Extractives from Neobeguea mahafalensis and Cedrelopsis grevei

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Submitted in partial fulfilment of the requirements for the degree of Master of Science in the Department of Chemistry, University of Natal, Durban 1997

In Memory of my Aunt Sofia,

an angel on earth.

Εις Μνήμην της Θείας μου της Σοφίας,

ένας άγγελος στην γη.

Λόγια δεν μπορούν να περιγράψουν την αγάπη μου για σένα.
Θα ζης πάντα μες στης σκέψης μου και βαθειά μες στην καρδιά μου.
Αιωνία σου η μνήμη, γλυκιά μου Θεία.

Το Βιβάκη σου.

27. 10. 47 – 27. 11. 97

Preface

The experimental work described in this thesis was carried out in the Department of Chemistry, University of Natal, Durban, from February 1997 to February 1998 under the supervision of Professor D. A. Mulholland.

This study represents original work by the author and has not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

Signed: 

M. P. Kotsos B.Sc.Hons (Natal)

I hereby certify that the above statement is correct.

Signed: 

Professor D. A. Mulholland Ph.D. (Natal)

Acknowledgments

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List of Abbreviations

Acetyl-CoA - acetyl coenzyme A

ADP - adenosine diphosphate

ATP - adenosine triphosphate

DAHP - 3-deoxy-D-*arabino*-heptulosonic acid-7-phosphate

DEP - D-erythrose-4-phosphate

DHB - dihydrobiopterin

DHQ - 3-dehydroquinic acid

DMAPP - dimethylallyl pyrophosphate

DSA - 3-dehydroshikimic acid

EPSP - 5-enolpyruvylshikimic acid-3-phosphate

FAD - flavin adenine dinucleotide

FPP - farnesyl pyrophosphate

GGPP - geranylgeranyl pyrophosphate

GPP - geranyl pyrophosphate

HMG-CoA - 3-hydroxy-3-methylglutaryl-CoA

IPP - isopentenyl pyrophosphate

Malonyl-CoA - malonyl coenzyme A

MVA - mevalonic acid

NAD^+ / NADH - nicotinamide adenine dinucleotide

$NADP^+$ / NADPH - nicotinamide adenine dinucleotide phosphate

NIH - National Institute of Health

P^i - inorganic phosphate

PAL - L-phenylalanine ammonia-lyase

PEP - phosphoenol pyruvate

PSPP - presqualene pyrophosphate

SA - shikimic acid

THB - tetrahydrobiopterin

^{13}C NMR - carbon-13 nuclear magnetic resonance

COSY - correlation spectroscopy

c - concentration

d - doublet

DEPT - distortionless enhancement by polarisation transfer

^{1}H NMR - proton nuclear magnetic resonance

HETCOR - heteronuclear chemical shift correlation

HMBC - heteronuclear multiple bond coherence

HMQC - heteronuclear multiple quantum coherence

Hz - Hertz

IR - infra red

m - multiplet

NOE - nuclear Overhauser effect

NOESY - nuclear Overhauser effect spectroscopy

q - quartet

s - singlet

br s - broad singlet

t- triplet

UV - ultra-violet

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Abstract

Neobeguea mahafalensis Leroy. and *Cedrelopsis grevei* Baill. are the two Madagascan species which were investigated in this work.

Neobeguea mahafalensis Leroy. belongs to the *Neobeguea* genus of the Meliaceae family and is a plant unique to Madagascar. It is commonly referred to as "Handy" by the native people of Madagascar, who use it as a medicinal plant. The stem-bark of *N. mahafalensis* which has been analysed in this work, was collected from the dry, thorny forests of the deep south of Madagascar. Most species found in this region are unique in the world and are highly specialised in adapting to the very dry climate. The hexane extract was found to yield a limonoid (compound I) which has not been previously reported as a natural product. The known pentacyclic triterpenoid, β-amyrin (compound II) and stigmasterol (compound III), were also isolated.

Cedrelopsis grevei Baill. is one of seven species of the genus *Cedrelopsis* which are confined to Madagascar. This species, commonly referred to as "Katrafay" by the Madagascan people, has undergone intensive chemical investigation as prior inclusion of this species in the Meliaceae family has always been questionable. The South African species *Ptaeroxylon obliquum* (Thung.) Radlk., is a member of the Ptaeroxylaceae family which is found to be so chemically similar to *Cedrelopsis* that the latter has been placed in the Ptaeroxylaceae family.

The stem bark of *Cedrelopsis grevei* was obtained from the dry southern part of Madagascar and yielded a variety of chromones and coumarins, as well as stigmasterol (compound III) and the pentacyclic triterpenoid, β-amyrin (compound II). Two chromones were isolated from *Cedrelopsis grevei*, namely ptaeroxylinol (compound IV) and ptaeroglycol (compound V). Ptaeroglycol has been previously isolated from this species as well as from *Ptaeroxylon obliquum* whereas ptaeroxylinol has only been isolated from *Ptaeroxylon obliquum*. Six coumarins were isolated from *Cedrelopsis grevei* in this study, all of which were 6,7-dioxygenated coumarins. These included the known compounds, scoparone (compound VI), O-methylcedrelopsin (compound VIII), norbraylin (compound X) and cedrelopsin (compound IX), as well as compound VII and compound XI which have not been reported previously. No limonoids were isolated from this species in this investigation.

Contents

Chapter 1

An Overview of Metabolic Pathways

1.1 INTRODUCTION

Primary metabolites are fundamental building blocks of living matter and are essential for life. They include polysaccharides, proteins, fats and nucleic acids. Such metabolites, as well as their metabolic pathways, are very similar in all organisms and are thus referred to as universal. These pathways proceed via various complex catalytic reactions using carbon dioxide and photosynthesis as their primary source. Many primary metabolites act as precursors in secondary metabolism[1, 2].

Secondary metabolites form an infinitely greater body of natural products, they have a restricted taxonomic distribution and are not formed under all circumstances. They include alkaloids, terpenes, polyenes, polyacetylenes, pigments, phenols and mycotoxins to name but a few, and are found mostly in plants and microorganisms[3]. Primary metabolite precursors facilitate their production via very specific and specialised enzyme mediated reactions. The type of secondary metabolites formed depends on the species, location and environment, age and even seasonal changes. In microorganisms they are associated with the life-cycle stages[4]. Such metabolites are clearly very specific and have found great importance in species characterisation in such cases where morphological classifications are not enough. For example, natural products isolated from *Cedrelopsis grevei* have proven to be vital chemotaxonomic markers and have resulted in placing this species in the Ptaeroxylaceae instead of the Meliaceae family. This point shall be elaborated on later, as *Cedrelopsis grevei* is one of the two species of interest in this study, the other being *Neobeguea mahafalensis*.

It has been argued that secondary metabolites are unimportant as they are not essential for life and are not ubiquitous[5]. However, such metabolites do serve a purpose as many have been found to act as plant defense mechanisms against viral and fungal attack and play a key role in the survival of one species over another[5]. Secondary metabolism has also been categorised as a plant detoxification process of poisons and overabundant metabolites which collect in the plant[2].

Various secondary metabolites have been found to have very specific uses. Flavonoids and carotenoids are coloured and serve to attract insects and bird pollinators. Volatile monoterpenoids repel potential invaders, and certain metabolites act as fungicides or antibiotics, protecting the plant from fungal and bacterial invasion. Saponins are known to have insecticidal properties and have thus been used by man on a large scale[5]. Many secondary metabolites, such as the alkaloids and

the cyanoglycosides, are toxic to animals and insects. Such compounds frequently have a bitter taste thus serving as feeding deterrents. Herbaceous plants, such as ragwort (*Senecio* and *Crotalaria* species), fall under this category as they contain pyrrolizidine alkaloids which are liver poisons. Certain epidemics have also been a result of fungal toxins, these including St. Anthony's fire, (ergot infestation of rye, *Secale cereale*) and milk sickness caused by *Eupatorium rugosum*. Such epidemics have resulted in innumerable human deaths[5].

