CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF THE LEAF SURFACE EXUDATE OF PSIADIA PUNCTULATA

BY

FREDRICK A. O. OWUOR

A thesis submitted in partial fulfilment for the degree of Master of Science at the University of Nairobi

FEBRUARY, 1994

This thesis is my original work and has not been presented for a Degree in any University

Signature .....

F. A. O. OWUOR
DEPARTMENT OF CHEMISTRY
UNIVERSITY OF NAIROBI

This thesis has been submitted for examination with our approval as project supervisors

Signature .....

PROF. J. O. MIDIWO
DEPARTMENT OF CHEMISTRY
UNIVERSITY OF NAIROBI

Signature Bhalendra Bhatt
.....

DR. B. M. BHATT
DEPARTMENT OF CHEMISTRY
UNIVERSITY OF NAIROBI

CONTENTS

CHAPTER 4

EXPERIMENTAL

LIST OF TABLES

LIST OF SCHEMES

APPENDIX

ACKNOWLEDGEMENT

I would like to express my heartfelt thanks to my project supervisors, Prof. J.O. Midiwo and Dr. B.M. Bhatt and the Chairman, Chemistry Dept., Prof. P.M. Gitu for the assistance and encouragement they accorded me.

But special thanks go to Prof. J.O. Midiwo for the guidance he accorded me during every stage of my research work. You were always there when I needed your help. I am once more very grateful. Thanks are also due to Dr. Siboe and Mr. Ogutu for bioassay results and Mr. Mathenge for having helped in plant identification. I am also grateful to Prof. Bruce Jarvis of University of Maryland at College Park for the NMR spectroscopic analysis.

My wife Mary Akinyi, parents, brothers, and friends especially Donald Adongo, you cannot be forgotten. I am very grateful for your encouragement and tolerance. May I also take this opportunity to thank Mr. James Owuoche for having allowed me to use his computer when I needed it most.

Finally, financial support by DAAD and Egerton University during the whole MSc. course is also gratefully acknowledged.

SUMMARY

There are about twenty species of *Psiadia* spread all over the world. The Kenyan *Psiadia punctulata* enjoys wide distribution on edges of disturbed bushland in evergreen woodland and in dry forest areas at an altitude of 1200-2300m.

The leaf decoction of *P. punctulata* is used in traditional medicine where it is claimed to relieve abdominal pains and cure stomach ulcers. Powdered leaves of the plant are used for the treatment of cold by the Maasai. The aerial parts on pressing leave yellow stain on paper due to epicuticular exudate pigment which covers the leaves. Cold extraction of aerial parts through washing technique with fresh portions of acetone and subsequent concentration using the rotatory evaporator resulted in a pale gummy solid. This crude extract showed a high cytotoxicity with an ED_{50} of about 10ppm when bioassayed with brine-shrimp nauplii (larvae). A 1000ppm solution of this crude extract sprayed on a sugar coated Whatman No. 1 filter paper gave a relative anti-feedant percentage (RAP) of 97.7% when presented to *Locusta migrotoria* (desert locust) previously starved for twenty four hours. Anti-fungal tests with *Fusarium oxysporum* and the coffee berry disease fungus, *Collectotrichum coffeanum* showed that the crude extract exhibited fungistatic activity at 1000ppm.

Gradient elution column chromatography of this crude extract on silica gel using n-hexane, chloroform and methanol as eluting solvents led to the isolation of three flavonoids (positive flavonoid test) and four terpenoids (positive terpenoid test) and concentrated the anti-brine shrimp activity in the terpenoid fraction with compound (22) giving a high anti-brine shrimp activity with an ED_{50} of about 40ppm and compound (20) showing marginal activity with an ED_{50} of about 920ppm.

Spectroscopic analysis led to the characterization of the flavonoids as:- 5-hydroxy-2',3,4',5',7-pentamethoxyflavone (16), 5,7-dihydroxy-2',3,4',5',-tetramethoxyflavone (17), and 5,7-dihydroxy-2',4',5'-trimethoxyflavonol (18) and the trachylobane diterpenes as:- 3,7,17-trihydroxytrachylobane (19), 7,17,19-trihydroxytrachylobane (20) and the 17, 19- dihydroxy-2, 3-epoxytrachylobane (21). These compounds are new natural products since they could not be traced in the literature except for compound (16) which had previously been isolated from *Distemonanthus benthamianus*.

CHAPTER ONE

1.0.0 INTRODUCTION

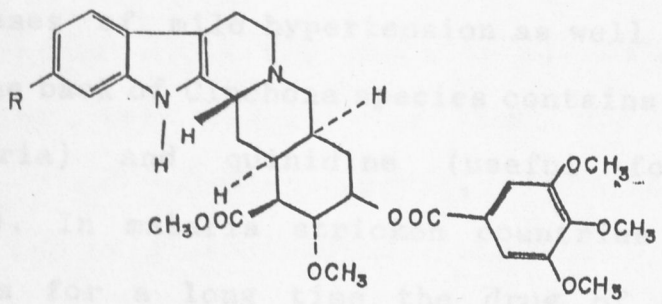
The world of nature and in particular the plant kingdom abounds in organic compounds of nearly every concievable structural class, the study of which constitutes a fascinating and fruitful area of scientific investigations. It offers us a diverse group of complex chemical structures with remarkable biological activities. Earlier on before systematic scientific studies evolved, natural products were recognised at once as those that have conspicuous physiological properties and indeed included medicinal, fragrant oils, food flavouring materials and poisonous substances. One of the earliest records of the use of herbal medicine is that of chaulmoogra oil from species of *Hydnocarpus gaertn*[1], which was known to be effective in the treatment of leprosy. Such a use was recorded in the pharmacopoeia of the emperor Shen Nung of China between 2730 and 3000 BC.

From time immemorial the root of *Rauwolfia serpentina* has been used in Indian traditional medicine to treat the mentally ill.[2] In Africa, a lot of knowledge about the use of plants medicinally has been passed through generations although very little was found in

literature. However, there is a lot of information today in the literature on the use of plant materials all over the world and in particular Africa. For instance Kokwaro(1976)[3] has highlighted some of these plants and their traditional medicinal uses in East Africa. In Kenya the leaves of *Asystasia gangetica*[3] when boiled and the decoction drunk, helps fight against intestinal worms. In Europe, *Polygonum avulcare*, a perenial herb has been used as a home remedy for lung complaints, hemorrhoids and rheumatism.[4] In China the use of crude plant extracts as traditional medicine are quite extensive and safe. For example *P. cuspidatum* is used in Chinese pharmacy, it contains cuspidatin and emodin.[5] In Nigeria the leaves of *Vernonia amygdaline* are eaten after macerating with water and are believed to help digestion.[6]

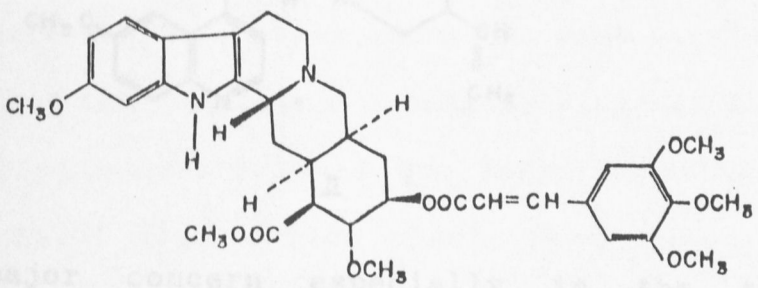
Research has developed from the screening of medicinal plants for bioactive agents to the development of drugs and dosage forms for natural products of merit. Studies on traditional medicine and medicinal plants are being carried out by medicinal anthropologists with a view to understanding the perception of disease in different populations. For example over 100 alkaloids have been isolated from the roots of *Rauwolfia* species including the therapeutically important

reserpine (1) deserpidine (2) rescinnamine (3) and yohimbine(4).[7]

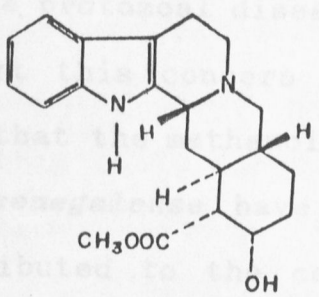


1 R = OCH_3

2 R = H

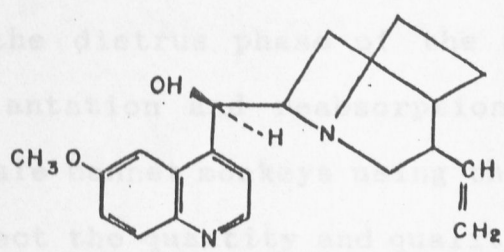


3



4

Reserpine(1) also exhibits antihypertensive properties and is used in modern medicinal practice to treat certian cases of mild hypertension as well as cases of anxiety. The back of Cinchona species contains quinine(5) (for malaria) and quinidine (useful for cardiac arrhythmia). In malaria stricken countries of Africa quinine was for a long time the drug of choice for treating this disease.[8]



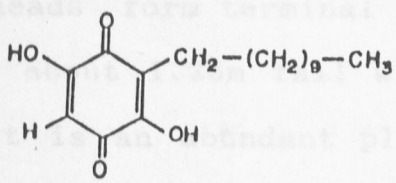
5

A major concern especially in the tropical countries, has been and still is a need to develop control measures against the parasitic diseases, in this connotation, the protozoal diseases. Several outstanding examples support this concern for example Dossaji and Kubo[9] reported that the methanolic extract of the leaves of *Polygonum senegalense* have molluscicidal activity which was attributed to the compound quercetin-3-(2"-galloylglucoside) which is found in the leaves. Isolated

compounds from this plant were found to be highly toxic towards *Lymnae natalensii*, *Biomphalaria pfeifferi* and *B. glabratus*. This activity was also reported with the Japanese *Polygonum nodosum*.[10] This snail control with the natural easily biodegradable compounds and thus Schistomiasis control has far reaching economic health implications for this region of the world where the disease is endemic. Fertility regulation of benzoquinones and related compounds from plants has been reported. From the berries of *E. ribes* anti-fertility activity tests were studied in the female rats and found to prolong the dietrus phase of the oestrus cycle and inhibit implantation and reabsorption of the foetus.[11] Study on female bonnet monkeys using the same berries was found to affect the quantity and quality of semen and the circulatory testosterone level was found to reduce. In China a compound from cotton plant *Gossypium* species (gossypol) is in use currently as a male contraceptive.[12] Today there is great emphasis on research work in this field of fertility regulation. W.H.O has embarked on an intensive programme of evaluating plants in different parts of the world for their pharmacological activity particularly in this field among others.

Anthraquinones have been found to be abundant in many plant species and have been evaluated exclusively for their anthelminthic and bacterial activities.[13]

Natural embelin (6) an active compound in Marsinaceae was found to be effective as an anthelminthic.[14] It was also found to be active against tapeworm. Anti-bacterial growth inhibition has been studied using natural extracted plant materials. Haungpu et al[15] found natural embelin (6) to be effective in inhibiting growth of *Mycobacterium tuberculosis* in vitro. Benzoquinones, embelin (6) and rapanone from Kenyan *Myrsinaceae* plant[16] were found to be active against gram-positive bacteria *Bacillus, Staphilococcus aureus* and gram-negative *Escherichia coli*. They are also active against *Streptococci*.[17] More biological active compounds



6

as ovicides and larval growth inhibitors have been isolated from *Asteraceae* plants in Japan.[18] Other useful utility of the plant products to man includes anti-plague,[19] anti-malarial,[20] analgesic activity etc. The compounds are also useful to the plants bearing them. For instance external flavonoids not only reflect radiation but have been known as good quenchers of singlet oxygen.[21 22] This indicate that they

protect plant internal soft tissues from harmful radiation. From this short review there is clear evidence that much awaits scientific investigations from plant species which should act as a pillar for human existence.

1.1.0 BOTANICAL DESCRIPTION OF *PSIADIA PUNCTULATA*

Psiadia species belongs to the compositae family. There are about 20 species of *Psiadia* spread all over the world. *Psiadia punctulata* is an erect round-topped shrub with entire lanceolate-ellyptic glossy leaves which produce a gum like secretion when young.[3] The bright yellow flower heads form terminal recemes. It is a many stemmed shrub, about 1.25m tall with alternate leaves which shine. It is an abundant plant on the edges of disturbed bushland in evergreen woodland and in dry forest areas at 1200-2300m. Typical habitats include Nairobi area, Western Kenya and lake areas.

1.2.0 FLAVONOID AGLYCONES AS LEAF EXUDATE CONSTITUENTS

The presence of a large number of flavoniod aglycone on the leaf surface of plants is a phenomenon that has drawn increasing attention in recent years. Their existence as externally deposited free aglycones has long been known for some conspicuous cases such as the bud excretion of poplars, or the whitish powder ("farina") on the leaves

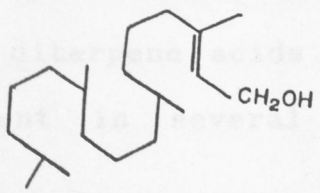
and inflorescences of primroses where some owe their names to this phenomenon e.g *Primular farinosa* and *Primular pulverulenta*.[23] The whitish or slightly yellowish powder on leaves and inflorescences is localised in distinct places as can be seen with a good hand-lens or a reflecting light microscope. Minute rod or needle-like crystals cover the head cell of a glandular trichome that excretes this material[24] in for instance Californian "sticky monkey flower" *Diplacus aurentiaca*. On many other plants the glands are not obvious, although an important excretion takes place, for instance *Citrus ladanifer* which produces the resin ladanum.[25] The amount and nature of the excretion causes the stickiness of its leaves. Another well known occurence of flavonoid aglycones is in the wax covering the leaves of *Eucalyptus* species where even the scanning electron microscope does not reveal the existence of glands.[25] It must be assumed that in such cases the flavonoids are extruded by epidermal cells, as is known for waxes. Even though the hydroxylated flavonoids are also found in the leaf resins, it is evident that the flavonoid aglycones encountered are mostly methylated derivatives leading to their increased solubility in terpenoid material. Typically flavonoids exist on the leaf at 2-3% dry weight and are common amongst plants that would have originated from arid or semi-arid habitats.[26] By there localization

on the leaf surface they appear especially suited for protective mechanisms. They probably play a part as antifungal, antibacterial, and antiviral agents.[27] External flavonoids have also been considered as a protective feature against phytophagous insects and against destruction by UV-radiation.[28] For example flavones and flavonol glycosides, becuase of there strong UV absorption both at 250-270nm and at 330-370nm, would seem to offer equally good protection against such radiation. Their presence in leaves, for example, would prevent mutagenesis of thymine (λmax 260nm) in DNA and also possible photodestruction of such coenzymes as NAD and NADP (λmax 340nm).[29] *Psiadia punctulata* as will be seen, has a surface exudate rich in flavonoid aglycones and terpenoids.

1.3.0 TERPENOIDS

The terpenoids are a group of substances that are widely distributed in the plant and the animal kingdoms. They are compounds whose structures are all based on the linking together of isoprene (C_5H_8) units and the majority contain 2,3,4,5,6,or 8 multiples of this basic structure. In general terpenoids form the major part of resinous excretions: monoterpenes, sesquiterpenes and diterpenes have been found, when these resins exhibit a more or less agreable scent. Diterpenes are found in

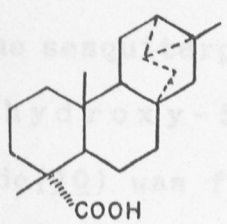
resins and in resinous high boiling fraction remaining after distillation of essential oils.[30] They can be cyclic or acyclic. The only important acyclic member is the alcohol phytol(7) which forms part of the chlorophyll molecule.



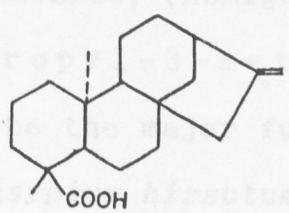
7

Many of the cyclic diterpenes may be regarded as derived from phytol by ring closures, but others do not show head-tail type of linkage. The group of diterpenoids includes some compounds of considerable physiological interest such as the group of plant hormones known as gibberellins.[31] Terpenoid compounds have been implicated as feeding detterents in a number of instances. A recent study on the nature of resistance in sunflower *Helianthus annuus* L.[32,33] to attack by larvae of the sunflower moth (Homeosoma electellum H) showed that resistant varieties contained large amount of two diterpenoid acids in extracts of their florets. Since first instar larvae of *Homeosoma electellum* consume florets as the major portion of their diet before burrowing into the immature seeds, the presence of the growth inhibiting chemicals was considered to be the

definitive factor in this example of host plant resistance. The two major substances trachiloban-19-oic acid(8) and the biogenetically related (-)-16-kaurene-19-oic acid(9) were isolated from the plant and incorporated into a suitable artificial diet in order to determine toxicity to *Homeosoma electellum* larvae. The two diterpene acids were found to inhibit larval development in several *Lepidoptera* species.



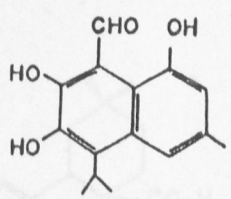
8



9

Two grindelic acid diterpenoids (18-hydroxygrindelic acid and 18-succinyloxygrindelic acid) isolated from Chrysothamnus nauseosus were found to exhibit significant anti-feeding behaviour against third-instar larvae of the Colorado potato beetle.[34] A bicarbonate-soluble portion of the chloroform extract of *C.*

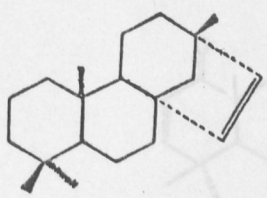
nauseosus also exhibited significant (threshold less than 40µg/cm^2) anti-feeding behaviour in third-instar Colorado potato beetle larvae. In addition a series of diterpene acids including 6-hydroxygrindelic acid, isolated from *Grindelia humilis* showed deterrent activity towards the aphid *Schizaphis graminum*.[35] The bioassay consisted of a synthetic diet to which substances to be tested were added. Each substance was tested at a series of concentrations so that a dose-response curve could be constructed. From this curve a concentration could be obtained at which half of the aphids would not feed, ED_{50}. The sesquiterpenoid aldehyde, (hemigossypol), 1,6,7-trihydroxy-5-isopropyl-3-methyl-8-naphthaldehyde(10) was found to be the major fungitoxin in diseased stele tissues of *Gossypium hirsutum*.[36]



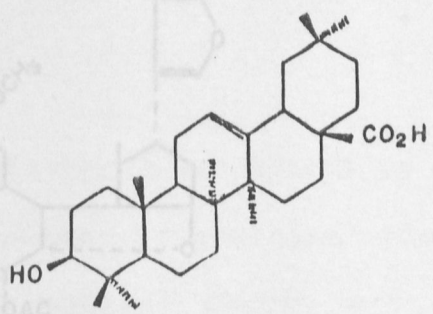
10

Anagallis arvensis has been reported to possess in vitro anti-viral activity against herpes simplex virus type 1 and poliovirus.[37] The anti-viral compounds were highly haemolytic, gave a cupious lather when they were shaken with water and produced positive colour tests for

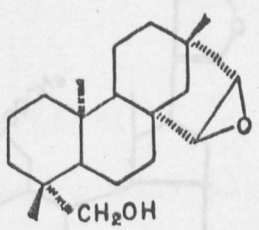
triterpenes. Two triterpene saponins were isolated from this plant. Investigation of four *Acritopappus* species (Compositae) afforded twenty-three new diterpenes of the labdane or kolavane type and three tetranoditerpenes as well as new benzofuran derivatives and a hydroxybicyclogermacrene.[38] From *Baccharies tola* (Compositae) five terpenoids were isolated and characterized. Three of the terpenoids were identified as erythroxylol-A (11), oleanolic acid (12) and erythroxylol-A-14,15-oxide.(13)



11



12

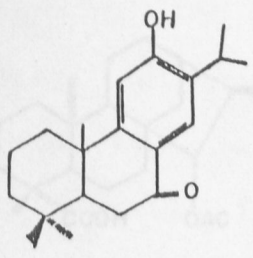


13

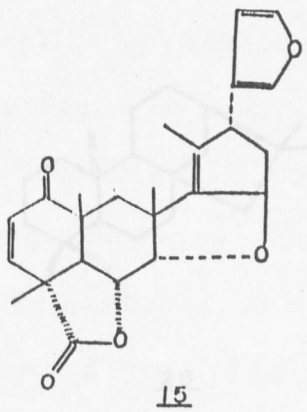
In view of the enormous therapeutic and the economic importance attributed to *Azadirachta indica*, also known as "neem", studies carried out by several

groups of workers on its various parts have led to the isolation of a series of new tri- and diterpenoidal constituents.[39] A new tricyclic diterpene named nimbolinin(14) was isolated from the root bark of the plant.

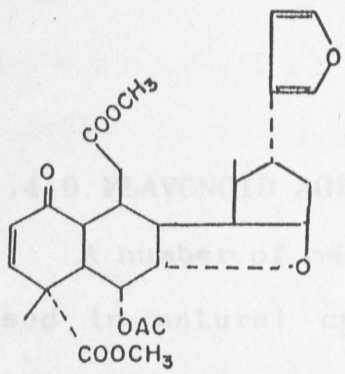
The alcoholic extract of leaves and stem bark yielded nimbolide(15), nimbin(23) and nimbadiol(24) and showed anti-inflammatory effects.



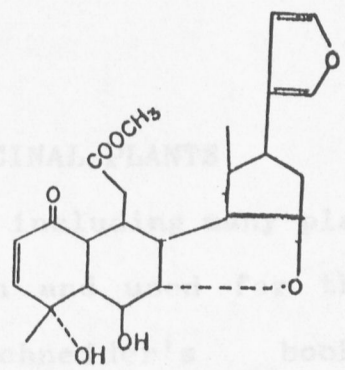
14



15

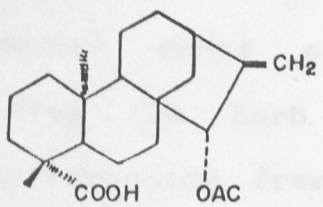


23

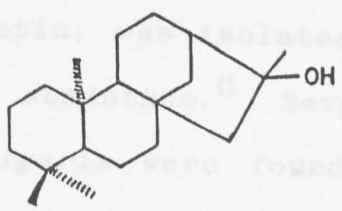


24

Ekong et al isolated from the fruits of *Xylopia aethiopica* (Dunal) A. Rich, diterpenes which include xylopic acid (25), Kauran-16α-ol (26) and their derivatives.[10] Xylopic acid, the major diterpene showed antimicrobial effects.



25

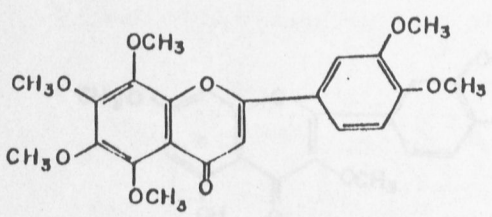


26

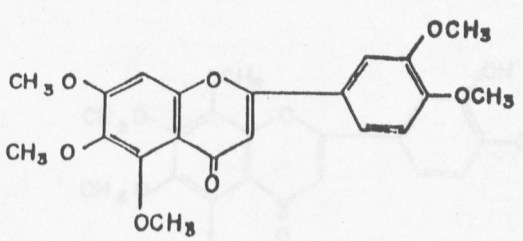
1.4.0 FLAVONOID AGLYCONES IN MEDICINAL PLANTS

A number of medicinal plants, including many plants used in natural cures, are known and used for their flavonoid contents. In Schneider's booklet "Pharmazeutische Biologie"[11] we find half a dozen of officinal drugs, such as birch-tree leaves or lime tree flowers and several others used in folk medicine. In Wagner's book,[12] lipophilic flavonoids are reported in

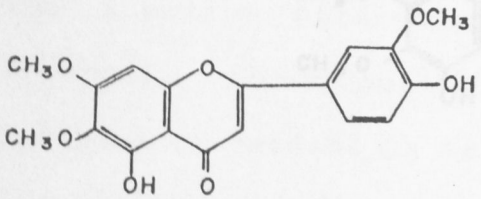
Citrus peel. The polymethoxylated products tangeretin (5,6,7,8,3',4'-hexamethoxyflavone)(27) had been isolated from the peel oil of *Citrus nobilis* as early as 1934/38.[43] While sinensetin (5,6,7,3',4'-pentamethoxyflavone)(28) was found later in the peel of *Citrus sinensis*.[44] The bulbs of *Populus nigra* (sometimes also of *P. balsamifera* and *P. candicans*) were used as diuretic and expectorants in folk medicine, and also for infections of the urinary organ.[42] Artemitin, the pentamethyl ether of quercetagetin, was isolated in 1898 from the herb *Artemisia absinthum*.[45] Several methoxyflavonoids from *Thymus vulgaris* were found to inhibit smooth muscular activity, due probably to effects on a non-competitive antagonism. Among the flavonoids causing this spasmolytic activity are circilineol(6-hydroxyluteolin-6,7,3'-trimethyl ether)(29), thymonin(5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone)(30).[46]



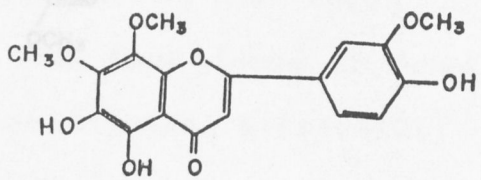
27



28

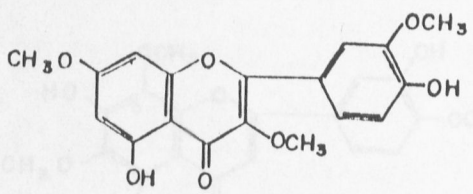


29

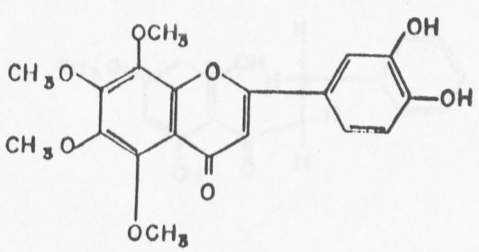


30

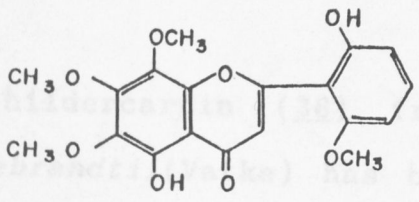
Quercetin-3,7,3'-trimethyl ether(31), a constituent of Chinese medicinal herb *Agastache rugosa* is found to have potent antiviral activity.[47] A series of 3',4'-dihydroxyflavones were assayed in vitro for inhibition of lens aldose reductase, an important enzyme in the pathogenesis of sugar cataract. 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone(32) was found to be the most potent inhibitor.[48] The compound 5,2'-dihydroxy-6,7,8,6'-tetramethoxyflavone(33), isolated from the roots of *Scutellaria baicalensis*, exhibits cytotoxic activity.[49]



31

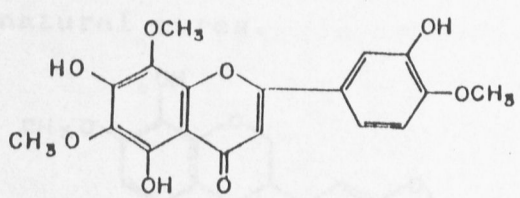


32

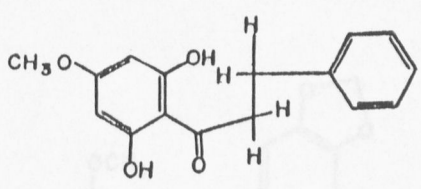


33

Acerosine (5,7,3'trihydroxy6,8,4'trimethoxyflavone)(34) identified from the seeds of *Vitex negundo* is reported to cause disruption of later stages of spermatogenesis in dogs.[50] The leaf surface exudate of *Polygonum senegalense* (Meisn) has strong toxicity and growth inhibition activity against the larvae of the mosquito, *Aedes aegyptii*.[51] The exudate flavonoid component that showed the highest activity amongst twelve others was 2',6'-dihydroxy-4'-methoxydihydrochalcone (35). The 3,6-dimethyl derivative of 6-hydroxy-kaempferol, first reported from the buds of sweet cherry[52], is found to be the lens aldose inhibitor in the Paraguayan medicinal plant *Acanthospernum australe*.[53]

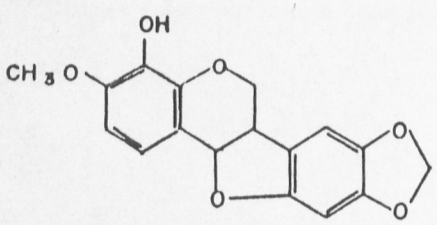


34

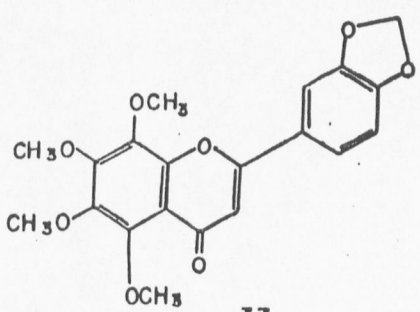


35

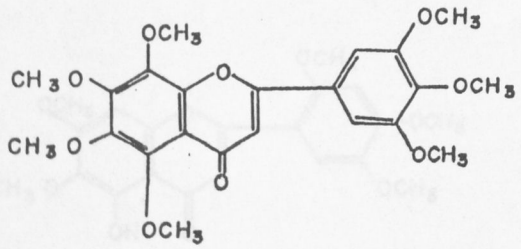
The rotenoid hildercarpin (36) from the roots of *Tephrosia hildebrandtii*(Vatke) has been found to have insect anti-feedant as well as anti-fungal properties.[54] Flavonoids have also been found to possess anthelminthic activities. One hundred and sixteen chalcones and analogues were evaluated on pinworms in mice, and found to be generally effective against pinworms, especially those with few hydroxyl substituents.[55] Adesogan and Okunde[56,57] isolated a chromone ketene acetal identified as conizorigun(37) and a another compound conyzagerin(5'-methoxynobiletin)(38) from *Ageratum conyzoides Linn* (Compositae). Both of these compounds showed a strong anti-bacterial effect. The extract and chemicals isolated from *Diospyros monbuffensis Gurke* inhibited the growth of *Saccharomyces* species and *Escherichia coli*.[58] These examples clearly indicate the importance of non-polar flavonoid aglycones as biologically active natural products. This emphasizes the importance of further studies on their distribution in medicinal plants and as natural cures.



36



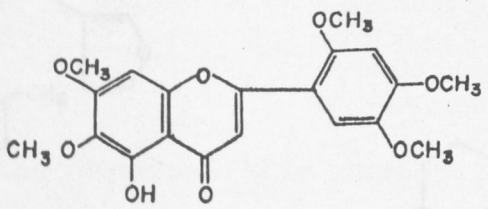
37



38

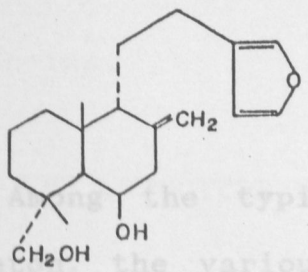
1.5.0 CHEMICAL AND PHARMACOLOGICAL BACKGROUND FOR *PSIADIA* SPECIES

Traditionally the leaf decoction of *P. punctulata* is drunk for abdominal pains and it is known to cure stomach ulcer. Powdered leaves are used for the treatment of colds by the Maasai of E. Africa.[3] Research work on other related species of *Psiadia* shows remarkable potentials of the genus as a source of useful compounds. *Psiadia trinervia*[59] shows anti-microbial activities. From its leaves thirteen, 3-methylated flavonols were isolated and their anti-microbial activities detected by bioassays using *Cladosporium cucumerinum* as the test organism. Recently, Al-Yahya Mohammed et al[60] isolated Psiadiarabin (39) from *Psiadia arabica*.

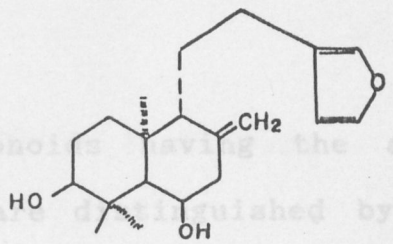


39

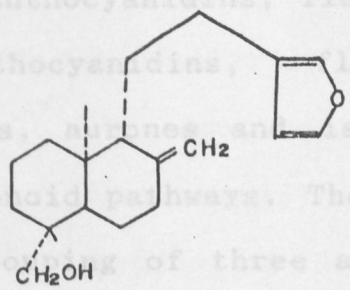
The leaves of *P. altissima*[61] were extracted with acetone and the extract worked up (chromatog.) to give labdane type diterpenes, Psiadiol (40), Isopsiadiol (41), 6-deoxypsiadiol (42) and Palmitic acid, stearic acid, oleic acid, linoleic aromadendrin and aromadendrin 7-methylether. In Madagasca the aerial parts of *P.salviifolia*[62] are used in local medicine as a specific for dysentry, liver disorders and hypertension. Utilizing GLC, IR, combined GC-MS, the following constituents were identified in the essential oil of *Psiadia salviifolia*: ß-pinene, limonene, γ-terpenene, *p*-cymene, α-copaene, linalool, ß-bourbonene, α-himachalene, γ-cadinene, γ-elemene, and a hydroxy derivative of calamene. A new monoterpene hydrocarbon was also isolated, which from MS and IR evidence was named as 7-methyl-3-methyleneocta-1,4-diene.



40



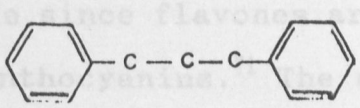
41



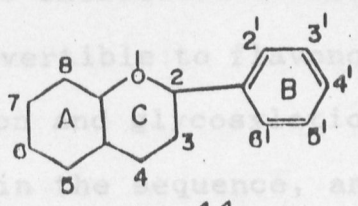
42

1.6.0 BIOSYNTHESIS OF FLAVONOIDS

The flavonoid group may be described as a series of C_6-C_3-C_6 (43) compounds; Their carbon skeleton consists of two C_6 groups (substituted benzene rings) connected by a three carbon aliphatic chain. The different classes within the group are ditinguished by additional oxygen-heterocyclic rings and by the hydroxyl groups distributed in different patterns. The largest group of flavonoids contain a pyran ring linking the three-carbon chain with one of the benzene rings(44).



43



44

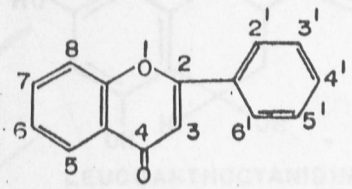
Among the typical flavonoids having the above skeleton, the various types are distinguished by the oxidation state of the C_3 carbon. The flavonoid compounds include leucoanthocyanidins, flavanones, flavononols, flavones, anthocyanidins, flavonols, chalcones, dihydrochalcones, aurones and isoflavones.[63] Scheme 1 shows the flavonoid pathways. The A-ring is formed by head-to-tail grouping of three acetate molecules. The aliphatic three carbon chain is probably added to ring-B before ring-A is formed to produce a C_6C_3 compound. This broad picture of flavonoid biosynthesis has been shown to hold for quercetin[64,65] cyanidin[66],phlorizin[67] and catechins.[68] It presumably also applies to other flavonoids. Tracer feeding experiments have shown that chalcones are converted to flavanones, anthocyanidins, flavonols and isoflavonols.[69,70] The location of hydroxyl groups is established at different stages in the synthesis e.g the hydroxylation pattern of ring-B in flavones and in flavonols is established at the flvanonol level, and the 3-hydroxyl must be introduced at the same stage since flavones are not convertible to flavonols or to anthocyanins.[71] The methylation and glycosylation are usually assumed to be the last in the sequence, and the genetic experiments of Harborne[72] support this idea.

Scheme 2 shows the biosynthetic pathways[73,74] of flavonoids.

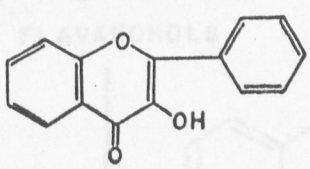
1.7.0 THE STRUCTURE DETERMINATION OF FLAVONOIDS

1.7.1 UV-ABSORPTION SPECTROSCOPY

The flavonoid spectrum is usually determined using a methanol or less satisfactorily an ethanol solution of the flavonoid. UV spectroscopy is particularly applicable to flavones(45) and flavonols(3-hydroxyflavones)(46) because of the direct conjugation of both the A- and B-rings to the carbonyl group.

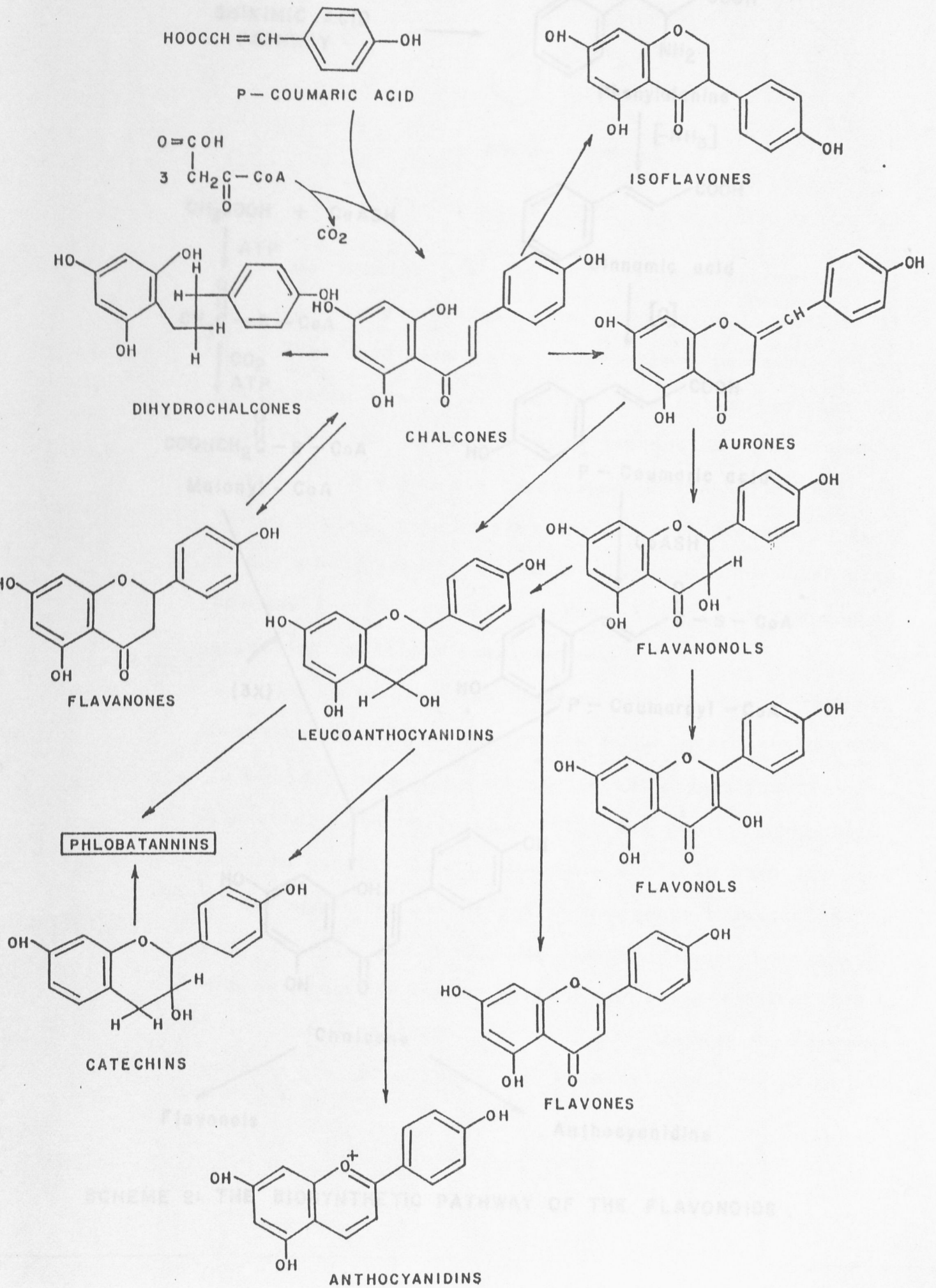


45

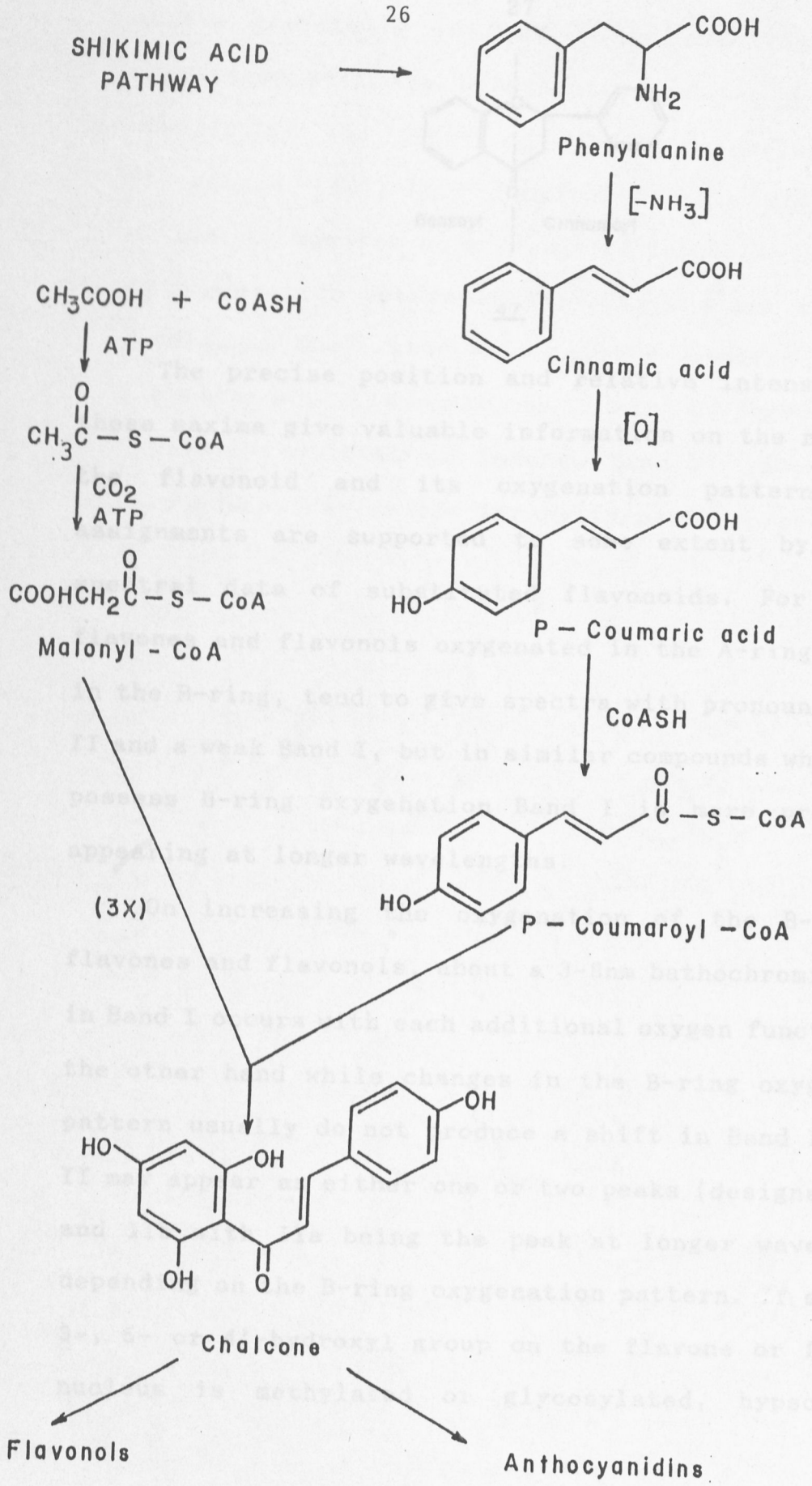


46

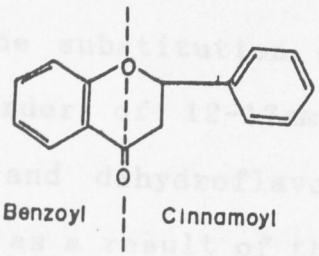
The methanol spectra of flavones and flavonols exhibit two major absorption peaks in the region 240-400nm. These two peaks are commonly referred to as Band I usually(300-380nm) and Band II usually (240-280nm). Band I is considered to be associated with the absorption due to the B-ring cinnamoyl system(47), and Band II with absorption involving the A-ring benzoyl system.[75]



SCHEME I: FLAVONOID PATHWAYS.



SCHEME 2: THE BIOSYNTHETIC PATHWAY OF THE FLAVONOIDS.

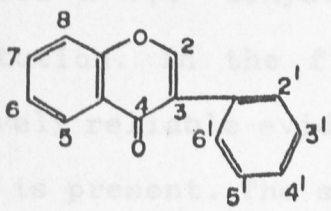


47

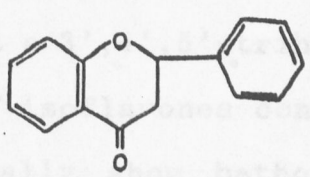
The precise position and relative intensities of these maxima give valuable information on the nature of the flavonoid and its oxygenation pattern. These assignments are supported to some extent by the UV spectral data of substituted flavonoids. For example flavones and flavonols oxygenated in the A-ring but not in the B-ring, tend to give spectra with pronounced Band II and a weak Band I, but in similar compounds which also possess B-ring oxygenation Band I is more pronounced appearing at longer wavelengths.

On increasing the oxygenation of the B-ring in flavones and flavonols, about a 3-8nm bathochromic shift in Band I occurs with each additional oxygen function. On the other hand while changes in the B-ring oxygenation pattern usually do not produce a shift in Band II, Band II may appear as either one or two peaks (designated IIa and IIb with IIa being the peak at longer wavelength) depending on the B-ring oxygenation pattern. If either a 3-, 5- or 4'-hydroxyl group on the flavone or flavonol nucleus is methylated or glycosylated, hypsochromic

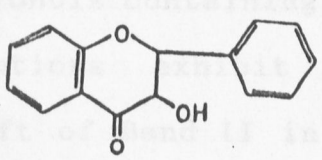
shifts especially in Band I are observed. The shift associated with the substitution of the 3-hydroxyl is usually of the order of 12-17nm. Isoflavones (48), flavanones (49), and dihydroflavonols (50) all give similar UV spectra as a result of their having little or no conjugation between the B-ring and the C-4 carbonyl function. They are all readily distinguished from flavones and flavonols by their UV spectra, which typically exhibits an intense Band II absorption with only a shoulder or low intensity peak representing Band I.



