mg, 0.04 mmol) prepared as above, in dichloromethane (10 mL) was added DBU (8 μL, 0.048 mmol) using a 10 μL Hamilton syringe. The

reaction mixture became turbid immediately. It was then stirred for two hours (TLC) while maintaining the temperature between 0-5°C, after which it was washed with water (3 × 5 mL) and aqueous HCl (1 %, 5 mL). The organic layer was further washed with water (10 mL), brine (5 mL) and dried over anhydrous sodium sulphate. The solvent was removed under vacuum and the residue was purified by flash column chromatography over silica gel (230-400 mesh size). Elution of the column with ethyl acetate and light petrol (30: 70 v/v) gave the α,β-enone derivative **8** as a white solid (4.9 mg, 29 %), mp. 153-157°C (dec.)

IR ν_{max} : 3421, 2962, 2856, 1747, 1683 (α,β-enone), 1624, 1379, 1260 and 1043 cm^{-1}.

PMR δ_H : 5.01 (d, 1H, C_6), 4.72 (m, 1H, C_3), 4.59 (dd, 1H, C_1), 4.21 (d, 1H, CH_2 at C_{28}), 4.03 (d, 1H, CH_2 at C_{19}), 3.81 (s, 3H, $COOCH_3$), 3.74 (d, 1H, CH_2 at C_{28}), 3.32 (br s, 1H, OH), 2.22 (m, 1H, CH_2 at C_2), 2.01 (ddd, 1H, CH_2 at C_2) and 1.98 (s, 3H, olefinic CH_3)

Analysis : Found C; 51.11 %, H; 5.87 %

Requires C; 55.33 %, H; 5.82 %; for $C_{19}H_{24}O_{10}$

Testing Biological Activity:

Insects

The adult insects of *Spodoptera litura* were collected from the field and confined in a cage (insectary) and were supplied with freshly cut castor twigs on which females after copulation laid eggs. The eggs were collected every day with the help of a moist hairbrush and kept separately. The larvae were reared on castor leaves and maintained at 27°C ± 2°C and 16: 8 (Light: Dark) photoperiod and 60 % relative

humidity. Three to four days old (second instar) larvae were used for the present bioassay work.

Plants

Fresh leaves of castor (*Ricinus Communis* L.) were obtained for bioassay from the plants grown in a model farm developed at Gujarat State Fertilizers & Chemicals Ltd. (Vadodara, India).

Bioassay studies were conducted on second instar larvae of *Spodoptera litura* obtained from a colony maintained on a common lepidopteran diet.[40,41] The bioassay trays and "PULL-N-PILL" TAB designed by (M/s. Patco Chem. Pvt. Ltd., Vadodara, India) were used. Each tray was bearing 128 wells of 225 sq. mm diameters.

Sample preparation

A 400 ppm stock solution of the crude (9.14 % Aza-A), pure Aza-A (91 %) and each of the derivatives [2-5, 7,8 (Scheme: IV. 1)], was prepared in 20 % ethanol in water (4 mg / 10 mL) with 0.05 per cent Triton X-100 as an emulsifier and stored between 0–4°C. Aliquots of the solutions were diluted with distilled water to give working solutions of 6.25 to 200 ppm.

Study of antifeedant activity

The test compounds were examined for antifeedant activity by leaf disc feeding bioassay.[13,42-46] Fresh leaves of castor were cut into 17 mm diameter leaf discs with a cork borer. The leaf discs were placed individually on the sterile moist sand, in each well. Solutions of 50 µL having different concentrations of each compound were applied uniformly on either side of castor leaf discs using a Gilson pipette. The discs were exposed under an electric fan for five minutes to allow drying of the solution and then weighed. Second instar larvae

of *S. litura* were starved for eight hours in order to equilibrate their level of hunger, released on surface of the leaf discs (one larva was released per leaf disc) treated with different concentrations of the each test compound. Seven concentrations were tested for each compound. Sixteen larvae were exposed individually to each concentration of each compound. The wells were covered with adhesive plastic lids, which had provision of micro fine holes for adequate ventilation and to prevent the escape of larvae. Similarly, in control, leaf discs were treated only with distilled water containing the emulsifier and larvae were released on leaf discs (one larva was released per leaf disc). The bioassays were terminated after 50 % of either disc was eaten or after 48 hours in control as well as in treated. The larvae were removed from the leaf discs in each well and then leaf discs were reweighed accurately. The Antifeedant Index (AFI) was calculated by the formula as adopted by Abivardi and Benz.[44,47]

$$AFI = \frac{C-T}{C} \times 100$$

where C and T represent the masses eaten of the control and treated leaf discs respectively. The antifeedant indices are presented in Table : IV. 1.

Insect Toxicity (LC_{50} Value) studies

The test compounds were determined for insect toxicity (Mortality) by an artificial diet feeding bioassay. The bioassay was run as a surface contamination method. The diet was prepared on the day of test, poured into wells, allowed to solidify and 50 μL of different concentrations of each compound was applied on the diet surface using a Gilson pipette to form a dry film. One-second instar larva, starved for eight hours was released in each well with the help of a

fine hairbrush. Sixteen larvae were exposed individually in each concentration of each test compound. The adhesive plastic lids were placed over the wells, which had a provision of micro fine holes for adequate ventilation. Parallel control was also run. The trays were incubated at 27 ± 2 °C and photoperiod of 16: 8 (L: D). Mortality was scored after seven days. The data on number of larvae responded in different concentrations of each test compounds and control were recorded and subjected to probit analysis using a computerized program developed by Dulmage, *et al.*[48] The LC_{50} values of each compound to *Spodoptera litura* are presented in Table: IV. 2.