Man has also found uses for secondary plant metabolites as many are essential vitamins, and others have important medicinal uses. Examples of those with medicinal properties include vinblastine, phusostigmine, quinine and quinidine to name but a few. These compounds are used to treat leukaemia and Hodgkin's disease, glaucoma, malaria and heart disease respectively[5]. The perfume industry relies on natural products for its existence, and various species are grown on a large scale for aroma extraction. These include *Rosa* (Rosaceae), *Citrus* (Rutaceae), *Viola* (Violaceae), *Lavandula* (Labiatae) and *Iris* (Iridaceae)[5].

Research into the biosynthesis of secondary metabolites, or natural products, as they are commonly referred to, has increased tremendously in the last thirty years with the introduction of isotopic tracer experiments. Such studies have provided information on the mechanism of precursor incorporation and the stereospecificity involved. The majority of these metabolites are found to be biosynthesised from a few common intermediates of primary metabolism, these being phosphoenolpyruvate (PEP), pyruvate, acetyl coenzyme A (acetyl-Co A), 3-phosphoglycerate, oxaloacetate and α-ketoglutarate[3]. Three major biogenetic natural product categories exist and are defined according to these limited precursors which link secondary metabolism to primary metabolism. These three categories each incorporate a specific pathway with numerous branch points, thus resulting in many groups of different secondary metabolites.

The three major routes are:

- the shikimate or shikimic acid pathway producing phenolics,
- the acetate-malonate pathway producing fatty acids and aromatics via polyketide intermediates, and
- the acetate-mevalonate pathway which produces terpenoids and sterols.

The above pathways are universal, producing the initial common primary metabolites from which the more specific secondary metabolites are formed[5]. The main flow of carbon metabolism is

outlined in figure 1.1.1 below, where the relationship between primary and secondary metabolism is illustrated. The secondary metabolites are indicated in bold.

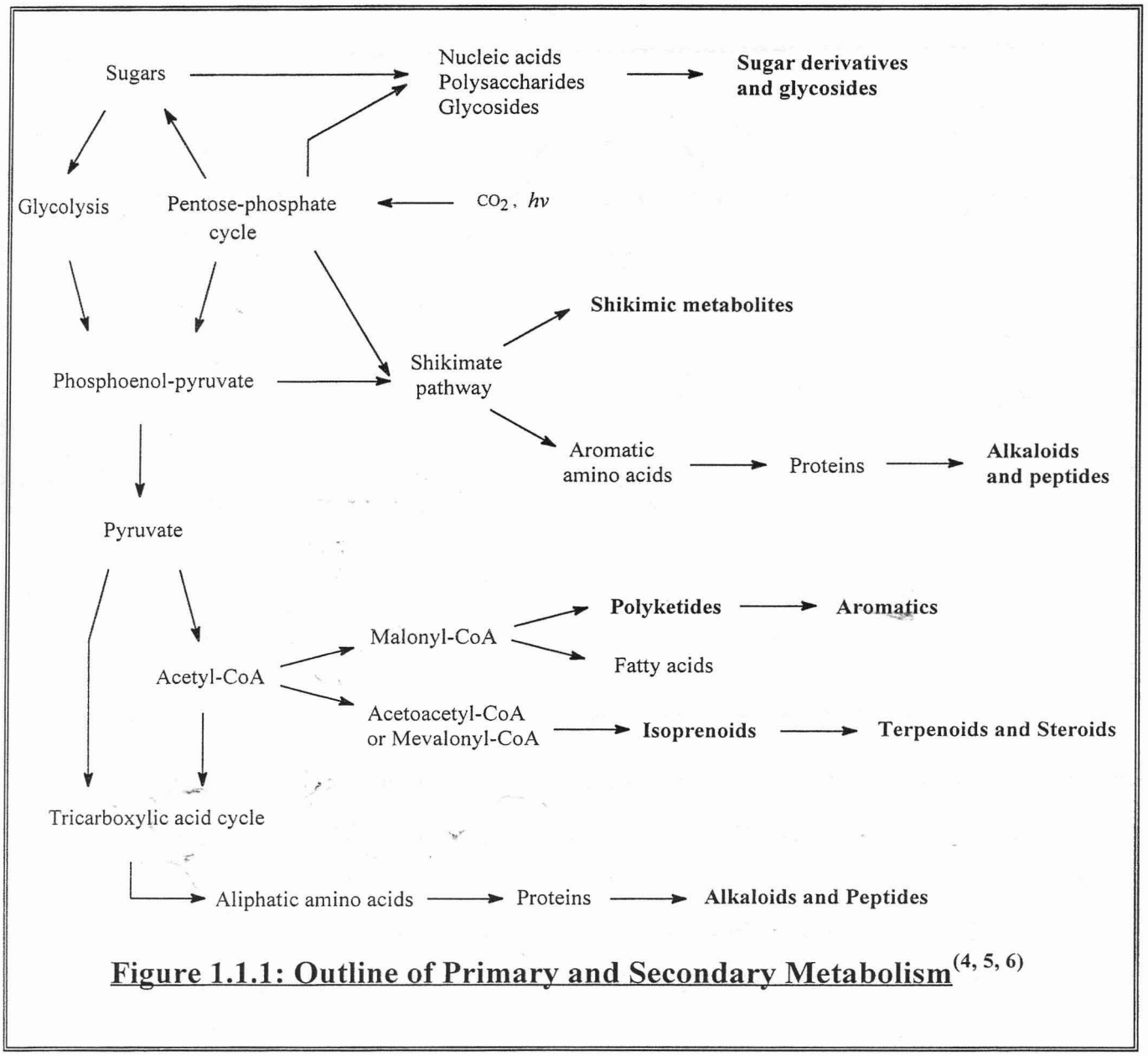
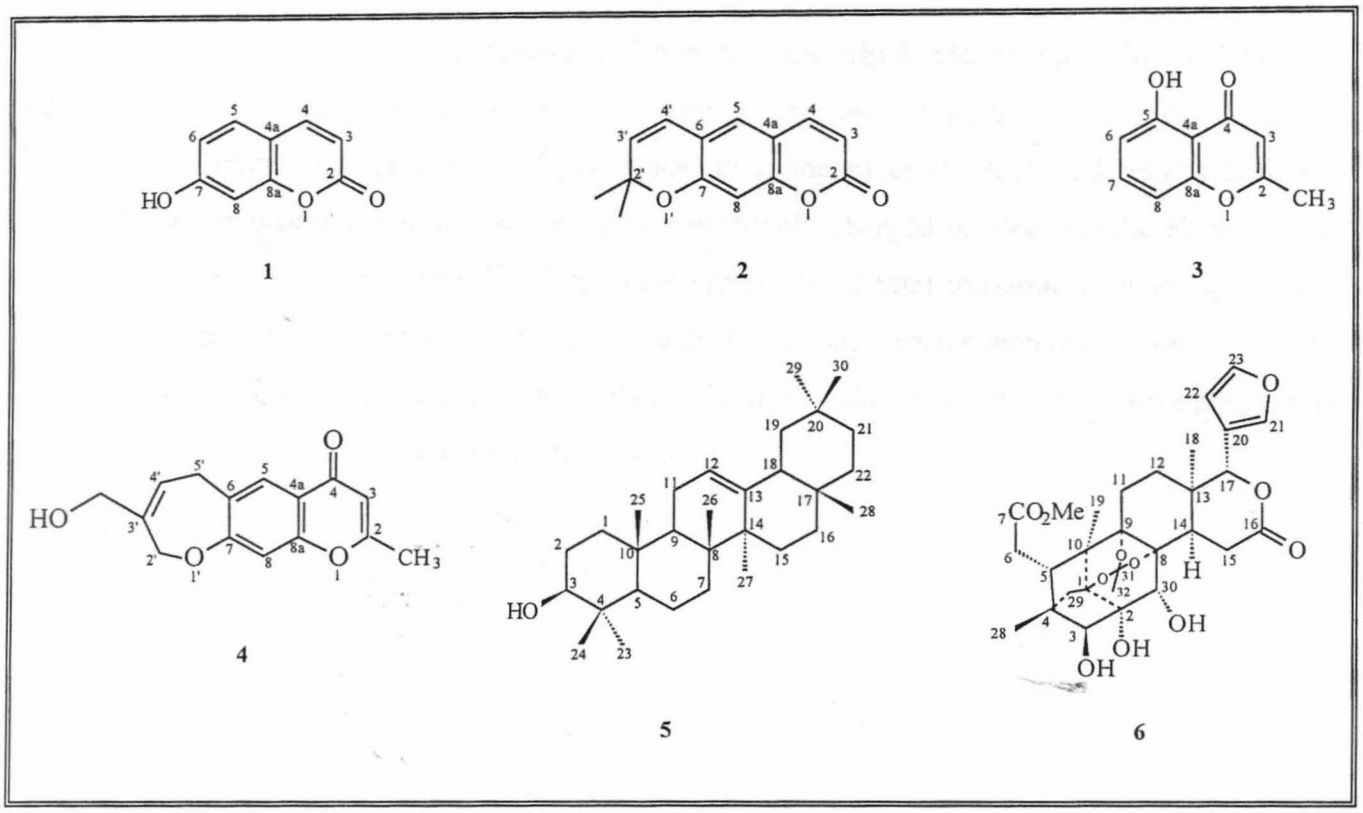