48



49



50

The Band II absorption of isoflavones usually occurs in the region 245-270nm, and is relatively unaffected by increased hydroxylation of the B-ring. It is however shifted bathochromically by increased oxygenation in the A-ring. Both flavanones and dihydroflavonols have their major absorption peak (Band II) in the range 270-295nm. The second step considers the changes induced in the spectrum by the various shift reagents. Degeneration of the UV absorption peaks of flavonols on addition of NaOMe indicates a 3,4'-dihydroxyl or a 3',4',5'-trihydroxyl substitution. In the flavone series this is the only relatively reliable evidence that a 3',4',5'-trihydroxyl system is present. The spectra of isoflavones containing the A-ring hydroxyl groups usually show bathochromic shifts of both Band I and Band II. Flavanones and dihydroflavonols with A-ring hydroxylation show bathochromic shifts for Band II. The UV spectra of flavones and flavonols containing free 7-hydroxyl groups with few exceptions exhibit a diagnostic 5-20nm bathochromic shift of Band II in the presence of NaOAc although when 6,8-oxygen substituents are present in flavones (but not in flavonols), the bathochromic shift with NaOAc is always small or imperceptible.[76]

Flavanones and flavononols containing 7-hydroxyl group give a bathochromic shift of 30-40nm for Band II, coupled with a two fold increase in intensity. In the

presence of NaOAc, boric acid will chelate with ortho-dihydroxyl groups at all locations on the flavonoid nucleus, except C-5, 6. Flavones and flavonols containing a B-ring ortho-dihydroxyl group show a consistent 12-30nm bathochromic shift of Band I. The $AlCl_3$ and $AlCl_3$/HCl reagents form acid-stable complexes between hydroxyls and neighbouring ketones and acid-labile complexes with ortho-dihydroxyl groups such that the groups can be detected. In 5-hydroxyisoflavones Band II undergoes a consistent 9-13nm bathochromic shift and with 5-hydroxyflavanones a consistent bathochromic shift (22-26nm) of Band II occurs. The $AlCl_3$ spectrum, thus represents the sum effect of all complexes on the spectrum, while the $AlCl_3$/HCl spectrum represents the effect of only the hydroxyl-keto complexes.

1.7.2 ^{1}H-NMR SPECTROSCOPY

The application of nuclear magnetic resonance (NMR) spectroscopy to the structure analysis of flavonoids is now well established because many flavonoid aglycones, in particular isoflavones and highly methylated flavones and flavonols are sufficiently soluble in the commonly used solvents.[77] Solvents used for NMR analysis of flavonoids include deuteriochloroform ($CDCl_3$) which dissolves isoflavones and highly methylated flavones and flavonols, and hexadeuteriodimethylsulfoxide (DMSO-d_6). Trimethyl

silyl (TMS) ether derivatives have been used for obtaining NMR spectra of flavonoids which are otherwise insoluble in $CDCl_3$. The proton signals obtained in the NMR spectra of trimethylsilylated flavonoids generally occur in the range 0-10ppm (δ-scale). The A-ring protons at C-6 and C-8 of flavones, flavonols and isoflavones which contain the common 5,7-dihydroxy substitution pattern give rise to two doublets (J=2.5Hz) in the range 6.0-6.5 ppm. The H-6 doublet occurs consistently at higher field than the signal for H-8. The only other proton of the flavonoid nucleus which gives a signal consistently in the same region of the NMR spectrum as those of C-6 and C-8 proton is the C-3 proton of flavones, which appears as a singlet near 6.3 ppm. The C-5 proton in flavonoids with only C-7 oxygenation in the A-ring appear as a doublet (J=9.0Hz) as a result of ortho coupling between the C-5 and C-6 protons. The signals for the protons on C-6 and C-8 both occur at lower field than in the 5,7-dihydroxyflavonoids.

The protons of ring-B appear in the range 6.7-7.9 ppm, which is downfield from the region where the A-ring protons usually absorb. If ring-B is oxygenated only at C-4', a typical four peak pattern of two doublets (each J=8.5Hz) is observed. The doublet for the C-3' and C-5'protons, which are shielded by the C-4' oxygen substituent always appear upfield from the C-2' and C-6'

protons and generally falls in the range 6.65-7.10 ppm for all types of flavonoids. The C-2' and C-6' proton signals of 3',4',5'-oxygenated flavonoids overlap in the region 6.5-7.5 ppm. The C-2 proton in isoflavones occur in the range 7.6-8.7 ppm, a region downfield from where most A- and B-ring proton signals appear. The signal for C-2 proton of flavanones appear as quartet (two doublets J_{cis}=5Hz, J_{trans}=11Hz) near 5.2 ppm as a result of the coupling of the C-2 proton with the C-3 protons. The C-3 protons couple with each other (J=17Hz) in addition to their spin-spin interaction with the C-2 proton, thus giving rise to two overlaping quartets centred around 2.8 ppm. In dihydroxyflavonols the C-2 proton signal occurs as a doublet (J=11Hz) near 5.2 ppm, while the C-3 proton doublet appears further upfield at about 4.3 ppm. Methyl proton signals occur in the range of 1.65-2.50 ppm.

1.7.3 ^{13}C-NMR SPECTROSCOPY

The information gained relates to the total number of carbon atoms per molecule. Carbon-13 resonances occur over a range of 0-200 ppm downfield from TMS compared with a range of only 0-10 ppm for ^{1}H-NMR. In proton-decoupled spectra each carbon atom is represented by one line and its chemical shift is determined primarily to be the electron density at the carbon atom.[78] Thus the

carbon resonances at lowest field are generally those of carbonyl carbons and oxygenated aromatic carbons, whereas those at highest field will represent non-oxygenated aliphatic carbons. The introduction of a new substituent into a molecule will normally cause changes in the chemical shifts of nearby ^{13}C-atoms. Such changes are referred to as substituent effects and a knowledge of the extent of these effects is of immense predictive value. Table 1 below shows a selection of substituent effects which are relevant to the flavonoid field.[79] Using this type of substituent effect data, it is possible to calculate with some accuracy the spectrum of an unknown flavonoid from that of a known similarly substituted flavonoid. To do this a wide range of reference spectra is necessary.

TABLE 1. SHIFT DATA FOR THE EFFECT OF NEW SUBSTITUENTS AT C-1, ORTHO, META, AND PARA POSITIONS.

SUBSTITUENT	C-1	ORTHO	META	PARA
OH	+26.9	-12.7	+1.4	-7.3
OCH_3	+31.4	-14.4	+1.0	-7.7
PHENYL	+13.1	-1.1	+0.4	-1.2
$COCH_3$	+9.1	+0.1	0.0	+4.2

1.7.4 MASS SPECTROSCOPY

Mass spectroscopy is an important technique which for flavonoids is used for the determination of the molecular weight. In electron impact process flavonoids are cleaved into a number of fragments according to certain pathways. For instance the fragmentation of flavanones and dihydroxyflavonols often involves a Retro-Diels-Alder processes.[77] During cleavage many intact A- and B-ring fragments are formed, the combination of which tend to be characteristic for the class of flavonoids. These fragments are of particular value in determining the distribution of substituents between the A- and B-rings.

Kingston[80] found intense $(M-CH_3)^+$ and $(M-CH_3CO)^+$ fragments to be significant for 6- and 8-methoxyflavonoids. Neilsen[81] also indicated that in 6-methoxyquercetin, M^+ appears as base peak, whereas in 8-methoxyquercetin the base peak is at M-15. More recently Goudard et al[82] confirmed that in underivatized 5,7-dihydroxyflavones with methoxyl at C-6, in general M^+ is more important than M-15 and mostly produces the base peak, whereas in those with methoxyl at C-8 the base peak is due to M-15. 6-O-methylflavonols exhibit a less important peak at M-15 than the corresponding flavonols. M-13 signals of less than 30% intensity might be indicative of underivatized 8-methoxy and/or 3-methoxy

flavonols, whereas M-43 signals of more than 30% are indicative for underivatized 6-methoxyflavones or 6-methoxyflavonols which have free 3-hydroxyl group. Isoflavones give a similar fragmentation pattern to that for flavones. Flavanones and flavanonols give similar fragments from the A-ring as the flavones, but the C-8 moieties from the B-ring have a double rather than a triple bond in between C-2 and C-3 and the oxygen of the flavanonols is restrained (Scheme 1).

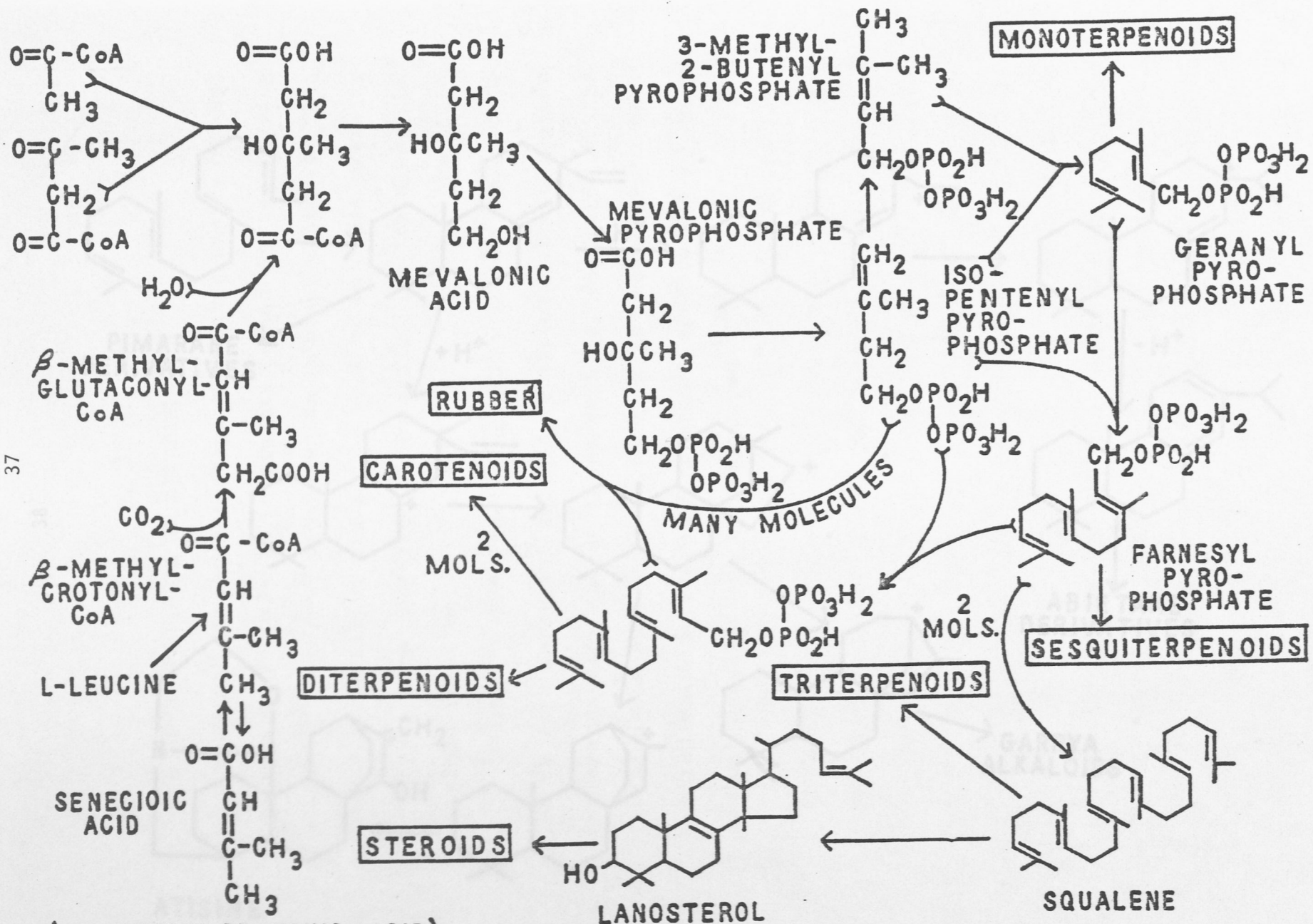
1.7.5 IR SPECTROSCOPY

Infra-red (IR) spectra of flavonoids form useful adjuncts to structural elucidation and are usually quoted in support of identities of newly isolated compounds. IR spectra is particularly useful in establishing the presence or absence of 2'- and 6'-hydroxyl groups in chalcones, 5- and 3-hydroxyl groups in flavonols and flavanonols, and 5-hydroxyl groups in flavones. The IR carbonyl absorption range for these compounds is usually determined prior to methylation or acetylation and again after methylation or acetylation. However very little development have taken place over the last years with regard to the use of IR spectra for diagnostic purposes in the flavonoid field. This is probably because the UV spectra are more informative, require much less material

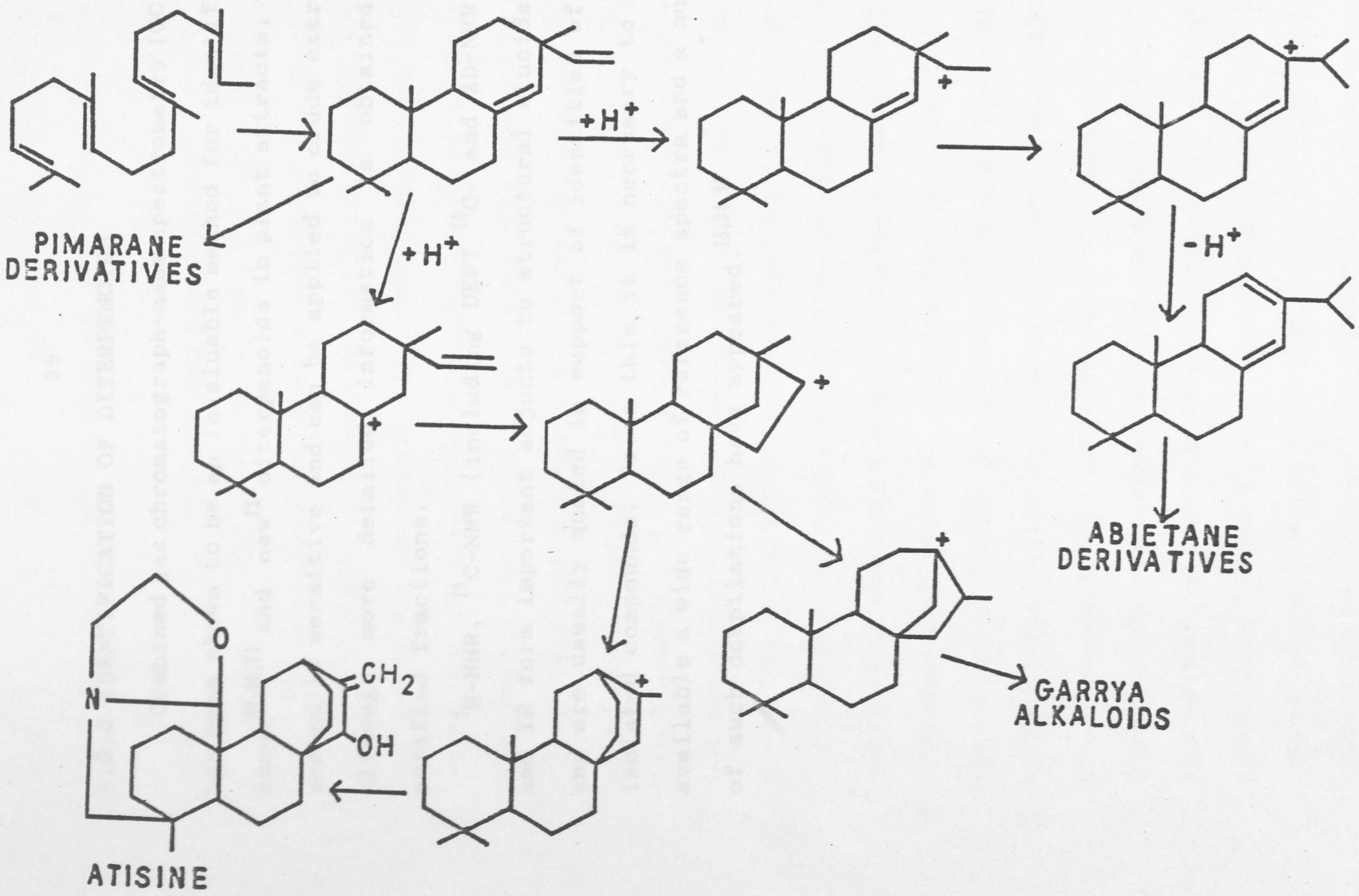
and are generally easier to carry out using polar solvents in which the flavonoids are fully soluble.

1.8.0 BIOSYNTHESIS OF DITERPENES

There is reasonable agreement regarding the early steps in the biosynthesis of isoprenoid compounds and some scattered evidence regarding biosynthesis of some of the major categories. The generally acceptable pathways are outlined in Scheme 3. The diterpenes are formed by the sequencial conversion of mevalonate through isopentenyl pyrophosphate and dimethylallyl pyrophosphate to geranyl pyrophosphate, farnesyl pyrophosphate and thence the C_{20} geranylgeranylpyrophosphate. Cyclization of the latter is thought to occur in a number of discrete stages through bicyclic and tricyclic intermediates to the tetracyclic diterpenes. In more complicated cases several different rearragements may occur before the final structure is attained so that profound modifications are introduced.[83] The diterpenoid rearrangement shown in Scheme 4 is for some diterpenoid structures including the diterpenoid alkaloids.[84,85,86] Mevalonic acid lactone acts as a precursor of the tricyclic diterpene, rosenonolactone and the tetracyclic diterpenes, gibberellic acid[87] and the kaurenolides. Geranylgeranyl pyrophosphate is converted to (-)-kaurene and then latter to gibberellic acid.[88]



SCHEME 3 *TERPENOID AND STEROID PATHWAYS*



SCHEME 4 DITERPENOID REARRANGEMENTS

1.9.0 IDENTIFICATION OF DITERPENOIDS

Combined gas chromatograph-mass spectrometry (GC-MS) has been shown to be an invaluable method for identifying known[89,90,91] and new[92] diterpenoids in plant extracts. This method is sensitive and can be applied to crude extracts although more detailed information is obtained on purified fractions.

^{1}H-NMR, ^{13}C-NMR (including DEPT ^{13}C- and 2D-NMR) UV and IR form important adjunts to structural elucidation and are usually quoted in support of identities of the isolated compounds. To do this it is necessary to have available a wide range of reference spectra and a number of such compilations have appeared.[93,94,95]

CHAPTER 2

RESULTS AND DISCUSSION

The aerial parts of *Psiadia punctulata* (D.C) Vatke were pressed with pieces of filter paper without rapturing the epidermal layer and left yellow stain on paper due to epicuticular exudate pigment which covers the leaves. The leaves were dipped into acetone in a two litre beaker for a period of fifteen seconds only to avoid extraction of internal tissue material as indicated by chlorophyll.[96] Acetone was initially coloured yellow but later the colour intensified to pale yellow. At this stage the extract was decanted into an erlenmeyer flask and subsequent extractions carried out using fresh portions of acetone. The extracts were combined, filtered and concentrated in vacuo using the rotatory evaporator resulting in a pale gummy solid. T.L.C analysis of this gummy extract using Merck aluminium precoated silica gel 60 plates developed in 4% methanol in chloroform and the dried chromatogram examined using visible light, UV light before and after exposing to fumes of concentrated ammonia and iodine quickly showed that it was composed of twelve pigment spots. The presence of flavonoids and terpenoids as externally deposited free aglycones on the crude extract[97], was found in both cases to be positive.

To achieve an applied meaning and significance, our work incorporated bioassays. The first bioassay was the cytotoxicity test using brine shrimp as described by Maclaughlin et al.[98] This bioassay determines the lethalities of materials toward brine shrimp nauplii (larvae) and in doing so predicts the ability to kill cancer cells in cell cultures, to kill various pests and exert a diverse range of pharmacological effects. In this bioassay initial concentrations of 10, 50, 100, 250, 500, and 1000µg/ml in sample bottles containing 5ml of brine and twenty shrimp in each of four replicates was used. The control mortalities of the larvae were usually less than 10% ; when greater than 10% of the controls died, the results were discarded. The bioassay was repeated three times, each time starting with a new batch of shrimp. The data was processed with a SAS Language Guide for Personal Computer Version six to estimate the ED_{50} values with 95% confidence intervals for statistically significant comparisons of potencies.[99] The results are in table 8 and show that the crude had very high activity with an ED_{50} of 11.2 ppm(µg/ml). From the results it became obvious that *P. punctulata* is a source of bioactive compounds. The results compare very well with the results of ethanolic extracts of seeds of some *Euphorbiaceae* species (see table 10).

The second bioassay model was anti-feedant test with mid 5th instar nymphs of *Locusta migrotoria* (desert locust) exposed to sugar soaked (and dried) whatman no.1 filter paper according to the procedure of Butterworth and Morgan.[100] The crude extract at 1000µg/ml gave a relative anti-feedant activity (RAP) of 97.7% (Table 11). Results for other concentrations are shown in the same table. The RAP values were calculated from the formular:

$$RAP = \frac{A_2 - A_1}{A_1 + A_2} \times 100$$

where A_1 = eaten area of sample treated filter paper.

A_2 = eaten area of sucrose treated filter paper.

The third bioassay model was that of growth inhibition towards pathogenic fungi (*Collectotricum coffeanum* and *Fusarium oxysporum*). In this bioassay the colony diameter of the treated plates compared with the control plates was taken as a relative measure of fungitoxicity. The crude extract inhibited the mycelial growth of coffee berry disease fungus *Collectotricum coffeanum* and *Fusarium oxysporum* as shown in tables 2-7. At 1000µg/ml the percentage inhibition for *C. coffeanum*

and *F. oxysporum* after five days were 47.7% and 60.4% respectively. The percentage inhibition was calculated using the formular :

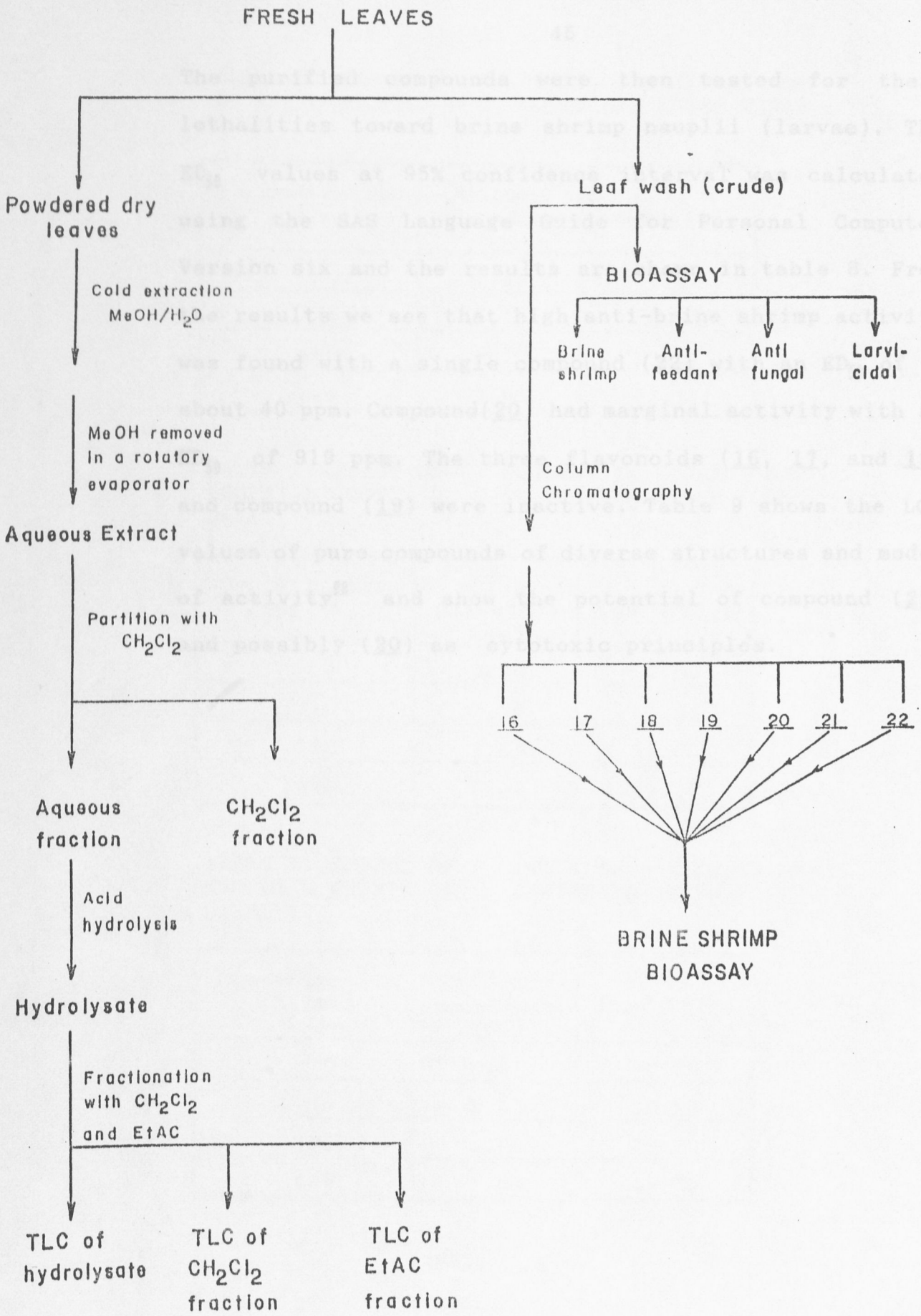
$$\% \ inhibition = \frac{C - T}{C} \times 100$$

where C= mycelial growth in control plates

T= mycelial growth in the treated plates.

The final bioassay model was the toxicity towards the 2nd instar larvae of the yellow fever vector *Aedes aegyptii*. The mortality rate was traced after 24 hours, 48 hours, upto a period of one week. The crude extract was found to have no larvicidal activity (Table 12). The larvae reached adulthood within eleven days as required.

Isolation of the components of the crude extract was carried out using step gradient elution column chromatography. Scheme 5 shows the silica gel chromatographic fractionation of *P. punctulata* leaves. The eluting solvents were n-hexane, chloroform, and methanol. Purification of the compounds was accomplished using preparative tlc, flash chromatography and fractional crystallization. The compounds isolated included the three flavonoids (positive flavonoid test) (16), (17), and (18) and the four terpenoids (positive terpenoid test), (19), (20),(21) and (22).



EME 5: Fractionation of P. punctulata leaves

The purified compounds were then tested for their lethalities toward brine shrimp nauplii (larvae). The ED_{50} values at 95% confidence interval was calculated using the SAS Language Guide for Personal Computer Version six and the results are shown in table 8. From the results we see that high anti-brine shrimp activity was found with a single compound (22) with an ED_{50} of about 40 ppm. Compound(20) had marginal activity with an ED_{50} of 919 ppm. The three flavonoids (16, 17, and 18) and compound (19) were inactive. Table 9 shows the LC_{50} values of pure compounds of diverse structures and modes of activity[98] and show the potential of compound (22) and possibly (20) as cytotoxic principles.

TABLE 2. EFFECT OF *P. PUNCTULATA* EXTRACT ON MYCELIAL GROWTH OF *C. COFFEANUM* AFTER THREE DAYS.

Concentration (µg/ml)	Colony diameters (cm)	% inhibition
0	1.46	0.00
10	1.45	0.68
100	1.27	13.01
1000	0.80	45.21

TABLE 3.EFFECT OF *P. PUNCTULATA* EXTRACT ON MYCELIAL GROWTH OF *C. COFFEANUM* AFTER FOUR DAYS.

Concentration (µg/ml)	Colony diameters (cm)	% inhibition
0	2.00	0.00
10	1.78	11.00
100	1.63	18.50
1000	1.06	47.00

TABLE 4. EFFECT OF *P. PUNCTULATA* EXTRACT ON MYCELIAL GROWTH OF *C. COFFEANUM* AFTER FIVE DAYS.

Concentration (µg/ml)	Colony diameters (cm)	% inhibition
0	2.41	0.00
10	2.15	10.90
100	1.94	19.50
1000	1.26	47.70

TABLE 5. EFFECT OF *P. PUNCTULATA* EXTRACT ON MYCELIAL GROWTH OF *F. OXYSPORUM* AFTER THREE DAYS.

Concentration (µg/ml)	Colony diameters (cm)	% inhibition
0	3.40	0.00
10	3.38	0.59
100	3.23	5.00
1000	1.51	55.60

TABLE 6. EFFECT OF *P. PUNCTULATA* EXTRACT ON MYCELIAL GROWTH OF *F. OXYSPORUM* AFTER FOUR DAYS.

Concentration (µg/ml)	Colony diameters (cm)	% inhibition
0	4.75	0.0
10	4.63	2.53
100	4.46	6.11
1000	1.93	59.37

TABLE 7. EFFECT OF *P. PUNCTULATA* EXTRACT ON MYCELIAL GROWTH OF *F. OXYSPORUM* AFTER FIVE DAYS.

Concentration (µg/ml)	Colony diameters (cm)	% inhibition
0	5.88	0.0
10	5.78	1.70
100	5.51	6.30
1000	2.33	60.37

TABLE 8. BRINE SHRIMP BIOASSAY RESULTS OF ACETONE EXTRACT AND PURIFIED COMPOUNDS OF THE AERIAL PARTS P.PUNCTULATA

SAMPLE	PERCENTAGE DEATHS AT 24 HOURS			ED_{50}
	10µg/ml	100µg/ml	1000µg/ml	
CRUDE EXT	23.8	85.7	100.0	11.2
COMPD(16)	9.4	15.0	19.0	Inactive
COMPD(17)	9.1	28.6	19.0	Inactive
COMPD(18)	23.8	28.6	23.8	Inactive
COMPD(19)	9.5	23.8	19.0	919.9
COMPD(20)	14.3	33.3	52.4	Inactive
COMPD(22)	14.2	81.0	95.2	39.5

TABLE 9. BRINE SHRIMP BIOASSAY RESULTS FOR KNOWN ACTIVE NATURAL PRODUCTS

NATURAL PRODUCT	LC_{50} (µg/ml)
Podophyllotoxin	2.4
Berberrine chloride	22.5
Strychine sulphate	77.2
Digitalin	151.0
Quinidine sulphate	215.0
Ephedrin sulphate	215.0
Arbutine	275.0
Caffeine	306.0
Atropine sulphate	686.0
Santonin	>1000

TABLE 10. SOME BRINE SHRIMP BIOASSAY RESULTS OF ETHANOLIC EXTRACTS OF SEEDS OF SOME *EUPHORBIACEAE* SPECIES AT 95% CONFIDENCE INTERVAL COMPARED WITH 9KB AND 9PS CYTOTOXICITIES.

SPECIES	PERCENTAGE DEATHS AT 24 HOURS					
	10 µg/ml	100 µg/ml	1000 µg/ml	LC_{50} µg/ml	9PS LC_{50} µg/ml	9KB LC_{50} µg/ml
E. lagascae	30	34	80	119	0.8	Inact
E. marginata	0	26	100	162	7.7	Inact
A. fordii	0	10	100	247	18.3	Inact
A. nigricans	0	4	100	286	Inact	Inact
J. spathulata	10	32	48	981	0.3	Inact
M. rubricaulus	0	0	38	>1000	55.8	Inact
E. paralias L.	0	0	46	>1000	20.2	Inact
M. isoloba S.	0	0	6	>1000	4.0	3.3
C. elaticus L.	0	0	0	>1000	33.5	31.2
E. medicaginea	0	0	0	>1000	4.0	59.9
S. montevidence	0	24	98	116	4.2	Inact

TABLE 11. PERCENTAGE PROTECTION AGAINST FEEDING BY MID 5TH INSTAR *L. MIGROTORIA* HOPPERS AFTER EIGHT HOURS

CONCENTRATION (µg/ml)	RAP (%)
1000	97.7
100	62.5
10	51.3

TABLE 12. NUMBER OF 2ND INSTAR LARVAE OF AEDES AEGYPTI SURVIVING AFTER TREATMENT WITH VARIOUS CONCENTRATIONS OF THE CRUDE LEAF WASH OF *P. PUNCTULATA*

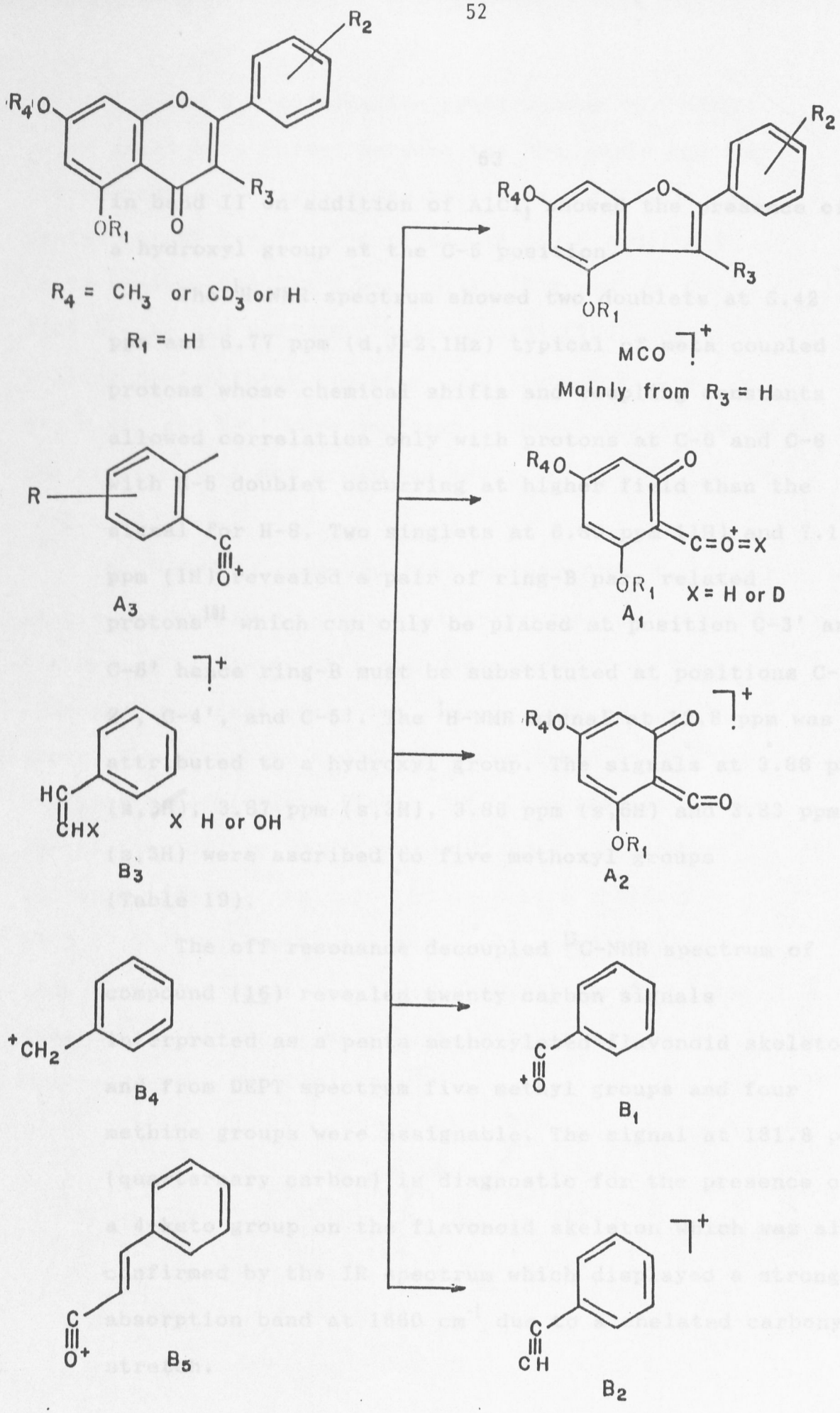
CONC. µg/ml	POST-TREATMENT DAYS 0	1	2	3	4	5	6		7		
							L	P	L	P	A
0	20	20	20	20	20	20	6	14	2	17	1
1	20	20	20	20	20	20	3	17	1	19	0
2	20	19	19	19	19	19	6	14	0	19	0
3	20	19	19	19	19	19	5	15	0	19	0
4	20	20	20	20	20	20	3	17	1	19	0
5	20	20	20	20	20	20	4	16	2	18	0
6	20	20	20	20	20	20	4	16	0	20	0
7	20	20	20	20	20	20	3	17	1	19	0
8	20	20	19	19	19	19	5	15	0	19	0
10	20	20	20	20	20	20	4	16	0	20	0

KEY: L = larvae P = pupa A = adult

2.1.0 CHARACTERIZATION OF CONSTITUENT COMPOUNDS OF *P. PUNCTULATA*

Compound (**16**) with an rf of 0.85 using chloroform as the developing solvent appeared yellowish purple on silica gel plate under UV-light. The UV spectrum in methanol displayed two major absorption peaks characteristic of either flavones or flavonols at λmax 331 and 267.5 nm associated with absorption due to B-ring cinnamoyl system (band I) and A-ring benzoyl system (band II) respectively.[97] The mass spectral analysis showed a molecular ion peak at m/e 388 (100%) corresponding to $C_{20}H_{20}O_8$ which was also in accordance with the elemental analysis. Scheme 6 shows the major mass spectral A- and B-ring fragments expected of flavones or flavonols. The prominent fragment peak at m/e 167 corresponds to the A-ring fragment holding one hydroxyl and one methoxy group. The presence of a prominent peak for the $[M-15]^+$ ion at m/e 373 indicated the loss of a methyl group. The prominent fragment peak at m/e 222 requires a trimethylated B-ring.

From the UV spectral analysis using shift reagents, the lack of shifts in all the bands when NaOAc was added to a methanolic solution of the compound indicated the absence of a C-7 hydroxyl group while a bathochromic shift of 15 nm in band I and 9 nm



SCHEME 6: Fragmentation processes from flavones and flavonols.

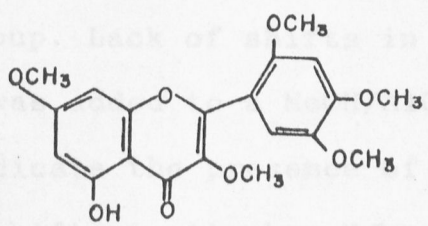
in band II on addition of $AlCl_3$ showed the presence of a hydroxyl group at the C-5 position.

The ^{1}H-NMR spectrum showed two doublets at 6.42 ppm and 6.77 ppm (d,J=2.1Hz) typical of meta coupled protons whose chemical shifts and coupling constants allowed correlation only with protons at C-6 and C-8 with H-6 doublet occurring at higher field than the signal for H-8. Two singlets at 6.80 ppm (1H) and 7.19 ppm (1H) revealed a pair of ring-B para related protons[101] which can only be placed at position C-3' and C-6' hence ring-B must be substituted at positions C-2', C-4', and C-5'. The ^{1}H-NMR signal at 12.8 ppm was attributed to a hydroxyl group. The signals at 3.88 ppm (s,3H), 3.87 ppm (s,3H), 3.86 ppm (s,6H) and 3.83 ppm (s,3H) were ascribed to five methoxyl groups (Table 19).

The off resonance decoupled ^{13}C-NMR spectrum of compound (16) revealed twenty carbon signals interpreted as a penta methoxylated flavonoid skeleton and from DEPT spectrum five methyl groups and four methine groups were assignable. The signal at 181.8 ppm (quarternary carbon) is diagnostic for the presence of a 4-keto group on the flavonoid skeleton which was also confirmed by the IR spectrum which displayed a strong absorption band at 1660 cm^{-1} due to a chelated carbonyl stretch.

From the H,C-correlation spectroscopy (H,C-COSY) cross peaks were formed between the ^{1}H-signals and the corresponding ^{13}C-signals and helped in confirming the assignment of H-6 (6.42 ppm) to C-6 (98.0 ppm), H-8 (6.77 ppm) to C-8 (92.8 ppm), H-3' (7.2 ppm) to C-3' (107.0 ppm) and H-6' (6.8 ppm) to C-6' (109.1 ppm). Placement of methoxy groups at positions C-7, C-3, C-2', C-4', and C-5' were confirmed through comparison of the experimental and predicted chemical shifts (Table 13).

The ^{1}H-NMR of compound (16) acetate gave a signal at 2.4 ppm ascribed to the acetoxy protons while the C-5 hydroxyl proton at 12.8 ppm disappeared from the spectrum thus confirming the presence of one hydroxyl group. The structure was further confirmed using ^{13}C-NMR spectroscopic data (Table 14). From the literature correlation of the ^{1}H-NMR values[110] and ^{13}C-NMR values[111] compound (16) was assigned the structure 5-hydroxy-2',3,4',5',7-pentamethoxyflavone. Compound (16) is being reported present in *P. punctulata* for the first time but it had previously been isolated from *Distemonanthus benthamianus*.[102]



16

Compound (17) is yellowish on silica gel tlc under UV light and its UV spectrum in methanol displayed two major absorption peaks characteristic of either flavones or flavonols at λmax 333 and 268nm associated with absorption due to the B-ring cinnamoyl system (band I) and with A-ring benzoyl system (band II) respectively. The mass spectral analysis showed a molecular ion peak at m/e 374 (100%) corresponding to $C_{19}H_{18}O_8$ which was also in accordance with the elemental analysis. The major mass spectral A- and B- fragments could be interpreted according to scheme 6. The prominent fragment peak at m/e 153 require a dihydroxylated A-ring while the presence of methoxyl groups was indicated by the prominent $[M-15]^+$ ion at m/e 359 (29.1%). A prominent fragment peak at m/e 222 requires a trimethylated B-ring.

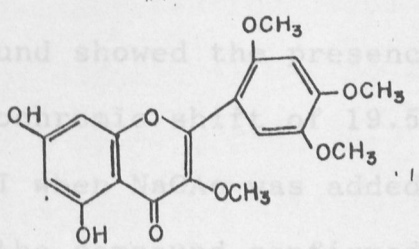
From the UV spectral analysis using shift reagents, a bathochromic shift of 14 nm in band I and 6 nm in band II when NaOAc was added to a methanolic solution of compound (17) confirmed the presence of a C-7 hydroxyl group. Lack of shifts in all absorption bands when HCl was added to a MeOH/$AlCl_3$ solution of the compound indicate the presence of a 5-hydroxyl group while no shift at all when H_3BO_3 was added to MeOH/NaOAc solution of the compound showed the absence of ortho-dihydroxyl groups.

The ^{1}H-NMR spectrum showed two doublets at 6.2 ppm (d,J=2.1Hz) and 6.5 ppm (d,J=2.1Hz) typical of meta coupled protons at C-6 and C-8 (ring-A) with the H-6 doublet occuring at higher field than the H-8. Two sharp singlets at 6.7 ppm (1H) and 7.1 ppm (1H) represent para coupled protons of ring-B which can only be ascribed to C-3' and C-6' positions. The signals at 10.9 ppm (s,1H) and 12.8 ppm (s,1H) were attributed to two hydroxyl groups in ring-A and signals at 3.88 ppm (s,3H), 3.87 ppm (s,3H), 3.86 ppm (s,3H), and 3.81 ppm (s,3H) were ascribed to the methoxyl protons (Table 19).

The off resonance decoupled ^{13}C-NMR spectrum of compound (17) revealed nineteen carbon signals due to a tetramethylated flavonoid skeleton. From the DEPT spectrum, four methyl and four methine groups were determined, while the signal at 181.6 ppm was diagnostic of a 4-keto group on the flavonoid skeleton and was confirmed further by the IR spectrum which displayed a strong band at 1650 cm^{-1} due to chelated carbonyl stretch.

From the H,C-correlation spectroscopy cross peaks were formed between the ^{1}H-signals and the corresponding ^{13}C-signals and helped in confirming the assignment of H-6 (6.2 ppm) to C-6 (98.9 ppm), H-8 (6.5 ppm) to C-8 (94.0 ppm), H-3' (7.1 ppm) to C-3' (107.0

ppm) and H-6' (6.7 ppm) to C-6' (108.9 ppm). The placement of methoxy groups at positions C-2', C-4', and C-5', and hydroxy groups at C-5 and C-7 positions were confirmed through comparison of the experimental and predicted chemical shifts (Table 15). Through literature correlation with ^{1}H-NMR and ^{13}C-NMR spectroscopic data (Table 17), compound (17) was assigned the structure 5,7-dihydroxy-2',3,4',5'-tetramethoxyflavone. Compound (17) is a new natural product since it could not be traced in the literature.



17

Compound (18) which also turned purple on silica gel tlc under UV light gave a UV spectrum which displayed two major absorption bands characteristic of either flavones or flavonols at λmax (MeOH) 344.5 nm associated with absorption due to the B-ring cinnamoyl system (band I) and λmax (MeOH) 265.0 nm associated with absorption by the A-ring benzoyl system (band II). The mass spectral analysis showed a molecular ion at m/e 360 (100%) corresponding to $C_{18}H_{16}O_8$ which was also confirmed by the elemental analysis. From the mass

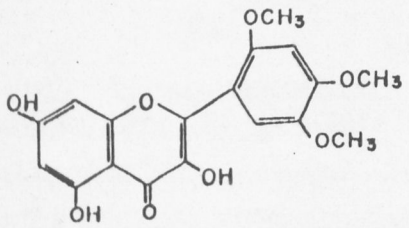
spectral analysis a prominent fragment ion at m/e 153 (31%) required a dihydroxylated ring-A (see scheme 6). The prominent fragment ion at m/e 345 (25%) was due to the loss of fifteen mass units corresponding to a methyl group. The fragment $[B_3-2]^{+\cdot}$ at m/e 208 require a trimethylated B-ring.

From the UV spectral analysis using shift reagents a bathochromic shift of 54 nm in band I and 5.5 nm in band II when NaOMe was added to a methanolic solution of the compound showed the presence of hydroxyl groups while a bathochromic shift of 19.5 nm in band I and 8 nm in band II when NaOAc was added to a methanolic solution of the compound confirmed the presence of C-7 hydroxyl group.

The ^{1}H-NMR of compound (18) showed two doublets at 6.2 ppm (d,J=2.1 Hz) and 6.5 ppm (d,J=2.1 ppm) typical of meta-coupled protons at C-6 and C-8 (A-ring) with the H-6 doublet occuring at higher field than the signal for H-8. The two sharp singlets at 6.7 ppm (1H) and 7.1 ppm (1H) represent para coupled protons of ring-B which can only be placed at positions C-3' and C-6'. Signals at 9.6 ppm (s), 10.6 ppm (s) and 12.8 ppm (s) were attributed to three hydroxyl groups while the signals at 3.88 ppm (s,3H), 3.86 ppm (s,3H) and 3.81 ppm (s,3H) were assigned to the methoxyl protons (Table 19).

The off resonance decoupled ^{13}C-NMR spectrum of compound (18) revealed eighteen signals due to a trimethylated flavonoid skeleton and ^{13}C- and DEPT ^{13}C-NMR helped in placing the three methyl and the four methine groups. The signal at 181.7 ppm was diagnostic for the presence of a 4-keto group on the flavonoid skeleton and was also confirmed by the IR spectrum which displayed a strong absorption band at 1650 cm^{-1} due to the C=O stretch.