Table: IV.1 Antifeedant Index (AFI) of Test Compounds against *Spodoptera litura* by Leaf disc Bioassay Method

Concentration applied to leaf disc (in ppm)			6.25	12. 5	25	50	100	200	400
Sr. No	Compound No.	Test Compound	Antifeeding index						
1	-	Crude (9.14 %) Aza-A containing material	87	89	91	93	95	95	97
2	1	Aza-A (91 % approx.)	82	84	88	90	92	94	95
3	2	11-Methoxy--azadirachtin	81	82	87	90	91	94	94
4	3	22,23-Dihydro-11-Methoxy--azadirachtin	81	83	87	90	93	93	94
5	4	Diketocarbonate	44	58	67	69	73	79	85
6	5	Decalin	42	54	63	67	70	75	80
7	7	α-Bromodecalin	40	51	59	62	66	71	78
8	8	α,β-enone decalin derivative	37	51	57	60	63	68	77

TABLE: IV.2 Insect Toxicity (Lc_{50} value) of Test Compounds against *Spodoptera litura* by Surface Contamination Method

Sr. No	Compound No	Test Compound	Insect toxicity[a]
			LC_{50} value[b]
1	-	Crude(9.14% approx.)Aza-A containing material	43.19 (25.30-67.42)[c]
2	1	Aza-A(90-95% approx)	65.83 (41.91-100.48)
3	2	11-Methoxy azadirachtin	59.50 (31.97-104.18)
4	3	22,23-Dihydro-11-methoxyazadirachtin	54.12 (32.50 - 87.60)
5	4	Diketocarbonate	95.37 (52.45- 206.65)
6	5	Decalin derivative	110.53(55.98- 215.32)
7	7	α-Bromodecalin	145.35(70.82- 227.01)
7	8	α,β-Enone decalin derivative	199.67(96.95-006.34)

[a]ng/well
[b]The probit analysis method was used to estimate LC_{50}, which represents the concentration required for causing 50 % mortality
[c]Number in parentheses are the 95 % confidence intervals