Figure 1.1.1: Outline of Primary and Secondary Metabolism[4, 5, 6]

The shikimic acid pathway has various branch points making it difficult to clearly distinguish between primary and secondary metabolites. This pathway shall be discussed in the following subchapter, as the compounds which were isolated in this work included coumarins, which are derived from the shikimate pathway. The types of coumarins isolated included 7-hydroxycoumarin or umbelliferone (**1**) derivatives, and pyranocoumarins related to xanthyletin (**2**). The acetate-malonate and mevalonate pathways both rely on acetyl-CoA as their main precursor. The compounds isolated in this study produced by the acetate-malonate pathway are chromones such as 5-hydroxy-2-methylchromone (**3**) derivatives and oxepin ring chromones such as ptaeroxylinol (**4**),

while those produced by the acetate-mevalonate pathway isolated in this work include pentacyclic triterpenoids such as β-amyrin (**5**) and limonoids similar to phragmalin (**6**), a group of compounds with a triterpenoid precursor.



Structural similarities between various natural products has resulted in the formulation of certain rules, which, today, play a vital role in biogenetic predictions. These include the following:

- Ruzika's Isoprene Rule: Biosynthesis of terpenoids via a head-to-tail linkage of isoprene units.
- Birch's Acetate Hypothesis: Biosynthesis of various compounds by repeated CH_2 - CO units based on acetate.
- Robinson's Skeletal Classifications: Include chromone and coumarin C_6 - C_3 skeletons composed of a C_9 nucleus.

Almost all *in vivo* reactions are catalysed by enzymes which are important in mediating such very specific reactions with controlled rates and yields. It has been found that enzyme catalysed reactions occur 10^6-10^{14} times faster than such non-enzymatic reactions[7]. All enzymes are proteins with very specific structural features. They all possess an active site composed of both protein (apoenzyme) and non-protein (cofactor) units where the former forms the bulk of the molecule. The

enzyme can have singular or multiple active sites which are three dimensional, asymmetric cavities on which the specificity of the enzymic reaction depends. This specificity results in the exclusive formation of only one stereoisomer[8, 9].

Coenzymes, referred to also as cofactors, are non-proteins which aid enzymes in catalysis. A primary function of some coenzymes is in providing electrophilic centers in enzyme catalysed reactions. Examples include pyridoxal phosphate, thiamine pyrophosphate and tetrahydrofolate, which all contain quaternary nitrogens and thus a positively charged or electrophilic electron sink essential in carbanion stabilisation[10]. Other coenzymes are of vital importance in group transfer reactions, oxidation and reduction reactions and molecular rearrangements. Such electron transferring systems can be pyridine based (NAD^+, $NADP^+$), flavin based (FAD), iron-porphyrins (cytochromes) as well as ubiquinones or coenzyme Q[8, 11].

1.2 THE SHIKIMATE PATHWAY

Benzenoid compounds in higher plants are biosynthesised by various pathways, one of which is the shikimate or shikimic acid pathway. It has been estimated that about one fifth of the carbon fixed by plants is done so via this route[12]. This biosynthetic pathway is responsible not only for the production of vital primary metabolites, but also for an array of secondary metabolites including simple and polyphenols, coumarins, flavonoids, alkaloids, quinones, lignans, lignins, betalains and pigments. Metabolic pathways from which all primary and secondary natural products are produced are enzymatically controlled and occur in the presence of certain essential cofactors, (e.g. NADPH, NADH). One of the classes of secondary metabolites of interest in this work is the coumarins, and thus the biosynthetic route leading to their production shall be elaborated on. It must be noted that **P**, in the biosynthetic mechanisms which follow, stands for PO_3^{2-} of a monophosphate unit (PO_4^{2-}).

Many natural phenolics are synthesised by the shikimate pathway. The most important of these are the three proteinogenic aromatic amino acids, L-phenylalanine, L-tyrosine and L-tryptophan. These amino acids are vital to all living systems, but since the shikimate pathway operates only in microorganisms and higher plants, animals are diet dependent for such aromatics[13, 14, 15]. Shikimic acid, after which this pathway is named, was first isolated in 1885 from the Japanese ashikimi plant *Illicium religiosum* by Eykman[15, 16]. It is a vital intermediate in the *de novo* biosynthesis of these amino acids, which, in turn, give rise to various other aromatic compounds constituting secondary plant metabolites.

The pathway can be divided into three main parts:

1. The prechorismate pathway,
2. The pathway from chorismate to phenylalanine and tyrosine, and
3. The pathway from chorismate to tryptophan.

Once chorismate has been produced, (end of part 1), the shikimate pathway branches into two paths, one producing phenylalanine and tyrosine and the other producing tryptophan. Only the first two paths shall be elaborated on, as only these are of interest in the present case. Figure 1.2.1 outlines the shikimate pathway just prior to amino acid production, or the prechorismate pathway, a route common in all organisms. This part of the pathway introduces the first two double bonds