The H,C-COSY spectrum helped in confirming the assignment of signals at H-6 (6.2 ppm) to C-6 (98.8 ppm), H-8 (6.5 ppm) to C-8 (94.0 ppm), H-3' (7.1 ppm) to C-3' (106.4 ppm) and H-6' (6.7 ppm) to C-6' (108.6 ppm) due to the formation of the cross peaks. Placement of methoxy groups at positions C-2'and C-4'and hydroxyl groups at positions C-5 and C-7 were confirmed through comparison of experimental and calculated chemical shifts except for the chemical shift for position C-5' which deviated slightly as observed in both compounds (16) and (17) (Table 16). Through the literature correlation with ^{1}H-NMR and ^{13}C-NMR spectroscopic data (Table 16), compound (18) was assigned the structure 5,7-dihydroxy-2',4',5'-trimethoxyflavonol and is also a new natural product since it could not be traced in the literature.



18

TABLE 13. ^{13}C-NMR CHEMICAL SHIFT POSITIONS FOR COMPOUND (16) (400MHz in DMSO, ppm)

POSITION OF C	$\delta_{experimental}$ (ppm)	$\delta_{predicted}$ (ppm)
2	157.4	-
3	145.6	-
4-keto	181.8	-
5	162.0	159.7
6	98.0	-
7	165.3	168.1
8	92.8	-
9	161.8	160.8
10	104.7	107.5
1'	119.3	116.9
2'	149.3	148.0
3'	107.0	102.0
4'	147.1	146.0
5'	146.3	138.9
6'	109.1	111.0
2'-OCH_3	61.2	-
3-OCH_3	60.7	-
4'-OCH_3	56.1	-
5'-OCH_3	61.0	-
7-OCH_3	56.3	-
5-OH	-	-

TABLE 14. A SELECTION OF FLAVONE AND FLAVONOL ^{13}C-NMR SPECTRA.

COMPOUND	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-1'	C-2'	C-3'	C-4'	C-5'	C-6'
Quercetin 7,4'-dimethyl ether	146.7	136.4	176.0	160.4	97.4	164.9	91.8	156.0	104.0	123.4	114.8	146.2	149.4	111.7	97.4
Kaempferol 4'-methyl ether	146.4	135.8	175.8	160.6	98.2	163.9	93.4	156.2	103.1	123.2	129.0	114.0	160.6	114.0	129.0
Quercetin 3,4'-dimethyl ether	155.3	138.2	178.1	161.6	98.8	164.4	93.8	156.6	104.5	122.6	115.2	146.6	150.3	111.8	120.9
Tricetin 3',4',5'-trimethyl ether	162.9	105.0	181.5	161.3	98.8	164.1	94.0	157.3	104.0	125.8	105.0	153.1	141.4	153.1	105.0
Compound (16)	161.1	145.6	181.8	162.0	98.0	165.3	92.8	157.4	104.7	119.3	149.3	107.0	147.1	146.3	109.1

TABLE 15. ^{13}C-NMR CHEMICAL SHIFT POSITIONS FOR COMPOUND (17) (400MHz in DMSO, ppm)

POSITION OF C	$\delta_{experimental}$ (ppm)	$\delta_{predicted}$ (ppm)
2	157.5	-
3	145.4	-
4-keto	181.6	-
5	161.8	159.7
6	98.9	98.6
7	164.3	163.9
8	94.0	96.1
9	161.3	161.8
10	103.7	107.7
1'	119.5	116.9
2'	149.3	148.0
3'	107.0	102.0
4'	147.1	146.0
5'	146.2	138.9
6'	108.9	111.0
2'-OCH_3	61.2	-
3-OCH_3	60.7	-
4'-OCH_3	56.2	-
5'-OCH_3	61.0	-
5-OH	-	-
7-OH	-	-

TABLE 16. ^{13}C-NMR CHEMICAL SHIFT POSITIONS FOR COMPOUND (18) (400MHz in DMSO, ppm)

POSITION OF C	$\delta_{experimental}$ (ppm)	$\delta_{predicted}$ (ppm)
2	157.5	-
3	141.7	-
4-keto	181.7	-
5	162.1	159.7
6	98.8	98.6
7	164.2	163.9
8	94.0	96.1
9	161.3	161.8
10	103.7	107.7
1'	119.3	116.9
2'	149.1	148.0
3'	106.4	102.0
4'	147.1	146.0
5'	106.4	138.9
6'	108.6	111.0
2'-OCH_3	61.2	-
4'-OCH_3	56.2	-
5'-OCH_3	61.0	-
3-OH	-	-
5-OH	-	-
7-OH	-	-

TABLE 17. A SELECTION OF FLAVONE AND FLAVONOL ^{13}C-NMR SPECTRA.

COMPOUND	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
MORIN	149.9	136.5	177.0	161.3	99.6	164.1	95.0	157.0	105.0	111.2	158.1	105.0	161.0	109.2	132.3
QUERCETIN	146.9	135.6	175.7	160.7	98.2	163.9	93.4	156.2	103.0	122.0	115.3	145.0	147.6	115.6	120.0
KAEMPFEROL	146.9	135.6	175.5	160.7	98.2	163.9	93.5	156.2	103.1	121.7	123.5	115.4	159.2	115.4	123.5
COMPD (17)	149.3	145.4	181.6	161.9	98.9	164.3	94.0	157.5	103.7	119.5	161.3	107.0	147.1	146.2	108.9
COMPD (18)	149.1	141.7	181.7	162.1	98.8	164.2	94.0	157.5	103.7	119.3	161.3	106.4	147.1	144.5	108.6

TABLE 19. ^{1}H-NMR CHEMICAL SHIFT POSITIONS OF COMPOUNDS (16), (17) AND (18) (400MHz in DMSO, ppm)

C-POSITION	(16) ^{1}H-NMR(ppm)	(17) ^{1}H-NMR(ppm)	(18) ^{1}H-NMR(ppm)
2	-	-	-
3	-	-	-
4-keto	-	-	-
5	-	-	-
6	6.43(d,J=2.1)	6.2(d,J=2.1Hz)	6.2(d,J=2.1Hz)
7	-	-	-
8	6.77(d,J=2.1Hz)	6.5(d,J=2.1Hz)	6.5(d,J=2.1Hz)
9	-	-	-
10	-	-	-
1′	-	-	-
2′	-	-	-
3′	7.2(s)	7.1(s)	7.1(s)
4′	-	-	-
5′	-	-	-
6′	6.8(s)	6.7(s)	6.7(s)
7-OCH_3	3.88	-	-
2′-OCH_3	-	-	-
4′-OCH_3	3.87	-	-
5′-OCH_3	-	-	-
7-OH	-	10.9	10.6
5-OH	-	12.8	12.8
3-OH	-	-	9.6

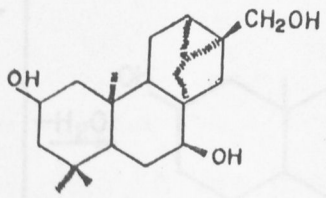
Compound (**19**) showed a positive terpenoid test. It did not fluoresce under UV light and showed no significant absorption in the UV region (λmax MeOH 203 nm). Through the MS fragmentation pattern and ^{1}H- and ^{13}C-NMR spectral correlation with literature[103,104,105,106,107] compound (**19**) was found to belong to the trachylobane diterpenoid group (Table 18).

The mass spectral analysis showed a molecular ion at m/e 320 corresponding to the molecular formula $C_{20}H_{32}O_3$ which was in accordance with the elemental analysis. The MS showed a series of prominent peaks, the genesis of which is well accounted for by the cation at m/e 302 and 284 as shown in scheme 7.

Two-dimensional ^{1}H- and ^{13}C-NMR direct chemical shift correlation experiments (Table 20) allowed all signals to be interrelated. The presence of cyclopropyl grouping in compound (**19**) was disclosed by the signals at 0.62 ppm and 0.82 ppm. This is due to the two non-equivalent hydrogens on the cyclopropane ring. A similar signal is observed for the cyclopropyl protons of trachylobanic acid, as a broad multiplet between 0.4 and 0.8 ppm[108]. The ^{1}H-NMR gave chemical shifts for H-9 to H-15 which compared very well with those of trachylobanol (**53**) (Table 21). The AB quartet centred at 3.39 ppm (J=10.4 Hz) was assigned to the primary hydroxyl group which was further confirmed by the IR

spectrum, displaying a broad peak at 3300 cm^{-1} due to the O-H stretch of primary hydroxyl group. The ^{1}H-NMR spectrum also showed signals at 1.22 ppm (s,3H), 1.11 ppm (s,3H), and 1.07 ppm (s,3H) for three tertiary methyl groups. The fact that no proton signals were present in the vinyl region of the ^{1}H- and ^{13}C-NMR spectrum indicates that compound (**19**) contains no carbon-carbon double bond.

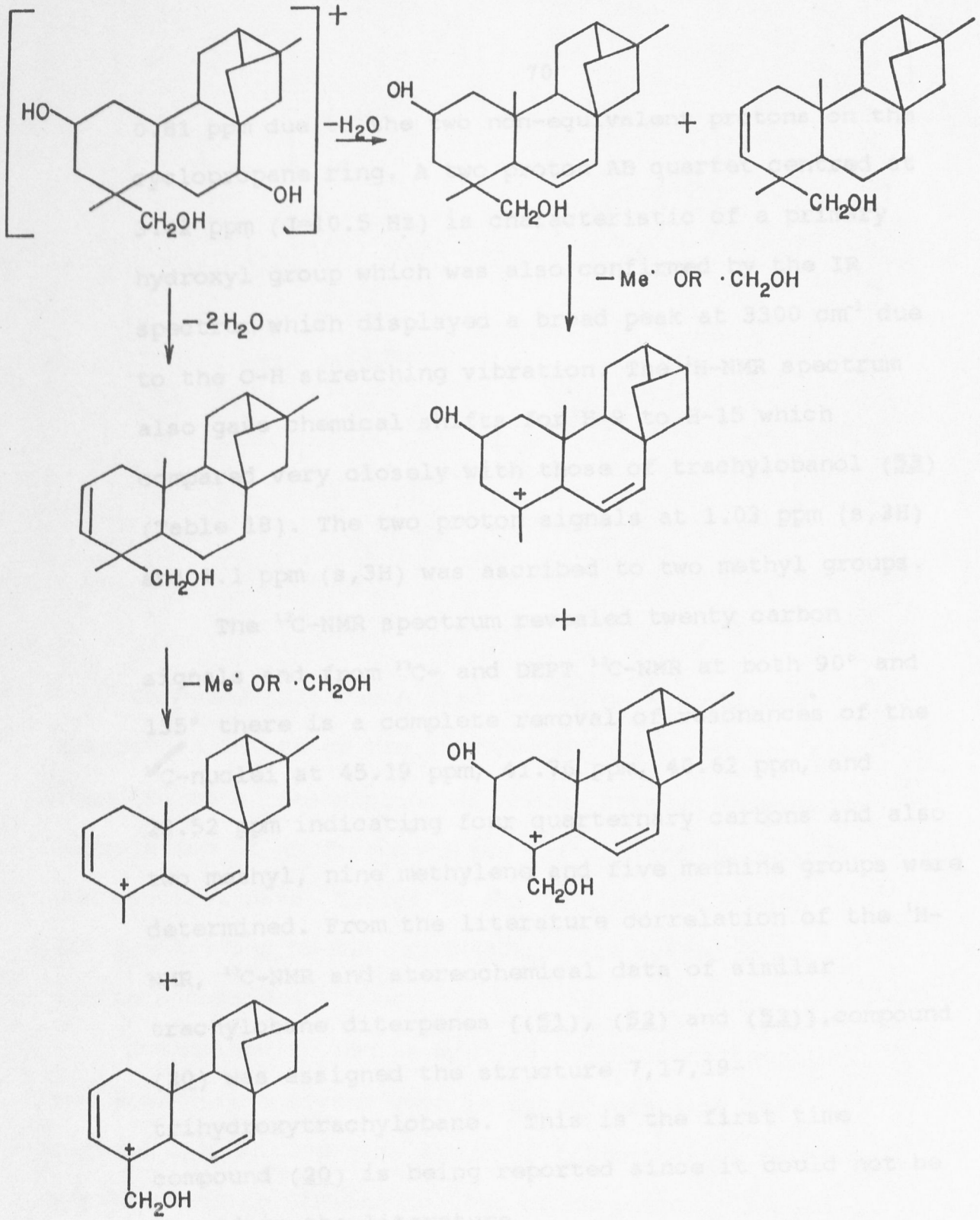
The ^{13}C-NMR spectrum revealed twenty carbon signals and from ^{13}C- and DEPT ^{13}C-NMR at both 90° and 135° there is complete removal of resonances of the ^{13}C-nuclei at 41.97 ppm, 42.04 ppm, 42.84 ppm and 23.58 ppm indicating four quaternary carbon atoms and also three primary, seven secondary, and six tertiary groups were present (table 23). From the literature correlation of the ^{1}H-NMR, ^{13}C-NMR and stereochemical data of similar trachylobane diterpenes, compound (**19**) was assigned the structure 3,7,17-trihydroxytrachylobane. Compound (**19**) is being reported for the first time since it could not be traced in the literature but similar trachylobane diterpenoids have been isolated from other Compositae where they occur quite widely particularly in sunflower (*Helianthus annus L.*).[109]



19

Compound (20) also did not show any significant absorption in the UV region (λmax MeOH 203 nm) and its MS fragmentation pattern and ^{1}H- and ^{13}C-NMR spectral correlation with literature showed that it was a trachylobane diterpenoid. The mass spectral analysis showed a molecular ion at m/e 320 corresponding to the molecular formula $C_{20}H_{32}O_3$ which was also confirmed by the elemental analysis. The most striking evidence of close similarity of compounds (19) and (20) was found in the mass spectra (Scheme 7) and it can be seen that they are nearly identical in all details of the fragmentation pattern. The near identity of the spectra indicate that initial fragmentations lead at once to the ions which then follow identical courses of further fragmentation.

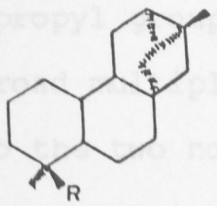
Two-dimensional ^{1}H- and ^{13}C-NMR direct chemical shift correlation experiments (Table 20) allowed all signals to be interrelated. The presence of cyclopropyl protons was disclosed by the signals at 0.58 ppm and



SCHEME 7 : Fragmentation processes of a trachylobane diterpenoid .

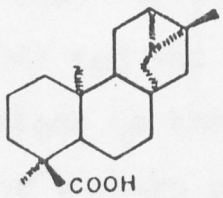
0.81 ppm due to the two non-equivalent protons on the cyclopropane ring. A two proton AB quartet centred at 3.42 ppm (J=10.5 Hz) is characteristic of a primary hydroxyl group which was also confirmed by the IR spectrum which displayed a broad peak at 3300 cm^{-1} due to the O-H stretching vibration. The ^{1}H-NMR spectrum also gave chemical shifts for H-9 to H-15 which compared very closely with those of trachylobanol (**53**) (Table 18). The two proton signals at 1.03 ppm (s,3H) and 1.1 ppm (s,3H) was ascribed to two methyl groups.

The ^{13}C-NMR spectrum revealed twenty carbon signals and from ^{13}C- and DEPT ^{13}C-NMR at both 90° and 135° there is a complete removal of resonances of the ^{13}C-nuclei at 45.19 ppm, 41.76 ppm, 40.62 ppm, and 23.52 ppm indicating four quarternary carbons and also two methyl, nine methylene and five methine groups were determined. From the literature correlation of the ^{1}H-NMR, ^{13}C-NMR and stereochemical data of similar trachylobane diterpenes {(**51**), (**52**) and (**53**)},compound (**20**) was assigned the structure 7,17,19-trihydroxytrachylobane. This is the first time compound (**20**) is being reported since it could not be traced in the literature.

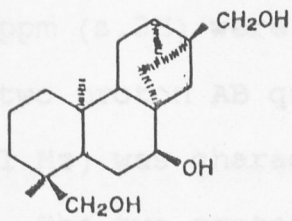


51 R = CH_3

52 R = CH_3OH



53



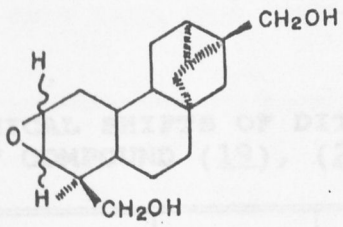
20

Compound (**21**) which also showed no significant absorption in the UV region (λmax MeOH 203 nm) was also found to be a trachylobane diterpene through literature correlation of MS fragmentation pattern and ^{1}H- and ^{13}C-NMR spectral data. Tables 18 and 20 show the ^{13}C-NMR (at 125MHz) and ^{1}H-NMR (at 500MHz) data respectively for this compound.

The molecular ion at m/e 318 in the mass spectra and elemental analyses were in accordance with the formula $C_{20}H_{30}O_3$. The mass spectral fragmentation pattern was found to be similar to that of compounds (**19**) and (**20**) (see scheme 7). Two-dimensional ^{1}H- and ^{13}C-NMR direct chemical shift correlation experiments allowed all signals to be interrelated. The presence of

cyclopropyl grouping in compound (**21**) was disclosed by the broad multiplet centred at 0.61 ppm and 0.84 ppm due to the two non-equivalent protons on the cyclopropane ring. The presence of a sharp peak in the IR spectrum at 3025 cm^{-1} due to the cyclopropane C-H stretch also confirmed this. The two signals at 1.20 ppm (s,3H) and 1.1 ppm (s,3H) were ascribed to two methyl groups. The two proton AB quartet at 4.00 ppm and 3.91 ppm (J = 11 Hz) was characteristic of an axial hydroxymethyl group. The two proton signals at 4.45 ppm and 5.00 ppm did not form cross peaks with ^{13}C-NMR and are the hydroxyl protons.

A medium peak in the IR spectrum at 1295 cm^{-1} is due to the C-O stretch of primary or secondary alcohols. A C-O stretch characteristic of epoxides was observed at 1210 cm^{-1} in the IR spectrum. The ^{13}C-NMR revealed the presence of twenty carbon signals. Signals at 23.1 ppm, 41.6 ppm, 41.9 ppm, and 44.7 ppm did not form cross peaks with ^{1}H-NMR and were ascribed to four quarternary carbons. From DEPT ^{13}C-NMR two methyl, eight methylene, and six methine groups were established. From the literature correlation of the ^{1}H-NMR, ^{13}C-NMR and stereochemical data of similar trachylobane diterpenes, compound (**21**) was assigned the trachylobanol epoxide. Compound (**21**) is a new natural product since it could not be traced in the literature.



21

The macerated leaves were extracted with 70% methanol in water for twenty four hours, filtered and the residue re-extracted with 50% methanol in water for another twenty four hours.[66] Methanol was removed from the extract at the rotatory evaporator (fraction I). The remaining aqueous extract was then fractionated with dichloromethane to remove any remnant aglycones (fraction II). The aqueous extract was hydrolysed with 2M HCl refluxed and the hydrolysis monitored using tlc for a period of three hours. The aqueous extract did not hydrolyse in 2M HCl after refluxing for three hours and monitoring with tlc by spotting against fraction I, II, and quercetin. What could be observed on the tlc plate were glycosides whose dark yellow colour intensified on exposure to fumes of concentrated ammonia solution. Also from the aqueous extract several compounds that fluoresced pink under UV light were observed.

TABLE 18. ^{13}C-NMR CHEMICAL SHIFTS OF DITERPENES (51-53) COMPARED WITH THOSE OF COMPOUND (19), (20), AND (21).

C-POSITION	(19)	(20)	(21)	(51)	(52)	(53)
C-1	49.3	40.1	48.3	39.2	39.4	39.7
C-2	69.7	20.0	65.8	18.3	17.9	19.4
C-3	48.8	39.5	68.1	42.2	35.7	38.5
C-4	41.9	45.2	41.9	33.0	38.3	43.6
C-5	54.4	54.7	54.1	56.2	56.8	54.0
C-6	49.4	49.3	36.8	20.2	20.4	24.9
C-7	64.2	64.4	47.9	39.1	39.5	74.2
C-8	42.0	41.7	44.7	40.8	40.7	47.4
C-9	61.5	50.0	53.2	53.4	53.5	52.2
C-10	42.8	40.6	41.7	38.4	38.3	39.3
C-11	20.9	21.0	20.4	19.7	19.9	22.3
C-12	21.4	21.0	21.5	20.7	20.6	24.0
C-13	25.3	25.4	25.4	24.4	24.3	21.0
C-14	35.1	34.4	34.1	33.5	33.4	32.2
C-15	51.9	51.5	51.4	50.6	50.4	45.4
C-16	23.6	23.5	23.1	22.5	22.4	20.1
C-17	20.7	64.8	68.2	20.6	20.6	20.9
C-18	32.1	21.7	21.5	33.5	26.9	29.3
C-19	67.7	68.9	65.4	21.7	65.5	179.9
C-20	17.0	16.7	20.6	14.6	15.1	13.1

TABLE 20. DEPT AND ^{1}H-NMR DATA FOR COMPOUNDS (19), (20), AND (21)

	(19)		(20)		(21)	
C- POSITION	DEPT	δ ^{1}H	DEPT	δ ^{1}H	DEPT	δ ^{1}H
1	CH_2	1.6,1.4	CH_2	1.4,1.3	CH_2	1.6,1.4
2	CH	4.0	CH_2	1.7,1.6	CH	3.92
3	CH_2	1.6,1.4	CH_2	1.9,1.1	CH	3.9
4	C	-	C	-	C	-
5	CH	1.2	CH	1.2	CH	1.3
6	CH_2	1.9,1.6	CH_2	1.4,1.7	CH_2	1.8,1.3
7	CH	3.7	CH	3.8	CH_2	1.4,1.2
8	C	-	C	-	C	-
9	CH	1.2	CH	1.2	CH	0.9
10	C	-	C	-	C	-
11	CH_2	1.9,1.6	CH_2	1.9,1.6	CH_2	1.9,1.7
12	CH	0.62	CH	0.58	CH	0.61
13	CH	0.82	CH	0.81	CH	0.84
14	CH_2	2.0,1.3	CH_2	2.0,1.1	CH_2	2.0,1.3
15	CH_2	1.4,1.3	CH_2	1.4,1.3	CH_2	1.4,1.3
16	C	-	C	-	C	-
17	CH_2	3.8,3.4	CH_2	3.42	CH_2	4.0,3.4
18	CH_3	1.22	CH_3	1.1	CH_3	1.2
19	CH_3	1.11	CH_2	3.5,3.4	CH_2	3.9,3.6
20	CH_3	1.07	CH_3	1.03	CH_3	1.1

Compound (19); H-1 dd (J=3.9,3.9Hz), dd (J=4.5,5.3Hz) H-6 dd (J=4.6,3.4Hz) dd (J=2.0,2.2Hz) H-3 dd (J=2.0,2.2Hz) dd (J=4.5,5.3Hz)

Compound (21); H-3 J=7.7Hz, H-9 J=10.4Hz, H-11 J=3.2Hz, H-17 J=4.4Hz, H-19 J=4.3,7.7Hz

TABLE 21. ^{1}H-NMR PARAMETERS OF TRACHYLOBANOL (52) COMPARED WITH (19), (20), AND (21) (500MHz $CDCl_3$,TMS AS INTERNAL STD.)

	(19)	(20)	(21)	(52)
	δ	δ	δ	δ
H-9	1.20	1.17	0.90	1.13
H-11α	1.90	1.91	1.94	1.87
H-11ß	1.70	1.74	1.74	1.74
H-12	0.62	0.58	0.61	0.56
H-13	0.82	0.81	0.84	0.81
H-14α	2.02	1.90	2.01	2.02
H-14ß	1.25	1.10	1.30	1.13
H-15α	1.43	1.36	1.45	1.38
H-15ß	1.32	1.23	1.30	1.26

J(Hz); compound (19): 9,11α=11.1, 9,11ß=6.2, 11α,13=2.8, 12,14ß=3.1 14ß,15ß=7.8

Compound (20): 11α,11ß=14.2, 9,11α=9.5, 12,14ß=3.2, 12,15ß=3.1, 12,13=2.5, 14ß,15ß=2.7

CHAPTER 3

3.0 COMMENT AND CONCLUSION

The resinous exudate covering the aerial parts of *P. punctulata* could be a potential source of several biologically active ingredients. The claimed herbivory deterance during the dry seasons and the observed cytotoxicity against the brine-shrimp nauplii, anti-feedant activity against *Locusta migrotoria* (desert locust) and anti-fungal activity against *F. oxysporum* and the coffee berry disease fungus *C. coffeanum* clearly justify a thorough study of this plant.

The resinous epicuticular exudate affords a rich source of trachylobane diterpenes which were the active principles in the anti-brine shrimp tests with pure compounds and can serve as lead compounds for synthetic modifications that can be used to kill cancer cells. The resinous material also affords a source of flavones and flavonols whose ring-B 2',4', and 5'-oxygenation pattern has been observed to be the same and were devoid of any anti-brine shrimp activity.

Since pesticides produced by plants are growing in demand, not only because of their derivation from a renewable source, but also because they are often highly specific and consequently cause minimum ecological disturbance, it is necessary to design field tests to evaluate the efficacy levels of the anti-feedant

principles in *P. punctulata* so that they may be incorporated into commercial formulations. The anti-feedant effects for this easy to extract mixture of flavonoids and diterpenoids offers *P. punctulata* as a shrub with great economic potential.

Inspite of the *in vitro* anti-fungal activity of the *P. punctulata* extract, it is not known if the anti-fungal substances from the plant would effectively reduce the severity or incidence of plant diseases in the field caused by *F. oxysporum* and the four species of *Collectotricum*. Therefore field experiments on its ability to control fungal diseases *in vivo* is required before definitive statements on its agricultural usefulness can be made.

CHAPTER 4

4.0.0 EXPERIMENTAL

4.1.0 GENERAL

For TLC, Merck aluminiun and polythene plastic precoated silica gel 60 PF-254, 2.5 x 7.5cm, 0.25mm layer thickness were used and Merck silica gel 60G was used for preparative tlc whereas silica gel 60 (0.04-0.063\230-400 mesh ASTM) was used for gradient elution chromatography and 0.07-0.3 mesh was used for flash chromatography. T.L.C spots were visualized under UV light and by developing in an iodine chamber.

^{1}H-NMR spectra (obtained at 500MHz), ^{13}C-NMR (obtained at 125MHz) and two dimensional direct chemical shift correlation for compounds (19-22) were obtained in $CDCl_3$ or CD_3OD at the University of Maryland at College Park (USA). ^{1}H- and ^{13}C-NMR of compounds (16), (17), and (18) were obtained at 400MHz using the Varian XL-300 and the Jeol FX-200 spectrophotometers at Meiji College of Pharmacy, Tokyo Japan. The spectra were recorded in DMSO. The DEPT experiments were performed using polarization transfer pulses at 90° to diagnose methine groups and 135° to diagnose positive signals for methine and methyl groups and negative signals for methylene groups.

Chemical shifts (δ) are recorded in parts per million (ppm) relative to TMS. Spin-spin coupling constant (J) are given in Hertz (Hz).

Melting points were recorded on Gallenkamp and Buchi SMP 20 melting point apparatus using glass capillary tubes and are uncorrected. Mass spectra (MS) were recorded with a mass Lab 12-250 gas chromatograph-mass spectrometer (GC-MS) system at an ionization potential of 70ev and are given as mass to charge ratios (m/e) in atomic mass units (a.m.u), with relative ion intensities in parentheses. Ultraviolet-visible (UV-VIS) spectra were determined using a pye-unicam SP8-150 and DMR 10-Zeis spectrophotometers.

PREPARATION OF SHIFT REAGENT STOCK SOLUTIONS AND SOLIDS

SODIUM METHOXIDE : Freshly cut metallic sodium (2.5g) was added cautiously in small portions to dry methanol (100ml). The resultant solution was stored in a plastic-stoppered glass bottle.

ALUMINIUM CHLORIDE : Five grams of fresh anhydrous reagent grade $AlCl_3$ were added catiously to dry methanol (100ml).

HYDROCHLORIC ACID : Concentrated reagent grade HCl (50ml) was mixed with deionised water (100ml).

SODIUM ACETATE : Anhydrous coarsely powdered reagent grade was used.

BORIC ACID : Anhydrous powdered reagent grade was used.

4.2.0 PLANT IDENTIFICATION AND COLLECTION

Psiadia punctulata (D.C.) Vatke was identified with the help of Mr Mathenge from the University of Nairobi Herbarium, Botany Department. Aerial parts were collected in September 1992 along open roadside around Nairobi National Park in Langata area. In this site it is a sub-shrub 1.0-1.5m tall. Pressed vouchers are at the University of Nairobi herbarium . More detailed locality information is present on the voucher specimens.

4.3.0 EXTRACTION OF P. PUNCTULATA LEAVES WITH ACETONE

The leaves collected from open roadside around Nairobi National Park in Langata area, were successively dipped into acetone in a two litre beaker for a period of about fifteen seconds. Acetone was initially coloured yellow but later the colour intensified to pale yellow. The extract was decanted into an erlenmeyer flask and subsequent extractions carried out using fresh portions

of acetone. The combined extracts were filtered, and concentrated using the rotatory evaporator, resulting in a pale gummy solid. The extracted leaves were spread on a bench and allowed to dry under the shade for two weeks. The dry leaves were then ground into a powder using an electrically powered grinder to give 394g of powder. This was stored for further extraction.

4.3.1 RE-EXTRACTION OF MACERATED P. PUNCTULATA LEAVES WITH METHANOL IN WATER

The powder of previously acetone-washed leaves of *P. punctulata* were placed in a one litre erlenmeyer flask. This was extracted with 70% methanol in water for twenty four hours after which it was filtered. The residue was re-extracted with 50% methanol in water. Each extraction was carried out overnight. All extracts were combined and then concentrated over a rotatory evaporator until most of the methanol had been removed. On cooling the aqueous extract (fraction I) was exhaustively re-extracted with dichloromethane in a separatory funnel. The dichloromethane fraction (fraction II) contained some flavonoid aglycones previosly established as present on the leaf surface. The aqueous extract was stored ready for hydrolysis.

4.3.2 ACID HYDROLYSIS OF METHANOL EXTRACT OF *P. PUNCTULATA* LEAVES

18ml of the aqueous residue, devoid of leaf surface flavonoid aglycones, was mixed with 3ml of concentrated HCl acid to make up a 2N HCl solution. The solution was refluxed for at least two hours. The resultant hydrolysate was exhaustively extracted with ethyl acetate in a separatory funnel. The ethyl acetate soluble fraction (fraction III) was concentrated and quantitatively analysed by spotting it on tlc plates against fraction I, fraction II, and quercetin using 60% ethyl acetate in hexane as the developing solvent.

4.4.0 PRELIMINARY TESTING

4.4.1 DETECTION OF FLAVONOIDS

1.0g of the defatted sample (by several washing with n-hexane) was dissolved in 30ml of 80% ethanol and the solution divided into three portions. To 3ml portion of one, 4ml of 1% aluminium chloride in methanol was added. The appearance of a yellow colour confirmed the presence of flavonoids.

(I) To 3ml of the second portion of ethanol solution, 4ml of 1% KOH solution was added. The development of dark yellow colour reconfirmed the presence of flavonoids.

(II) To 3ml of the third portion of ethanol solution, a little of concentrated HCl was added followed by magnesium turnings. A colour change from pink to red further confirmed the presence of flavonoids.

4.4.2 DETECTION OF TERPENES AND STEROLS

1.5g of the crude extract was defatted with n-hexane and the residue was extracted with anhydrous sodium sulphate. To 5ml of the dichloromethane portion, a 0.5ml of acetic anhydride followed by two drops of concentrated sulphuric acid was added. Presence of terpenes and sterols was confirmed by the gradual appearance of green-blue colour.

4.5.0 EXAMINATION OF THE CRUDE EXTRACT

The complexity of the mixture in the crude extract was examined on tlc using methanol and chloroform at various percentages. The best separation occurred with 4% methanol in chloroform. The dried chromatogram was examined by visible light and the UV light both before and after exposing to fumes of conc. ammonia and developed in an iodine chamber. The number, position and visible and fluorescent colour of the individual spots were conveniently recorded on the plate with a soft pencil.

4.6.0 SEPARATION AND PURIFICATION OF ACETONE EXTRACT OF P. PUNCTULATA LEAVES

Column chromatography was performed with silica gel 60 (0.04-0.063mm\230-400 mesh ASTM). The diameters of the columns used were 4.5cm and 2.0cm. The columns were packed with the silica gel (100g) in hexane and a portion of the acetone extract (10g) adsorbed onto an equal amount of silica gel (10g) was introduced onto the column and the separation carried out by step gradient elution using chloroform and methanol. Commercial analytical Merck silica gel G plates were used for monitoring the eluants. Eight fractions were collected . Solvent removal from the fractions was carried out in vacuo using a rotatory evaporator. The solvents used for developing the plates were :-

chloroform (100%)	(solvent A)
1%methanol\chloroform	(solvent B)
2%methanol\chloroform	(solvent C)
3%methanol\chloroform	(solvent D)
4%methanol\chloroform	(solvent E)
6%methanol\chloroform	(solvent F)
7%methanol\chloroform	(solvent G)
8%methanol\chloroform	(solvent H)

The compounds crystallized in chloroform and hexane solvent system. This was accomplished by addition of hexane which turned the solution cloudy. Addition of a

few drops of chloroform cleared the cloudiness. The process was repeated with heating at the water bath until the cloudiness disappeared and crystals started forming. The compounds proved to be very crystalline. Fraction I showed a single spot on tlc plate. This was purified to give 5-hydroxy-2',3,4',5',7-pentamethoxyflavone (16)

5-hydroxy-2',3,4',5',7-pentamethoxyflavone (16)

Yellow needles, m.pt 122-124°C. UV MeOH (nm): 267.5, 331.0 : NaOMe 281.0, 350.0 : NaOAc 267.5, 331.5 : NaOAc + H_3BO_3 267.5, 332.0 : $AlCl_3$ 278.0, 295.6Sh., 346.0 : $AlCl_3$ + HCl 278.0, 294.0Sh., 346.0. MS m/e (rel. int.) 388 (M^+ ,100), 373 (M-Me,23.5), 167 (28.5), 194 (3.24), 222 (4.57). IR (KBr plate) 1660cm^{-1} (C=O streching). ^1H- and ^{13}C-NMR data are in tables 13 and 19.

Purification of fraction II gave 5,7-dihydroxy-2',3,4',5'-tetramethoxyflavone (17).

5,7-dihydroxy-2',3,4',5'-tetramethoxyflavone (17)

Yellow crystals m.pt 228-232°C. UV MeOH (nm) : 268.0, 333.0 : NaOMe 274.0, 361.5 : NaOAc 274.0, 347.0 : NaOAc +H_3BO_3 268.0, 333.0 : $AlCl_3$ 277.0,292.6Sh., 346 : $AlCl_3$ + HCl 277.5, 291.0Sh., 346.0. MS m/e (rel. int.) 374 (M^+,100), 359 (M-Me,29.1), 153 (30.5). IR (KBr plate) 1650cm^{-1} (C=O stretching). ^1H- and ^{13}C-NMR data are in tables 15 and 19.

Purification of fraction III resulted in 5,7-dihydroxy-2',4',5'-trimethoxyflavonol (18).

5,7-dihydroxy-2',4',5'-trimethoxyflavonol (18)

Yellow crystals, m.pt 245-250°C. UV MeOH (nm) : 265.0, 344.0 : NaOMe 268.0, 400.5 : NaOAc 270.5, 362.0 : NaOAc + H_3BO_3 267.0,344.5 : $AlCl_3$ 273.5, 300.5Sh. 383.5 : $AlCl_3$ + HCl 275.5, 298.5Sh., 346.0. MS m/e (rel. int.) 360.0 (M^+,100), 208.0 (6.0), 153 (31.2), 152 (1.6), 345 (M-Me,24.8), 222 (5.44). IR (KBr plate) 1650cm^{-1} (C=O stretching). ^1H- and ^{13}C-NMR data are in tables 16 and 19.

Purification of fraction IV afforded 7,17,19-trihydroxytrachylobane (20).

7,17,19-trihydroxytrachylobane (20)

White needles, m.pt 227-229°C. UV MeOH (nm)203.0. MS m/e (rel. int.) 302 (M^+,18.4), 284 (M^+,-$2H_2O$,62.6) 254 (M^+-2Me-$2H_2O$,100) 202 (32.2) 187 (77.6) 133 (41.4) 119 (58.4) 105 (89.0). ^1H- and ^{13}C-NMR data are in tables 18, 20, and 21.

Purification of fraction V afforded 17,19-dihydroxy-2,3-epoxytrachylobane (21).

17,19-dihydroxy-2,3-epoxytrachylobane (21)

White crystals, UV MeOH (nm) 203. MS m/e (rel. int.) 300 (M^+-H_2O,**) 289 (M^+-Me-H_2O,10.3) 271 (M^+-Me-$2H_2O$,25.5) 187 (21.8) 173 (17.3) 164 (9.6) 161 (18.6) 159 (46.6) 157 (31.3) 152 (12.6) 145 (42.7) 143 (27.5) 135 (43.7) 134 (28.0) 123 (12.2) 121 (32.1) 120 (27.2) 119 (62.4) 105

(95.8). ^{1}H- and ^{13}C-NMR data are in tables 18,20, and 21.

Purification of fraction VI afforded 3,7,17-trihydroxytrachylobane (19).

3,7,17-trihydroxytrachylobane (19)

White crystals, m.pt 181-185°C. MS m/e (rel. int.) 302 ($M^{+}-H_2O$,8.4) 284 ($M^{+}-2H_2O$,100) 271 ($M^{+}-Me-2H_2O$,25.2) 159 (14.6) 152 (1.7) 135 (19.1) 133 (60.3) 119 (28.4) 105 (57.2). ^{1}H- and ^{13}C-NMR data are in tables 18, 20, and 21.

4.7.0 ACETYLATION OF COMPOUND (16)

40mg of this compound were mixed with 10ml of acetic anhydride and 2ml of pyridine in a 100ml round bottomed flask. Acetylation was allowed to continue for 24 hours after which some ice cold water was added into the reaction flask. The resultant whitish suspension was filtered and eventually dissolved in diethyl ether and some anhydrous sodium sulphate was added for drying. Once filtered, most of the diethyl ether was vapourised and crystalization set up in a diethyl ether-hexane mixture. White needle like crystals with a melting point of 118-119°C were obtained.

4.8.0 ACETYLATION OF COMPOUND (22)

Acetylation of compound (22) was carried out by adding 20mg of it to 6ml of acetic anhydride and 1ml of pyridine in a 100ml round bottomed flask. The reaction

was allowed to continue for 24 hours. The contents of the reaction flask were then drained into ice cold water. The resultant suspension was filtered and dissolved in diethyl ether to which enough anhydrous sodium sulphate was added for drying. Once filtered most of the diethyl ether was vapourised and crystalization set up in a diethyl ether-hexane mixture. White crystals of compound (22) acetate crystallized out from diethyl ether-hexane mixture.

4.9.0 BIOASSAYS

4.9.1 Anti-feedant Tests

Using the procedure of Butterworth and Morgan (1968), anti-feedant was tested for the crude acetone extract against *Locusta migrotoria*. This was carried out by impregnating filter papers (whatman no. 1) with dichloromethane solution of the extract at various dilutions. The papers were momentarilly dipped in the solution, allowed to dry and then sprayed with 0.25M of sucrose solution and all papers were dried in an oven at 40°C. These test papers were presented to separate groups of mid-5th instar hoppers of *L. migrotoria* which had been starved for about 24 hours. The data collected after 8 hours is summarised in table 11.

4.9.2 Cytotoxicity tests using brine shrimp

Sample preparation

Samples were prepared by dissolving 25mg of the crude extract, compound (16), compound (17),and compound (18) in 25ml of chloroform (solution A) while 25mg of compound (19), compound (20), and compound (22) was dissolved in 25ml of methanol (solution A). Solution B was prepared by diluting 5ml of A to 10ml of chloroform for the crude extract, compound (16), compound (17), and compound (18) and 10ml of methanol for compound (19), compound (20), and compound (22), while solution C was prepared by diluting 2.5ml, D by diluting 1ml, E by diluting 0.5ml and F by diluting 0.1ml to 10ml in each case for the concetrations of 1000µg\ml, 500µg\ml, 250µg\ml, 100µg\ml, 50µg\ml, and 10µg\ml respectively. Desired quantities of solution was added to the sample bottles and the solvent allowed to evaporate at room temperature. Four replicates were prepared for each concentration as well as for the control.

4.9.3 Hatching the brine shrimp

The brine shrimp eggs were hatched in a modified soap dish (10x7x3cm) filled with artificial sea water which was prepared with commercial salt mixture and deionised water. A plastic divider was glued across the

centre of the soap dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened while the smaller compartment was illuminated from the bulb in the box (26x23x29cm with a bulb socket fitted inside). The modified soap dish with eggs was placed in the box with 25W bulb fitted inside. The box was closed and the light put on. A thermometer was put in the box to measure the temperature inside the box which was 28°C. The eggs were incubated at 28°C for 22 hours. About 20 nauplii were transfered to previously prepared sample bottles using 1ml pipette. The nauplii were counted macroscopically in the stem of the pipette against a lighted background. The number of active nauplii were then counted and estimated at intervals of 4 hours after commencement of the experiment for 24 hours. The results are in table 8.

4.9.4 Anti-fungal Test

4.9.5 Media preparation

Potato Dextrose Agar (PDA)

Materials and procedure:

potatoes	200g
Agar	20g
Dextrose	15g
Distilled water	1 litre

The potatoes were boiled till soft and was then filtered through cheese cloth. To the filtrate, agar was added and heated to dissolve, after which dextrose was added. The volume was adjusted with distilled water to one litre. This was then sterilized in the autoclave for 25 minutes at 121°C.

Fungal pathogens

The fungi used in the study were *Collectotricum coffeanum* and *Fusarium oxysporum*. *C. coffeanum* was isolated from coffee beries with the help of Dr. Siboe of Botany Department. The pathogens were cultured in potato dextrose agar (PDA).

4.9.6 Preparation of sample

The crude extract was dissolved in 10ml of 5% Tween 20 solution (previously autoclaved) and mixed with 70ml of autoclaved potato dextrose agar cooled to 40°C., to prepare 1000µg\ml, 100µg\ml, and 10µg\ml. This was then dispensed into petri dishes and allowed to solidify under the microflow chamber in accordance with Shukla et al (1972). For each concentration level four replicates were made. For the control plates 10ml of sterile distilled water and 10ml of 5% tween 20 solution were respectively added to 70ml Of potato dextrose agar. The volume of PDA used in the petri dish bioassay was constant throughout

the experiment. Thus the effect of media on the dilution of the extract did not affect the experimental results.

4.9.7 Inoculation

Using a sterile cork borer, 8mm diameter mycelial plugs were cut from the margines of actively growing culture of the test fungi. With a sterile inoculating loop one plug was transfered asceptically and placed upside down in the centre of each of the PDA plates. Plates were filled with parafilm wax paper and incubated on the laboratory benches at room temperature (23-27)°C. Colony diameters were recorded daily. The measuring was done on the same axis throughout the experiment unless there was a contaminant. Colony diameters were recorded till fungal growth in one of the treatments filled the plate. The colony diameter of the treated plates compared with that of the control was taken as relative measure of fungitoxicity.

4.9.8 Larvicidal Tests

Test for activity of crude acetone extract against *Aedes aegyptii* mosquito larvae was performed. Eggs of *Aedes aegyptii* were flooded with NaCl solution (30g\25l deionised water) and stimulated to hatch by adding ca. 0.1g of finely ground dog biscuits. One day after hatching the larvae were distributed at the same larval

density, with twenty of them being placed into each rearing tray. Stock solutions were made by dissolving the extract in ethanol. Test solutions were prepared by serially diluting the stock solutions with deionised water. Untreated water and ethanol treated water served as controls. Each test series was repeated at least three times. The mortality rate was observed after 24 hours, 48 hours, upto a period of one week.

REFERENCES

1. LE STRANGE, R.; A History of Herbal Plants, Angus and Robertson, London (1977).

2. TAYLOR, N.; Plant Drugs that changed the world. George Allen and Unwin Ltd, London (1965).

3. KOKWARO, J.O.; "Medicinal Plants of East Africa", *East Africa Lit. Bureau*, 1976, Nairobi.

4 UPHOLF, J.C.; The "Dictionary of Economic plants" Edited by J. Cramer, Lehre (2nd ed.) (1968).

5 YEH, S.F.; CHOU, T.C.; and LIU, T.S.; *Planta Med.* 54(5), 413 (1988).

6 ARENE, E.O.; *Phytochemistry* II, 2886-2887 (1972).

7. DALZIEL J.M.; Useful Plants of West Tropical Africa. Crown Agents for Overseas Government, London (1956).

8. EKANEM, J.O.; "Socio-economic impact of malaria on our nations". In malaria chemotherapy *(ed Adenika, F.B) West African Pharm.* Federation, Lagos(1978).

9. DOSSAGI, S.; KUBO, I.; *Phytochemistry* 19(3), 1111(1980).

10. ISOBE, T.; FUKUSHINGE, T. and NODA, T.; *Chem. Lett.* I 27 (1979).

11. BARGARA, S.K.; *Fitoterapia* 55(5), 201-206 (1984).

12. BELL, A.A.; *Phytopathology* 57, 1111 (1967).

13. MIDIWO, J.O.; and RUKUNGA, G.M.; *Phytochemistry* 24 pp 1390 (1985).

14. PARANJE, A.S.; and GOKHALE, G.K.; *Arch Intern Pharmacodynamie* 42 212-222 (1932).

15. HUANGPU, Han et al; *Indian Grassland Fodder Res. Institute* 46 (5) 80 (1980).

16. MIDIWO, J.O.; and MANGURO, L.A.; *Bull Chem. Soc.* Ethiopia 2 (2) 83-85 (1988).

17. MANGURO, L.A.; (1988) "Phytochemical Analysis of Kenyan Medicinal Myrsinaceae Plant". MSc. Thesis submitted to the University of Nairobi Jan 1988.

18. NAKAJIMA, S.; "Search for biologically active substances in plants" *Natural Product Chemistry* (Okayama, 700 Japan) (1980).

19. TSUNEO, N et al.; Dentrifies Containing embelin for prevention of dental carries. *Drissan Chem. Indust.*Ltd. Japan (1985).