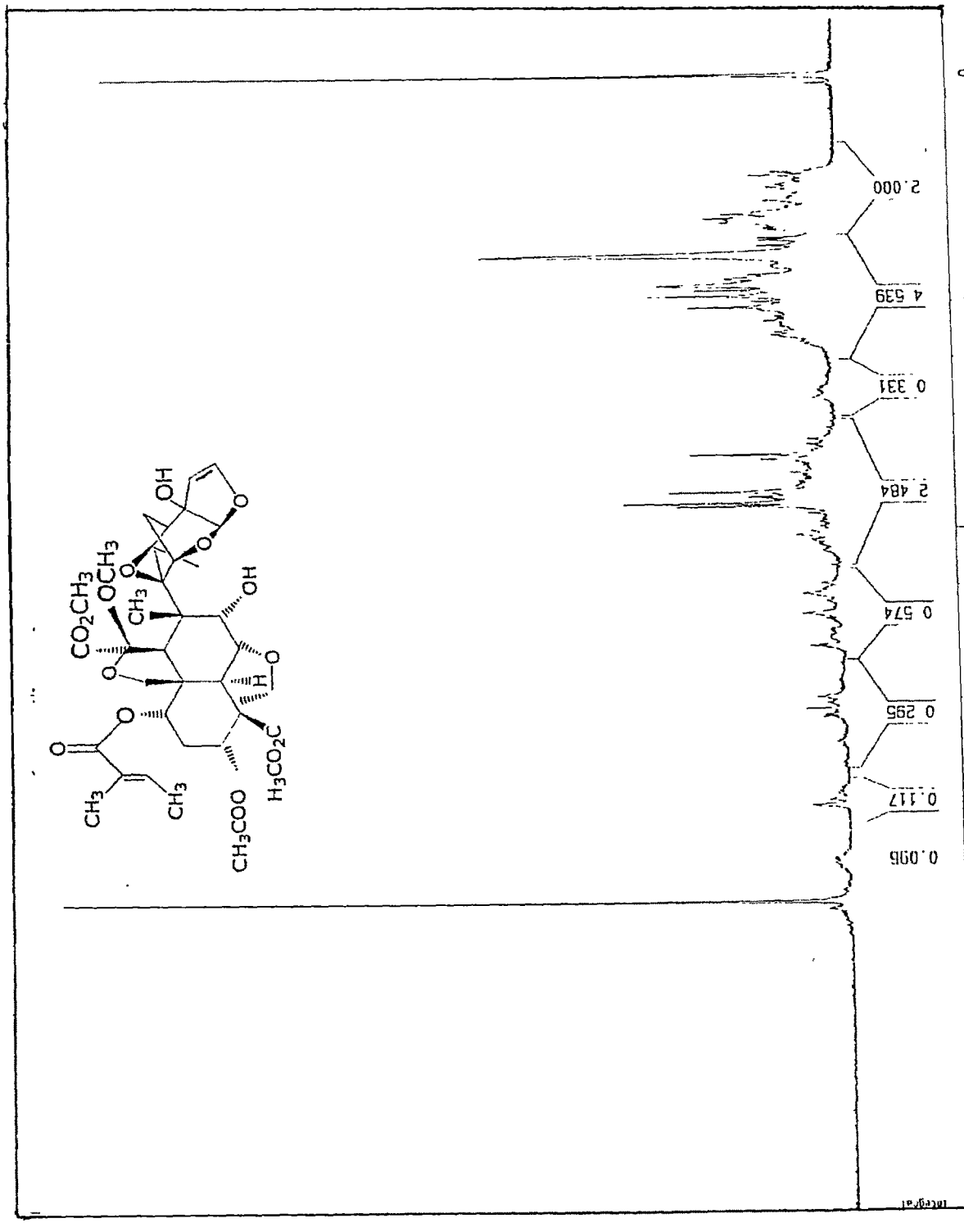


Figure IV.1: PMR Spectrum (200MHz) of the Compound **2**

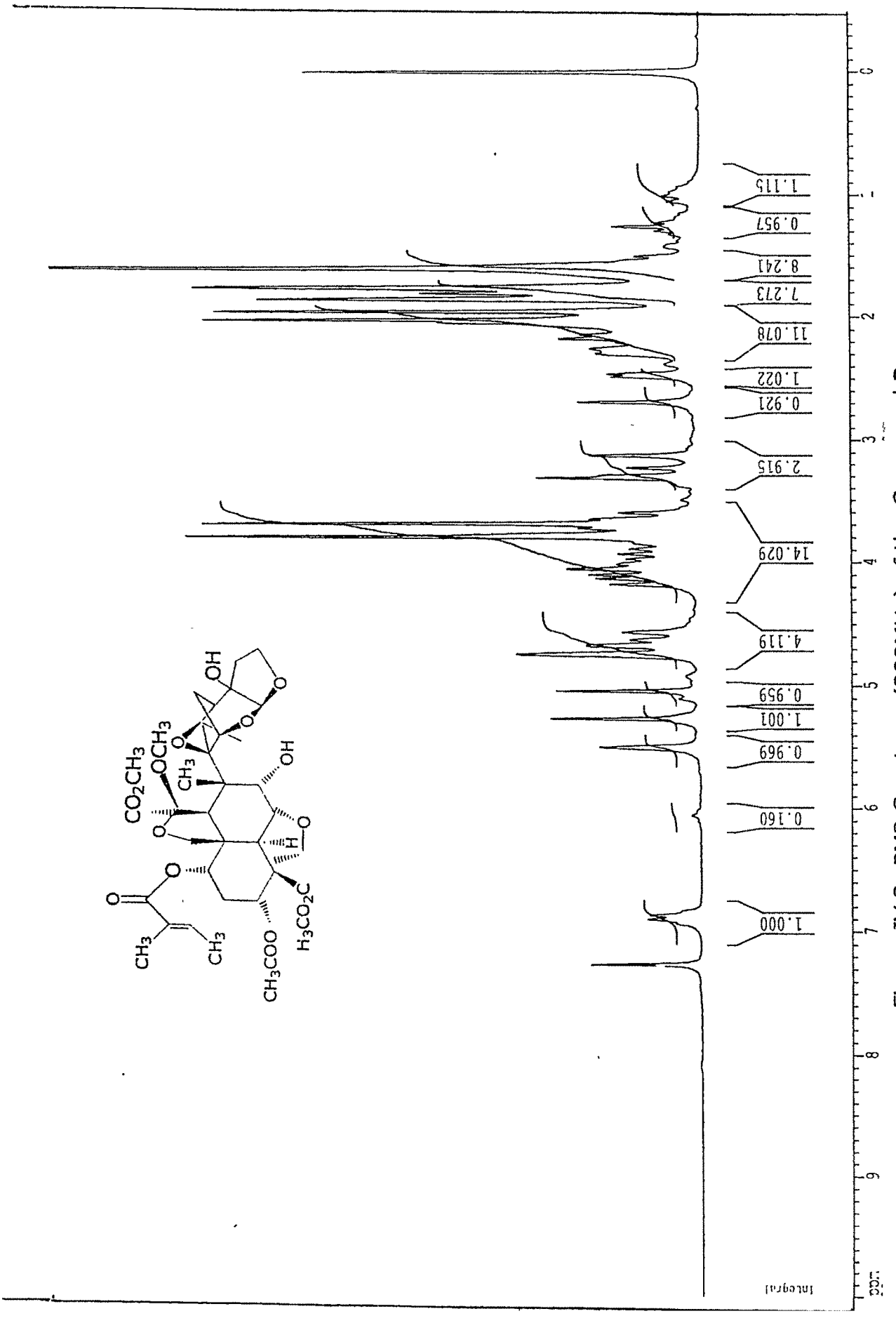


Figure IV.2: PMR Spectrum (200MHz) of the Compound **3**

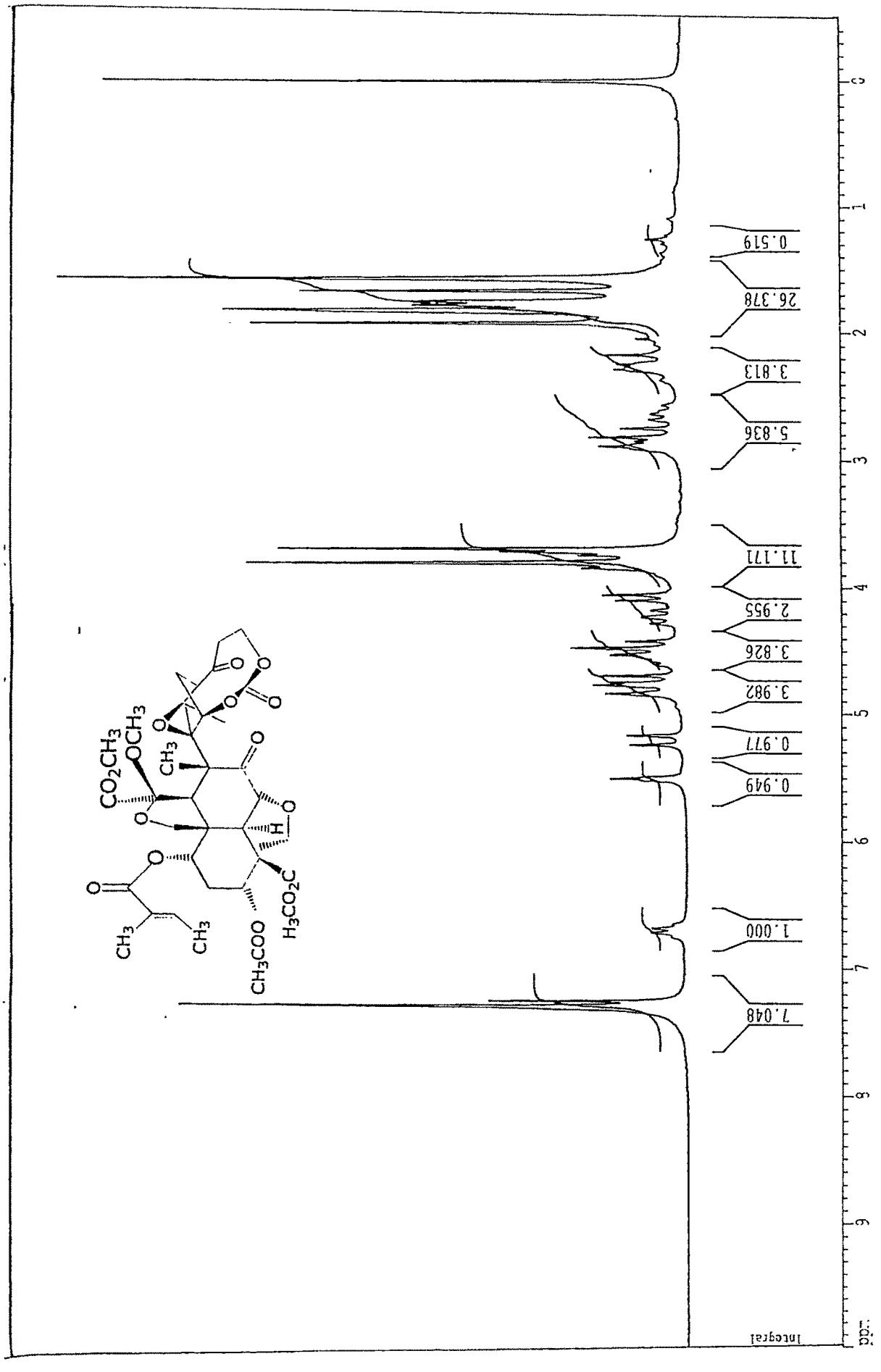


Figure IV.3: PMR Spectrum (200MHz) of the Compound **4**

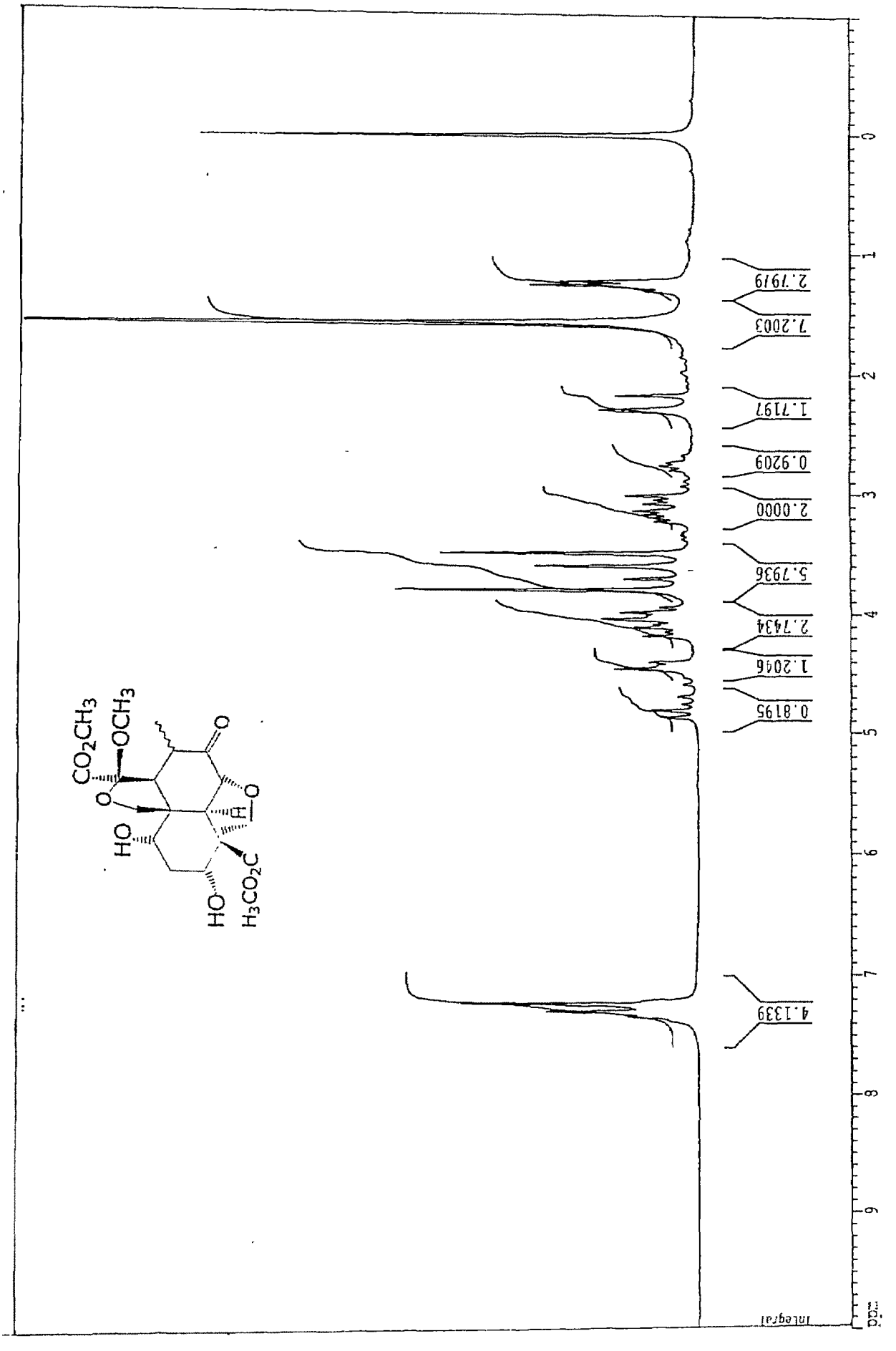


Figure IV.4: PMR Spectrum (200MHz) of the Compound **5**