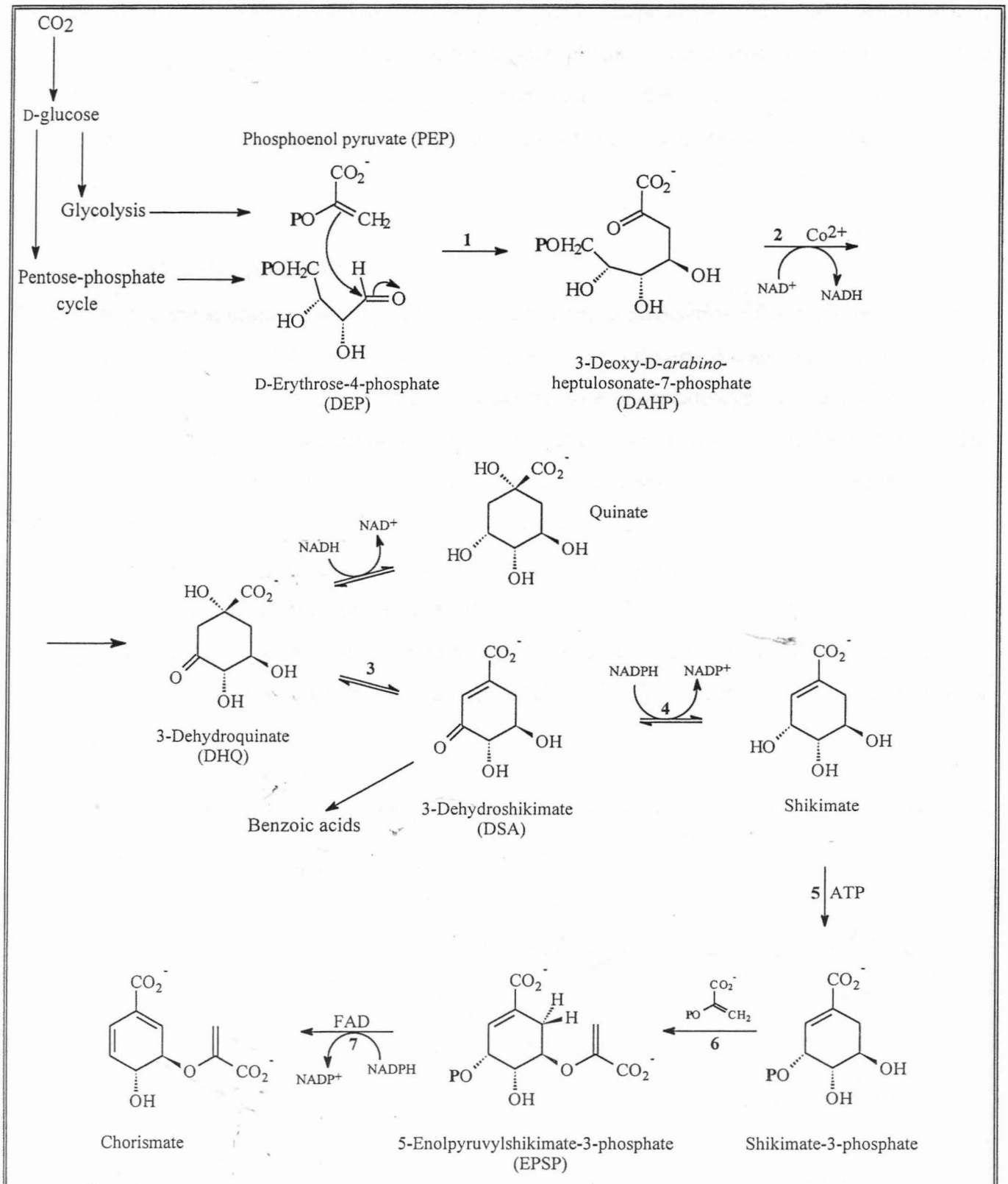


Figure 1.2.1: The Prechorismate or Common Shikimate Pathway[15, 17, 18, 19]

Enzymes: 1. 3-Deoxy-D-*arabino* - heptulosonate-7-phosphate (DAHP) synthase, 2. 3-Dehydroquinatesynthase, 3. 3-Dehydroquinase, 4. Shikimate dehydrogenase, 5. Shikimate kinase, 6. 5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase, 7. Chorismate synthase

into the basic ring system. Paths 2 and 3 vary depending on the organism and conditions but the general route observed in higher plants is illustrated in figure 1.2.7. The participating enzymes also play a very important role in the route taken. Aromatisation, (i.e. introduction of a third double bond into the cyclohexadiene system), occurs in both the latter pathways.

The Prechorismate pathway

The first step in the shikimate pathway (figure 1.2.1), is the condensation of phosphoenol pyruvate (PEP) and D-erythrose-4-phosphate (DEP), which are both primary metabolites produced from glucose-6-phosphate via glycolysis and the oxidative pentose phosphate cycle respectively[15]. This is a stereospecific aldol condensation where nucleophilic addition occurs via the *si*-face of the double bond in PEP to the *re*-face of the carbonyl in D-erythrose-4-phosphate as is evident by the C-4 configuration of the product[16, 17]. DAHP synthase (3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase) catalyses this addition reaction, resulting in the formation of 3-deoxy-D-*arabino*-heptulosonic acid-7-phosphate (DAHP), a seven carbon sugar[20]. The two methylene hydrogens of PEP do not lose their identity and by kinetic examinations of the reaction, the mechanism has been shown to be an ordered sequential one[14]. The mechanism for this reaction is illustrated in figure 1.2.2 below.

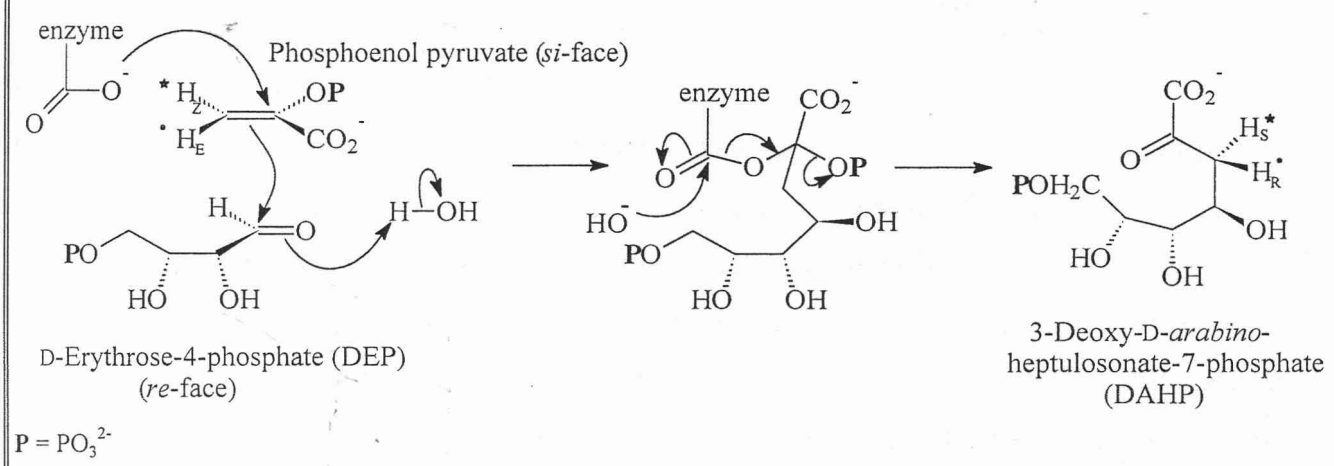


Figure 1.2.2: Reaction mechanism for DAHP formation[14, 17]

The cyclisation (step 2, figure 1.2.1) of DAHP to 3-dehydroquinic acid (DHQ), requires cobalt(II) and nicotinamide adenine dinucleotide (NAD^+) as cofactors and occurs via a complex sequence of reactions, resulting in the first alicyclic intermediate of the shikimate pathway. Oxidation of

the hemiketal form of DAHP at C-5 facilitates *syn*-β-elimination of inorganic phosphate at C-7. The two methylene hydrogens at C-7 retain their identity[12, 21]. Reduction then occurs at C-5 followed by hemiketal ring opening and an intramolecular aldol condensation via a chair transition state, where the C-7 enolate attacks the carbonyl group at C-2[12]. Whether cyclisation occurs before or after reduction is questionable[16]. This ring closure has been found to be spontaneous, while the oxidation and reduction steps are catalysed by the enzyme 3-dehydroquinate (DHQ) synthase[17, 18]. The reaction mechanism is illustrated in figure 1.2.3 , where the change in numbering on the formation of the carbocyclic product, must be noted.