20. SASTRY, B.S. et al; *Pharmacie* 42(10), 33 (1986).

21. ELLER, B.M.; *Flora* 168 146 (Eng) (1979).

22. KNOX, J.P.;and DODGE, A.D.; *Phytochemistry* 24(5), 889-896 (Eng)(1985).

23. BARTHLOTT, W.; and WOLLENWEBER, E.; *Trop. Subtrop. Pflanzenwelt* (Akad. Wiss. Lit. Mainz). 32, 35 (1981).

24. WOLLENWEBER, E.; Chemistry and Distribution of Flavonoids in leaf resins of desert plants, F.E.C.S. Third International Conference on chemistry and Biotechnology of Biologically Active Natural Products pp 99-110 (1985a).

25. JUNIPER, B.E.; and JEFFREE, C.E.; Plant Surfaces. Edward Arnold London, (1983).

26. WOLLENWEBER, E.; Plant Systematics and Evolution 150 83-88 (1985).

27. KELSEY, R.G.; REYNOLDS, G.W.; and RODRIGUEZ, E.; Biology and Chemistry of plant trichomes. Plenum press, New York, p. 187 (1984).

28. WOLLENWEBER, E.; HRADETSKY, D.; MANN, K.; ROITMAN, J.N.; YATSKIEVYCH, G.; PROKSCH, M.; and PROKSCH, P.; *J. Plant Physiol* 131, 37-43 (1985).

29. GOODWIN, T.W.; Chemistry and Biochemistry of plant pigments 1, 769 (1976).

30. WEISSMAN, G.; and BRUNS, K.; *Naturwissenschaften* 52, 185 (1965).

31. VARNER, J.E.; and HO D. T-H; "In The Molecular Biology of Hormone Action" (D. Brown Ed) (1976).

32. MABRY, T.J.; GILL, J.E.; **Sesquiterpene lactones and other terpenoids.** Herbivores: Their interaction with secondary plant metabolites. Academic Press, New York. pp 501-537 (1979).

33. ELLIGAR, C.A.; ZINKEL, D.F.; CHAN, G.B.; WAISS, A.C.; **Diterpene acids as larval growth inhibitors.** *Experimentia* 32, 1364-1366 (1976).

34. ROSE, A.; *Phytochemistry* 19, 2689-2693 (1980).

35. ROSE, A.; JONE, K.; HADSON, W.; DREYER, D.; *Phytochemistry* 20, 2249-2253 (1981).

36. ALOIS, A.B.; ROBERT, D.S.; CHARLES, R.H.; and PAUL, A.F.; *Phytochemistry* 14, 225-231 (1975).

37. AMOROS, M.; and GIRRE, R.L.; *Phytochemistry* 26 (3), 787-789 (1987).

38. BOHLMAN, F.; ZDERO, C.; GUPTA, R.K.; KING, R.M.; and ROBINSON, H.; *Phytochemistry* 19, 2695-2705 (1980).

39. ARA,I.; SIDDIQUI, B.S.; FAIZI, S.; and SIDDIQUI, S.; *J. Nat. Prod.* 52(6), 1209-1213 (1989).

40. BOAKY, Y.K.; FIAGBE, N.I.Y.; and AYIM, J.S.K.; Antimicribial properties of some West African medicinal plants. Antimicrobial activity of *Xylopia aethiopica Lioydia* 40, 543 (1977).

41. SCHNEIDER, G.; *"Pharmazeutische Biologie".* Mannheim-Wein-Zurich: B.I. Wissenschaftsverlag (1975).

42. WAGNER, H.; BLADT, S.; ZGAINSKI, E.M.; *"Drogenanalyse. Dunnschicht Chromatographische Analyse Von Arzneidrogen".* Berlin-Heidelberg. New York: Springer Verlag (1983).

43. KARRER, W.; **Uber einen Flavonfarbstoff Helv** *Chim Acta* 17, 1560-1565 (1984).

44. BORN, R.; *Chem. Ind.* 264-265 (1960).

45. GEISSMANN, T.A.; "The chemistry of flavonoid compounds". Oxford-London-New York-Paris: Pergamon Press. p.428 (1962).

46. VAN DEN BROUCKE, C.O.; LEMLI, J.A.; **Spasmolytic activity of the flavonoids from Thymus vulgaris.** *Pharmac. Weebl. Sci.* Ed: 9-14 (1983).

47. ISHITSUKA, H.; OHSAWA, C.; OHIWA, T.; UMEDA, I.; SUHARA, Y.; **Antipicornavirus flavone Ro 09-0179.** *Antimicr. Agents Chemother.* 22, 611-616 (1984).

48. OKUDA, J.; MIWA, I.; INAGAKI, K.; HORIE, T.; NAKAYAMA, M.; *Chem. Pharm. Bull.* 32, 767-772 (1984).

49. RYU, S.H.; AHN, B.Z.; PACK, M.Y.; *Planta Med*, 462 (1985).

50. BHARGAVA, S.K.; *Plantes Med. Phytother.* 20, 188-198 (1986).

51. WANJAU, O.M.; **Further Chemical Analysis of Kenyan Polygonum species.** MSc. Thesis submitted to the University of Nairobi, March 1990.

52. WOLLENWEBER, E.; LEBRETON, P.; CHADENSON, M.; **Flavonoide im Knospen-Exkret Von Prunus-und Rhamnus-Arten.** *Z. Naturforstch.* 27b, 567-570 (1972).

53. SHIMIZU, M.; HORIE, S.; *Chem. Pharm. Bull.* 35, 1234-1237 (1987).

54. LWANDE, W.; BENTLEY, M.D.; and HASSANALI, A.; *Insect Sci. Applic.* 7, 501-503 (1985).

55. LALIBERT, R.; CAMPBELL, D.; and BRUDERLIN, F.; *Can. J. Pharm. Sci.* 2, 37 (1967).

56. ADESOGAN, E.K.; and OKUNDE, A.L.; *J. Chem. Soc. Chemical communications,* 150 (1978).

57. ADESOGAN, E.K.; and OKUNDE, A.L.; *Phytochemistry* 18, 1863-1864 (1979).

58. ADESOGAN, E.K.; Anti-infective agents of plant origin. In E.E. Essien, A.O. Proceedings of the 5th international symposium on medicinal plants (D.R.P.U.) pp. 13-17 (1983).

59. WANG, Y.; HAMBURGER, M.; GUEHO, J.; HOSTETT, M.K.; (EC Pharm. Univ. Lausanne CH-1005 Lausanne Switz), *Phytochemistry* 28(9), 2323-2329 (1989).

60. AL-YAHYA, M.A.; HIFNAWY, M.S.; MACCHPHAIL, D.R.; MACPHAIL, A.T.; *Phytochemistry* 26(9), 2648-2649 (1987).

61. PELIZZONI, F.; *Corsi. Semin. Chim.* Univ. Milano, Milan Italy II 53-56 ital (1968).

62. FERDINAND, B.M.; and CHRISTA, Z.; *Phytochemistry* 21(7), 1697-1700 (1982).

63. GOODWIN, T.W.; **"Chemistry and Biochemistry of plant pigments"** 1, 428-429,. Academic Press Inc. London (1976).

64. GEISSMAN, T.A.; and SWAIN, T.; *Chem. and Ind.* 984 (1957).

65. WATKIN, J.E.; UNDERHILL, E.W. and NEISH, A.C.; *Can. J. Biochem. Physiol.*35, 229 (1957).

66. GRISEBACH, H.Z.; *Naturforsch.* 13b, 335 (1958).

67. HUTCHINSON, A.; TAPER, C.D.; and TOWERS, G.H.N.; *Can. J. Biochem. Physiol.* 37, 901 (1959).

68. ZAPROPMETER, M.N.; *Biokhimiya.* 27, 366 (1962).

69. GRISEBACH, H.; and PATSCHKE, L.; *Chem. Ber.* 93, 2326 (1960).

70. GRISEBACH, H.; and PATSCHKE, L.Z.; **Naturforsch. 16b**, 645 (1961).

71. PATSCHKE, L.; BARZ, W.; and GRISEBACH, H.Z.; *Naturforsch.* 216, 45 (1966).

72. HARBONE, J.B.; *Biochem. J.* 74, 262 (1960).

73. SMITH, H.; *Nature Lond.* 227, 665 (1970).

74. SMITH, H.; In "Phytochrome", Symposium of the N.A.T.O. Advanced Study Institute, Eritria, Greece,1971. Academic Press, New York and London (1972).

75. JURD, Z.; In **"The Chemistry of flavonoid compounds"** (T.A. Geissman, Ed.) pp.107-155, Pergamon Press Oxford (1962).

76. GOODWIN, T.W.; **"Chemistry and Biochemistry of plant pigments"** 2, 185-187. Academic Press Inc. London (1976).

77. MARKHAM, K.R.; and MABRY, T.J.; In **"The Flavonoids"** (Harborne, J.B., Marbry, T.J., and Mabry, H. Eds),45-77. Chapman and Hall London (1975).

78. KALABIN, G.A.; POGODAEVA, N.N.; TYUKAVKINA, N.A.; and KUSHNARER, D.F.; *Khim. Prir. Soed.* 4, 513 (Eng. Transl. p.429) (1977).

79. STOTHERS, J.B.; **Carbon-13 NMR Spectroscopy.** vol 24 of Organic Chemistry. A series of monographs. Academic Press, New York (1972).

80. KINGSTON, D.G.T.; *Tetrahedron* 30, 2811 (1971).

81. NIELSEN, J.G.; and MOLLER, J.; *Acta. Chem. Scand.* 24, 2665 (1970).

82. GOUDARD, M.; PAVRE-BONVIN, J.; LEBRETON, P.; and CHOPIN, J.; *Phytochemistry* 17, 145 (1978).

83. ACHILLADELIS, B.; and HANSON, J.R.; *Phytochemistry* 7, 589-598 (1968).

84. WENKERT, E.; *Chem. and Ind.* 282 (1955).

85. VALENTA, Z.; and WIESNER, K.; *Chem. and Ind.* 354 (1956).

86. WHALLEY, W.B.; *Tetrahedron* 18, 43 (1962).

87. BIRCH, A.J.; RICKARDS, R.W.; SMITH, H.; HARRIS, A.; and WHALLEY, W.B.; *Tetrahedron* 7, 241 (959) (1962).

88. CROSS, B.E.; GALT, R.H.B. and HASON, J.R.; *J. Chem. Soc.* 295 (1964).

89. BINKS, R.; MACMILLAN, J.; and PRYCE, R.J.; *Phytochemistry* 8, 271-284 (1969).

90. PRYCE, R.J.; MACMILLAN, J.; and MACCORMICK, A.; *Tetrahedron Letters* 5009 (1967).

91. PRYCE, R.J.; and MACMILLAN, J.; *Tetrahedron Letters* 4173 (1967).

92. **MACMILLAN**, J.; and PRYCE, R.J.; *Tetrahedron Letters* 1537 (1968).

93. **BANSAI**, R.K.; MARIA, C.G.; KRISHNA, C.J.; BENJAMIN, R.; and RENUKA, P.; *Phytochemistry* 19, 1979-1983 (1980).

94. **AURELIO**, S.M.; JUANA, R.; RODOLFO, B.; and MARIANO, C.; *Phytochemistry* 19, 1985-1987 (1980).

95. **FERDINAND**, B.M.; and CHRISTA, Z.; *Phytochemistry* 21, 1697-1700 (1982).

96. **SILVER**, F.A.M.S.; BAKER, E.A.; MARTIN, J.T.; *Ann. Appl. Biol.* 53, 43 (Eng) (1964).

97. **HARBORNE**, J.B.; and SWAIN, T.; Phytochemical society. Perspectives in Phytochemistry p.34 (1975).

98. **MACLAUGHLIN**, J.L.; NICHOLS, D.E.; JACOBSEN, L.B.; PUTNAM, J.E.; FERRRIGNI, N.R.; MEYER, B.N.; Brine Shrimp: a convenient general bioassay for active plant constituents. *Planta Medica* 45, 31-34 (1982).

99. **SAS Institute Inc.**, SAS Language Guide for Personal Computer Version 6; Cary, North Carolina (1985).

100. **BUTTERWORTH**, J.H.; and MORGAN, E.D.; *Chemical Communications* 1, 23-24 (Eng) (1968).

101. **DREYER**, D.L.; and BERTELLI, D.J.; *Tetrahedron* 23, 4607 (1967).

102. **ELFRANCO**, M.; and ROUX, D.G.; *J.C.S.* pp.2696-2703 (1978).

103. **ARNONE**, A.; and MONDELLI, R.; *Org. Res. Spec* 12(7), 429-431 (1979).

104. **BOHLMAN**, F.; JAKUPOVIC, J.; AHMED, M.; GRENZ, M.; SUDING, H.; ROBINSON, H.; and KING, R.M.; *Phytochemistry* 20, 113-116 (1981).

105. **HASSAN**, C.M.; HEALEY, T.M.; and WATERMAN, P.G.; *Phytochemistry* 21(1), 177 (1982).

106. **RIZZO**, J.C.; WOOD, J.L.; FURST, G.T.; and SMITH, A.B.; *J. Nat. Prod.* 53(3), 735-739 (1990).

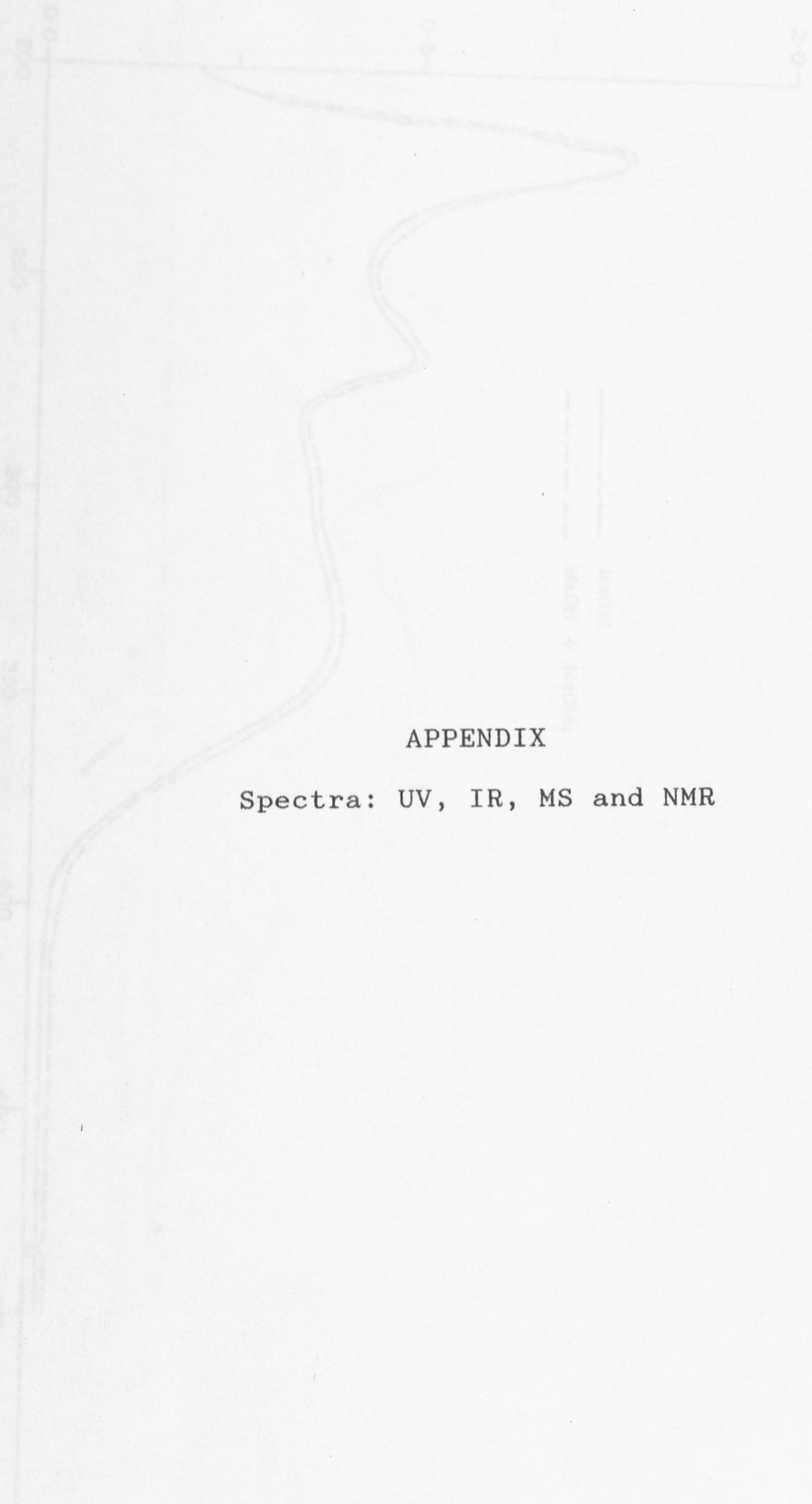
107. **HUFFORD**, C.; OGUNTIMEIN, B.O.; and MOHAMMED, I.; J. Nat. Prod. 55(1), 48-52 (1992).

108. **HUGEL**, G.; LODS, L.; MELLOR, J.M.; THEOBALD, D.W.; and OURISSON, G.; *Bull. Soc. Chim. Fr.* 2882 (1965).

109. **PYREK**, J.S.; *Tetrahedron* 26, 5029-5032 (1970).

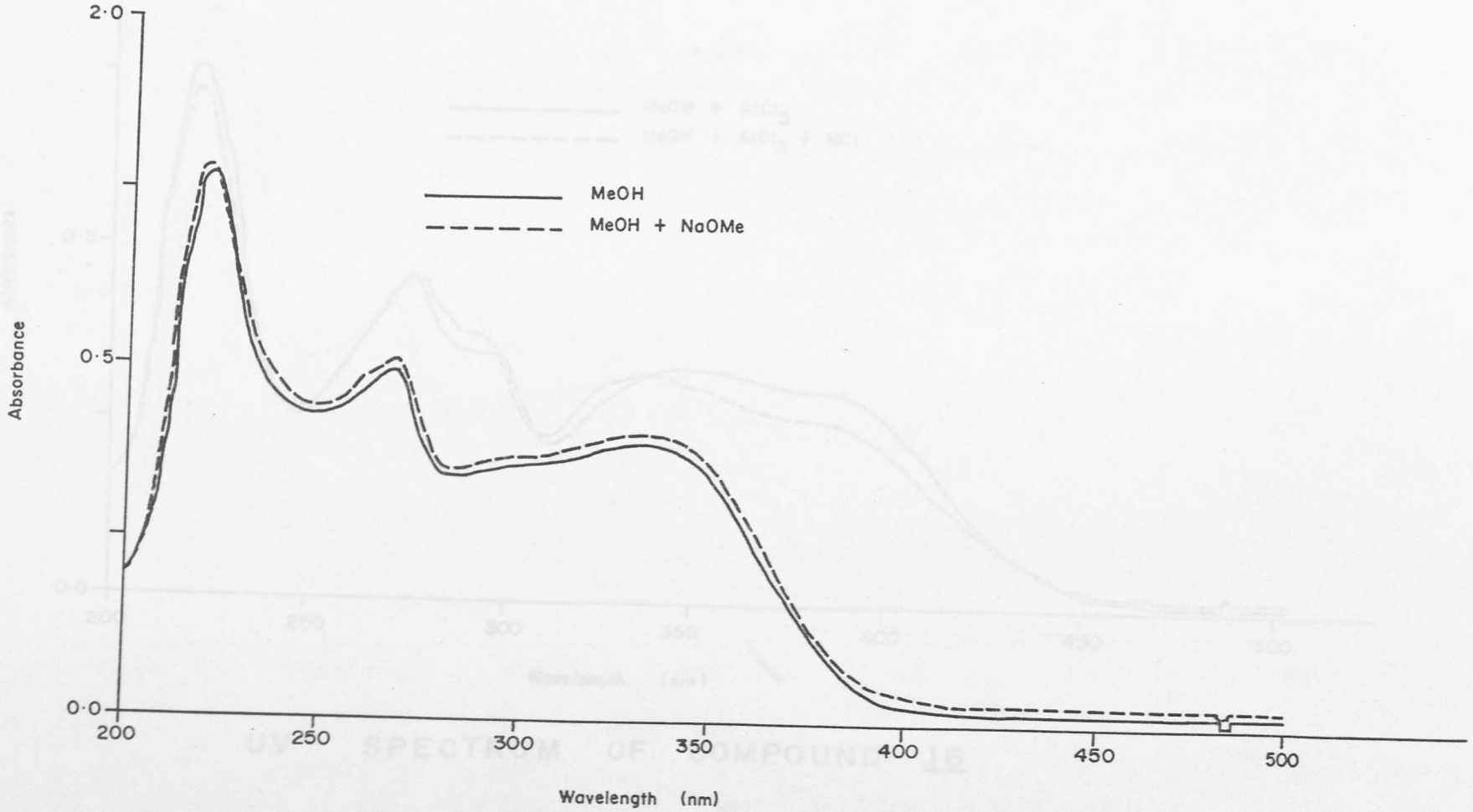
110. **RODRIGUEZ**, E.; VELDE, V.; and MABRY, T.J.; *Phytochemistry* 11, 2821-2826 (1972).

111. **HARBORNE**, J.B.; and MARBRY, T.J.; "The flavonoids, Advances in reaserch". Chapman New York pp. 29-32 (1982).