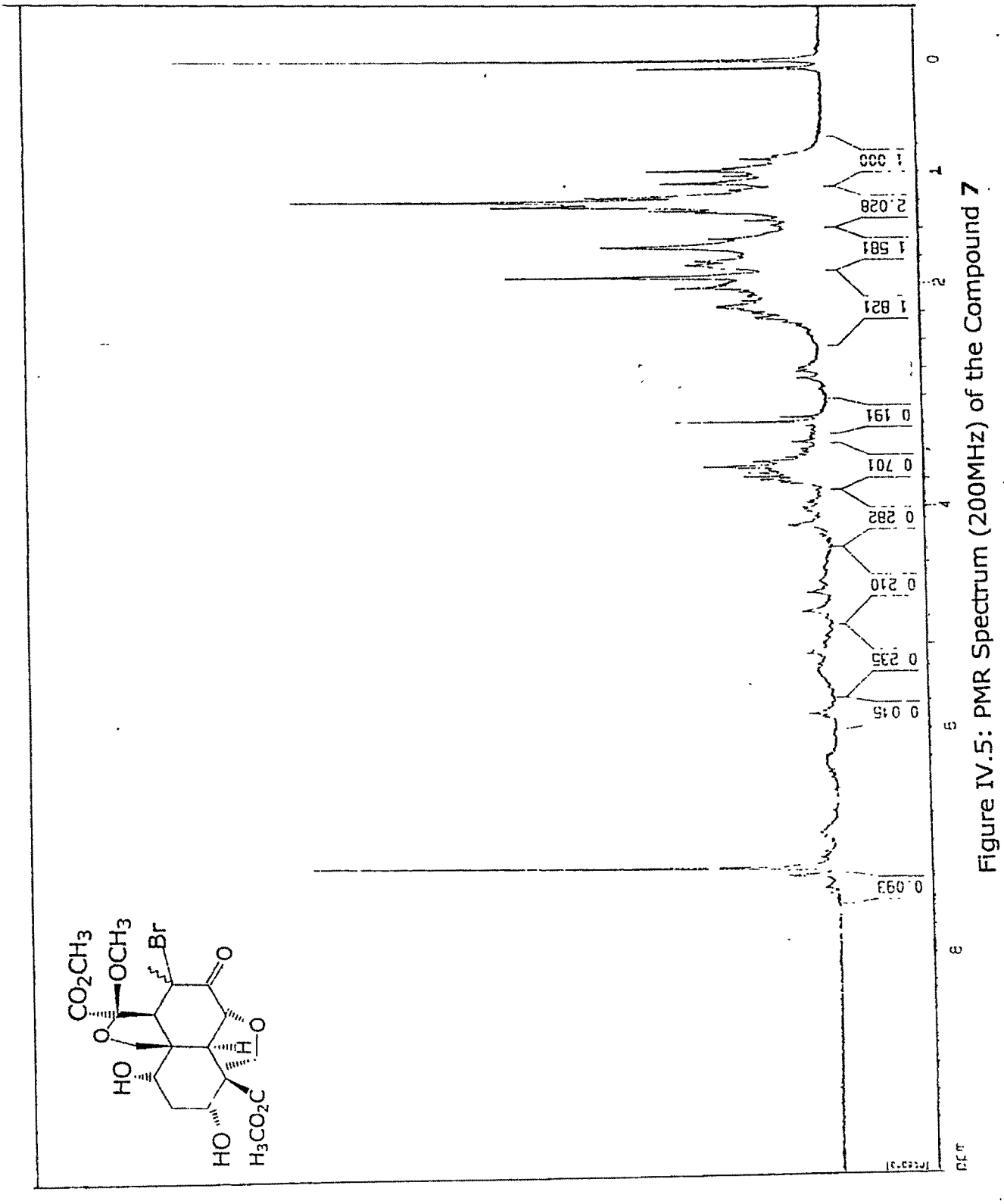


Figure IV.5: PMR Spectrum (200MHz) of the Compound **7**

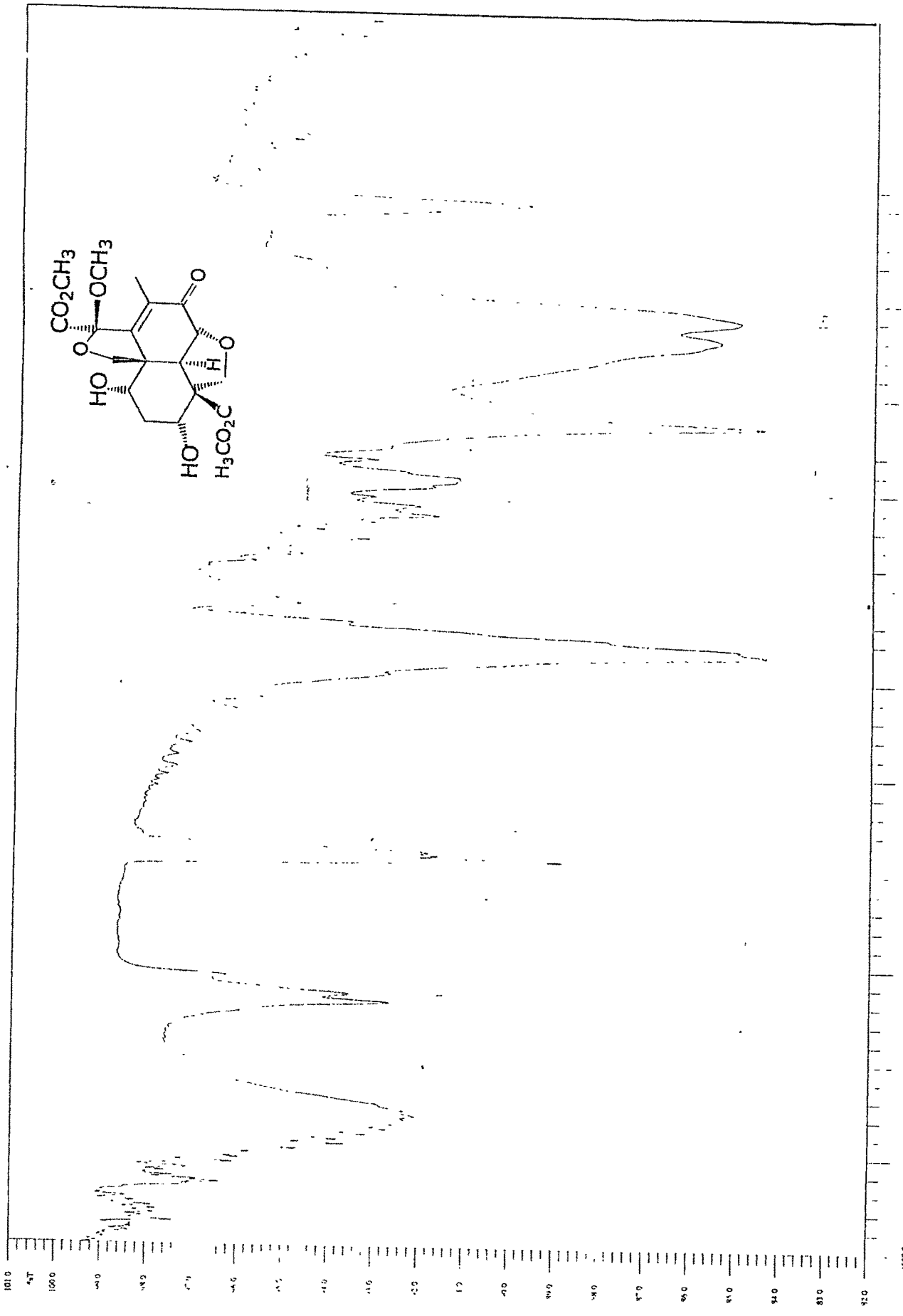


Figure IV.6: IR Spectrum of the Compound **8**

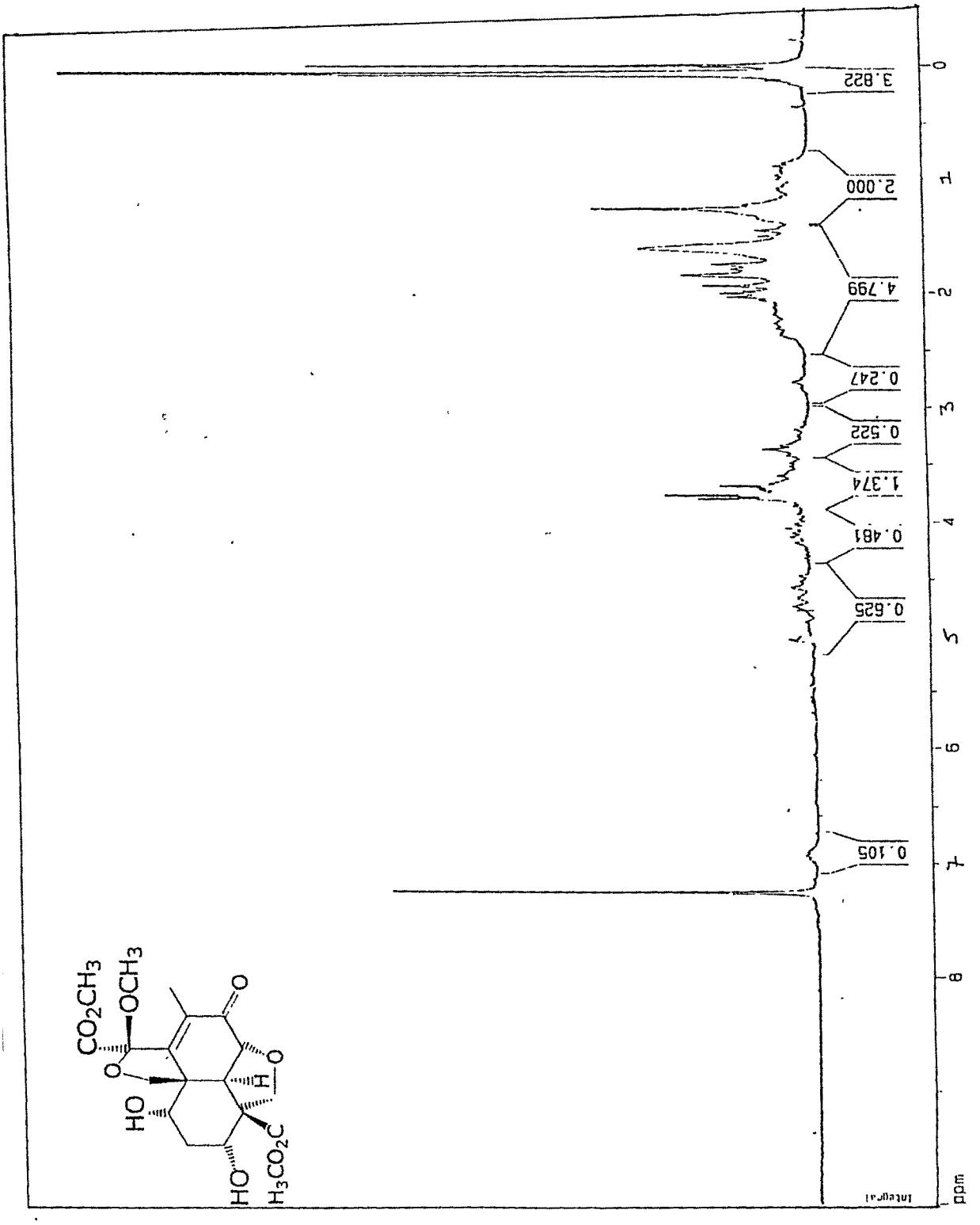


Figure IV.7: PMR Spectrum (200MHz) of the Compound **8**

IV.5 REFERENCES

1. P. B. Tanzubil, Potential for Neem (*Azadirachta indica* A. Juss) in armyworm control in Africa, in: R. P. Singh, M. S. Chari, A. K. Raheja and W. Kraus (eds.) Neem & Environment, Vol: **I**, pp: 91-101, Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi (1996).
2. K. M. S. Sundaram and J. Curry, Chemosphere. **32**(4), 649, (1996).
3. K. Kirsch, Studies of the efficacy of Neem extracts in controlling major insect pests in tobacco and cabbage. Proc.3rd int. Neem conf., Nairrobi, pp: 495-517, (1986).
4. H. Rembold, Neem and its general development for pest control, in: R. P. Singh, M. S. Chari, A. K. Raheja and W. Kraus (eds.) Neem & Environment, Vol: **I**, pp: 3-10, Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi (1996).
5. K. Munakata, Pure. Appl. Chem., **42**, 57, (1975).
6. S. V. Ley and P. L. Toogood, Chem. Bri., **26**, 31, (1990).
7. S. V. Ley, A. A. Denholm and A. Wood, natural Products reports, 109, (1993)
8. A. A. Denholm, L. Jennes, S. V. Ley and A. Wood, Tetrahedron, **51** (23), 6591, (1995).
9. H. Rembold, Azadirachtins. Their structure and mode of action, in: J. T. Arnason, B. J. R. Philogene and P. Morand (eds.). Insecticides of plant origin, Amer. Chem. Soc Symp., Series 387, pp: 150-163, Amer. Chemical Soc., Washington D.C. (1989).
10. H. Schmutterer, Annu. Rev. Entomol., **35**, 271, (1990).