DHQ then undergoes reversible dehydration, producing dehydroshikimic acid, (DSA). The dehydration mechanism is seen as an overall two step sequence involving the formation of an enamine via the keto group of DHQ[16, 17]. The active site of the enzyme has a lysine residue, thus acting as a strong nitrogen nucleophile which can form a Schiff's base, or imine, with the C-3 carbonyl of DHQ[21, 22]. The intermediate that is formed is a zwitterion. The positively charged nitrogen loses a proton and the negatively charged oxygen gains one, forming the tetrahedral addition product. The 1,2-elimination of water involves an enamine which subsequently isomerises and eliminates a hydroxyl moiety via a stereospecific *cis*-mechanism with *pro-R* hydrogen loss[22, 23, 24, 25]. This reversible dehydration process is catalysed by the enzyme 3-dehydroquinase (H_2O elimination) / shikimate dehydrogenase (H_2O addition), which is bifunctional only in higher plants. It is the first known example of an enzymatic *syn*-elimination of water[14, 15]. Step 3 in figure 1.2.1 illustrates the dehydration process for which the mechanism is shown in figure 1.2.4.

Once DSA has been produced, reduction of the C-3 keto group occurs in the presence of NADPH to produce shikimic acid (SA), or shikimate as evident in step 4 of figure 1.2.1. Shikimate dehydrogenase catalyses this reversible reduction. NADH is found to be inactive in this case[26] . Shikimate phosphorylation follows resulting in the shikimate-3-phosphate derivative. This phosphorylation is vital in forming a better leaving group for later elimination[16]. Shikimate kinase, which is ATP dependent, catalyses the phosphate transfer from ATP to the C-3 hydroxyl group of shikimate (step 5, figure 1.2.1). Condensation of shikimate phosphate with PEP, in the presence of enolpyruvateshikimate phosphate synthase (EPSP synthase), results in the attachment of a C_3 side chain, producing 5-enolpyruvylshikimic acid-3-phosphate (EPSP) and thus the basic C_6 - C_3 nucleus[27, 28], (step 6, figure 1.2.1). The reaction proceeds by the nucleophilic attack of the 5-hydroxyl of the phosphoshikimate derivative onto C-2 of PEP. The mechanism of this addition-

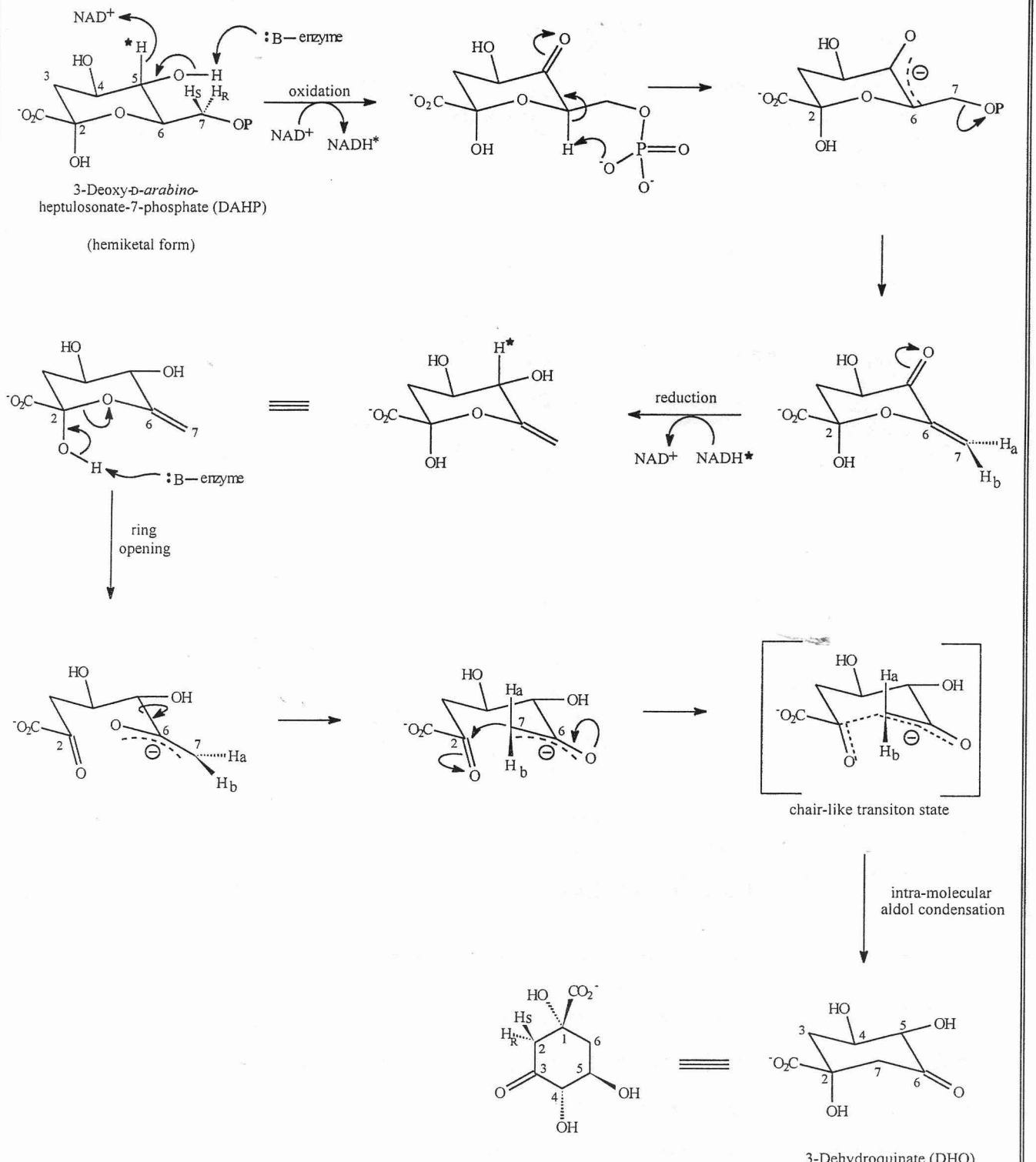


Figure 1.2.3: DHQ formation from DAHP[12, 17, 18]