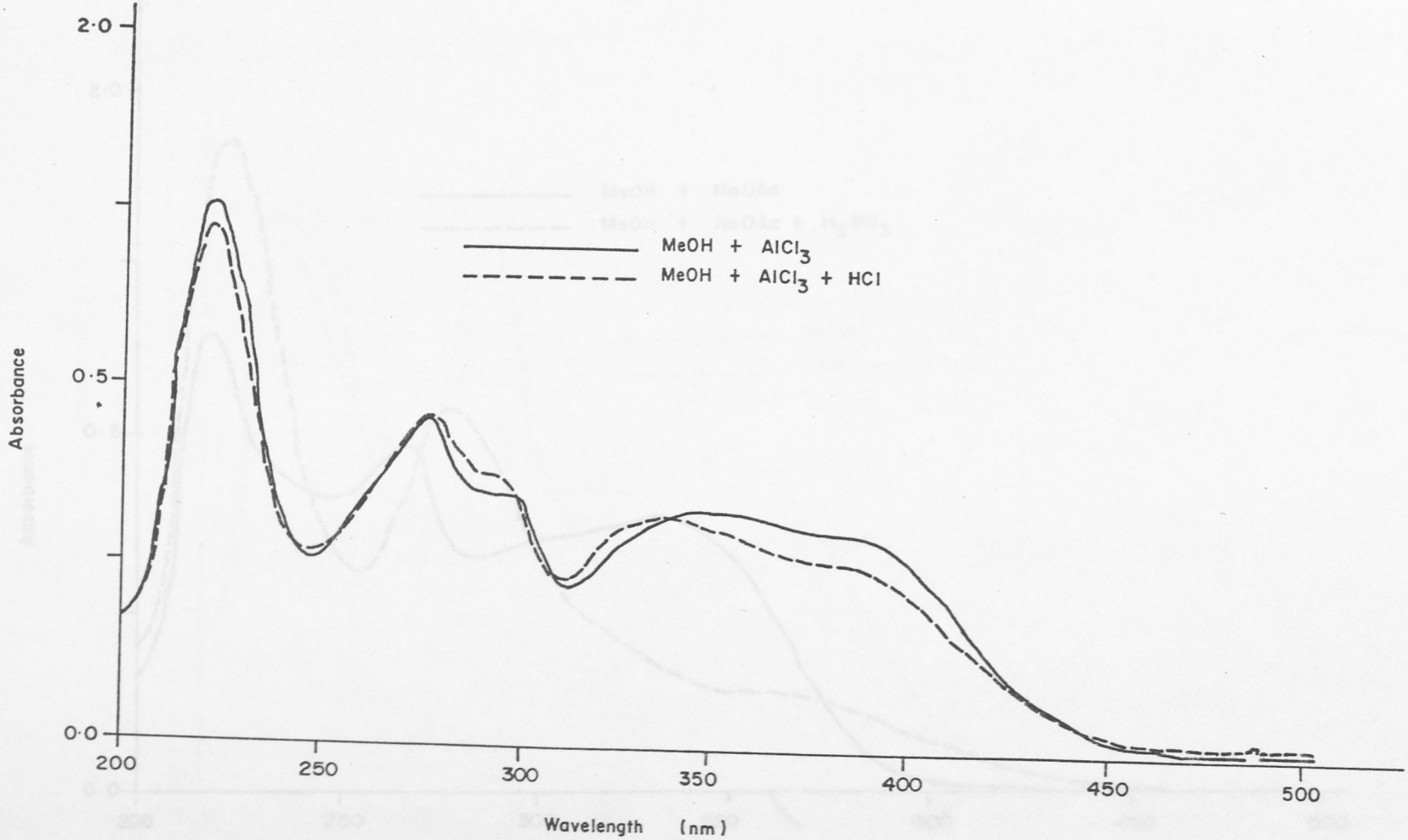


APPENDIX

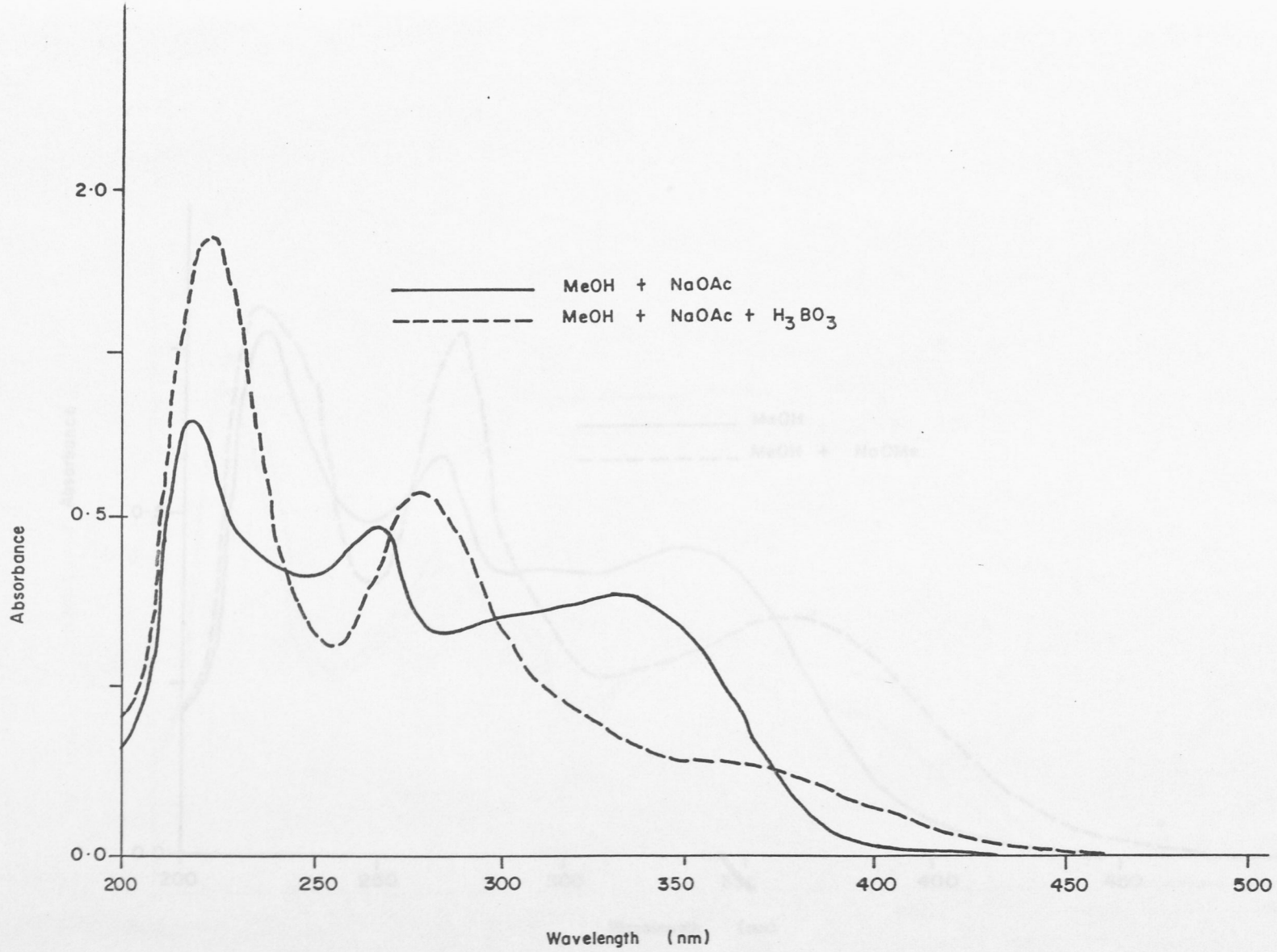
Spectra: UV, IR, MS and NMR



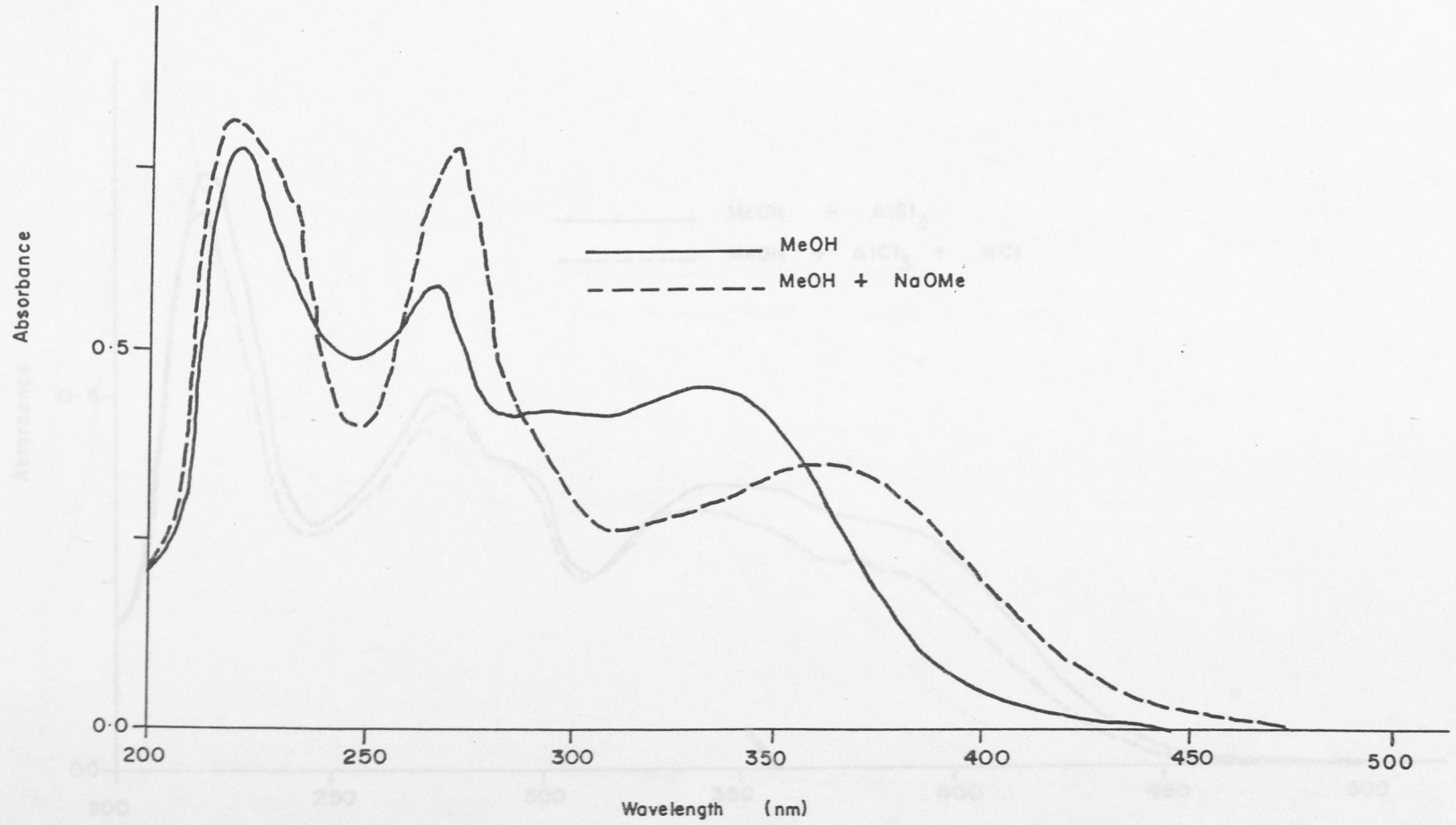
UV SPECTRUM OF COMPOUND 16



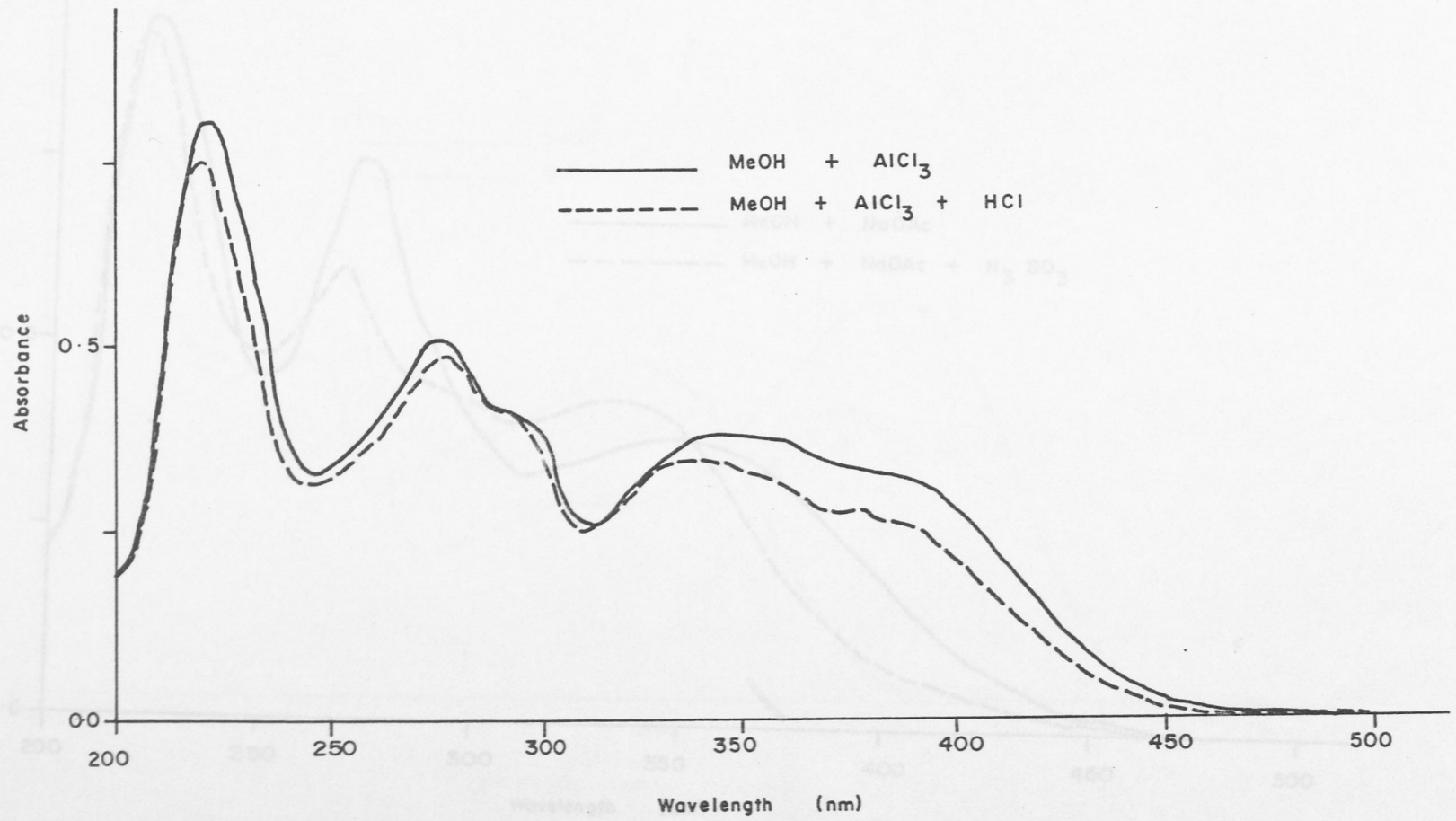
UV SPECTRUM OF COMPOUND 16



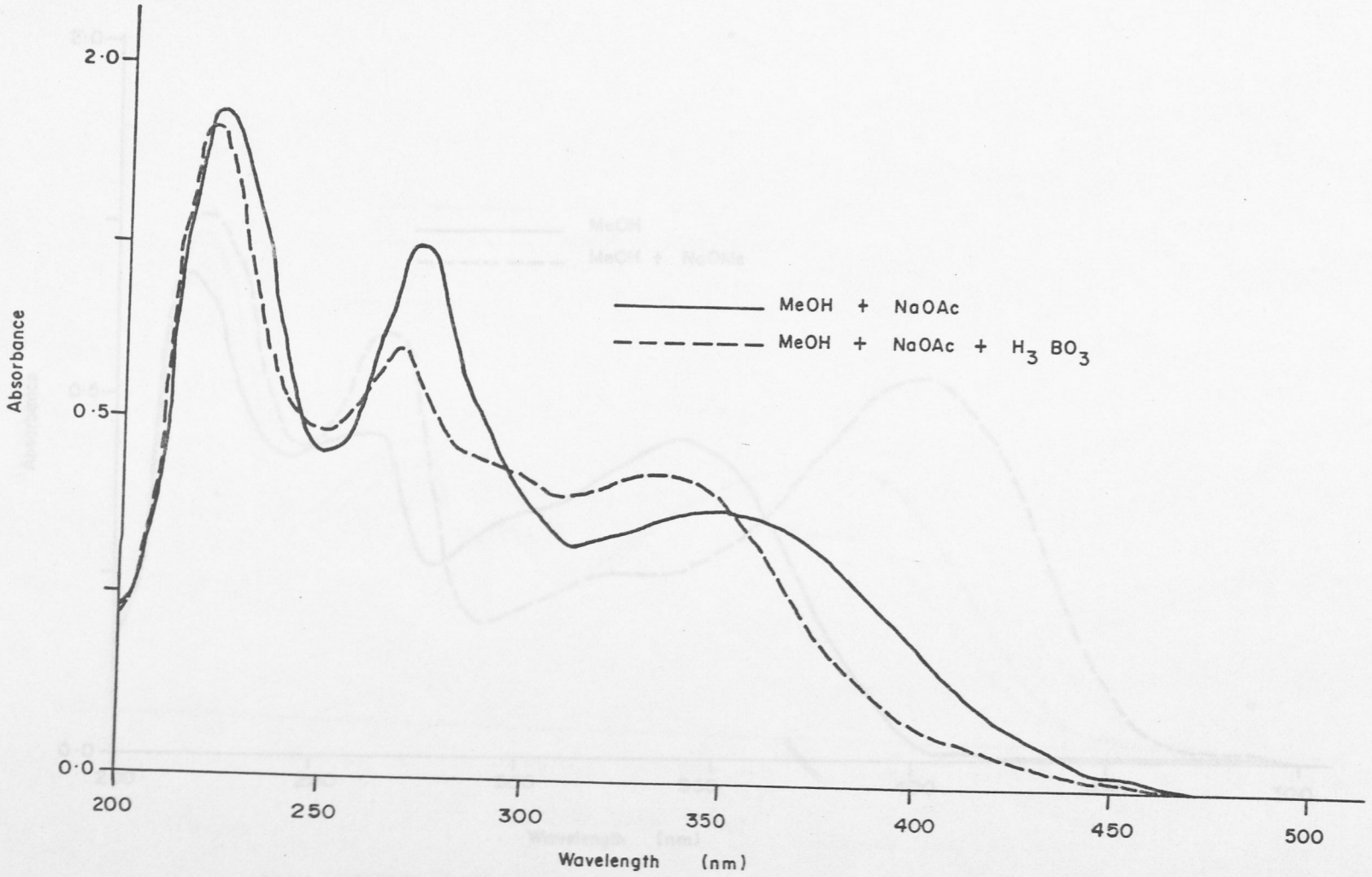
UV SPECTRUM OF COMPOUND 16



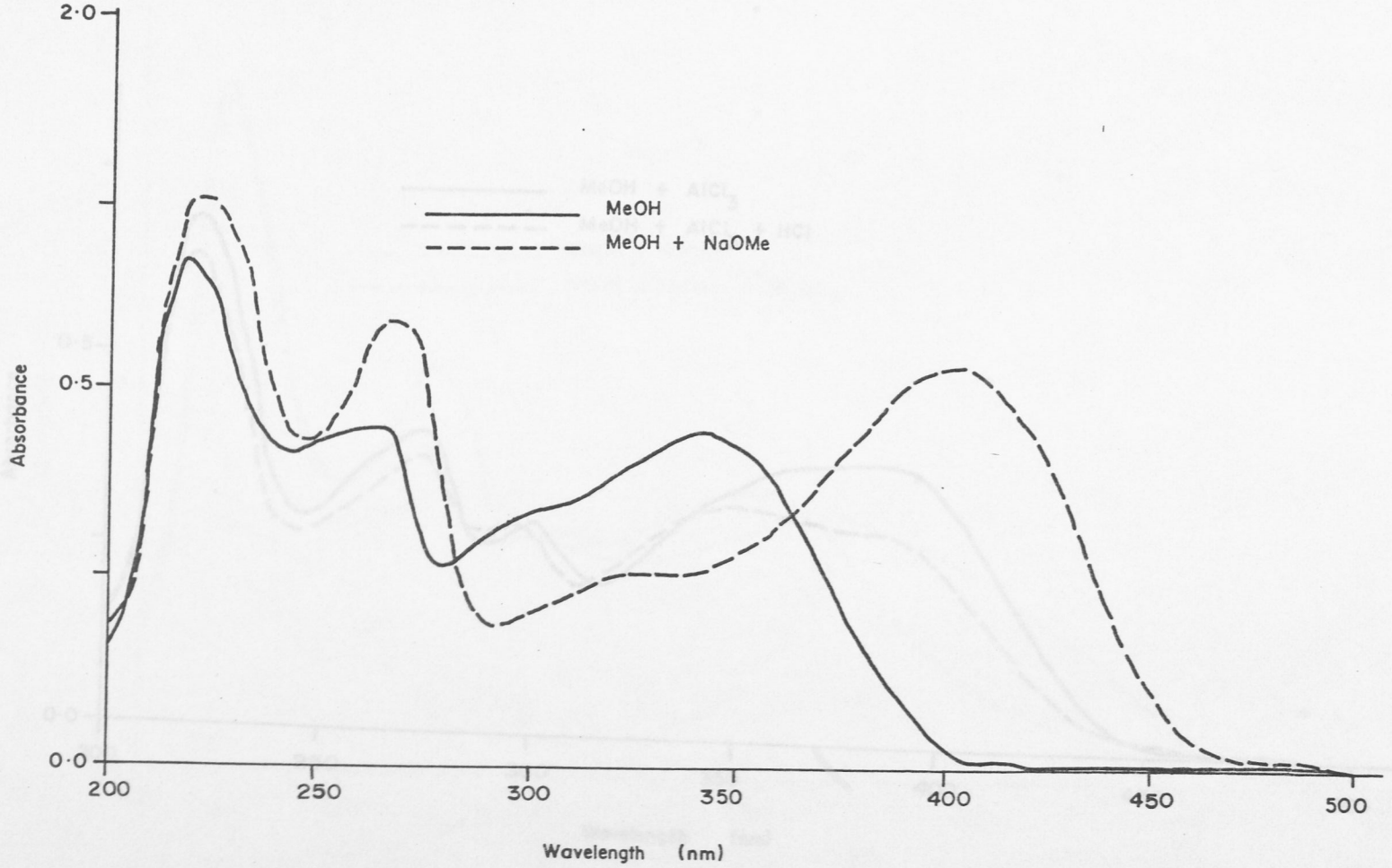
UV SPECTRUM OF COMPOUND 17



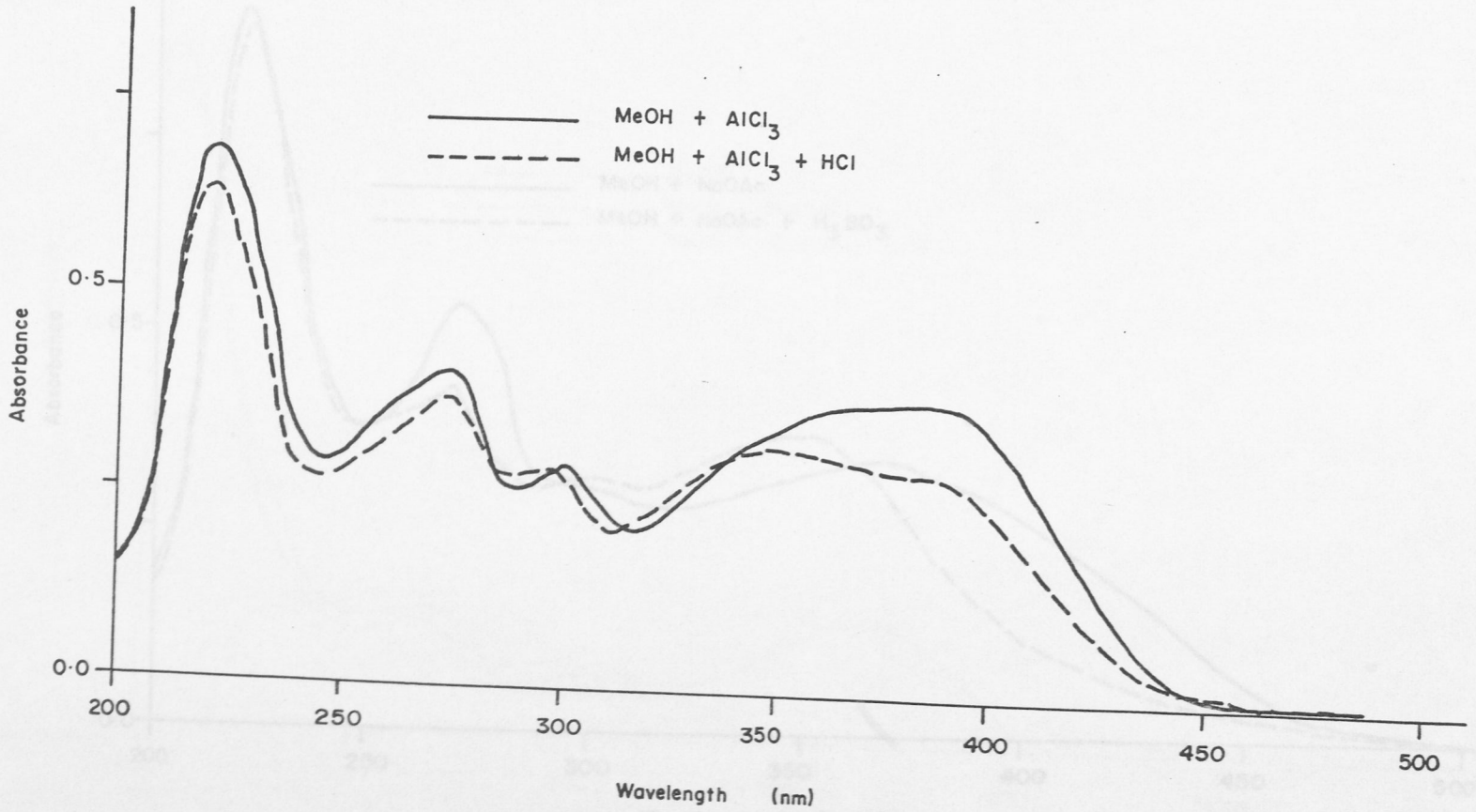
UV SPECTRUM OF COMPOUND 17



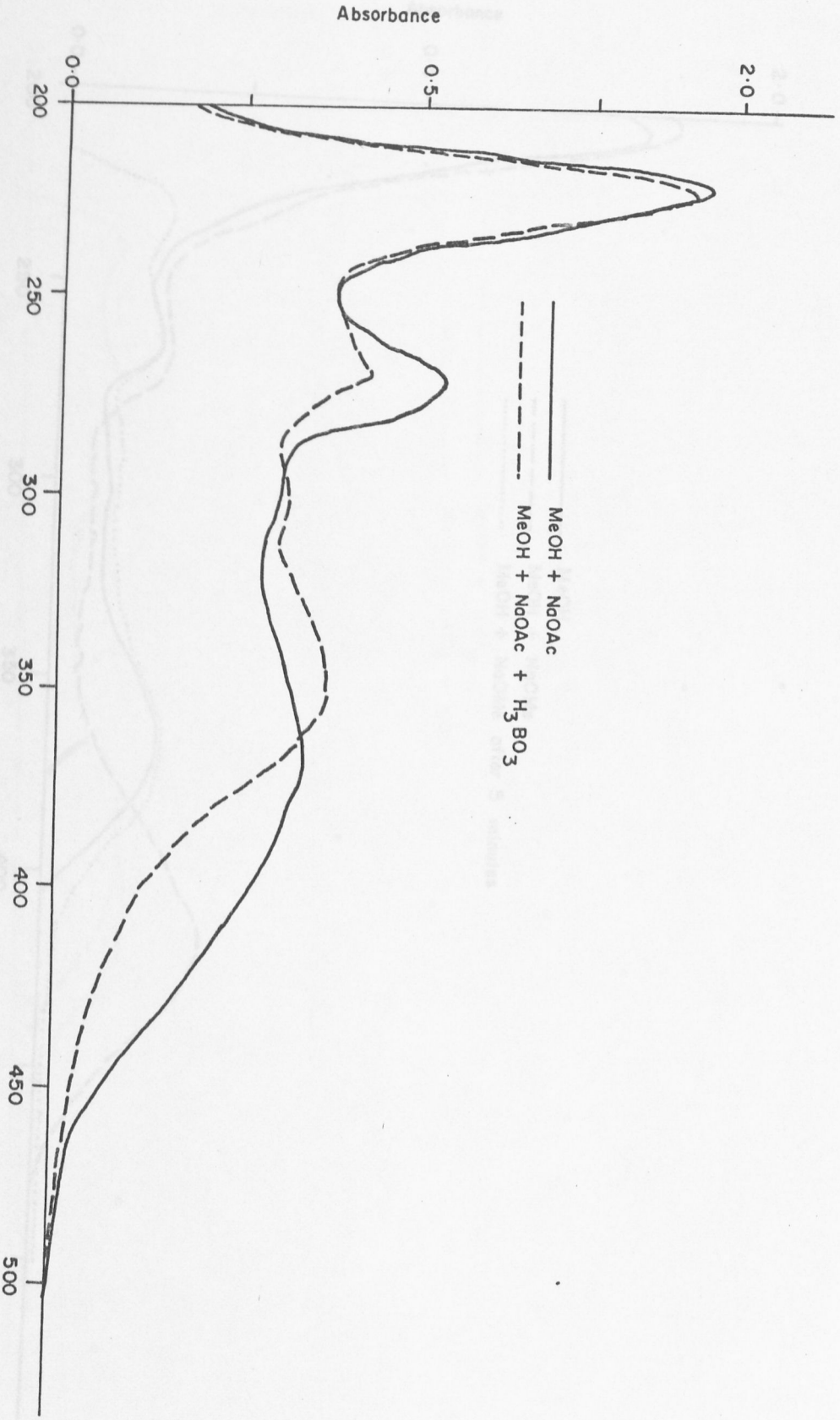
UV SPECTRUM OF COMPOUND 17



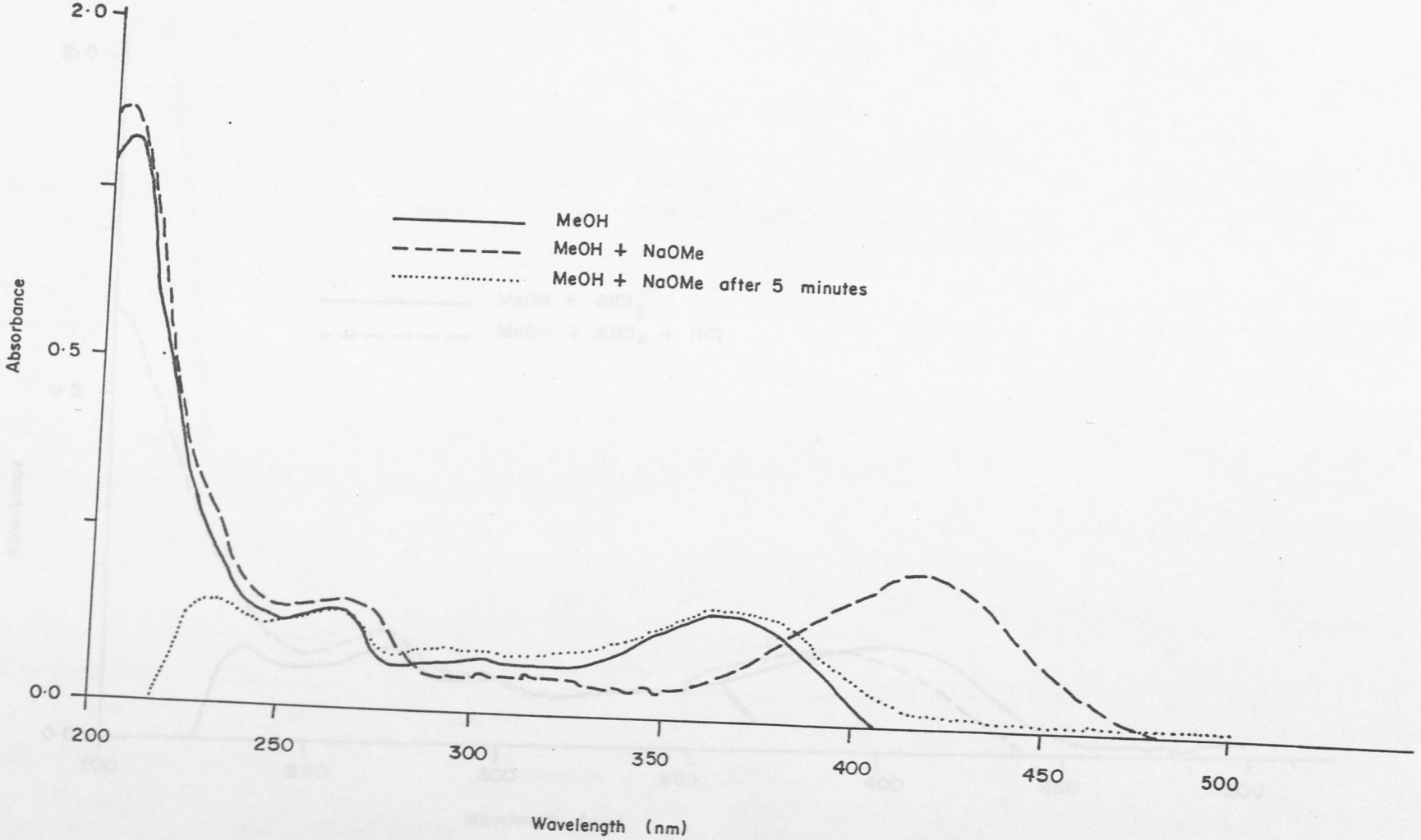
UV SPECTRUM OF COMPOUND 18



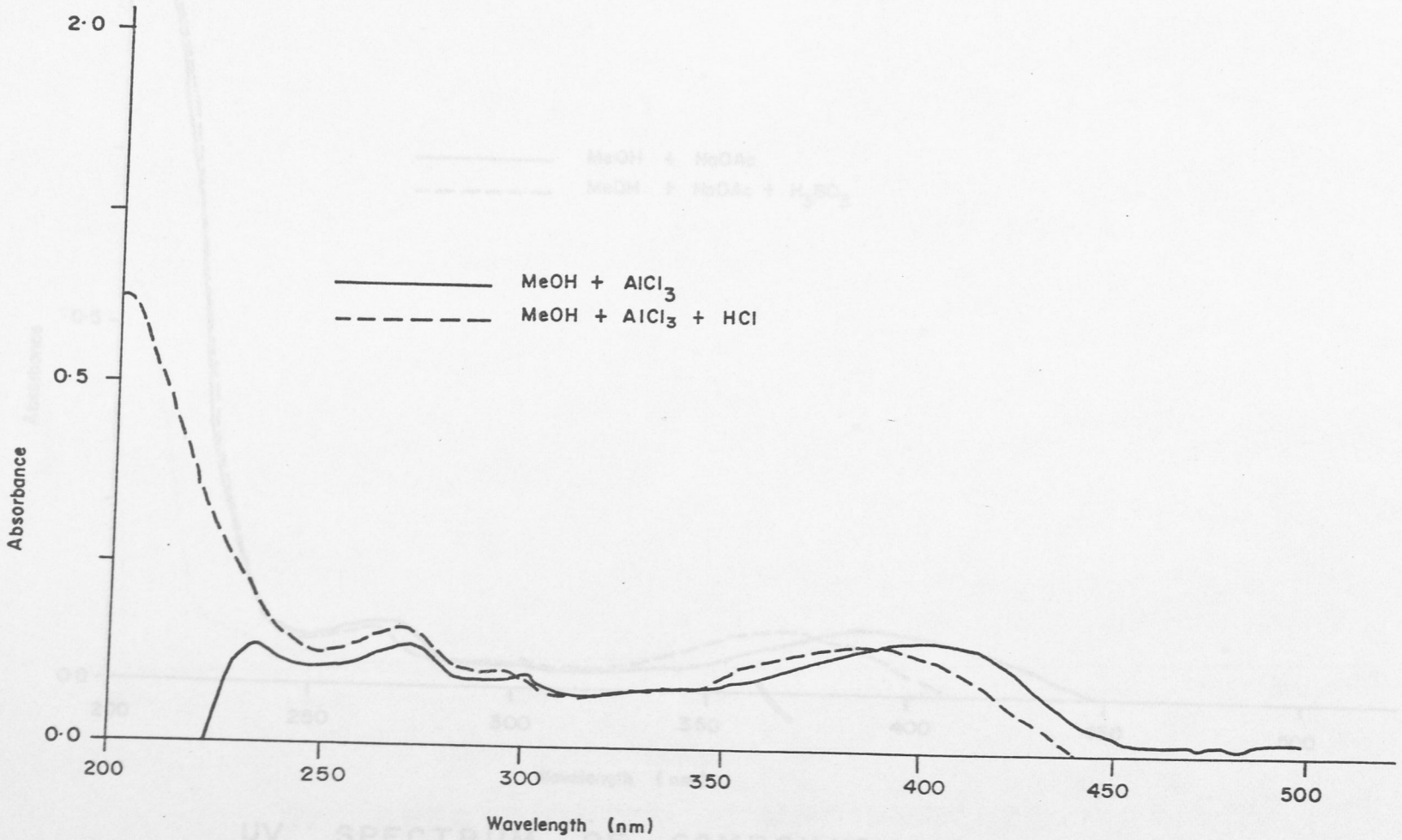
UV SPECTRUM OF COMPOUND 18



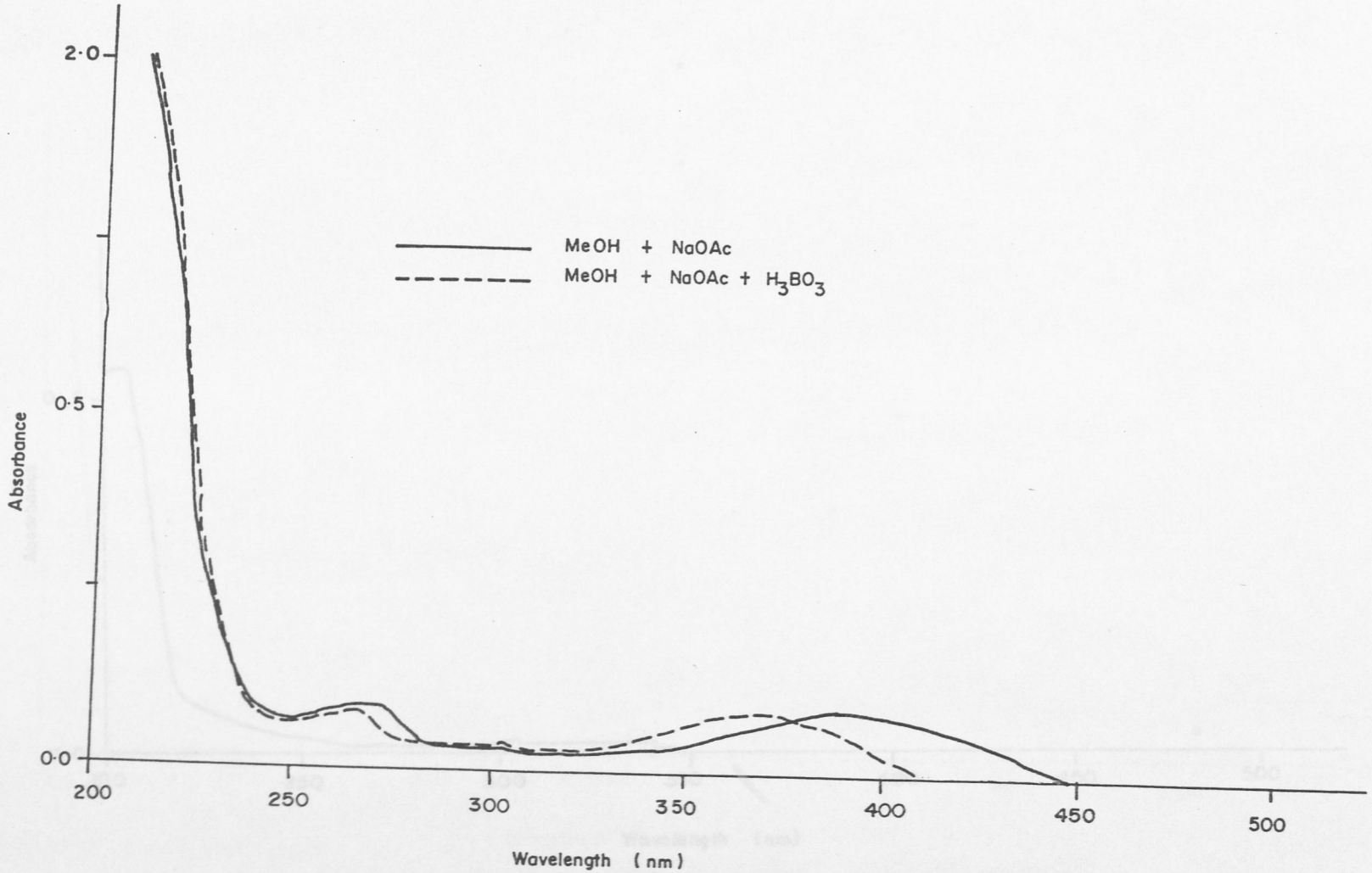
UV SPECTRUM OF COMPOUND 18



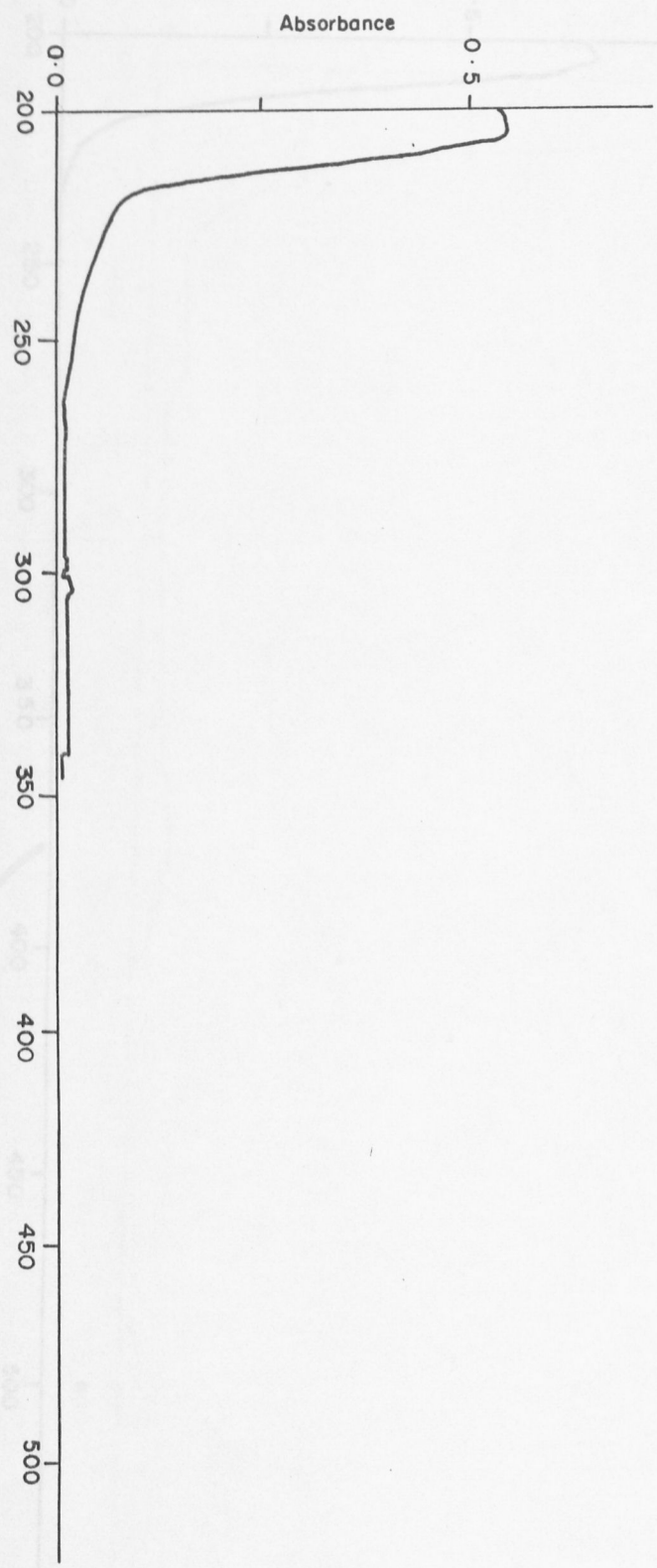
UV SPECTRUM OF COMPOUND 19



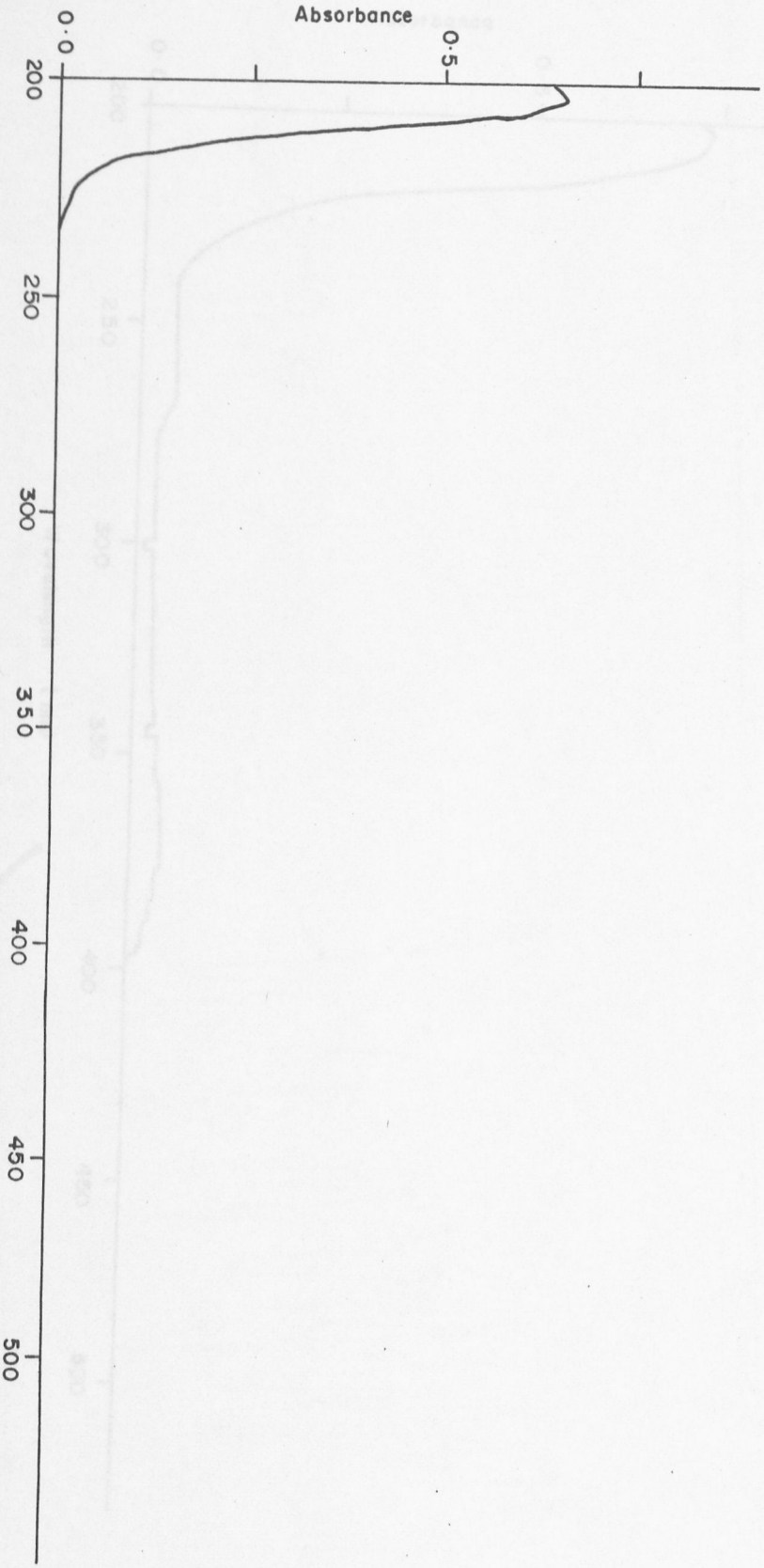
UV SPECTRUM OF COMPOUND 19



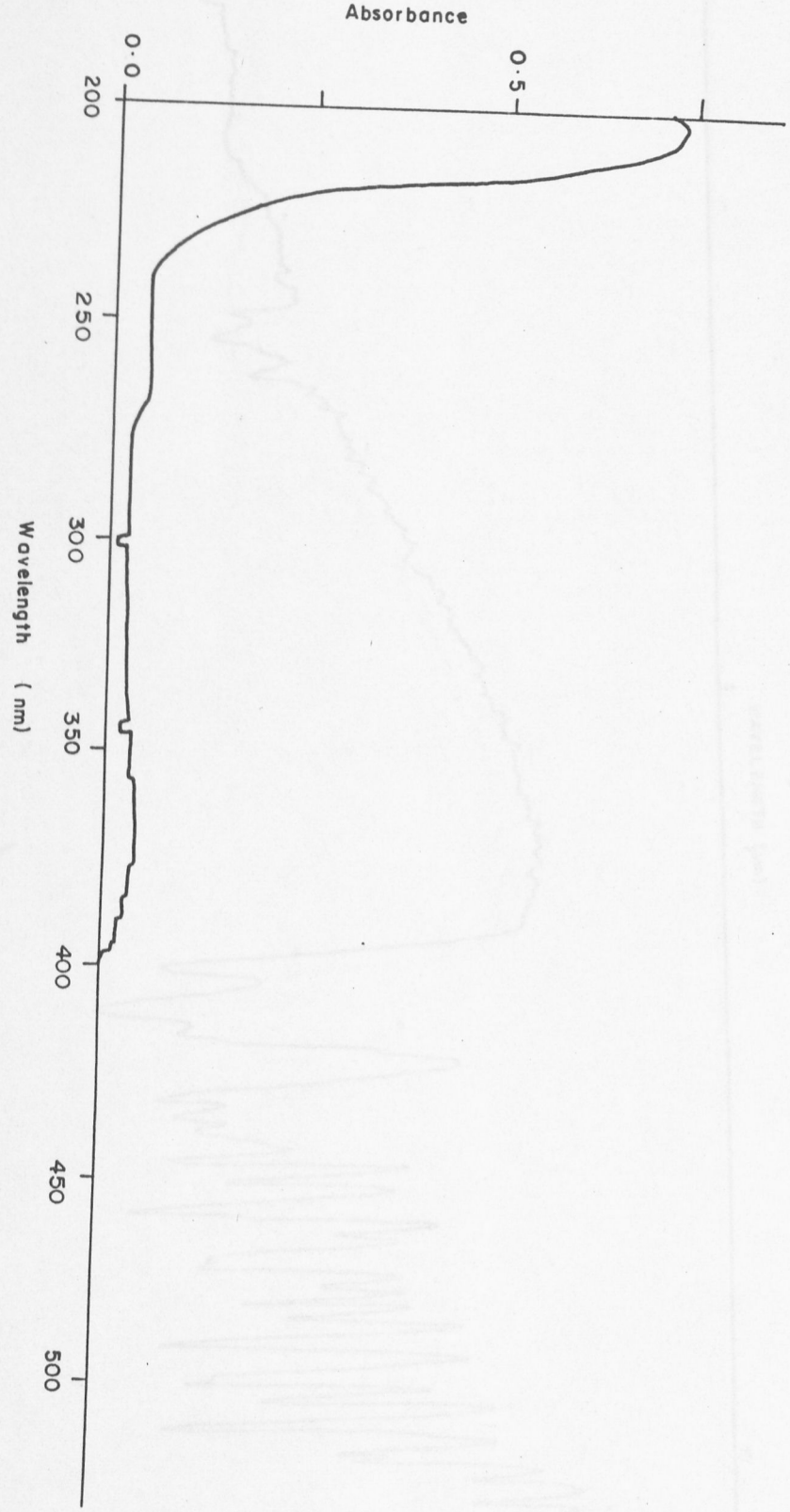
UV SPECTRUM OF COMPOUND 19



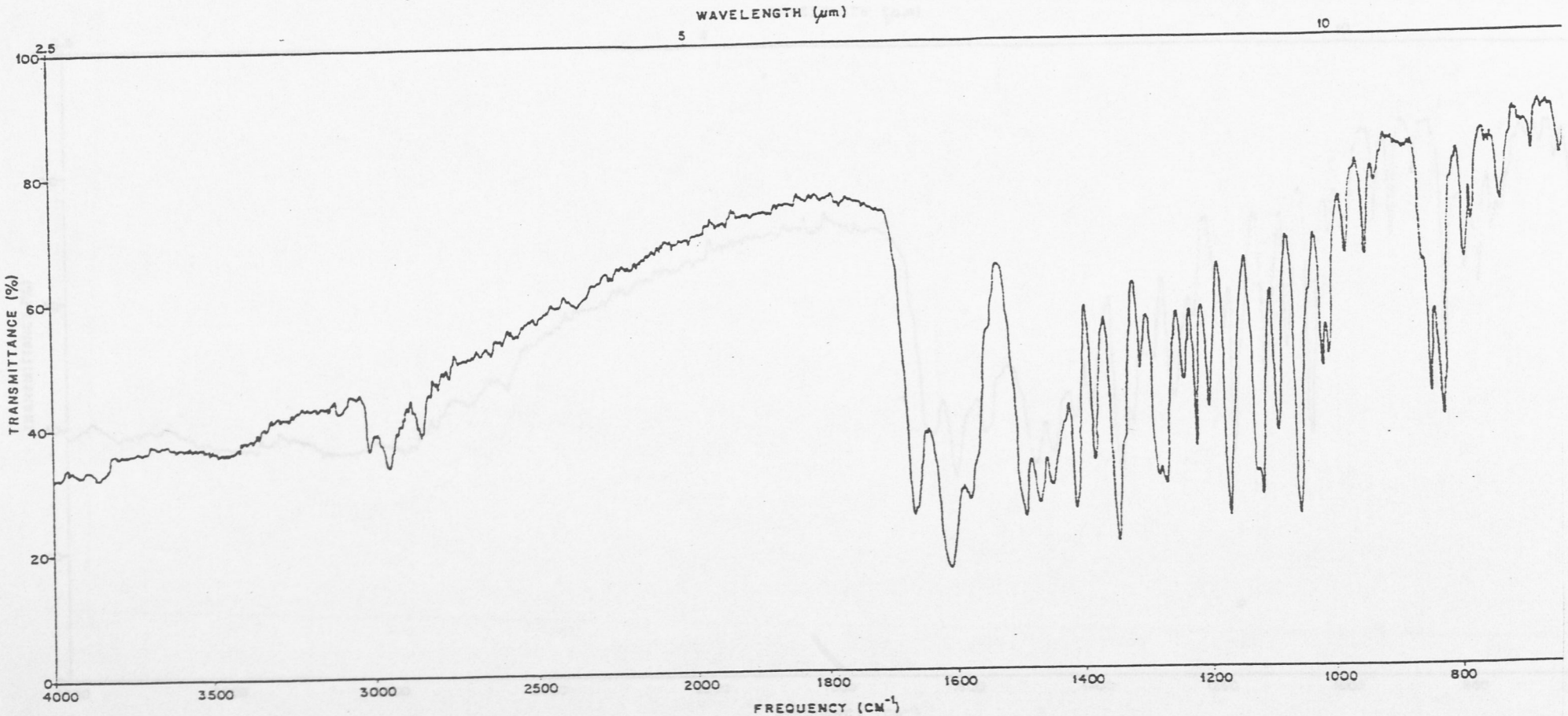
UV SPECTRUM OF COMPOUND 20



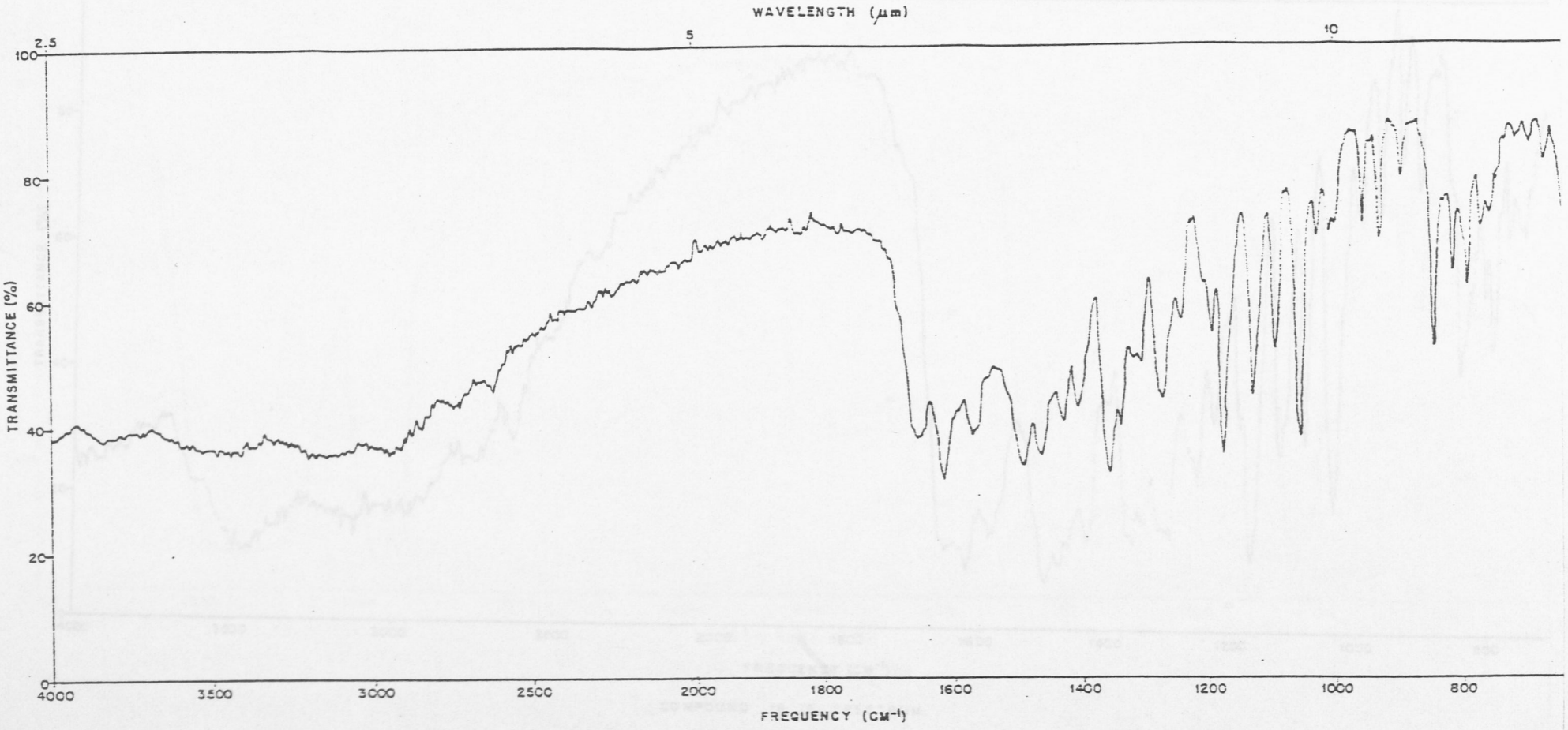
UV SPECTRUM OF COMPOUND 21



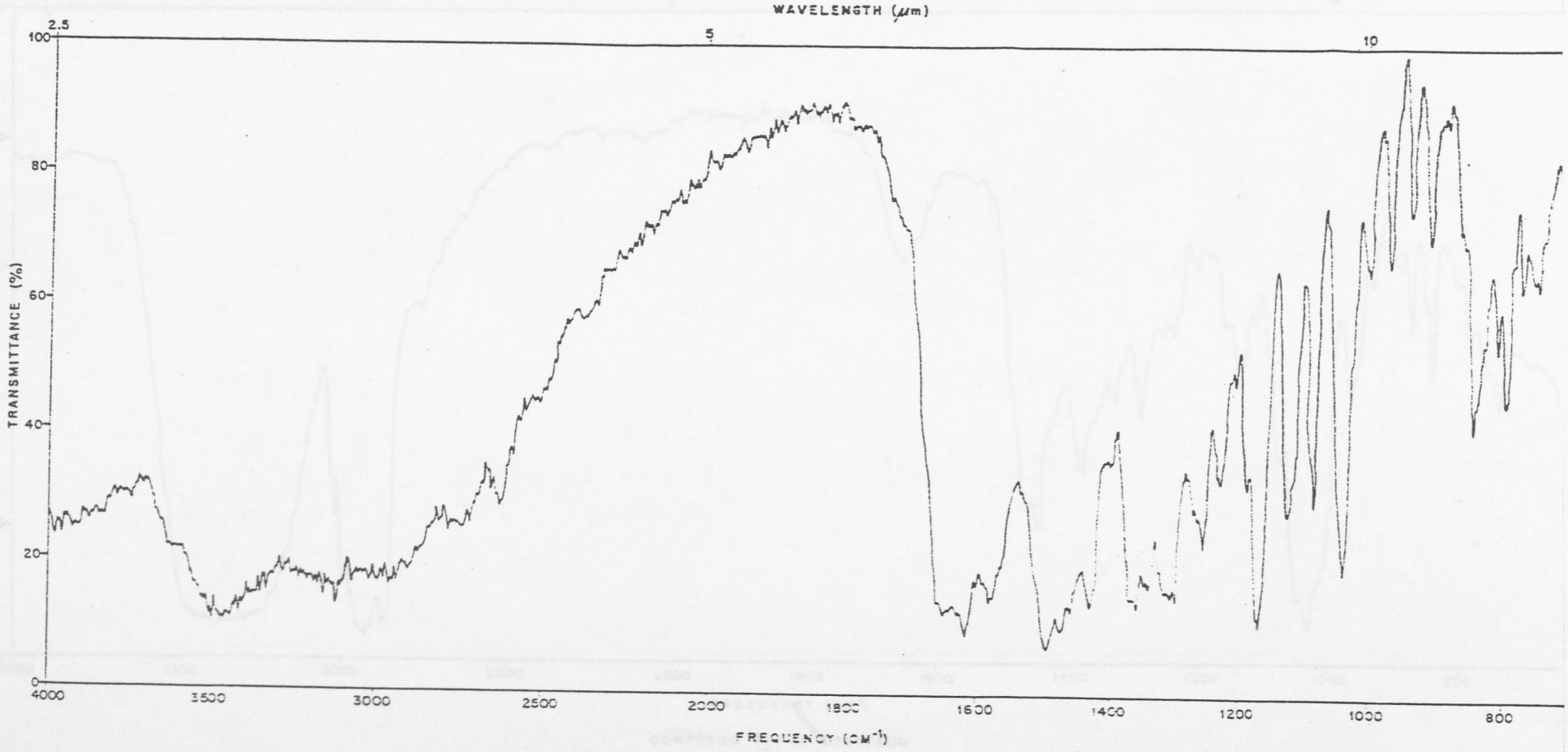
UV SPECTRUM OF COMPOUND 22



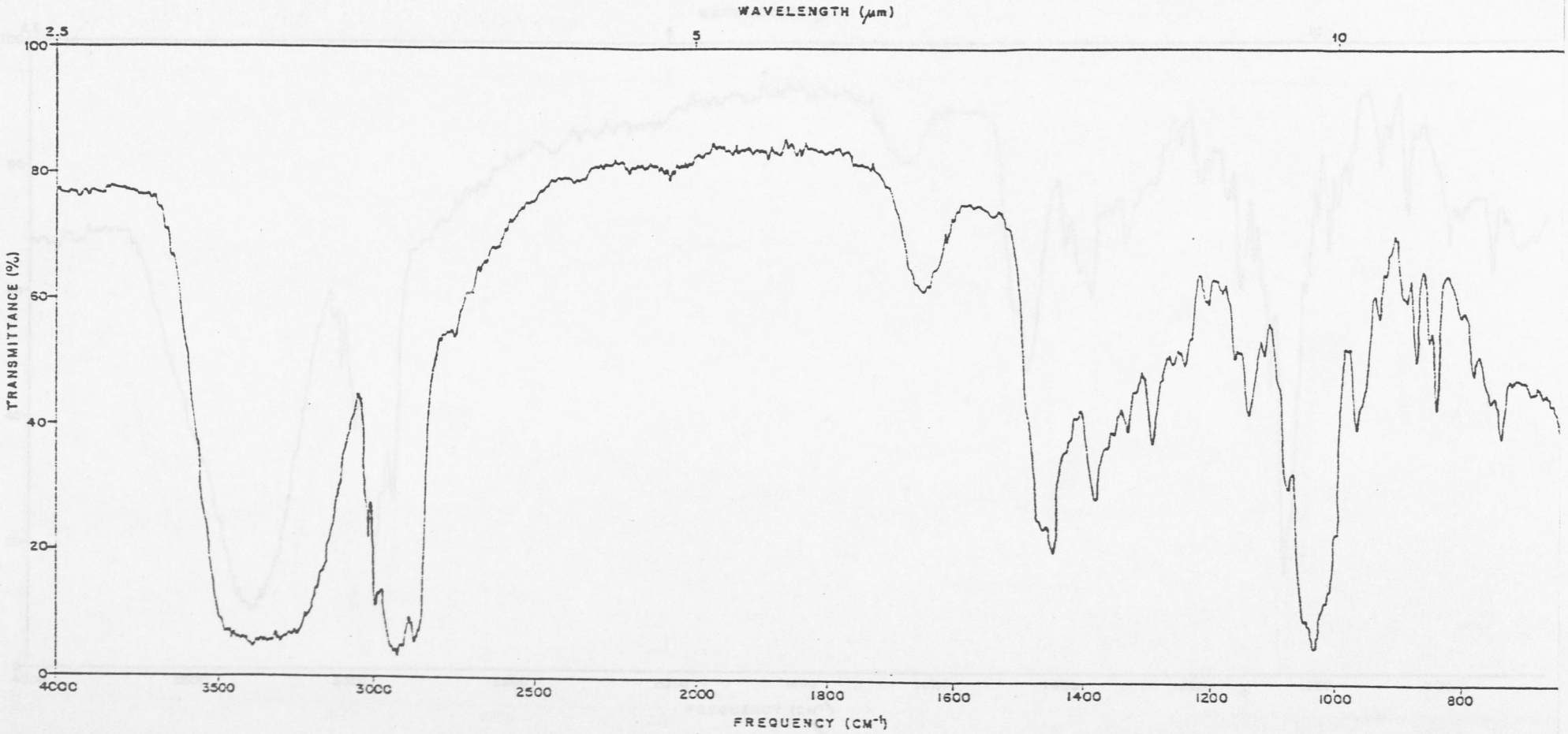
COMPOUND 16 IR SPECTRUM



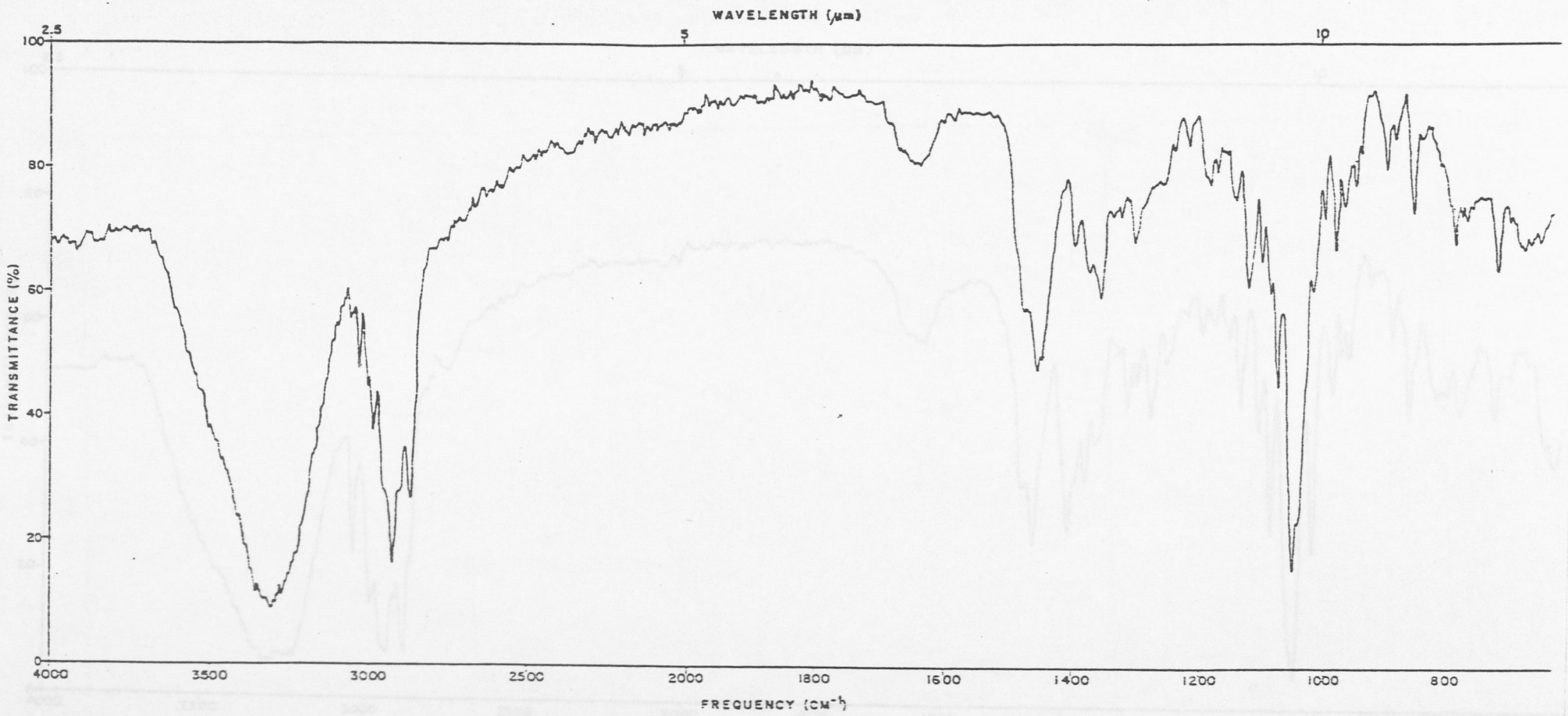
COMPOUND 17 IR SPECTRUM



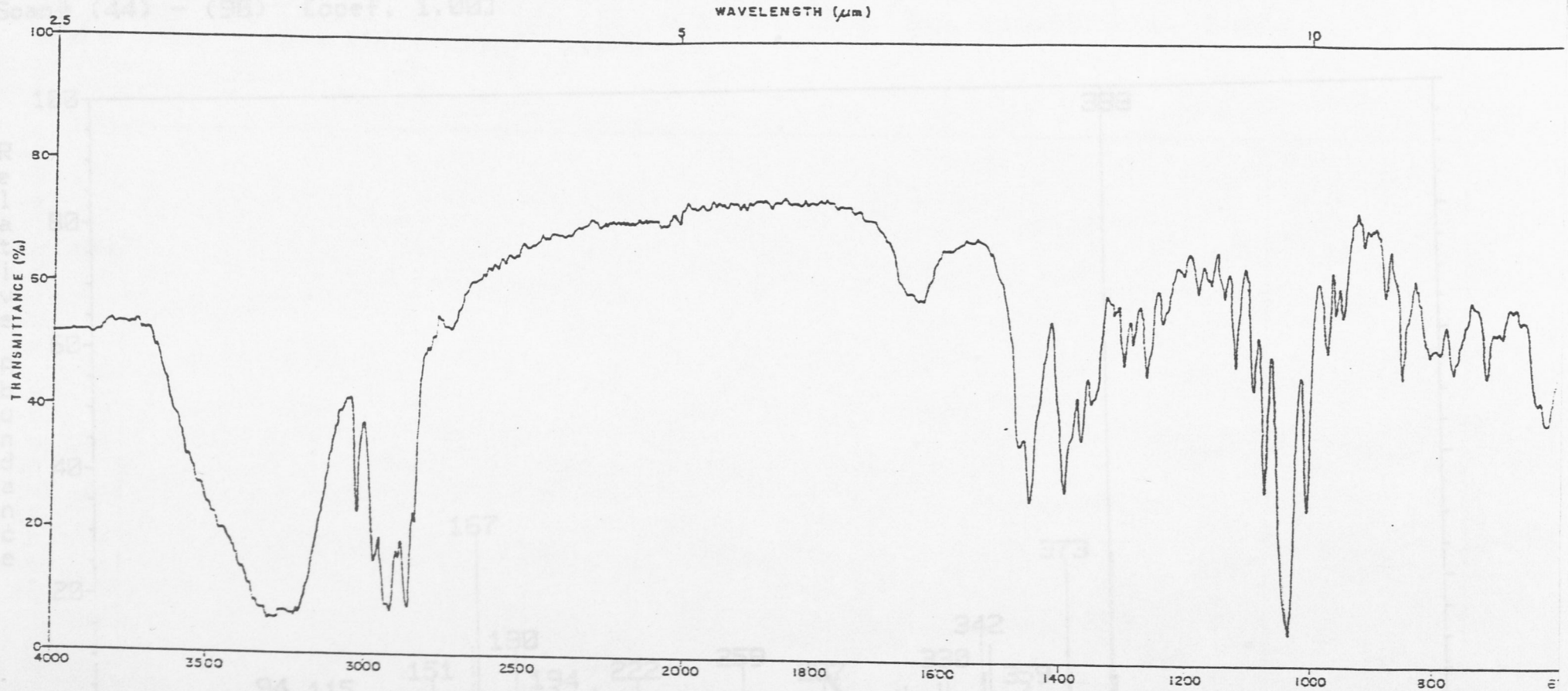
COMPOUND 18 IR SPECTRUM



COMPOUND 19 IR SPECTRUM

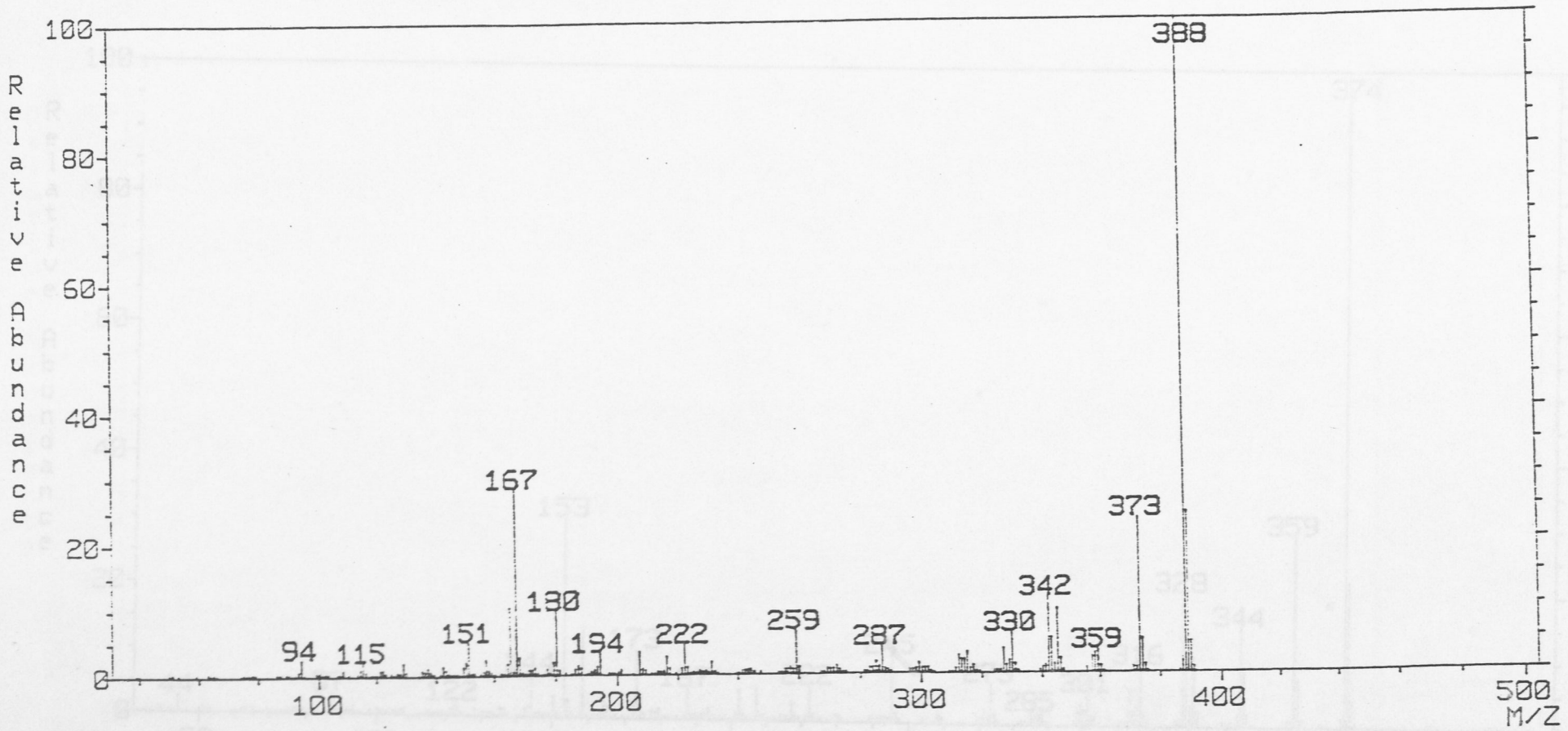


COMPOUND 20 IR SPECTRUM



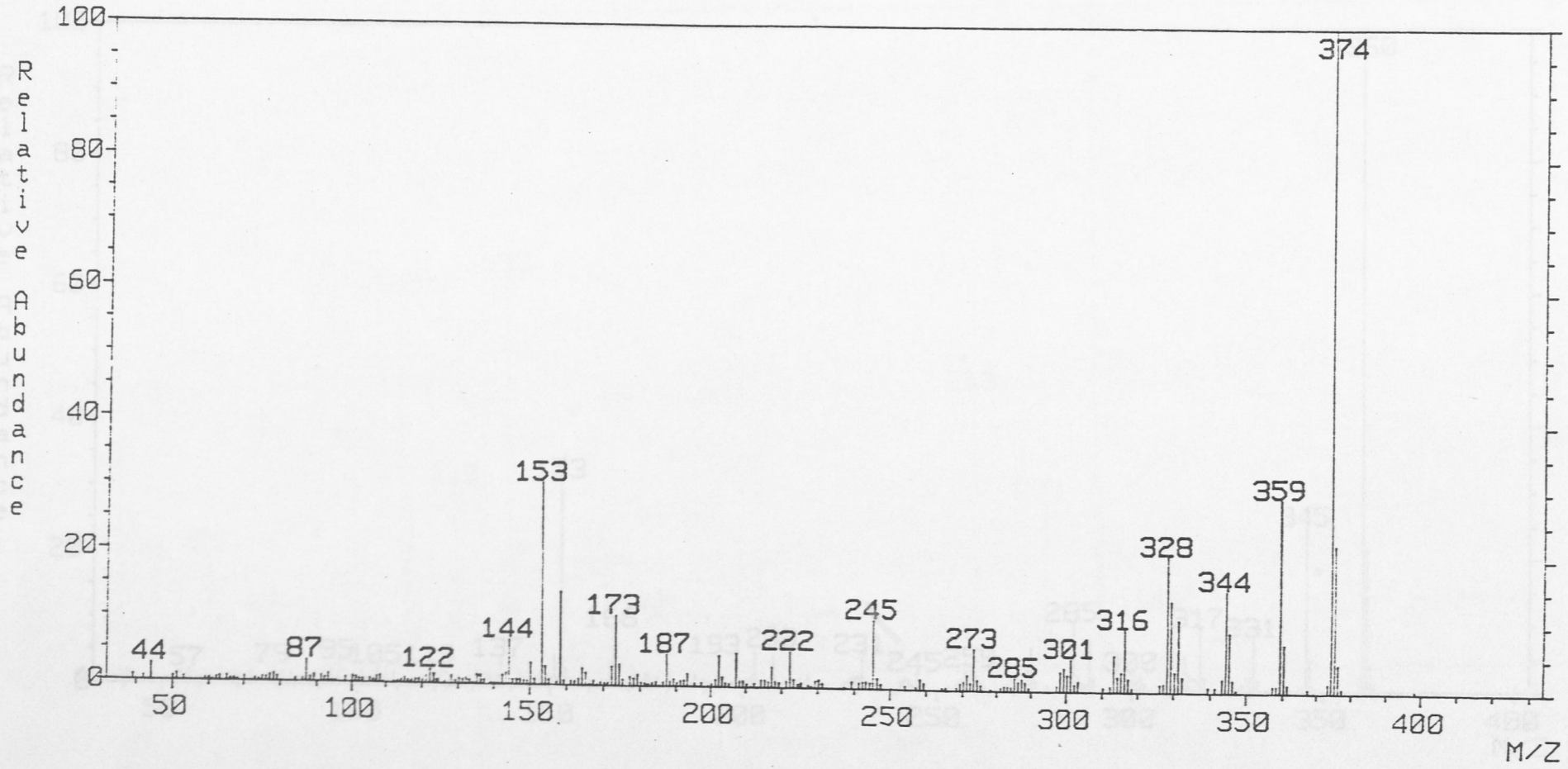
COMPOUND 21 IR SPECTRUM

MASS SPECTRUM Data File: 578 15-FEB-93 17:18