11. A. J. Mordue (Luntz) and A. Blackwell, J. Insect Physiol., **39** (11), 903, (1993).
12. S. Pradhan, M. G. Jotwani and B. K. Rai, Indian Farming., **12**, 7, (1962).
13. W. M. Blaney, M. S. J. Simmonds, S. V. Ley, J. C. Anderson and P. L. Toogood, Entomol. Exp. appl., **55**, 149, (1990).
14. M. S. J. Simmonds, W. M. Blaney, S. V. Ley, J. C. Anderson and P. L. Toogood, Entomol. exp. appl ., **55,** 169, (1990).
15. R. J. Aerts and A. J. Mordue (Luntz), J. Chem. Ecol., **23** (9), 2117, (1997).
16. K. M. S. Sundaram and J. Curry, Pestic. Sci., **41**,129, (1994).
17. J. H. Butterworth and E. D. Morgan, J. Insect Physiol., **17**, 969, (1971).
18. H. Schmutterer and H. Rembold, Angew. Entomol., 89, 179, (1980).
19. H. Schmutterer, K. R. S. Ascher and H. Rembold, Natural pesticides from the Neem Tree, Proc. 1st Int. Neem Conf., Rottach Egern., pp. 297, (1981).
20. H. Schmutterer, K. R. S. Ascher and H. Rembold, Natural pesticides from the Neem Tree and other tropical plants, Proc. 2nd Int. Neem Conf., Rauischholzhausen, GTZ, Germany pp. 587, (1984).
21. M. Jacobson, Focus on Phytochemical Pesticides, Vol: **I**, The Neem Tree, CRC Press Inc., Florida, 178, (1988).
22. R. B. Yamasaki and J. A. Klocke, J. Agric. Food Chem., **35**, 467, (1987).
23. M. C. Kolb, S. V. Ley, A. M. Z. Slawin and D. J. Williams, J. Chem. Soc. Perkin Trans, **I**, 2735, (1992).

24. J. B. Stokes and R. E. Redfern, J. Environ. Sci. Health, **17**, 57, (1982).

25. W. M. Blaney and M. S. J. Simmonds, J. Insect. Physiol., **36**,743, (1990).

26. H. Rembold, B. Subrahmanyam and T. Muller, Experientia, **45**,361, (1989).

27. I. W. Jones, A. A. Denhlom, S. V. Ley, H. Lovell, A. Wood and R. E. Sinden, FEMS Microbiology Letters, **120**, 267, (1994).

28. J. H. Butterworth, E. D. Morgan and G. R. Percy, J. Chem. Soc., Perkin Trans, **I**, 2445, (1972).

29. S. V. Ley, P. J. Lovell, S. C. Smith and A. Wood, Tetrahedron Lett., **32** (43), 6183, (1991).

30. S. V. Ley, J. C. Anderson, W. M. Blaney, E. D. Morgan, S. C. Smith, R. N. Sheppard, M. S. J. Simmonds, A. M. Z. Slawin and A. Wood, Tetrahedron, **47** (44), 9231, (1991).

31. J. N. Bilton, H. B. Broughton, P. S. Jones, S. V. Ley, Z. Lidert, E. D. Morgan, H. S. Rzepa, R. N. Sheppard, A. M. Z. Slawin and D. J. Williams, Tetrahedron, **43**, 2805, (1987).

32. S. V. Ley, P. J. Lovell, A. M. Z. Slawin, S. C. Smith, D. J. Williams and A. Wood, Tetrahedron, **49** (8), 1675, (1993).

33. W. M. Blaney, M. S. J. Simmonds, S. V. Ley, J. C. Anderson, S. C. Smith and A. Wood, Pestic. Sci., **40**, 169, (1994).

34. M. L. Dela Puente, R. B. Grossman, S. V. Ley, M. S. J. Simmonds and W. M. Blaney, J. Chem. Soc., Perkin Trans, **I** 1517, (1996).

35. R.B. Grossman and S. V. Ley, Tetrahedron, **50** (39), 11553, (1994).

36. E. J. Corey and J. W. Suggs, Tetrahedron Lett., 2647, (1975).

37. C. Djerassi and C. R. Scholz, J. Am. Chem. Soc., **70**, 417, (1948).

38. C. Djerassi, Chem. Revs., **43**, 271, (1948).

39. S. Wolff. M. E. Huecas and W. C. Agosta, J. Org. Chem., **47**, 4358, (1982).

40. S. Wakamura Jpn. J. Appl. Entomo, Zool., **32**, 329 (In Japanese with English summary) (1988).

41. H. T. Dulmage, A. J. Martinez and T. Pena U. S. Department of Agriculture Technical Bulletin No.1528, (1976).

42. M. Narayanrao, K. Nagaiah and G. Srimannarayana, Antifeedant and insecticidal activity of some neem fractions,in: R. P. Singh, M. S. Chari, A. K. Raheja and W. Kraus (eds.), Neem & Environment, Vol*:* **1**, pp. 237-241. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi (1996).

43. K. R. S.Ascher, M. Streloke, G. H. Schmidt and J. D. Warthen Jr, *Phytoparasitica,* **17** (3)**,** 167, (1989).

44. Y. K. Mathur and S. Nigam, insecticide, antifeeding and juvenilizing effects of neem (*Azadirachta indica* A juss.) oil against *Corcyra cephalonica* staint and *Epilachna vigintioctopunctata* F. in: R. P. Singh, M. S. Chari, A.K. Raheja and W. Kraus (eds.), Neem & Environment, Vol*:* **1**, pp. 335-341. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, (1996).

45. I. Kubo and J. A. Klocke, Plant Resistance to Insects, in: P. A. Hedin (ed.), Amer. Chem. Soc. Symp., Series 208, pp. 329-346. Amer. Chemical Soc., Washington, D.C. (1983).

46. I. Kubo and J. A. Klocke Agric. Biol. Chem., **46,** 1951, (1982).

47. C. Abivardi and G. C. Benz, Mitt. Schweiz. Entomol. Besell., **57**, 383, (1984).