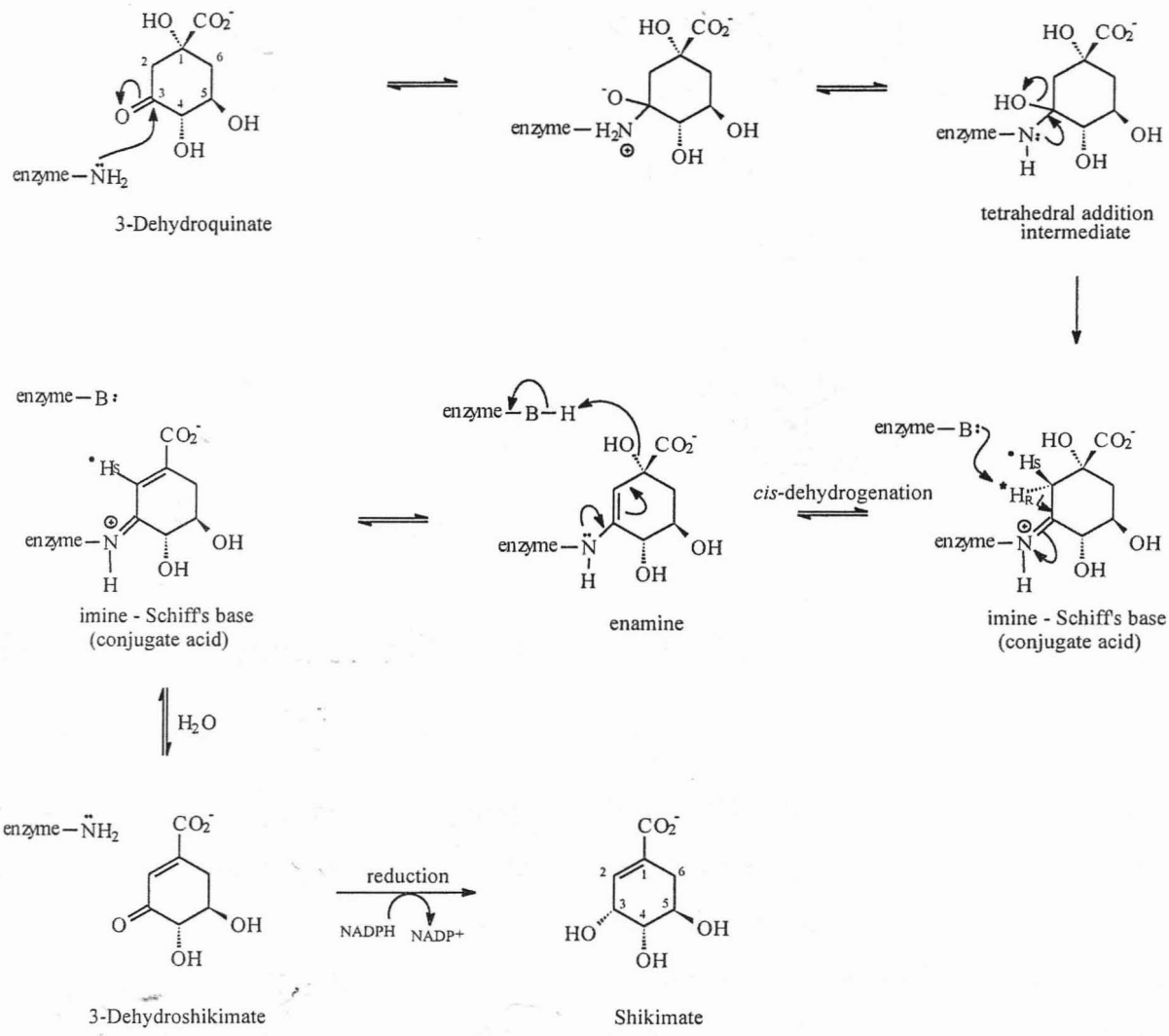
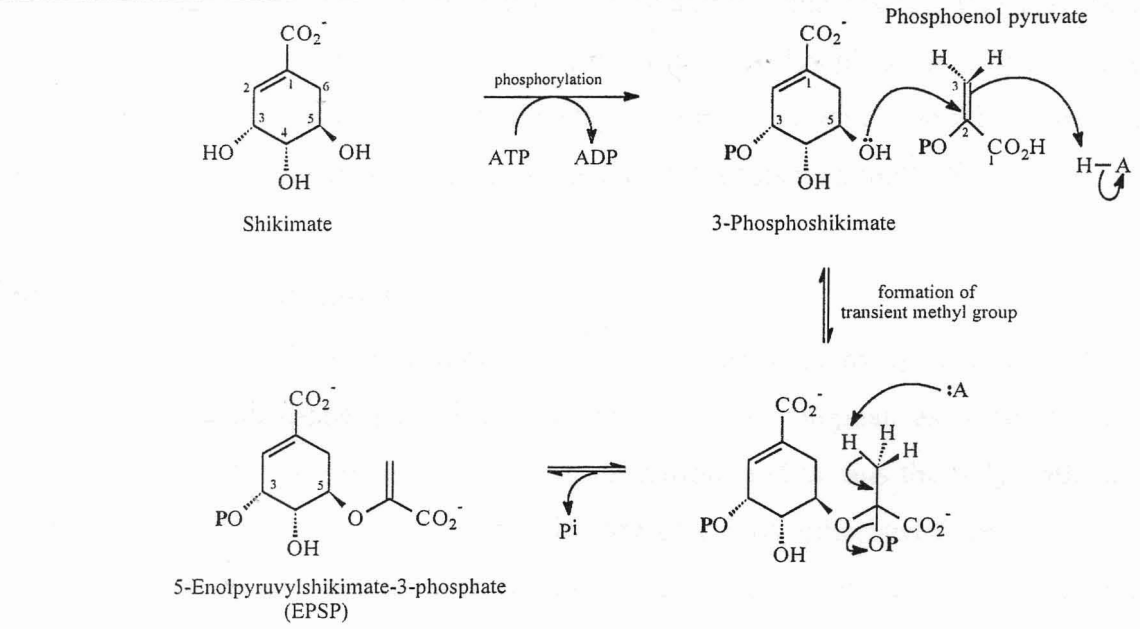


Figure 1.2.4: Shikimate production via a Schiff's base [14, 22, 24, 30]

elimination reaction, first proposed by Levin and Sprinson[12, 29], is illustrated in figure 1.2.5 and has been proven to proceed to give a product with opposite stereochemistry. Thus the reaction will proceed by either an *anti / syn* or *syn / anti* mechanism. The C-3 terminal methylene group of PEP becomes a transient methyl group in the enzyme bound tetrahedral intermediate[20, 30, 31]. This intermediate has been isolated[12]. By making use of labelled PEP, it has been found that the side chain configuration of EPSP is the same as PEP thus proving that the addition-elimination process must occur with opposite stereochemistry[32].

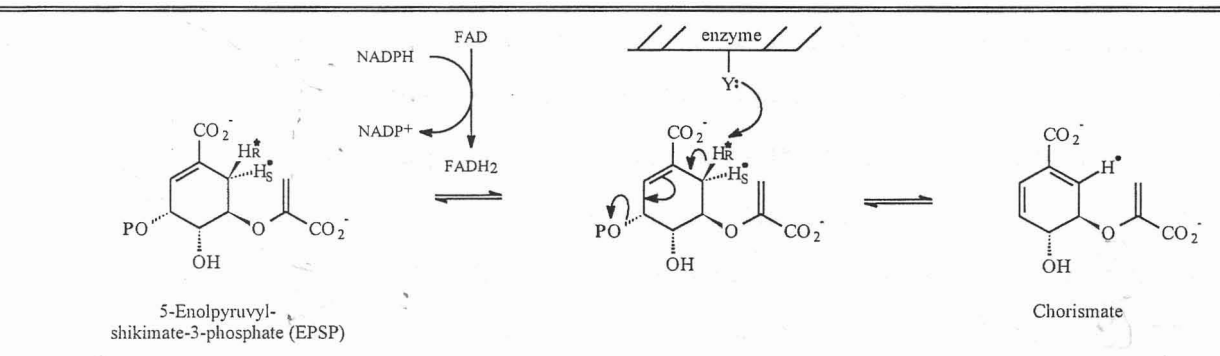
The final step in the common part of the shikimate pathway involves the production of chorismic acid where a second double bond is introduced into the ring system by the



P = PO_3^{2-}

Figure 1.2.5: Condensation of shikimic acid-3-phosphate with PEP[12, 16, 17]

trans-1,4-conjugated elimination of phosphoric acid. This reaction (step 7, figure 1.2.1) is catalysed by chorismate synthase (5-enolpyruvateshikimate-3-phosphate lyase) and requires a reduced flavin cofactor although its overall redox state remains unchanged[12, 26]. Labelling experiments have shown the elimination to involve the removal of the *pro*-R hydrogen from C-6 of EPSP, and it is thus formally a conjugated *trans*- or *anti*-1,4-elimination, and not a *cis*- or *syn*-1,4-elimination[14]. It would thus be thought that the mechanism should not be a concerted one, (as for *syn*-elimination), but rather a step-wise one. This is not so in this unique case. Although various two-step mechanisms have been proposed, it is the concerted mechanism illustrated in figure 1.2.6 which is the most widely accepted.



P = PO_3^{2-} , FAD = Flavin adenine dinucleotide

Figure 1.2.6: Chorismate formation via a concerted mechanism[14, 15, 19, 26, 33]

Once EPSP binds to the enzyme, distortion is caused in the ring system. This ring distortion is large enough to allow for a concerted *anti*-1,4-elimination. Although such a concerted mechanism is chemically unfavorable as compared to the step-wise reaction which would require less energy, it is postulated that the involvement of the enzyme facilitates this reaction[26, 33].