SamPle: COMPD 16
RT 2'52" EI (Pos.) GC 117.0c BP: m/z 388.0000 Int. 336.2857 Lv 0.00
Scan# (44) - (90) [coef. 1.00]



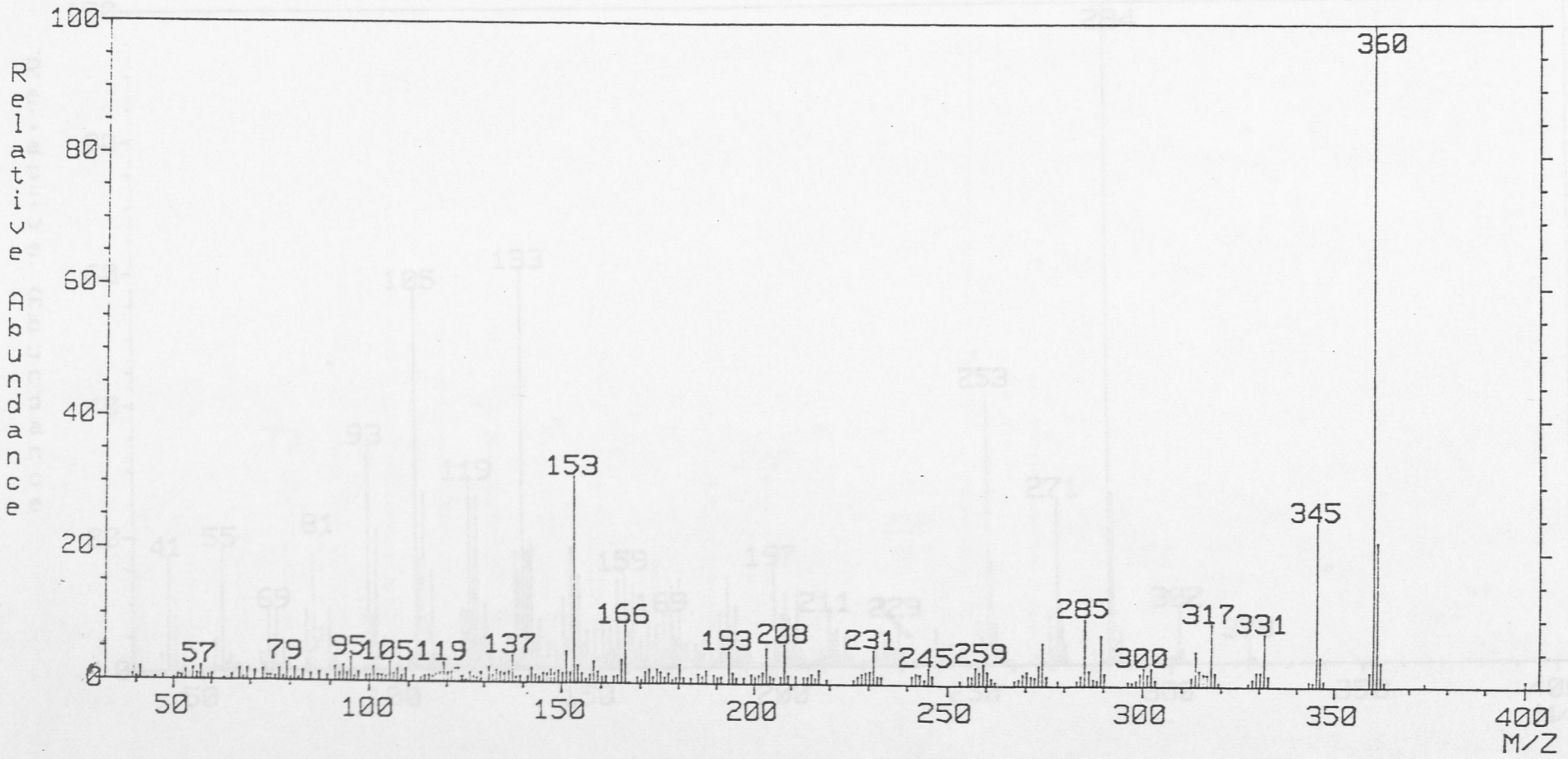
MASS SPECTRUM OF COMPOUND 16

MASS SPECTRUM Data File: 579 17-FEB-93 14:13
Sample: COMPD 17
RT 7'00" EI (Pos.) GC 191.2c BP: m/z 374.0000 Int. 119.9525 Lv 0.00
Scan# (106) - (57, 117) [coef. 1.00]



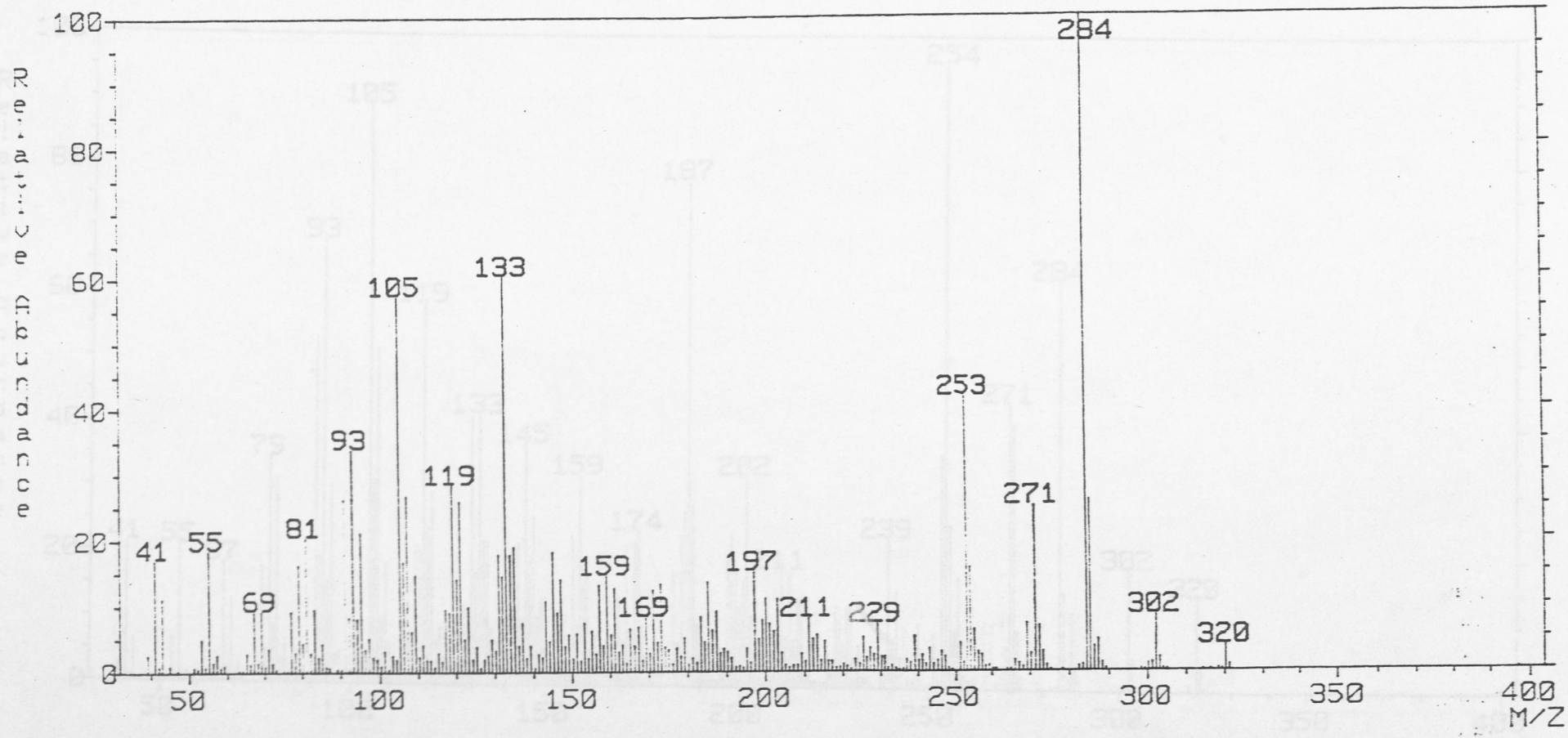
MASS SPECTRUM OF COMPOUND 17

MASS SPECTRUM Data File: 580 16-FEB-93 19:43
Sample: COMPD 18
RT 4'40" EI (Pos.) GC 316.5c BP: m/z 360.0000 Int. 100.1161 Lv 0.00
Scan# (71) - (101) [coef. 1.00]



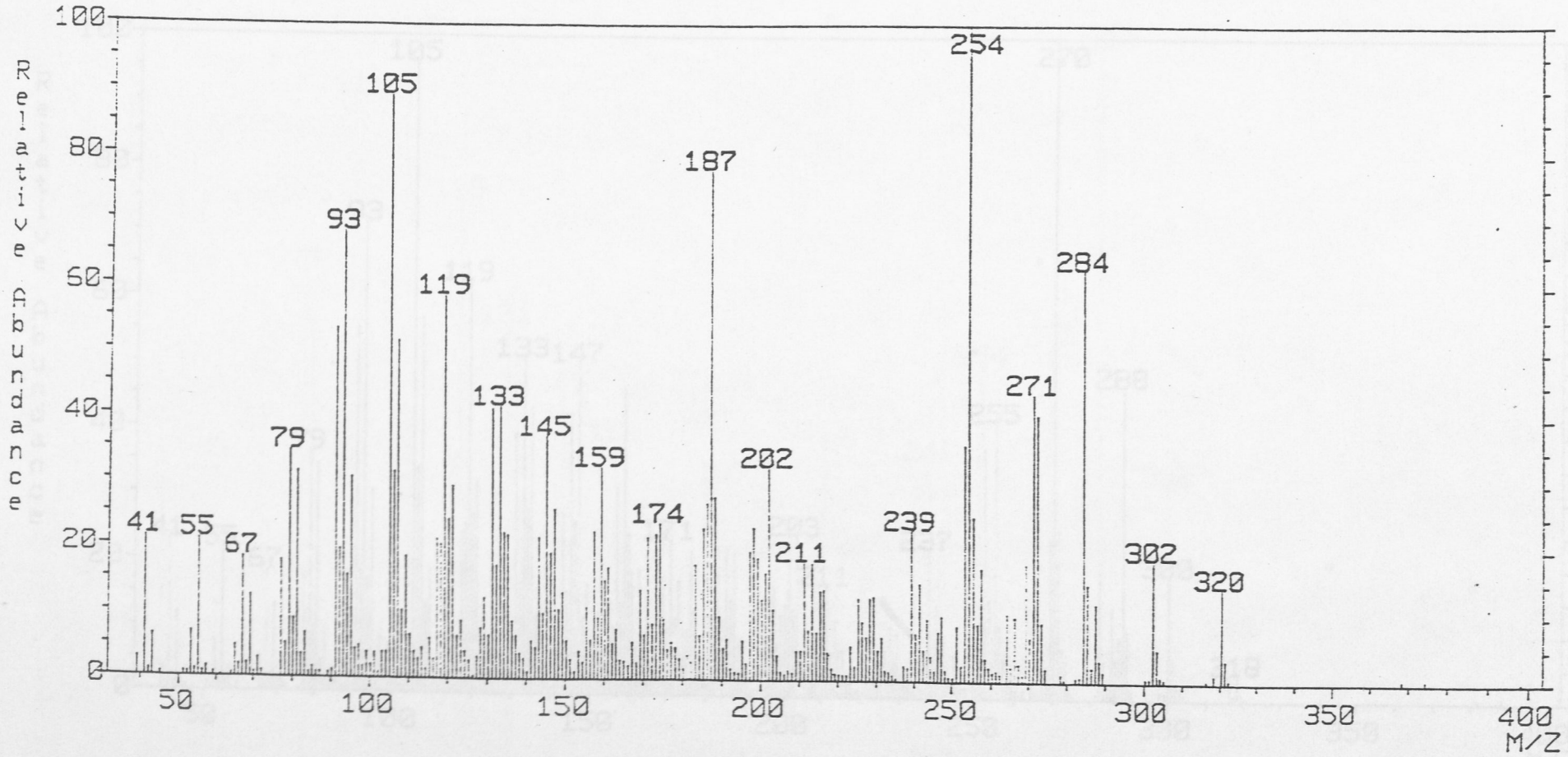
MASS SPECTRUM OF COMPOUND 18

MASS SPECTRUM Data File: 584 17-FEB-93 13:47
Sample: COMPD 19
RT 9'16" EI (Pos.) GC 150.2c BP: m/z 284.0000 Int. 337.9334 Lv 0.00
Scan# (140) - (29, 163) [coef. 1.00]



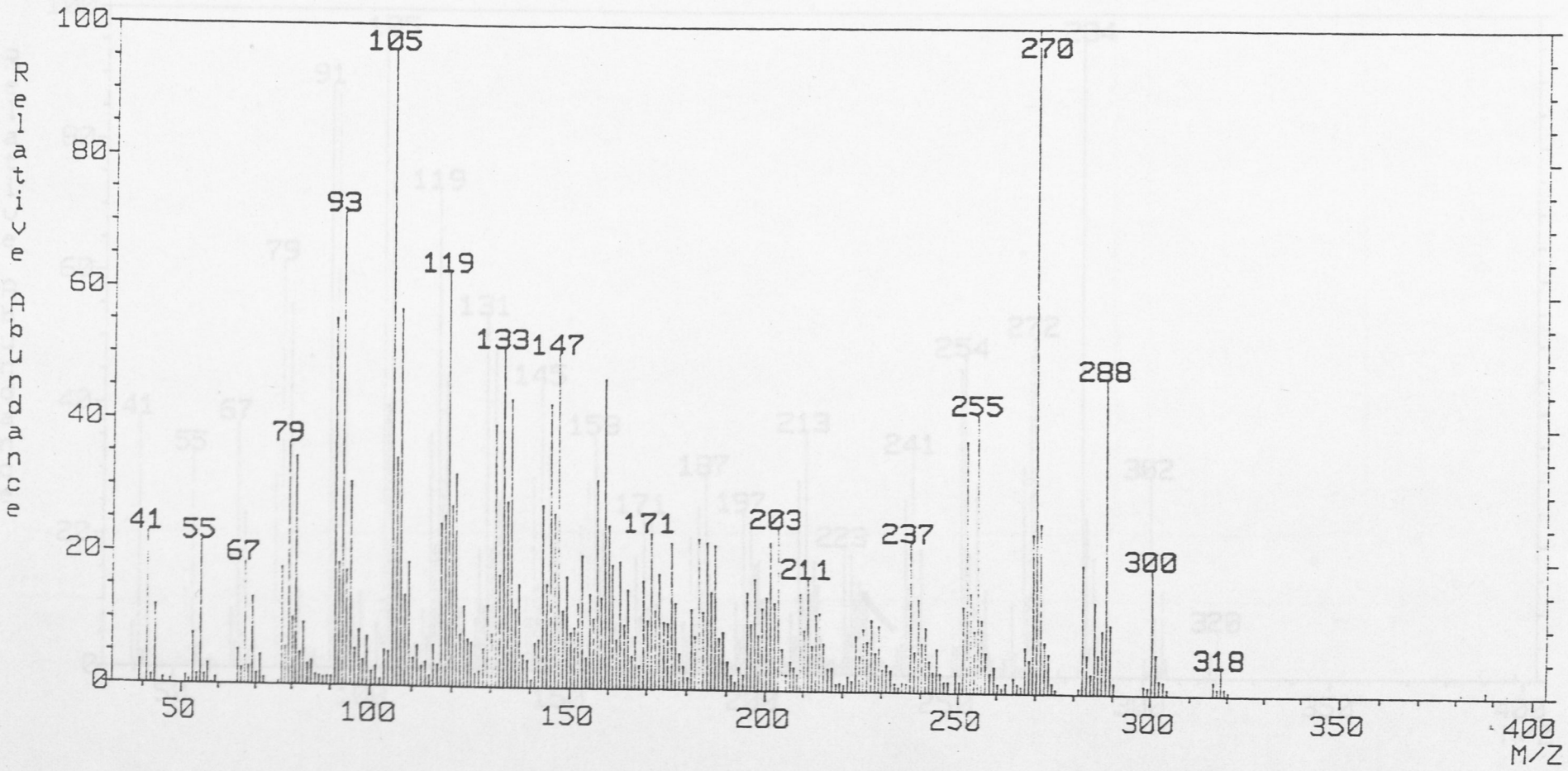
MASS SPECTRUM OF COMPOUND 19

MASS SPECTRUM Data File: 582 17-FEB-93 14:41
Sample: COMPD 20
RT 6'44" EI (Pos.) GC 201.3c BP: m/z 254.0000 Int. 122.7143 Lv 0.00
Scan# (102) - (72, 112) [coef. 1.00]



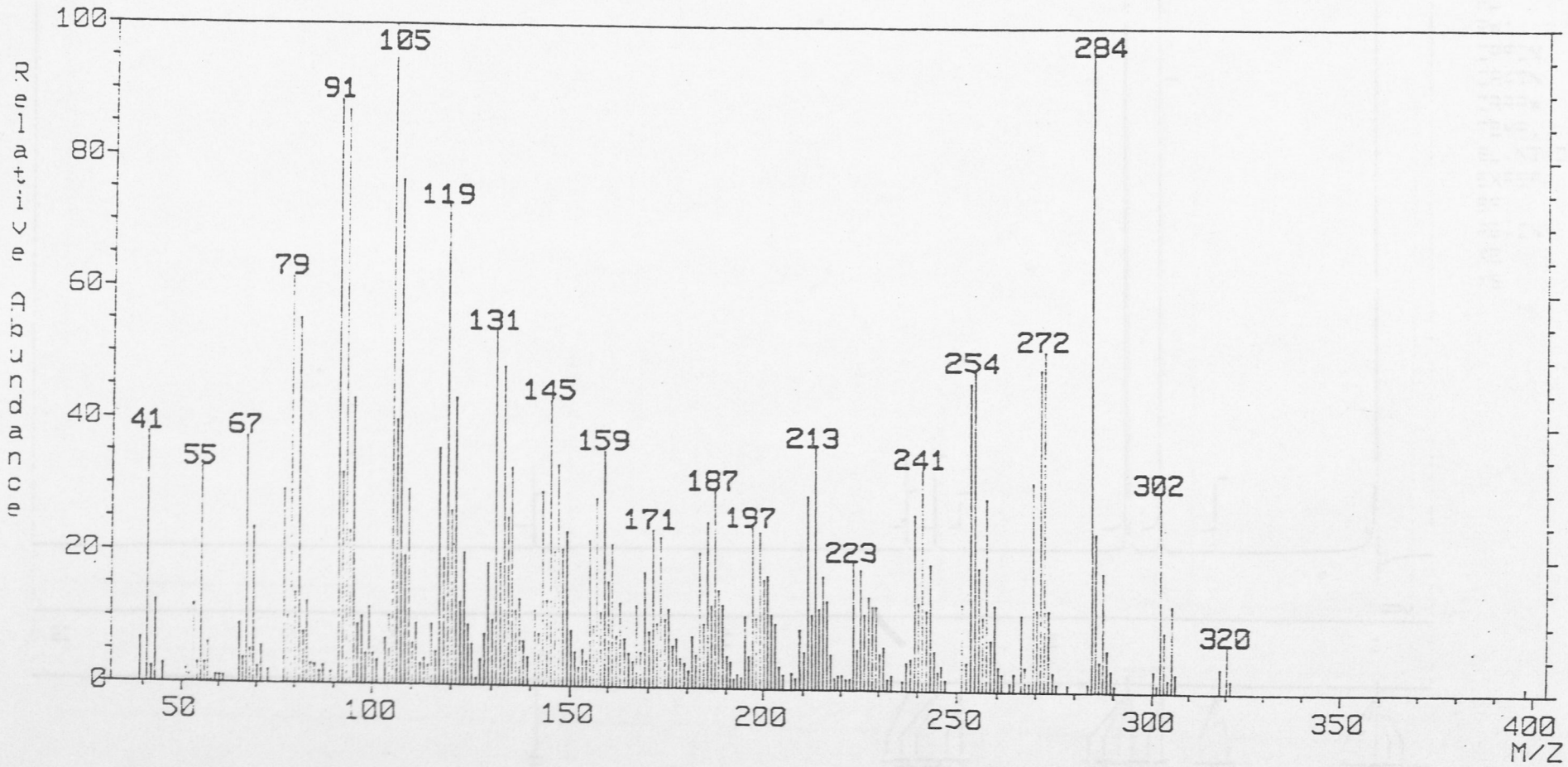
MASS SPECTRUM OF COMPOUND 20

MASS SPECTRUM Data File: 583 15-FEB-93 18:44
Sample: COMPD 21
RT 6'52" EI (Pos.) GC 185.4c BP: m/z 270.0000 Int. 131.0346 Lv 0.00
Scan# (104) - (61, 119) [coef. 1.00]



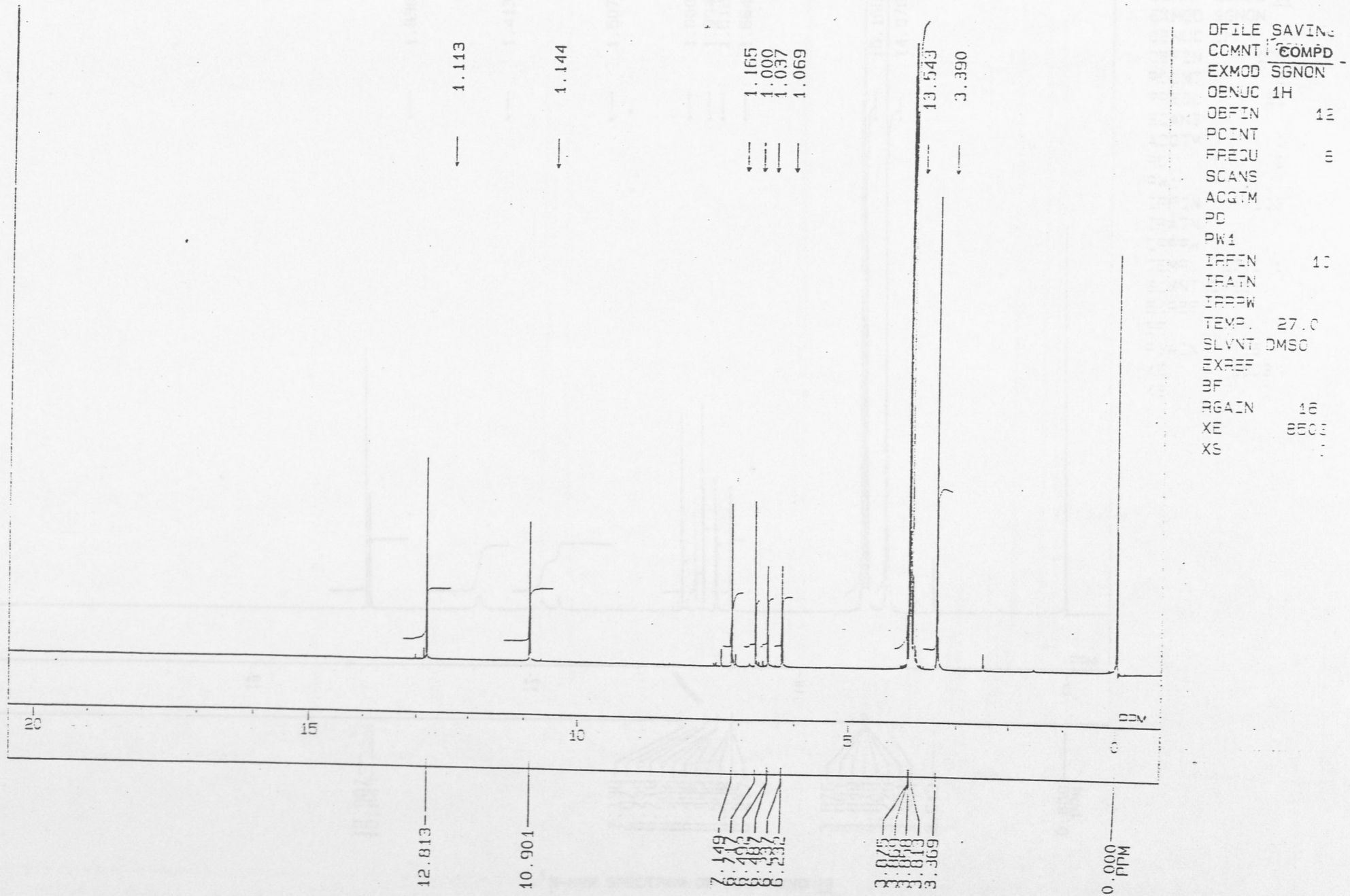
MASS SPECTRUM OF COMPOUND 21

MASS SPECTRUM Data File: 585 16-FEB-93 18:42
SamPle: ~~COMPD~~ 22
RT 8'56" EI (Pos.) GC 165.8c BP: m/z 105.0000 Int. 56.3462 Lv 0.00
Scan# (135) - (55, 165) [coef. 1.00]

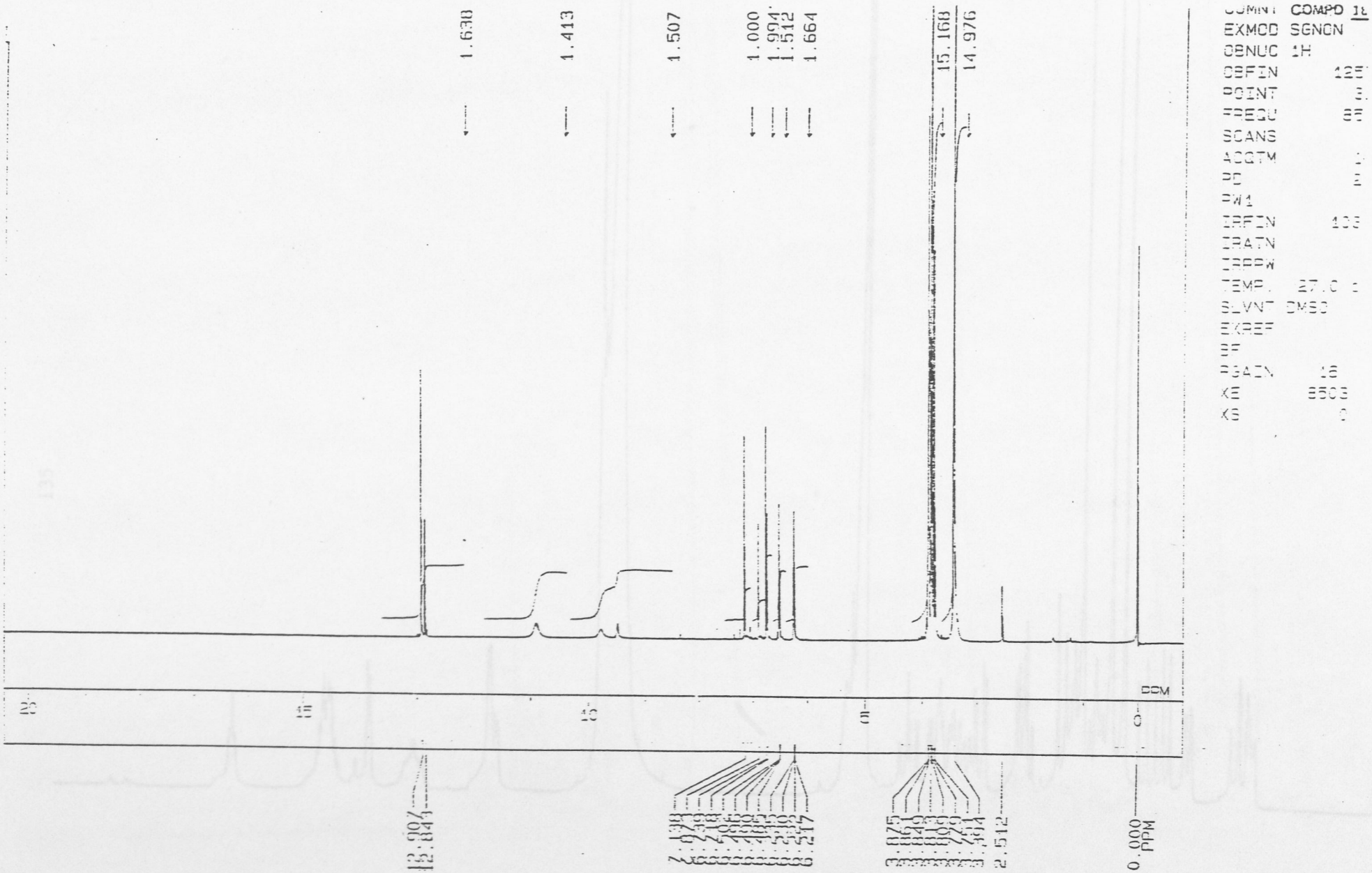


MASS SPECTRUM OF COMPOUND 22

^{1}H-NMR SPECTRUM OF COMPOUND 16

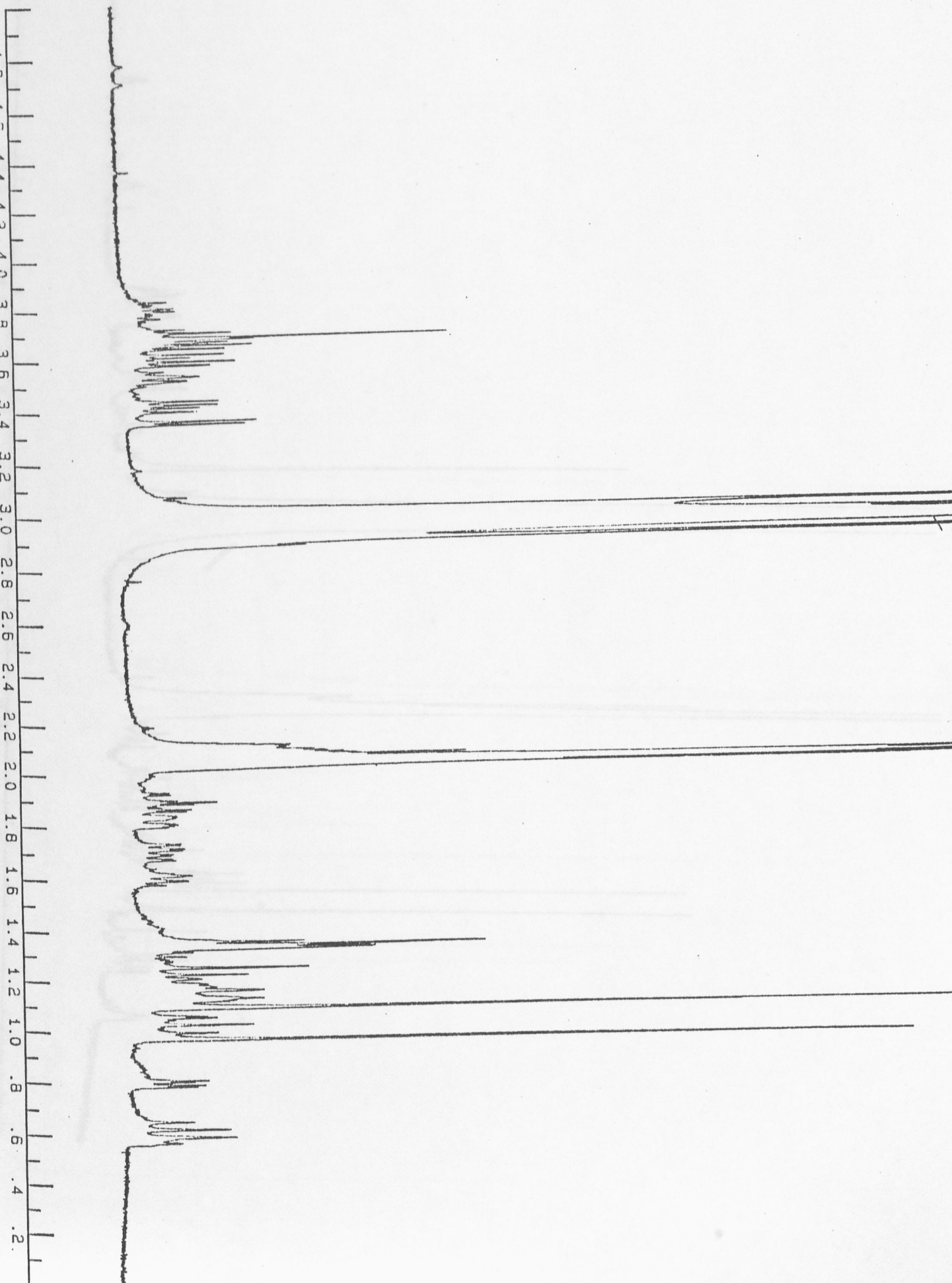


^{1}H-NMR SPECTRUM OF COMPOUND 17

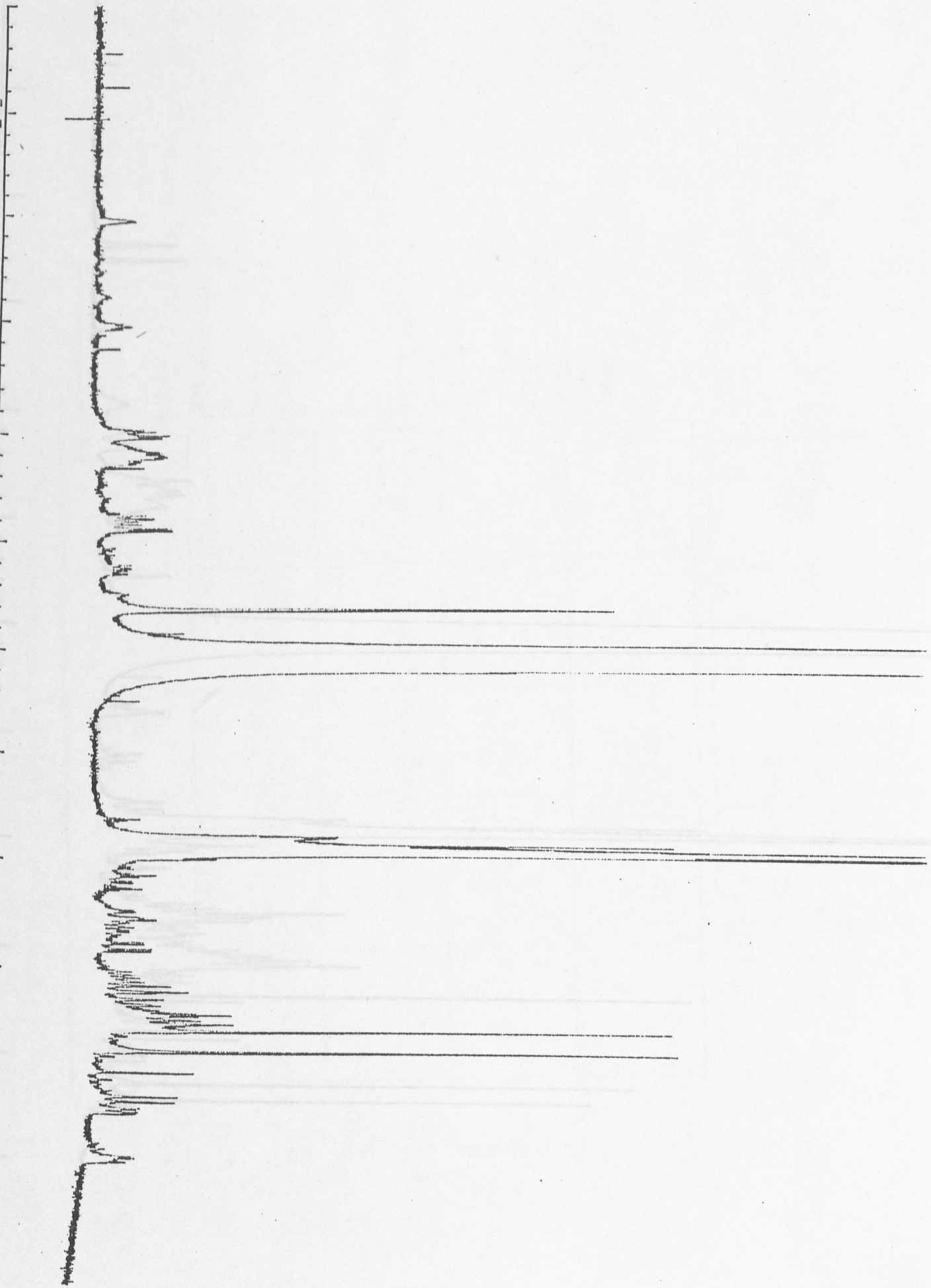


^{1}H-NMR SPECTRUM OF COMPOUND 18

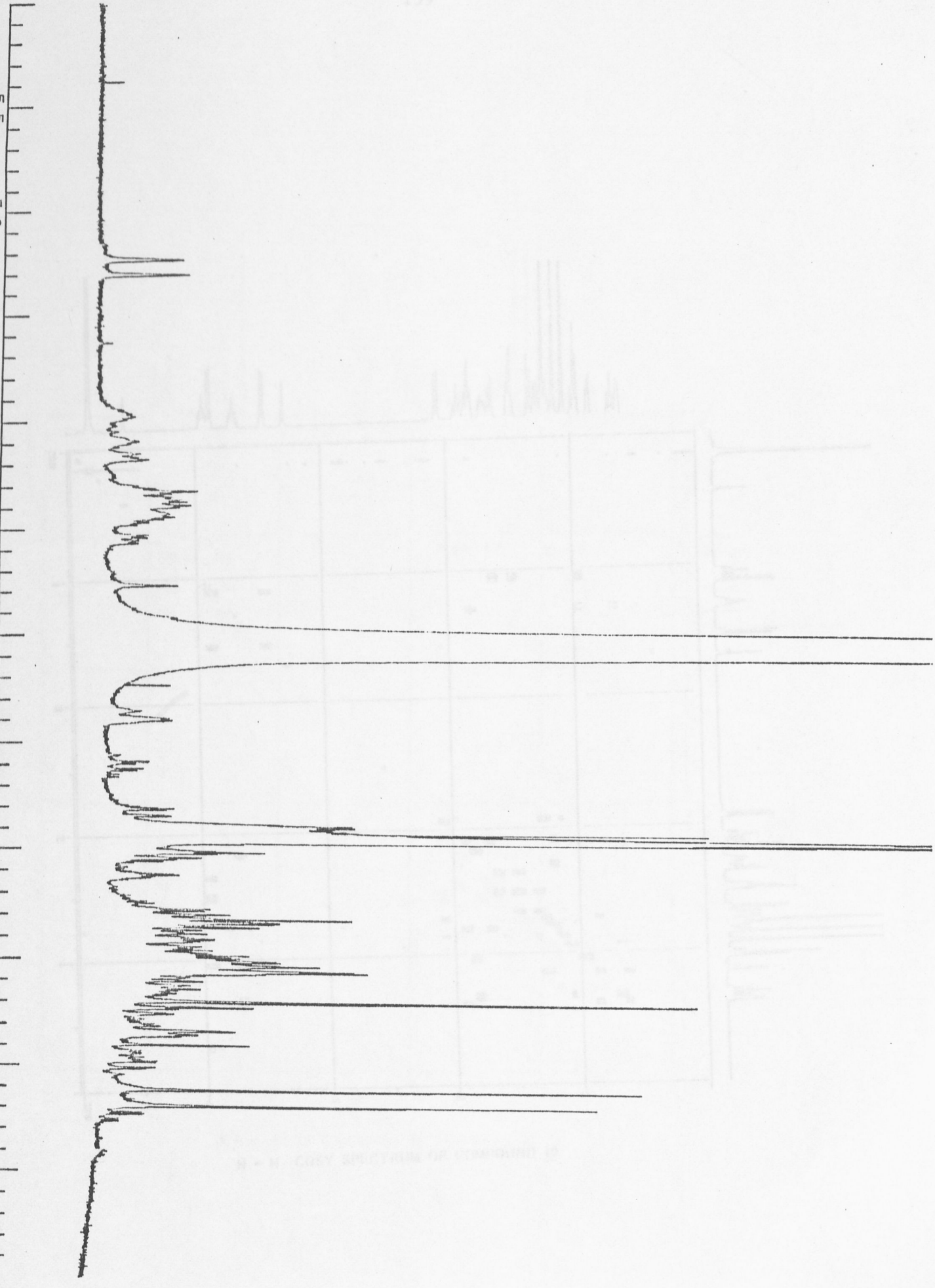
^{1}H-NMR SPECTRUM OF COMPOUND 19



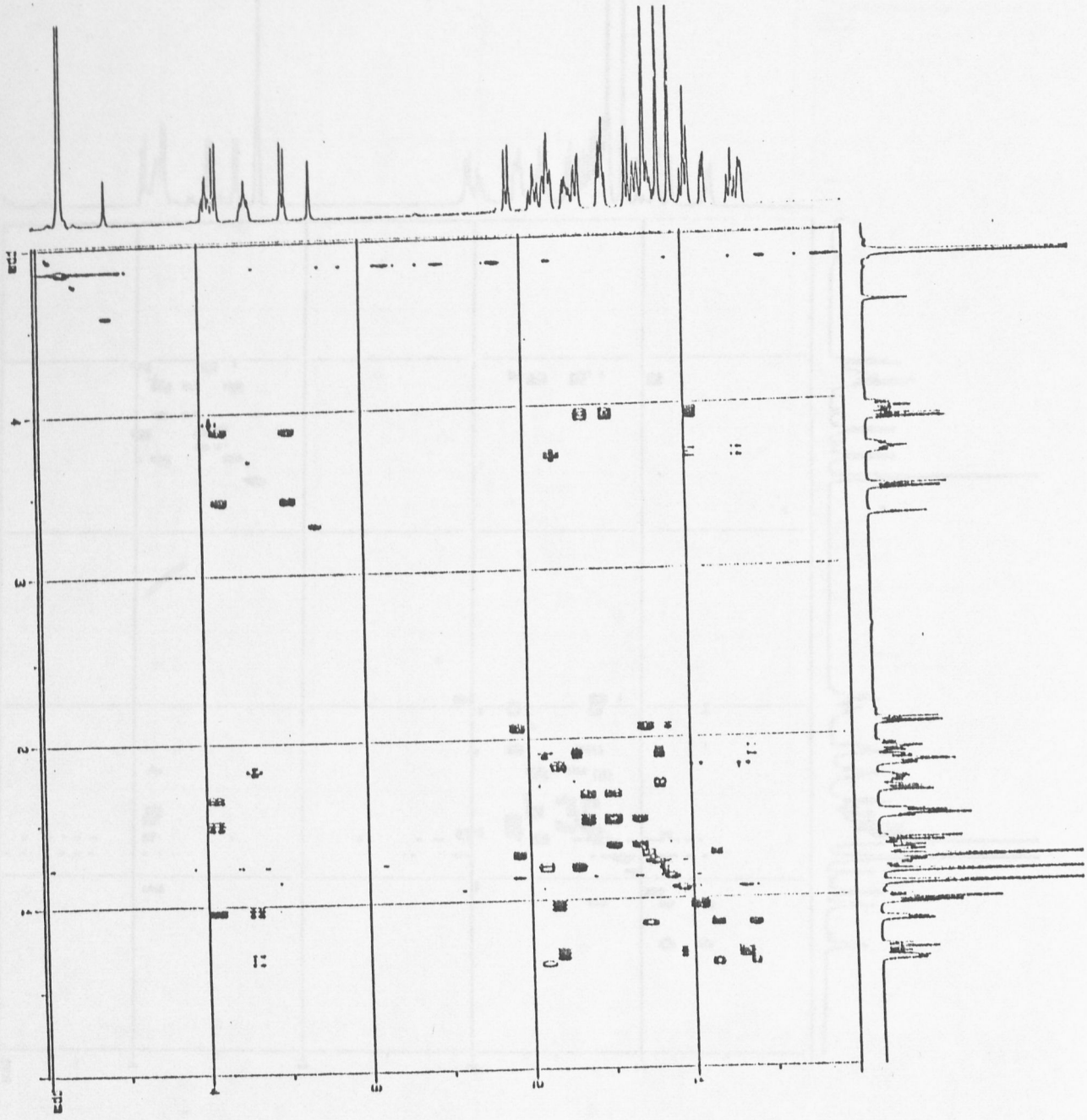
^1H - NMR SPECTRUM OF COMPOUND 20



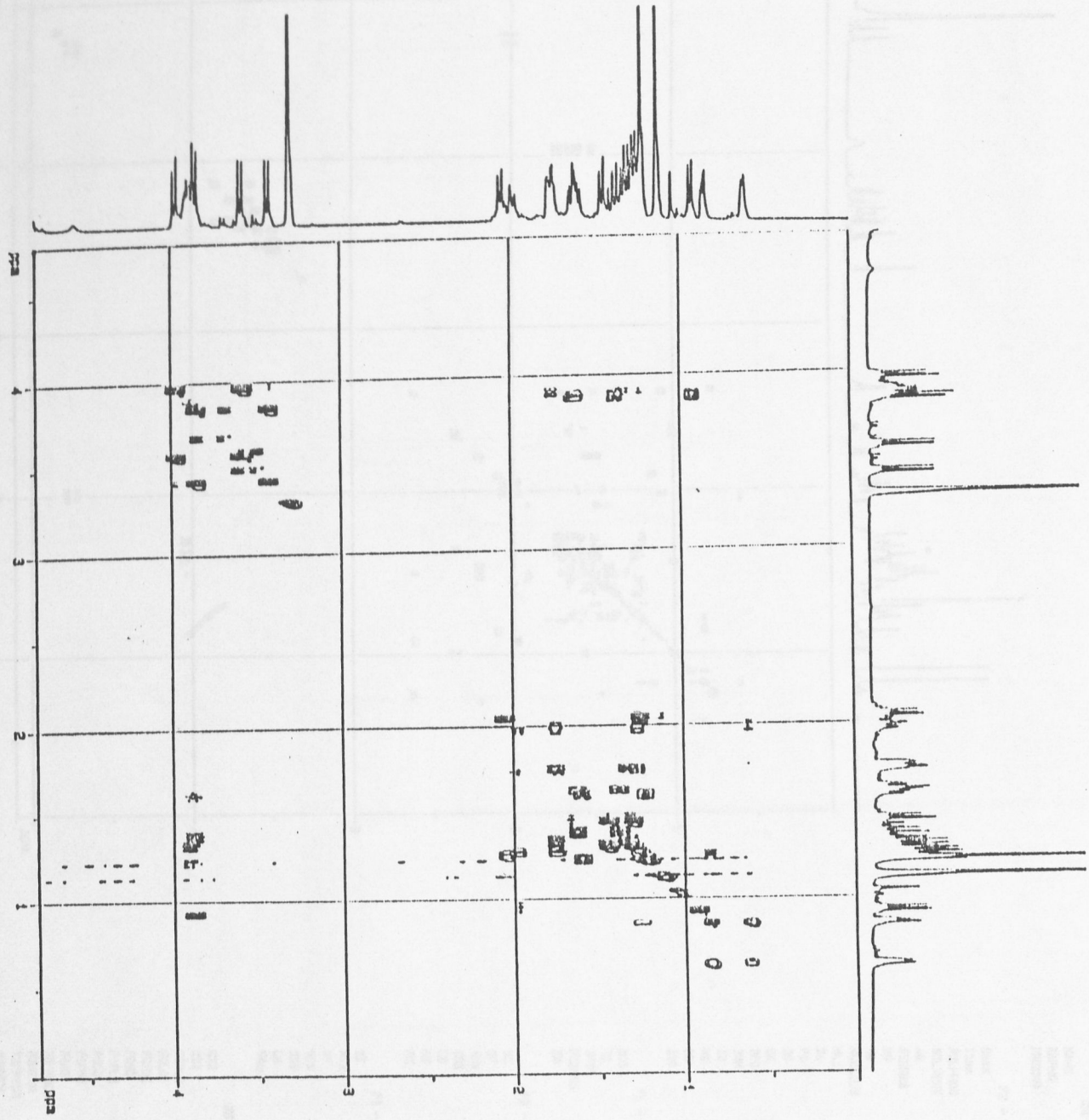
^{1}H-NMR SPECTRUM OF COMPOUND 21



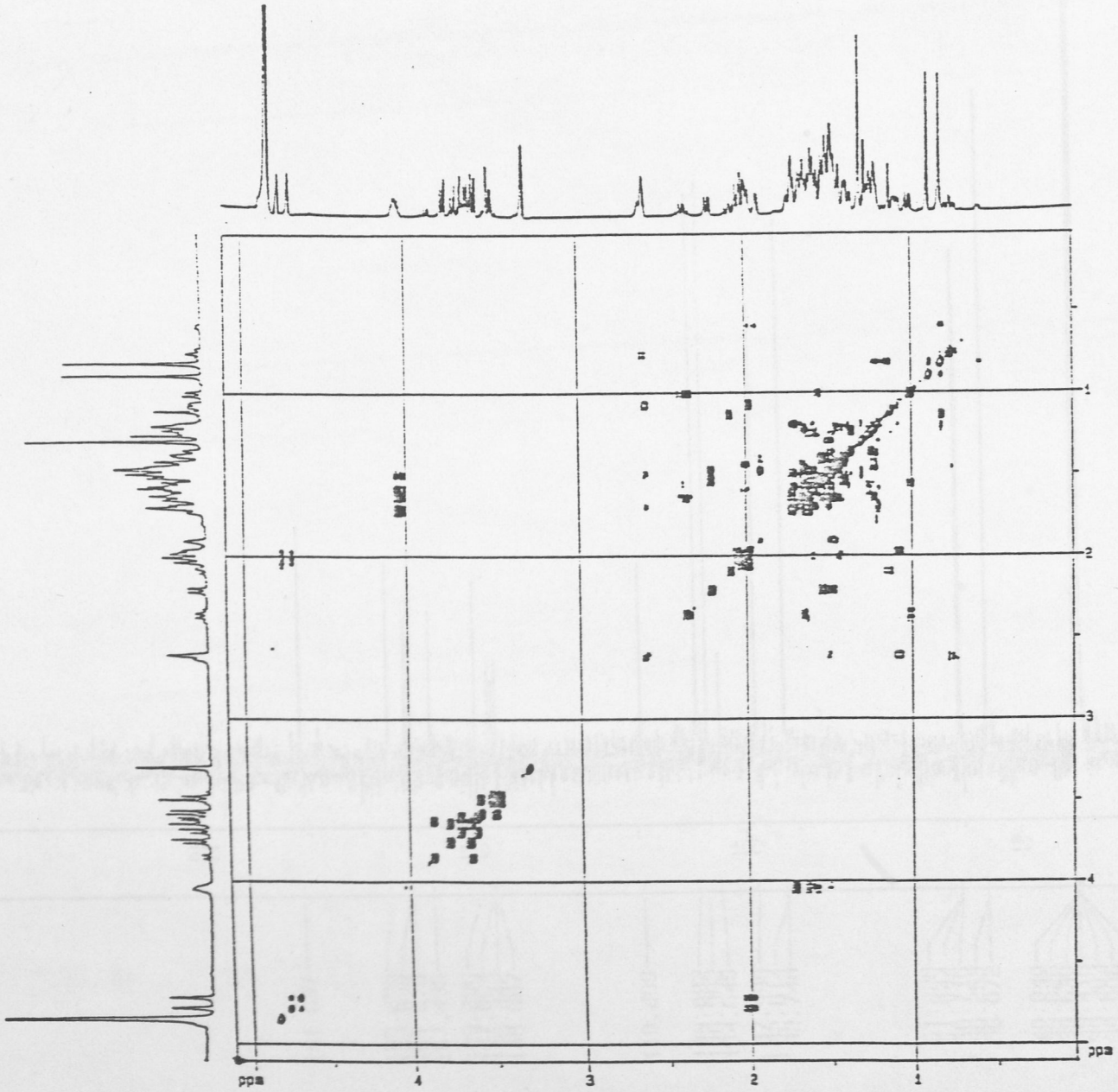
^1H - NMR SPECTRUM OF COMPOUND 22



H – H COSY SPECTRUM OF COMPOUND 19



H – H COSY SPECTRUM OF COMPOUND 21



H - H COSY SPECTRUM OF COMPOUND 22

Current Data Parameters

NAME	FA012-2d
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date	930629
Time	19.23
PULPROG	cosy90
SOLVENT	dmfno01
AQ	0.4034760 sec
FIDRES	1.239293 Hz
DW	157.1 usec
RG	128
NUCLEUS	1H
HL1	4 dB
D1	3.0000000 sec
P1	11.5 usec
D0	0.0000030 sec
DE	246.3 usec
SF01	500.1366784 MHz
SWH	2538.07 Hz
TD	2048
NS	24
DS	8
IN0	0.0003340 sec

F1 - Acquisition parameters

ND0	1
TD	512
SF01	500.1375 MHz
FIDRES	4.357125 Hz
SW	5.075 ppm

F2 - Processing parameters

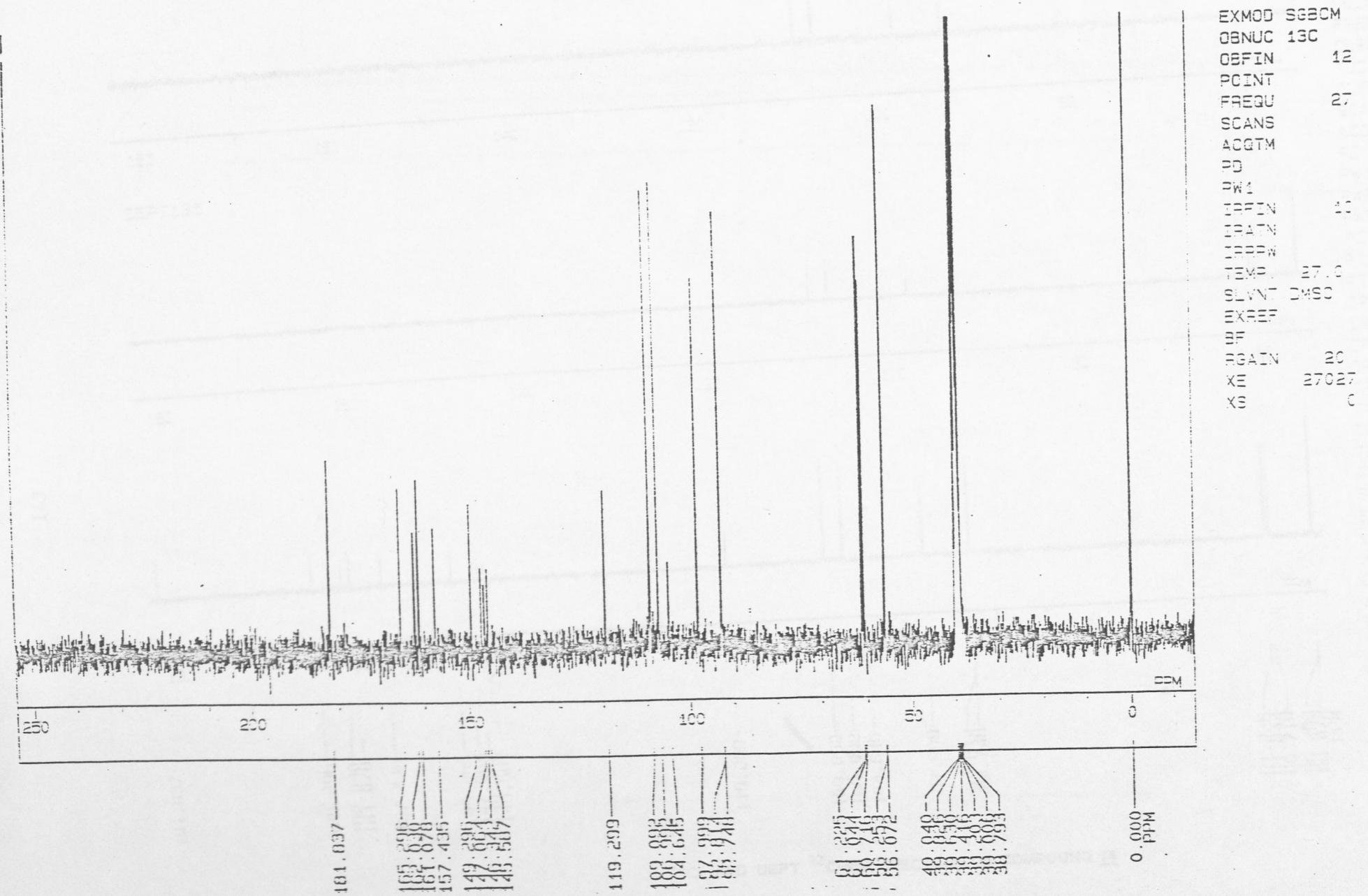
SI	2048
SF	500.1373941 MHz
WDW	QSINE
SSB	1.5
LB	0.00 Hz
GB	0
PC	4.00

F1 - Processing parameters

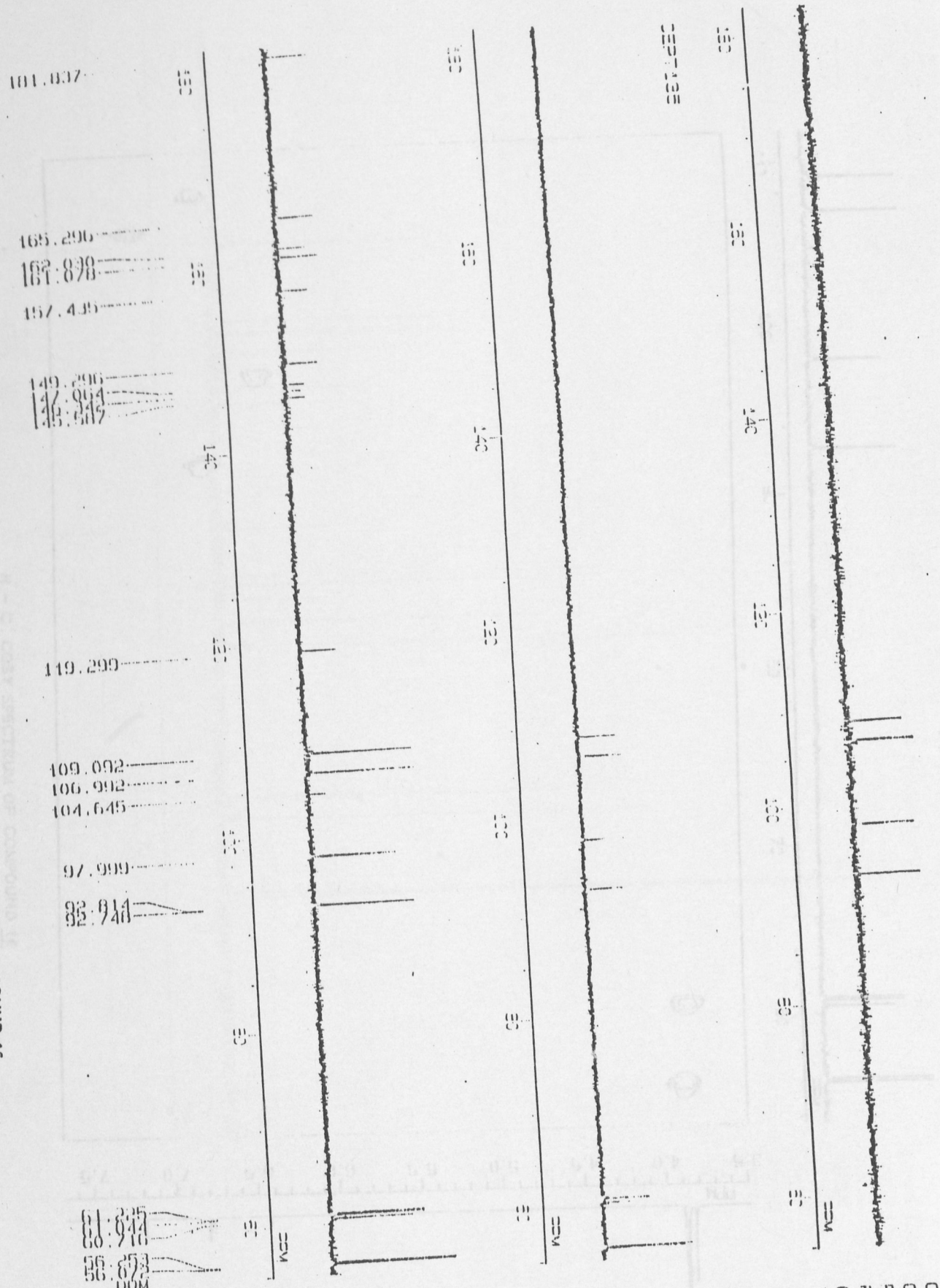
SI	512
MC2	QF
SF	500.1382388 MHz
WDW	QSINE
SSB	1.5
LB	0.00 Hz
GB	0

2D NMR plot parameters

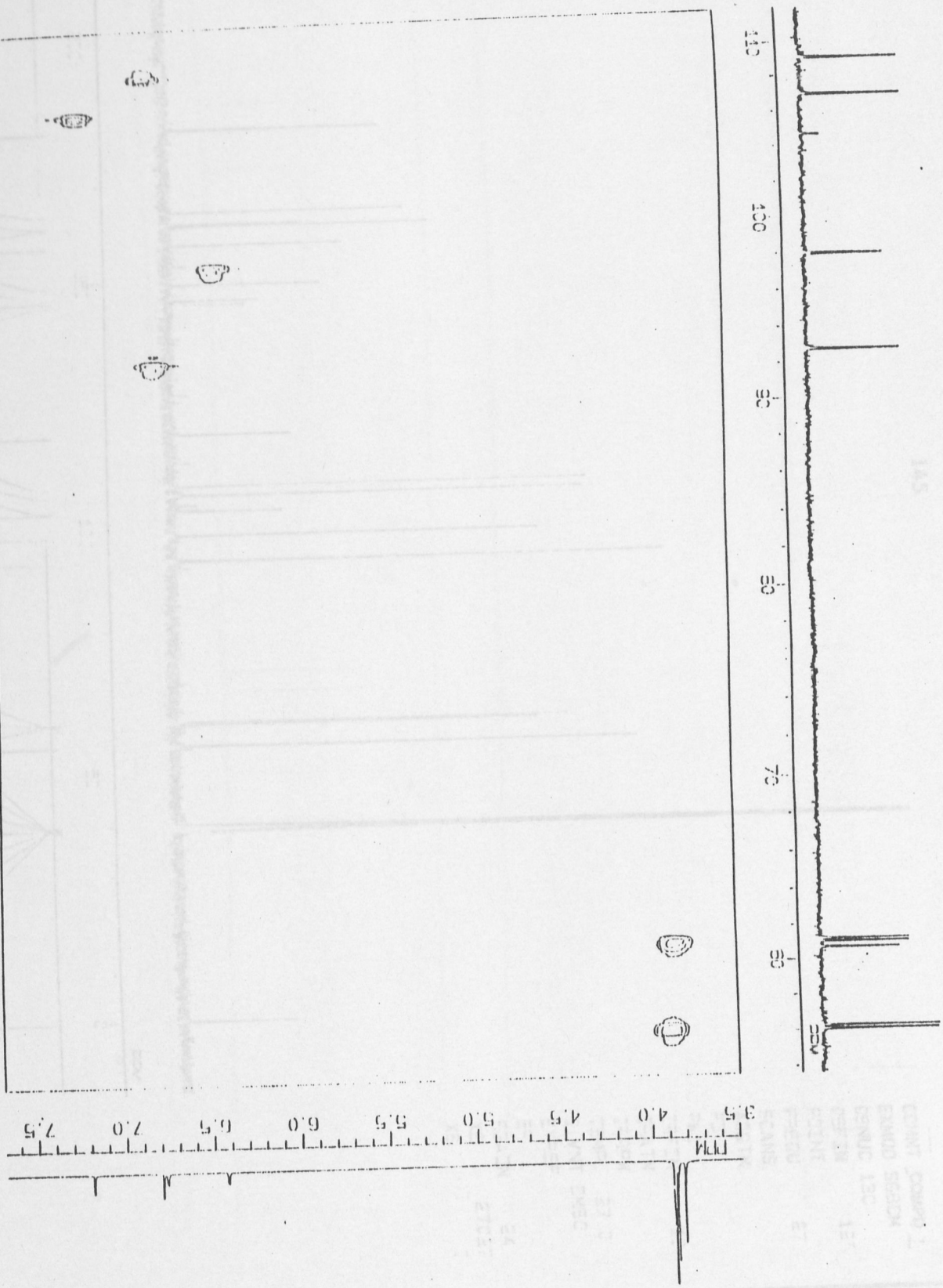
CX2	19.00 cm
CX1	19.00 cm
F2PLO	5.105 ppm
F2LO	2553.41 Hz
F2PHI	0.031 ppm
F2HI	15.23 Hz
F1PLO	5.105 ppm
F1LO	2553.20 Hz
F1PHI	0.030 ppm
F1HI	15.15 Hz
F2PPMCM	0.28193 ppm/cm
F2HZCM	141.00395 Hz/cm
F1PPMCM	0.28193 ppm/cm
F1HZCM	141.00267 Hz/cm



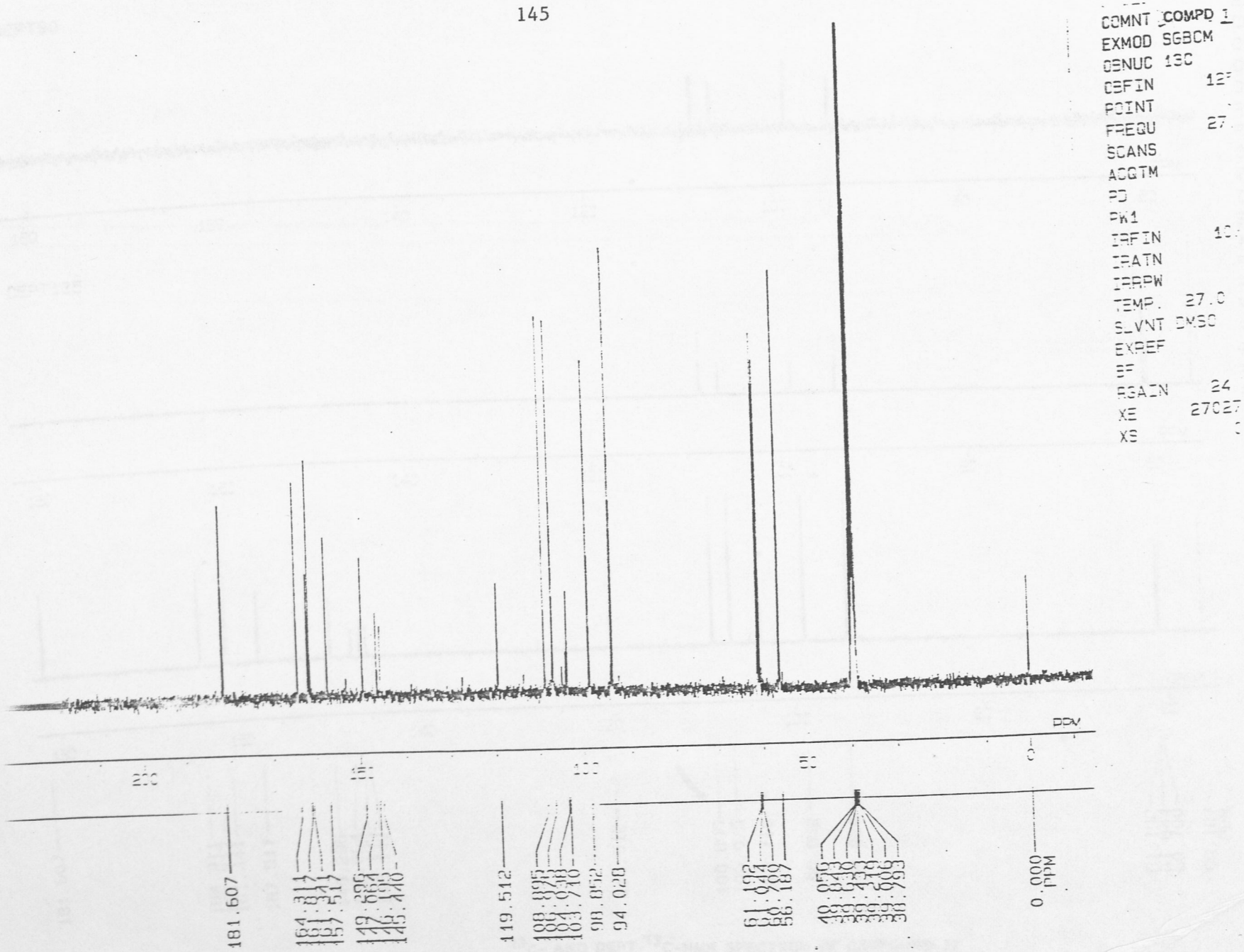
^{13}C-NMR SPECTRUM OF COMPOUND 16



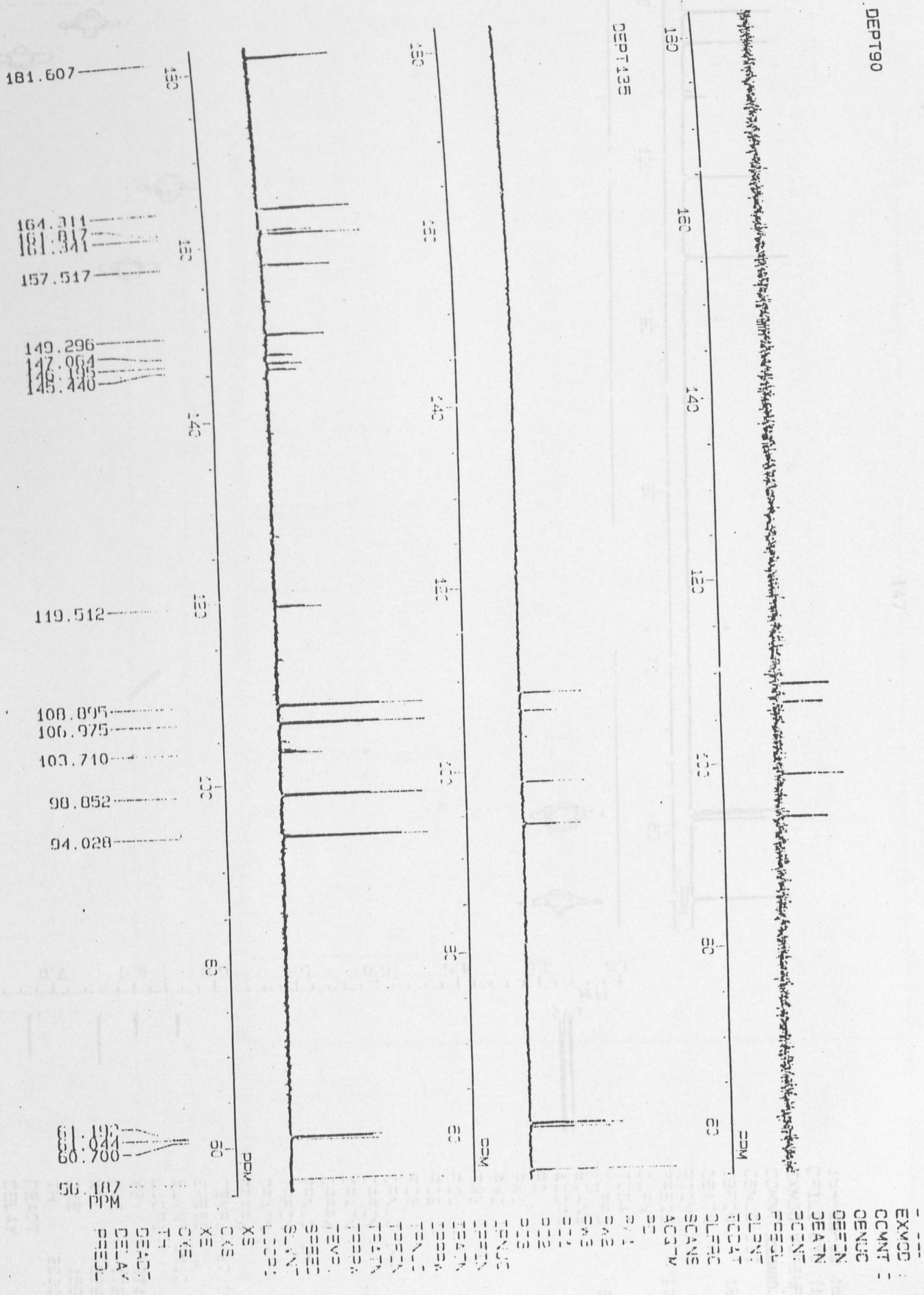
^{13}C- AND DEPT ^{13}C-NMR SPECTRUM OF COMPOUND 16



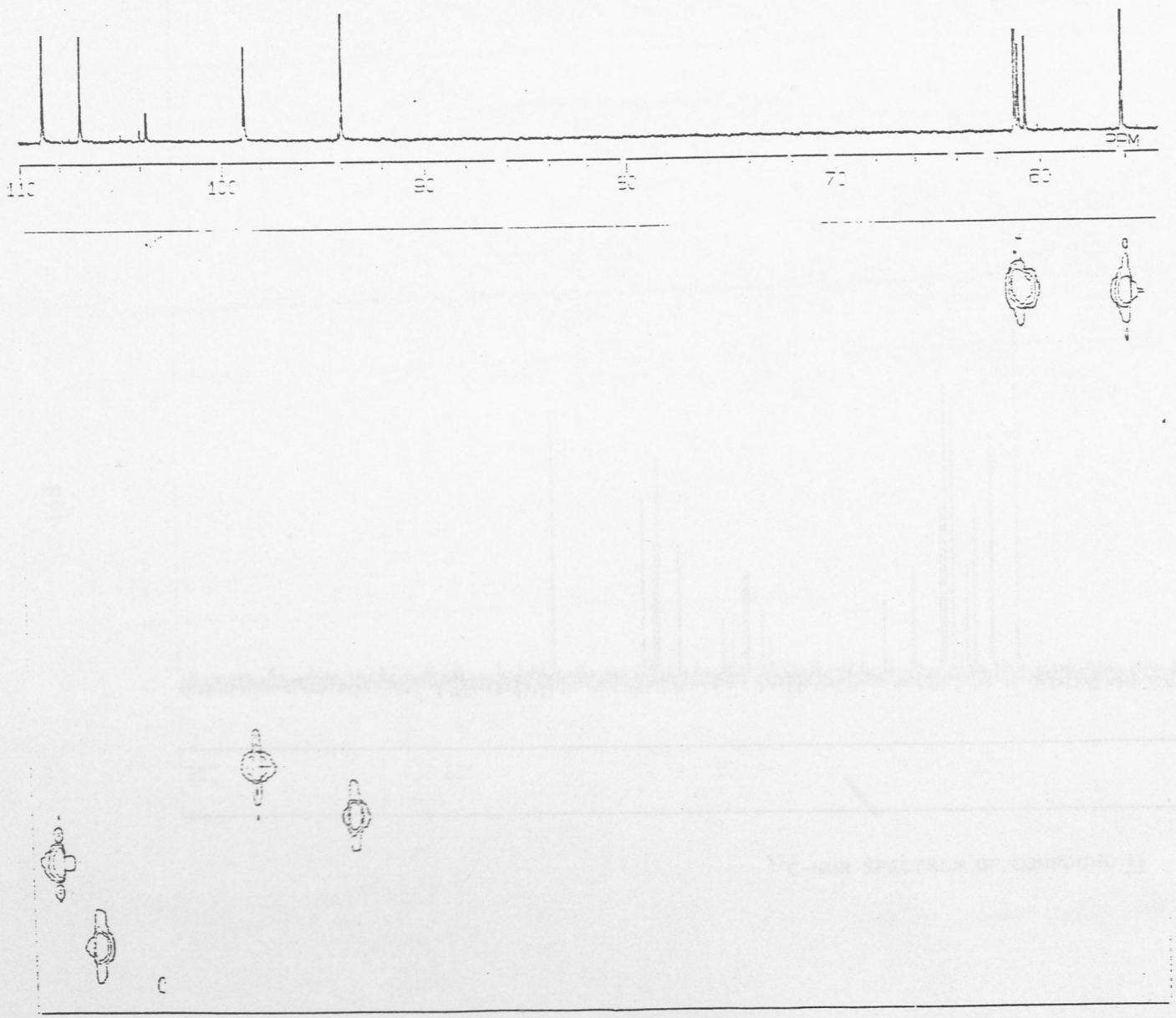
H - C. COSY SPECTRUM OF COMPOUND 16

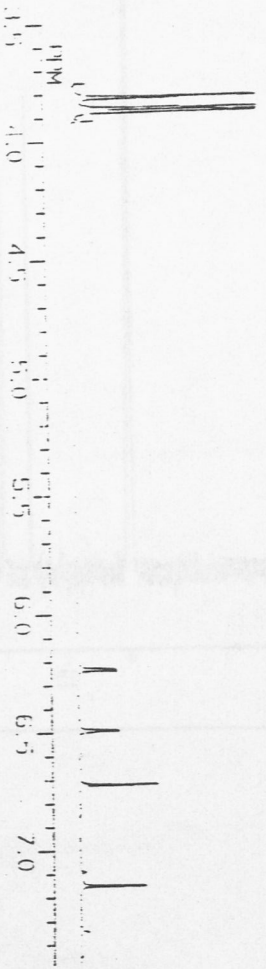


^{13}C-NMR SPECTRUM OF COMPOUND 17



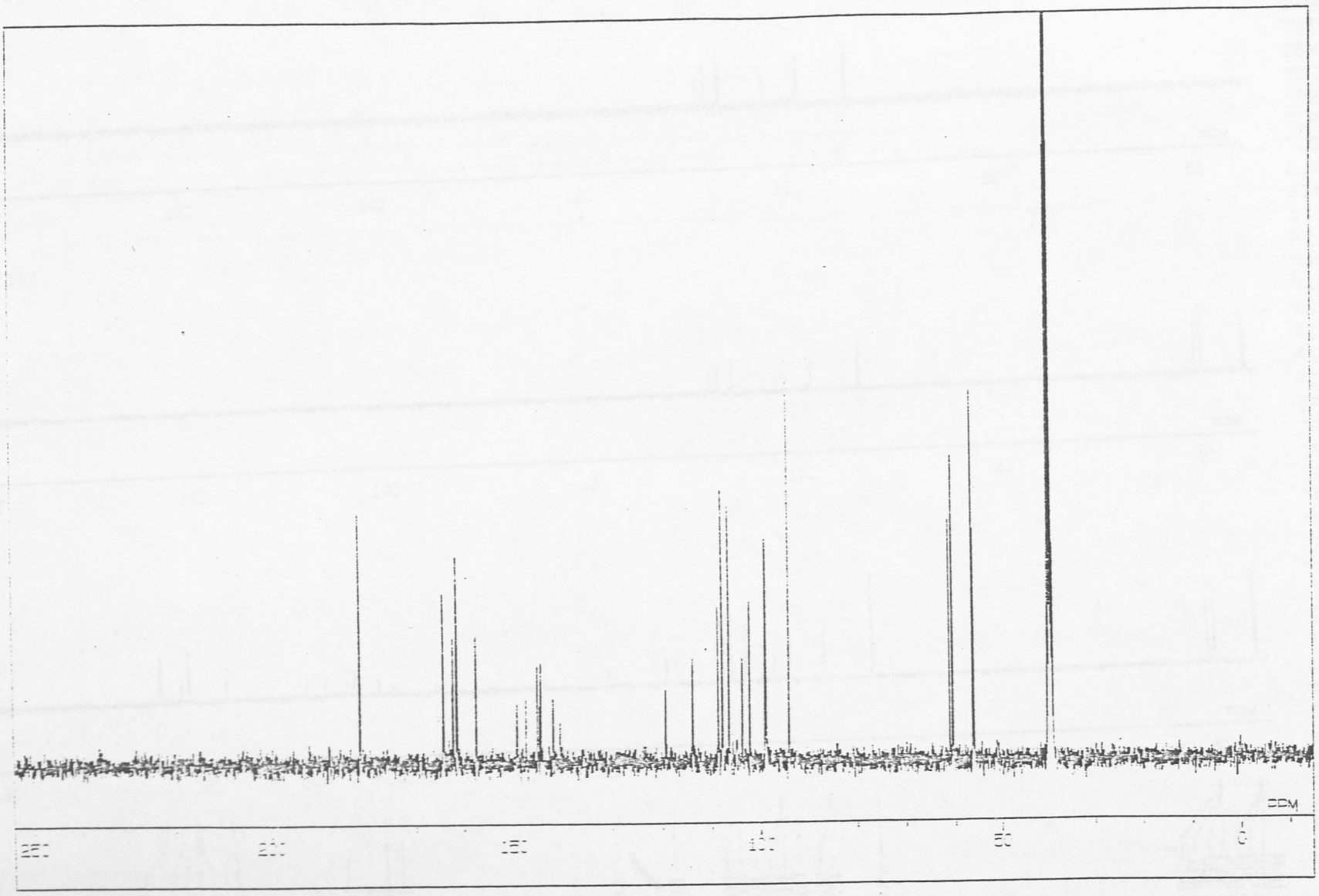
^{13}C- AND DEPT ^{13}C-NMR SPECTRUM OF COMPOUND 17



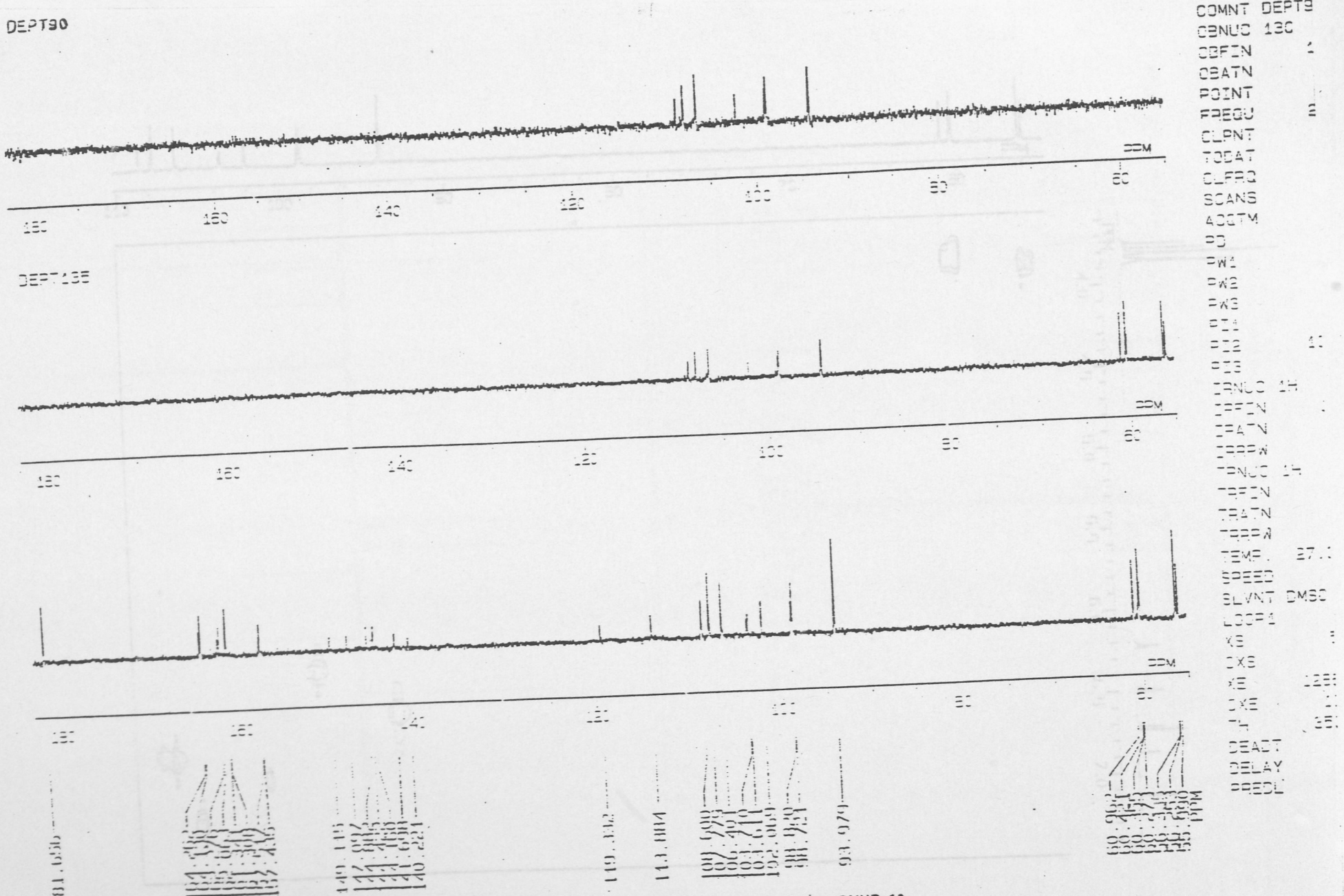


```
15-FEB-93 16.2
DFILE DYC. [100
EXMOD VC45HF
COMNT COMPD 17
OBNUC 13C
OBFIN      1250
OBATN
POINT         2
FREQU      3712
CLPNT
TODAT
SLPEW       85.
SCANS
ACQTM         0.
PD            1.
PW1
PW2           1
PW3           1
PS1           4.
PS2           .
PS3           2.
IRNUC 1H
IRFIN      1285
IRATN
IRPPM
TRNUC 1H
TRFIN
TRATN
TRPPM
TEMPL    27.0 C
SPEED
SLVNT CHCl3
LOCF1
XS         3747.9
CXS        1727.1
XE         3595.4
CXE        1594.8
TH         2000.0
PEADT         4
DELAY         4
PREDL       0.2
```