The Chorismate Pathway

Chorismic acid acts as the main branch point in the shikimate pathway resulting in the production of *p*-aminobenzoic acid, L-phenylalanine, L-tyrosine and L-tryptophan as illustrated in figure 1.2.7. L-Phenylalanine is the precursor to coumarin formation, and is thus the only pathway that shall be elaborated on. L-Phenylalanine and L-tyrosine are both biosynthesised from chorismic acid via the intermediate, prephenate, produced in the presence of chorismate mutase. A reaction scheme for their production is illustrated in figure 1.2.8. The basic biosynthesis of L-phenylalanine involves Claisen rearrangement of chorismic acid into prephenic acid (step 1, Figure 1.2.8), followed by the production of phenylpyruvic acid via prephenate aromatisation (step 2, figure 1.2.8). Transamination then results in L-phenylalanine formation (step 3, figure 1.2.8) and makes use of glutamate as the nitrogen donor in the presence of aminotransferase[12].

Studies have shown that L-phenylalanine can also be produced by another precursor, L-arogenate, which is itself produced from prephenate. L-Arogenate dehydrogenase with NAD^+ cofactor catalyses the conversion of L-arogenate into L-tyrosine (step 6, figure 1.2.8), while arogenate dehydratase catalyses phenylalanine production (step 5, figure 1.2.8). The pathway taken depends on the organism, although more than one route can operate at once. The most common route to phenylalanine in higher plants is via the phenylpyruvate intermediate, while conversion via L-arogenate is less frequent[20].

Chorismate mutase is the only enzyme characterised as catalysing a pericyclic process in the shikimate pathway, namely the uni- and intramolecular rearrangement of chorismate to prephenate[12]. This 3,3-sigmatropic rearrangement is of the Claisen type and is unique in the production of primary metabolites (step 1, figure 1.2.8). The mechanism involved is illustrated in figure 1.2.9 and has been proven by labelling experiments[20, 32]. Chorismate, in the enzyme bound pseudo-diaxial conformation, rearranges to form prephenate via a chair-like transition state. Transfer of the enylpyruvate side-chain occurs such that it becomes directly bonded to the carbocycle generating the basic phenylpropanoid skeleton [26, 34, 35]. This reaction has also

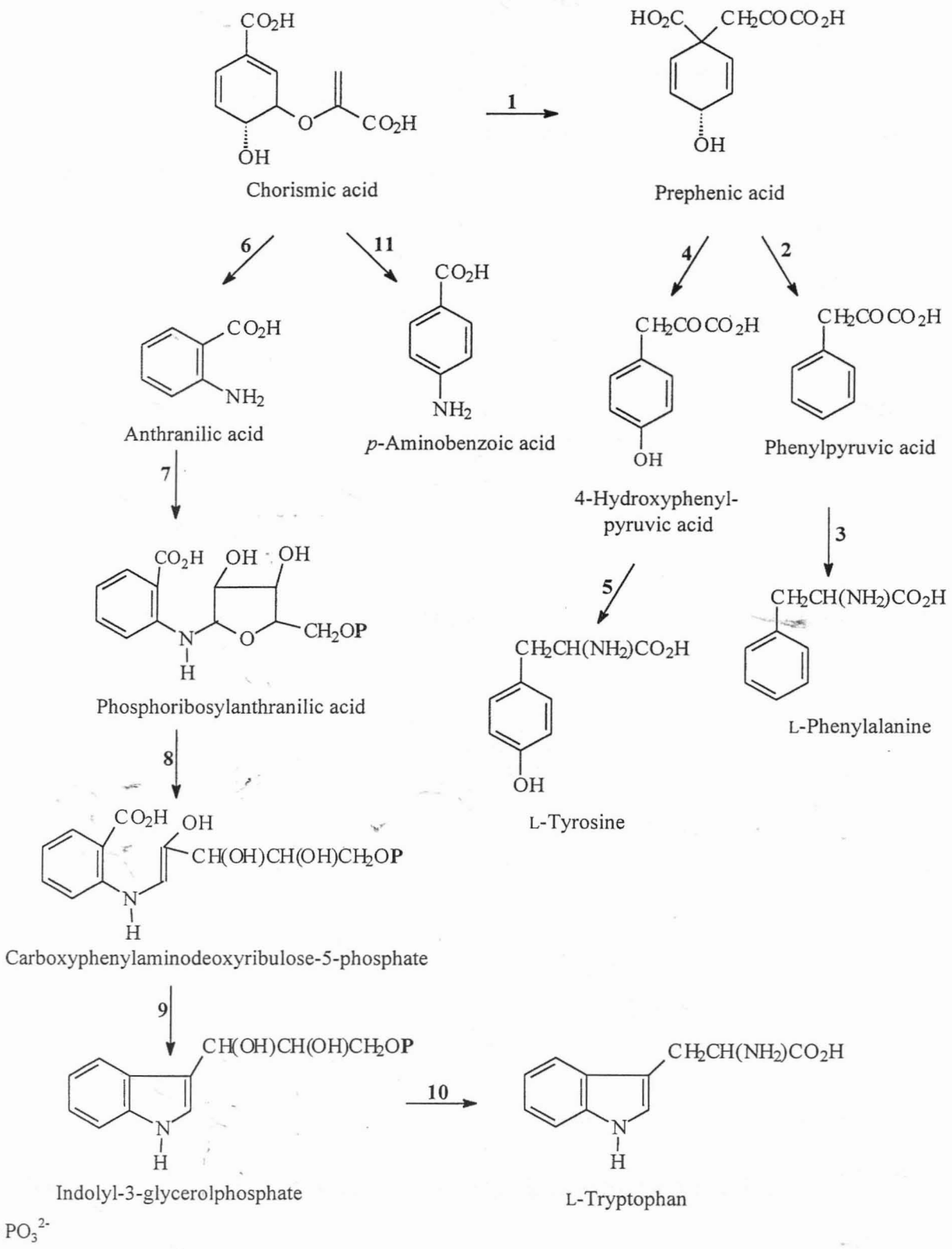


Figure 1.2.7: Aromatic amino acid production via the Shikimate Pathway[18]

Enzymes: **1. Chorismate mutase, 2. Prephenate dehydratase, 3. Phenylalanine aminotransferase, 4. Prephenate dehydrogenase, 5. Tyrosine aminotransferase, 6. Glutamine, 7. Anthranilate phosphoribosyltransferase, 8. Phosphoribosylanthranilate isomerase, 9. Indolylglycerol phosphate synthase, 10. Tryptophan synthase, 11. Glutamine.**