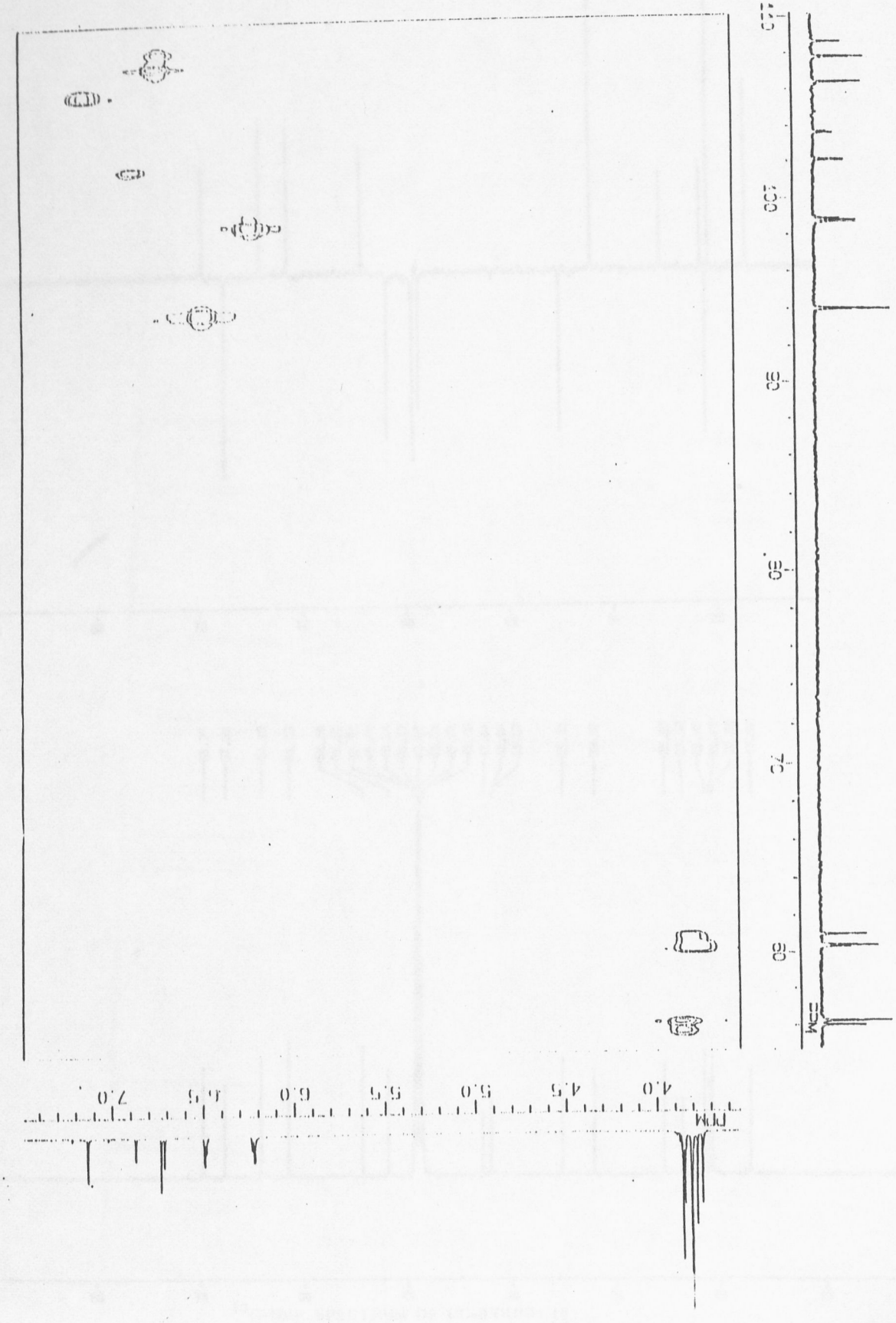
H-C COSY SPECTRUM OF COMPOUND 17



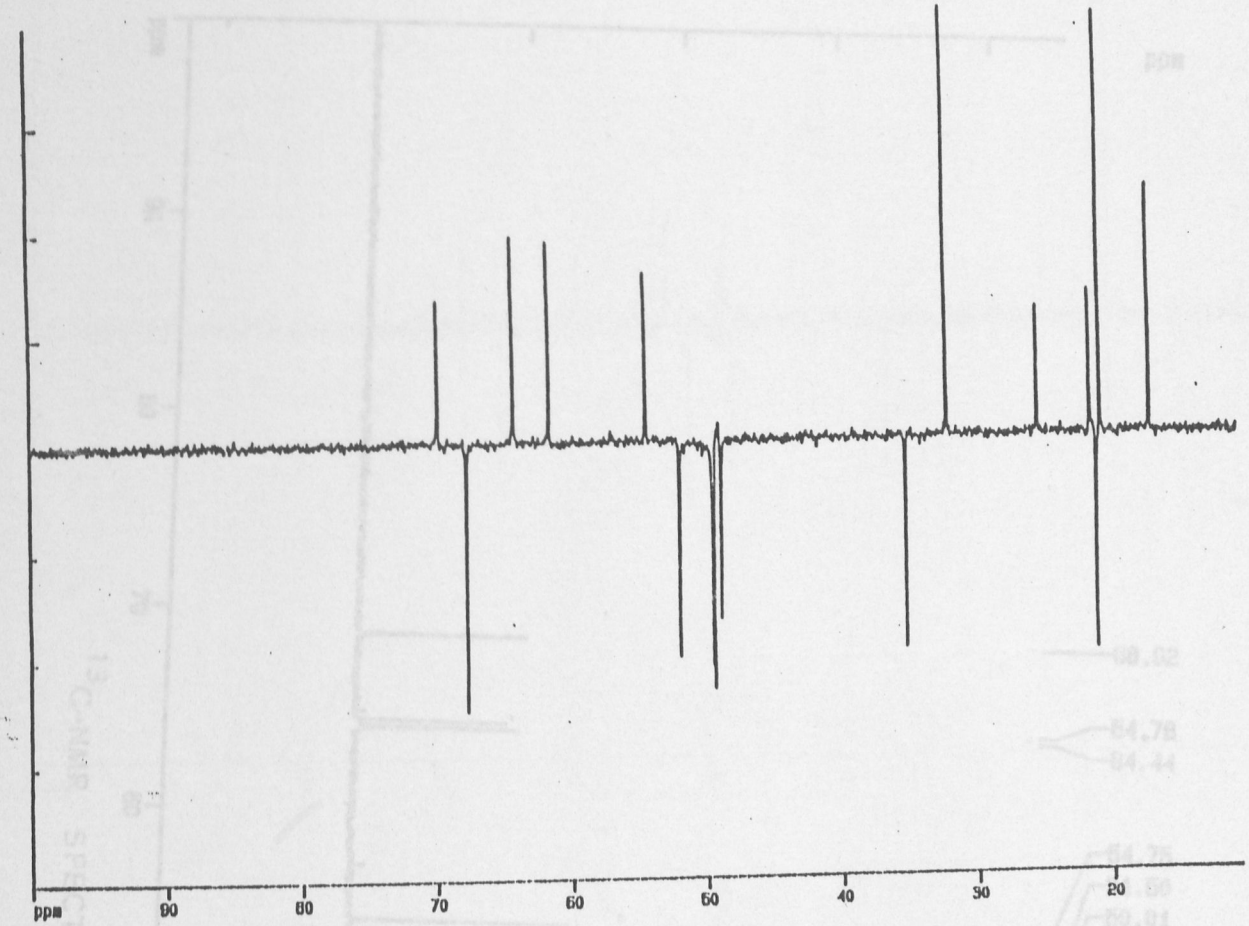
^{13}C-NMR SPECTRUM OF COMPOUND 18

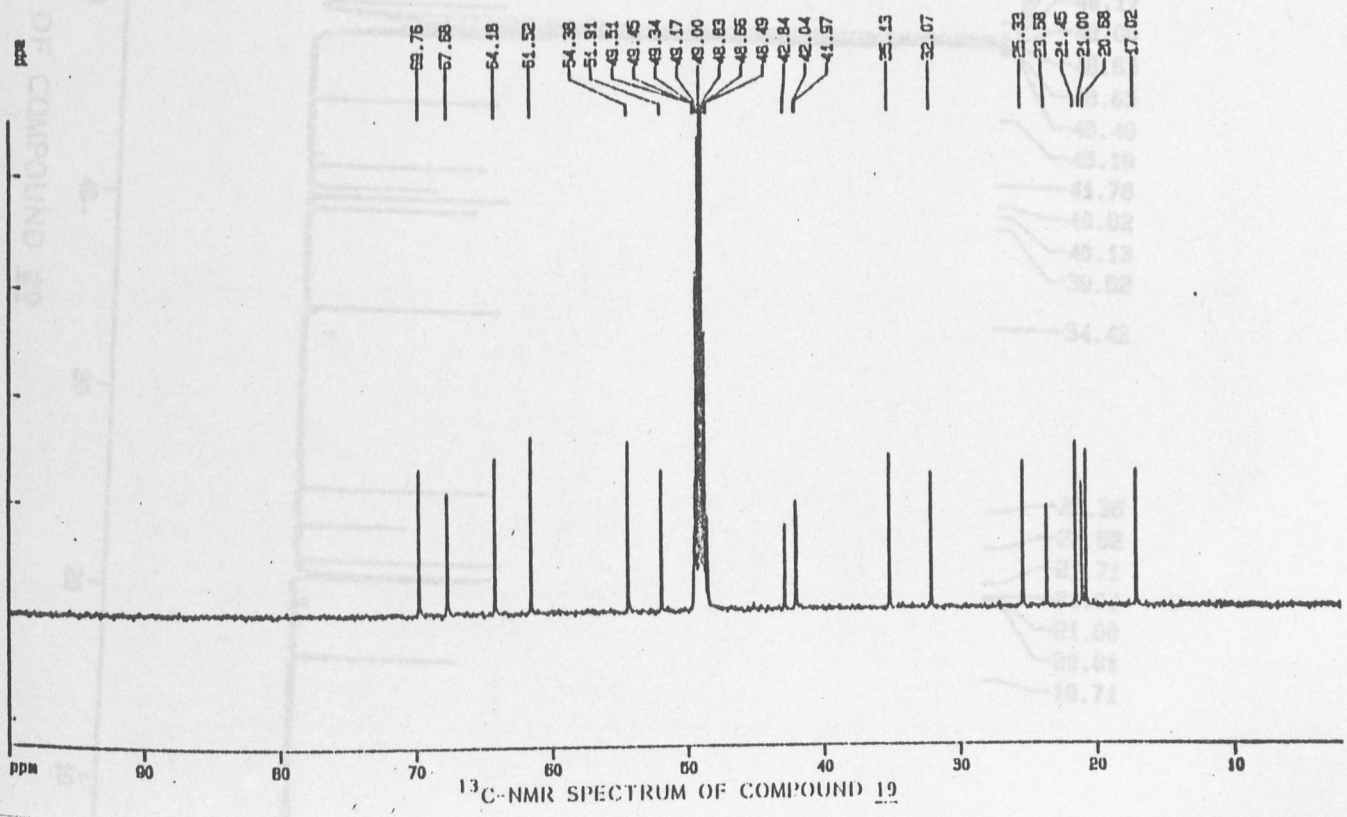


^{13}C- AND DEPT ^{13}C-NMR SPECTRUM OF COMPOUND 18

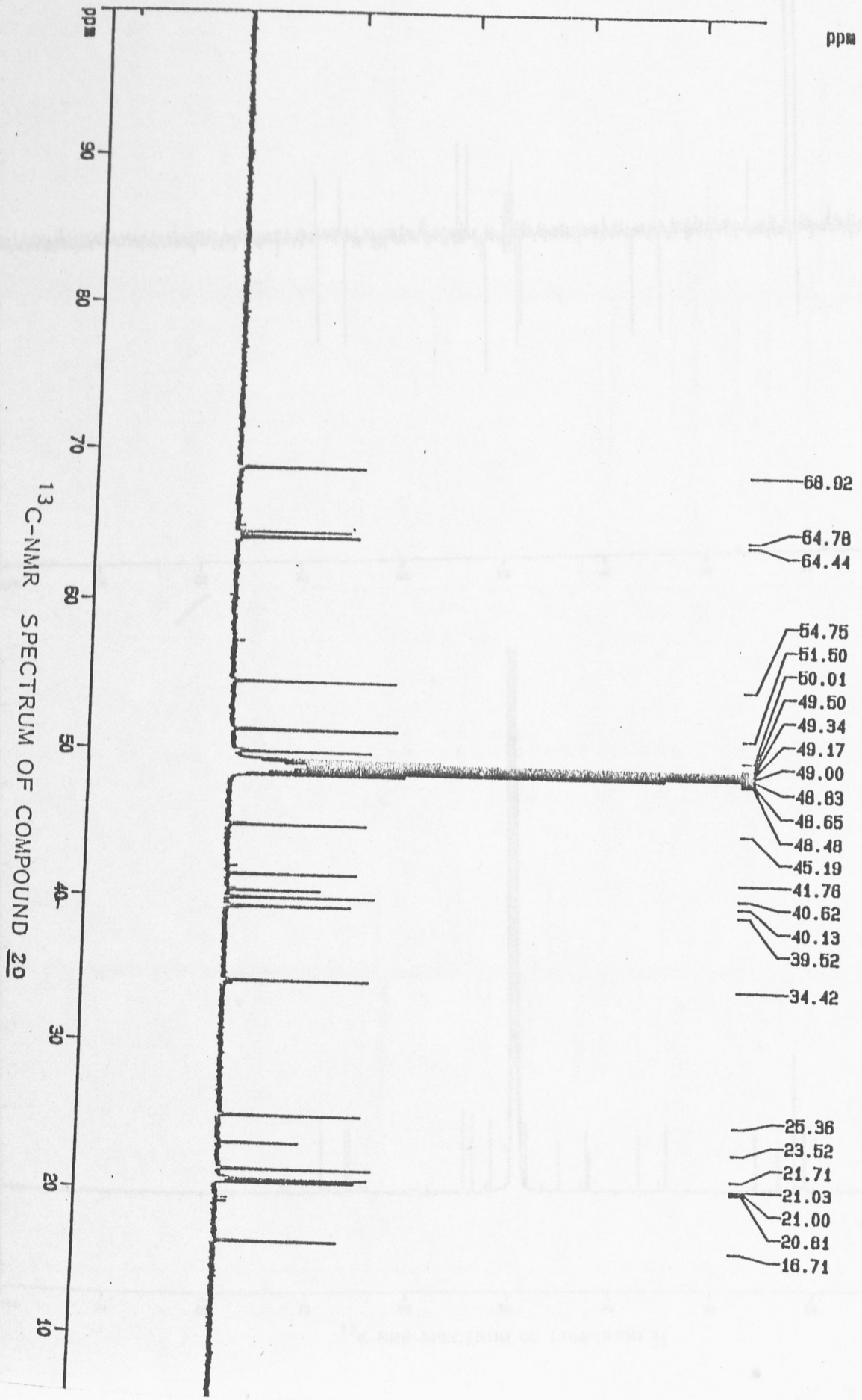


H - C COSY SPECTRUM OF COMPOUN 18

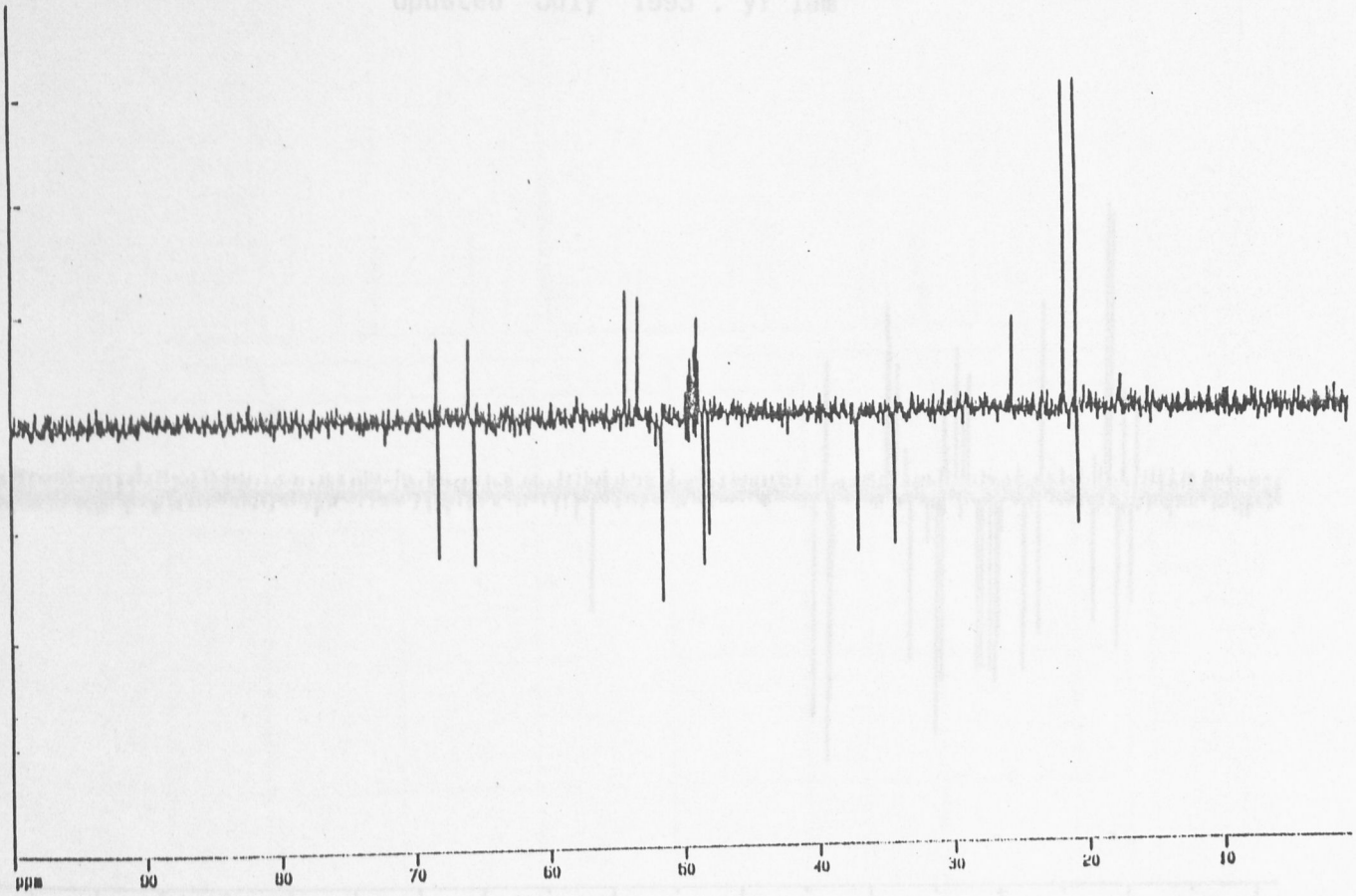


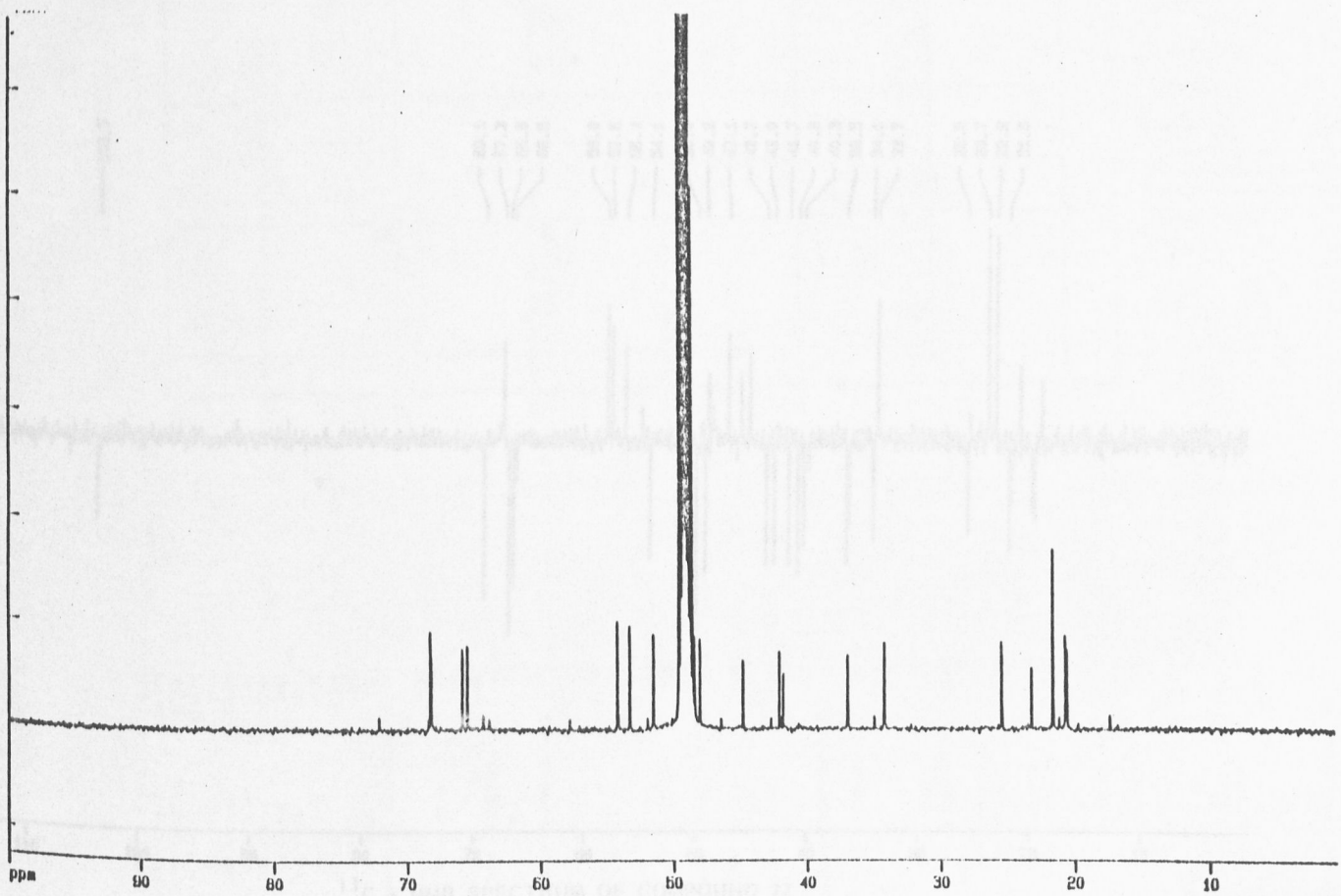


^{13}C-NMR SPECTRUM OF COMPOUND 19



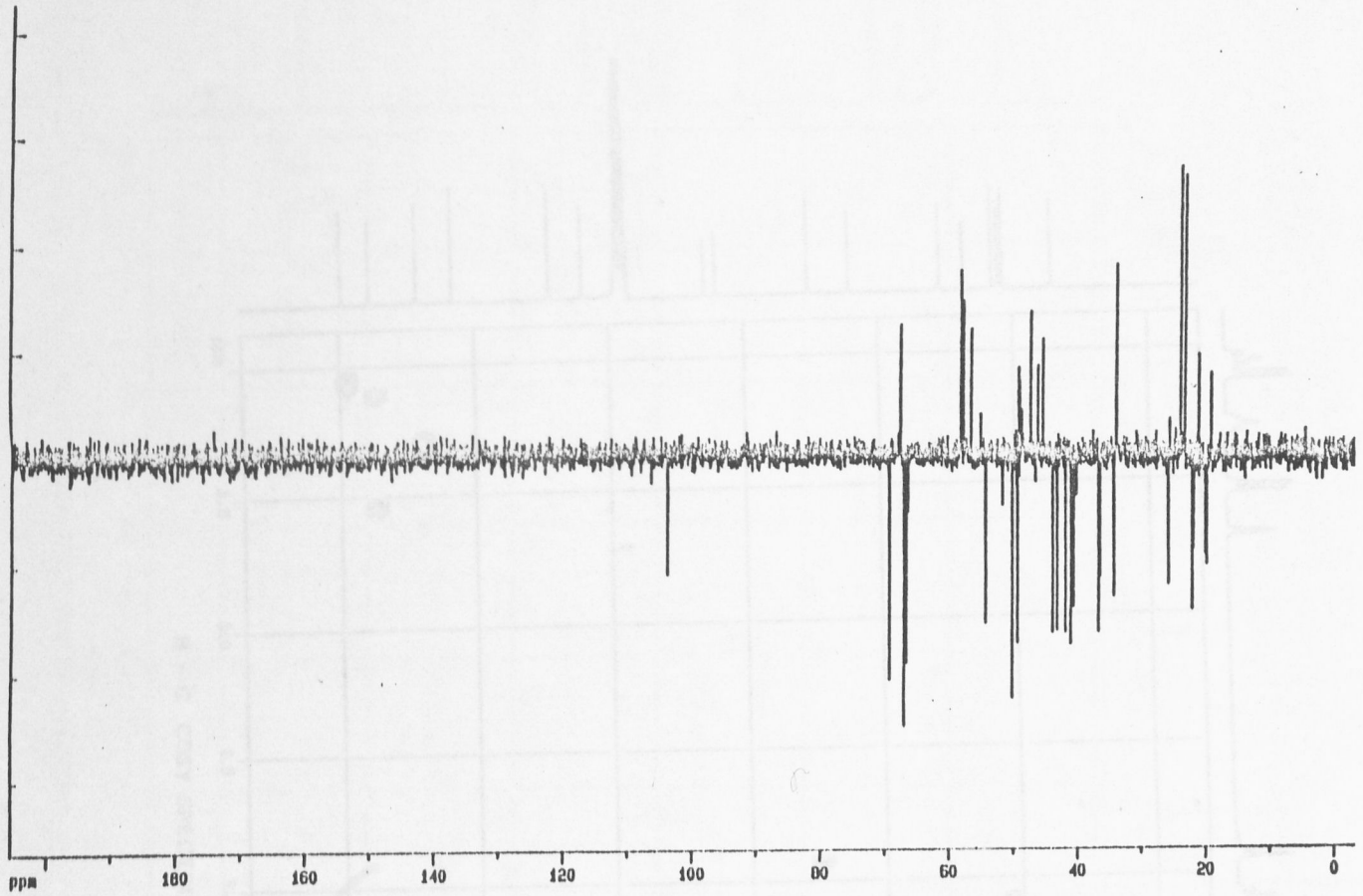
^{13}C-NMR SPECTRUM OF COMPOUND 20

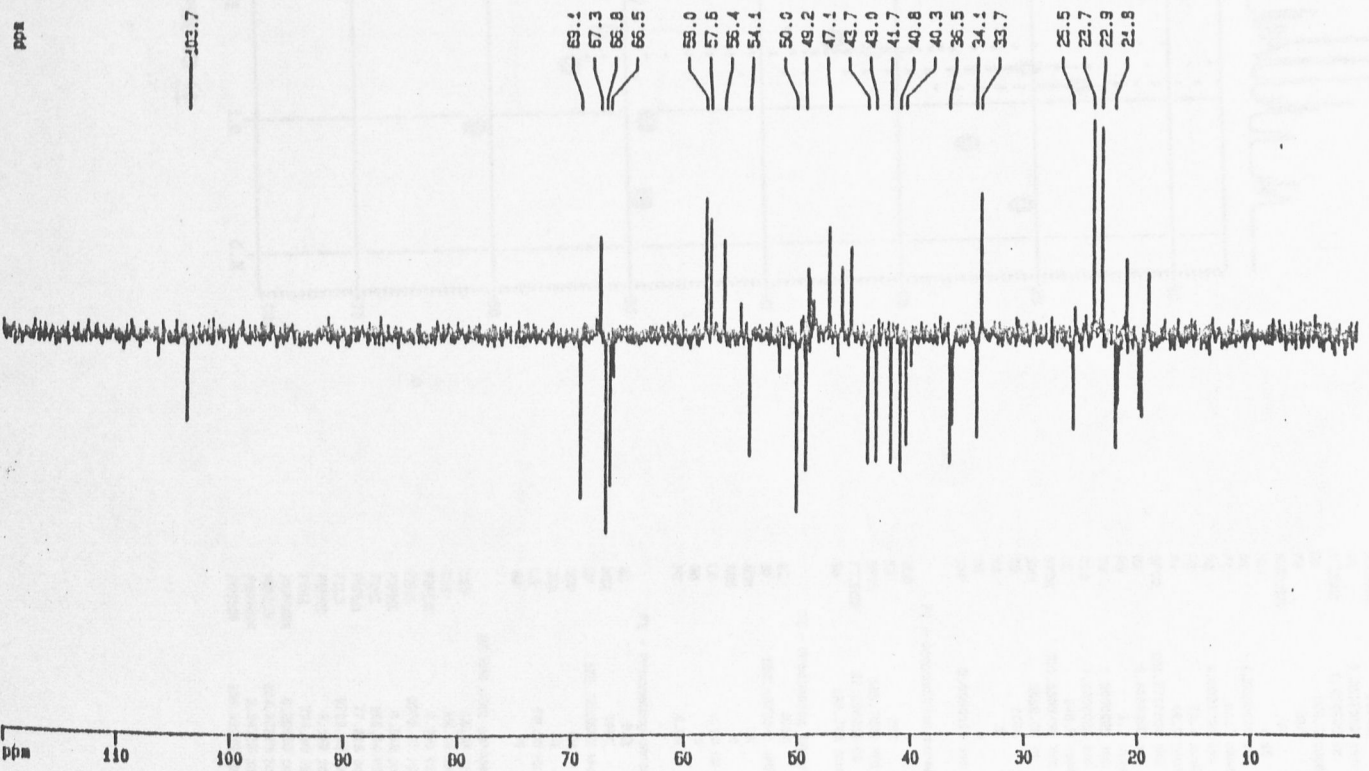




^{13}C-NMR SPECTRUM OF COMPOUND 21

deptme.500
updated July 1993 . yf lam





^{13}C - NMR SPECTRUM OF COMPOUND 22

H - C COSY SPECTRUM OF COMPOUND 19

EXPNO	2
PROCNO	1

F2 - Acquisition Parameters

Date	930712
Time	2.41
PULPROG	invtb
SOLVENT	cdcl3
AQ	0.2037960 sec
FIDRES	2.453675 Hz
DW	199.0 usec
RG	512
NUCLEUS	1H
HL1	4 dB
D1	1.5000000 sec
P1	11.7 usec
D2	0.0031000 sec
P2	23.4 usec
P4	18.0 usec
SFO2	125.7700000 MHz
D7	0.4000000 sec
P3	9.0 usec
D0	0.0000025 sec
D13	0.0000030 sec
DE	248.8 usec
SFO1	500.1386498 MHz
SW	2512.56 Hz
TD	1024
NS	32
DS	4
D40	0.0000100 sec

F1 - Acquisition parameters

NDO	4
TD	256
SFO1	125.7639 MHz
FIDRES	97.656250 Hz
SW	158.795 ppm

F2 - Processing parameters

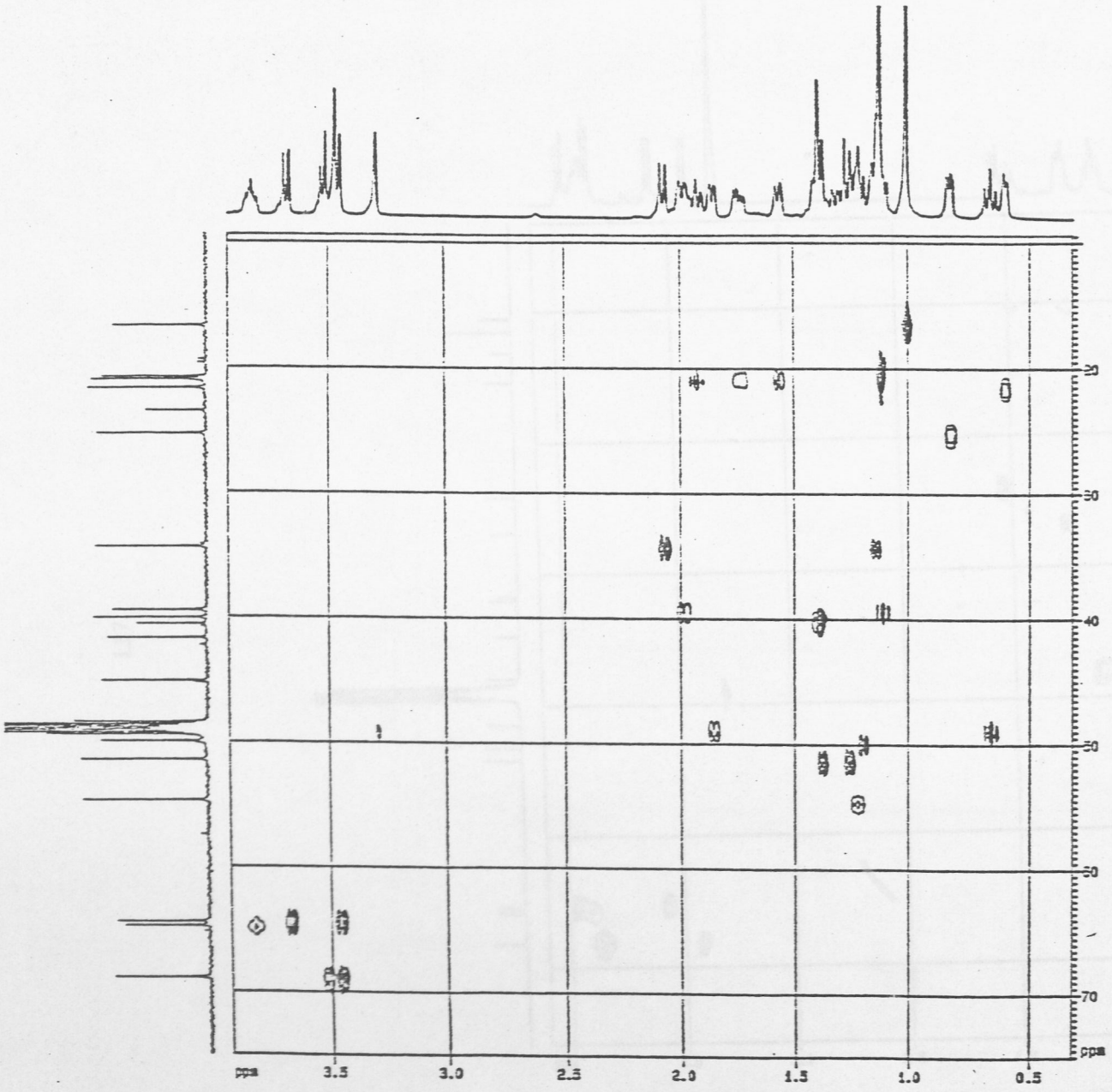
SI	1024
SF	500.1373941 MHz
WDW	EM
SSB	0
LB	3.00 Hz
GB	0
PC	4.00

F1 - Processing parameters

SI	512
MC2	TPPI
SF	125.7533852 MHz
WDW	EM
SSB	0
LB	50.00 Hz
GB	0

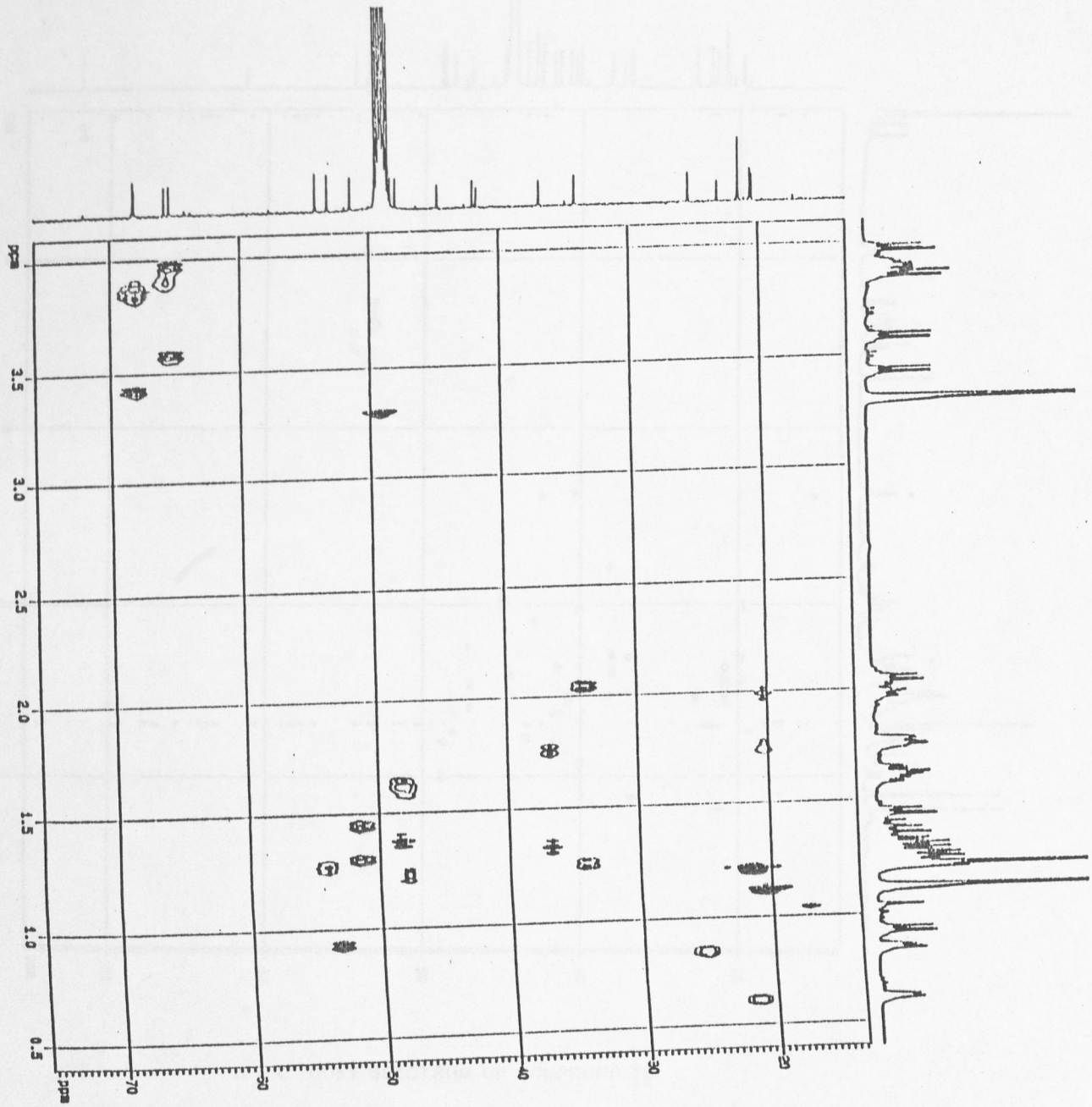
2D NMR plot parameters

CX2	18.00 cm
CY1	18.00 cm
F2PPLO	4.140 ppm
F2LO	2070.37 Hz
F2PPHI	0.362 ppm
F2HI	181.04 Hz
F1PPLO	77.218 ppm
F1LO	9710.41 Hz
F1PPHI	5.152 ppm
F1HI	774.86 Hz
F2PPMCM	0.20987 ppm/cm
F2HZCM	104.96275 Hz/cm
F1PPMCM	3.94756 ppm/cm
F1HZCM	496.41922 Hz/cm

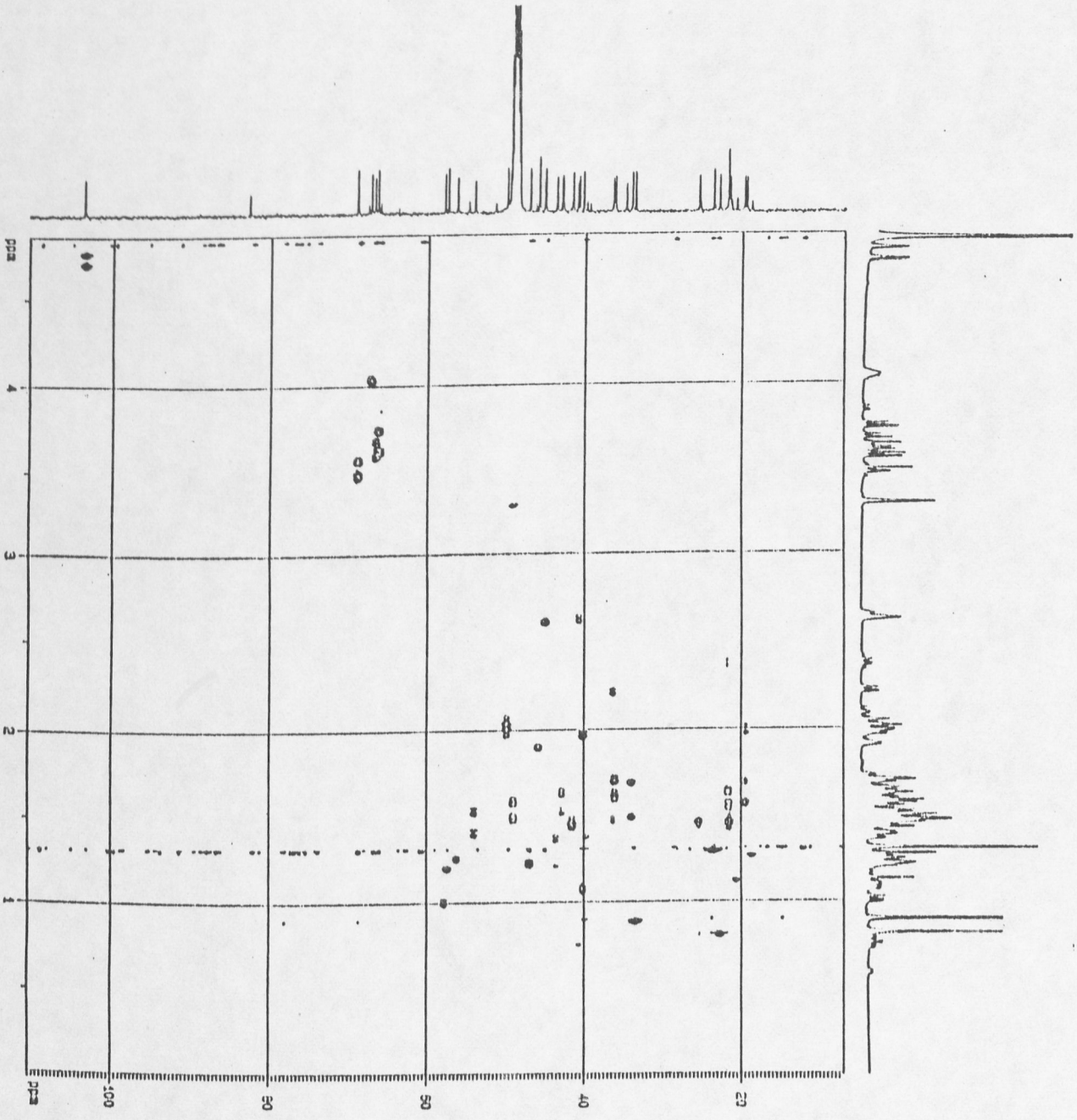


H - C COSY SPECTRUM OF COMPOUND 20

```
NAME            fac-19-24
EXPNO                   1
PROCNO                  1

F2 - Acquisition Parameters
Date_          20060[illegible]
Time           15.38
PULPROG        inv4gs
SOLVENT        cdcl3
NS             0.2289150 sec
FIDRES         2.[illegible] Hz
AQ             224.3 msec
RG             512
NUCLEUS        1H
P1             4 dB
D1             1.5000000 sec
D4             11.3 msec
D2             0.0031000 sec
P2             22.5 msec
P4             18.[illegible] msec
SFO2           125.7700000 MHz
D7             0.4000000 sec
D9             9.[illegible] msec
D0             0.0000025 sec
D13            0.0000020 sec
TE             225.[illegible] msec
SFO1           500.1385543 MHz
SWH            2450.38 Hz
TD             1024
NS             16
DS             4
D20            0.0000098 sec

F1 - Acquisition parameters
NO3            4
TD             255
SFO1           125.7742 MHz
FIDRES         38.281451 Hz
SW             200.000 ppm

F2 - Processing parameters
SI             1024
WF             500.1373841 MHz
WDW            EM
SSB            0
LB             3.00 Hz
GB             0
PC             4.00

F1 - Processing parameters
SI             256
MC2            TPPI
WF             125.7585803 MHz
WDW            SM
SSB            0
LB             100.00 Hz
GB             0

2D NMR plot parameters
CX2            18.00 cm
CY1            19.00 cm
F2PLO          3.336 ppm
F2LO           1669.57 Hz
F2PHI          0.322 ppm
F2HI           160.15 Hz
F1PLO          78.346 ppm
F1LO           9438.11 Hz
F1PHI          9.415 ppm
F1HI           1184.18 Hz
F2PPMCM        0.25100 ppm/cm
F2HZCM         [illegible].52548 Hz/cm
F1PPMCM        3.54814 ppm/cm
F1HZCM         [illegible].65175 Hz/cm
```



H - C COSY SPECTRUM OF COMPOUND 21



H - C COSY SPECTRUM OF COMPOUND 22