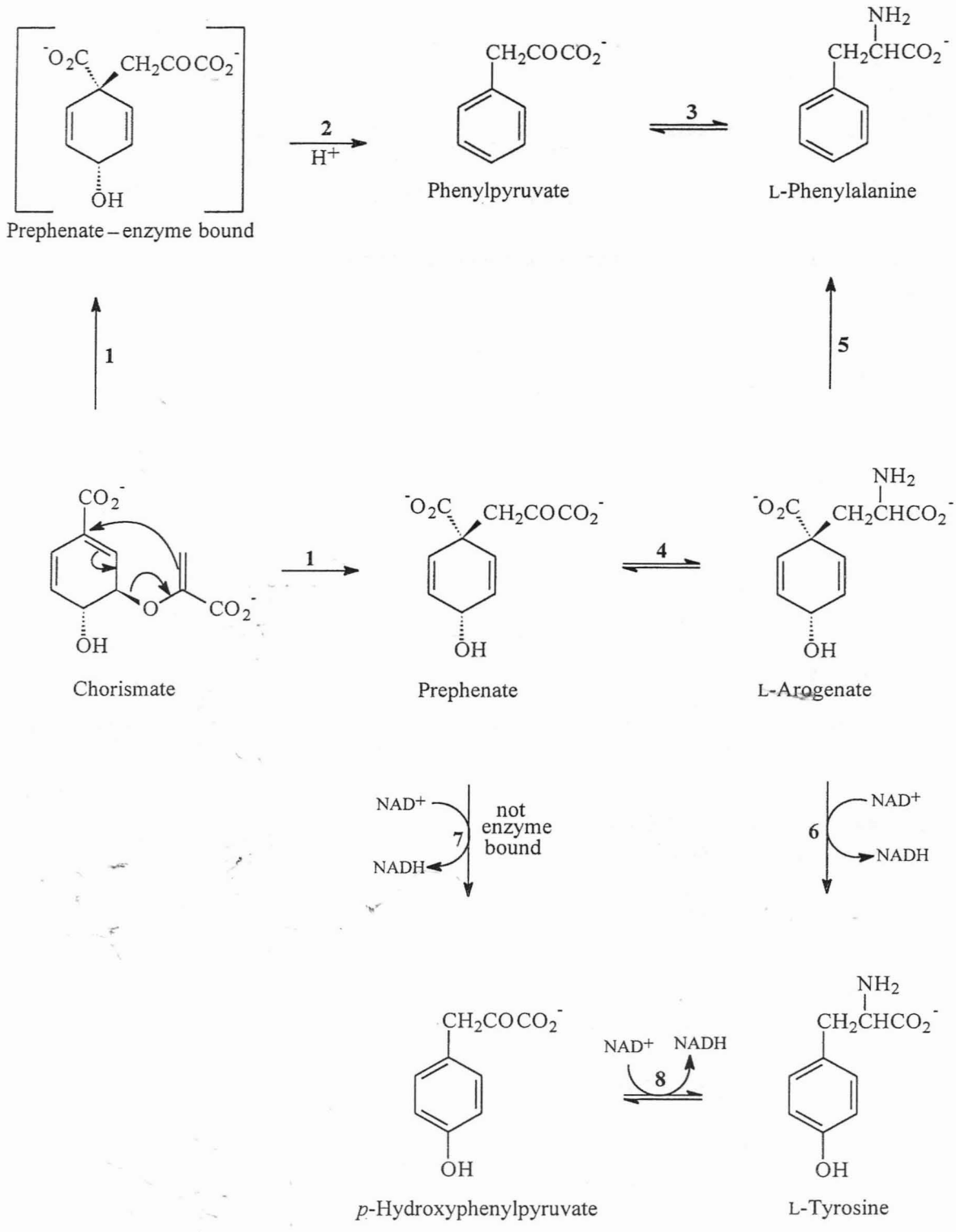


Figure 1.2.8: Various routes to L-Phenylalanine production [12, 13, 15, 26, 31]

Enzymes: **1. Chorismate mutase, 2. Prephenate dehydratase, 3. Phenylpyruvate aminotransferase, 4. Prephenate aminotransferase, 5. Arogenate dehydratase, 6. Arogenate dehydrogenase, 7. Prephenate dehydrogenase, 8. 4-Hydroxyphenylpyruvate aminotransferase.**

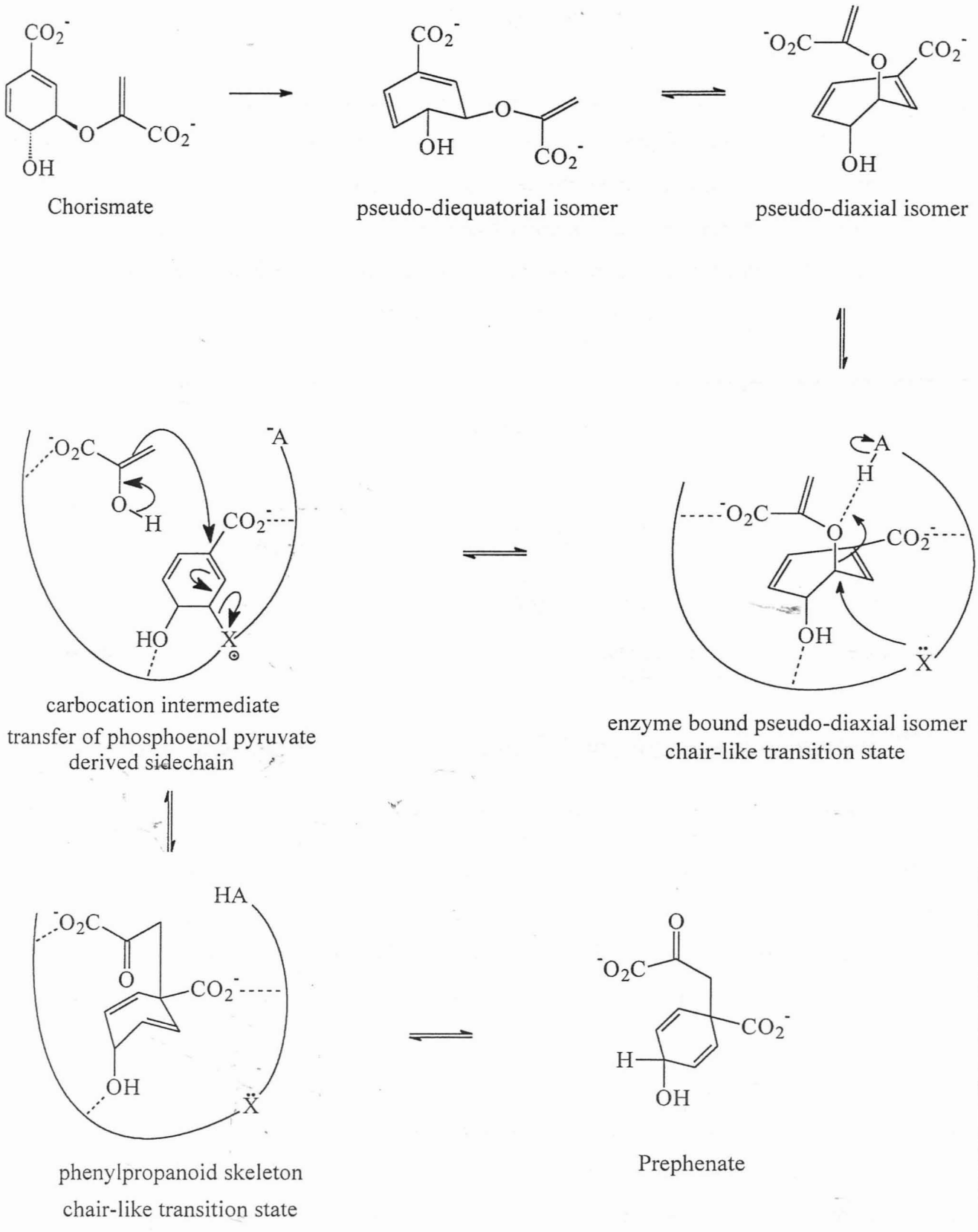


Figure 1.2.9: Prephenate production via pseudo-diaxial Chorismate isomer[35]

been found to proceed thermally, again via a Claisen-type rearrangement, in the absence of chorismate mutase[20].

Phenylpyruvate is produced from chorismate mutase bound prephenate (step 2, figure 1.2.8), via the stepwise mechanism illustrated in figure 1.2.10 below. Dehydroxylation of prephenate results in the formation of a carbocation intermediate. Decarboxylation then results in phenylpyruvate formation. If the reaction is catalysed by phenylpyruvate tautomerase, the phenylpyruvate enol isomer will also be produced. If, on the other hand, the process is simply acid catalysed, (in the absence of enzyme and NAD^+), only the keto isomer will form[32].

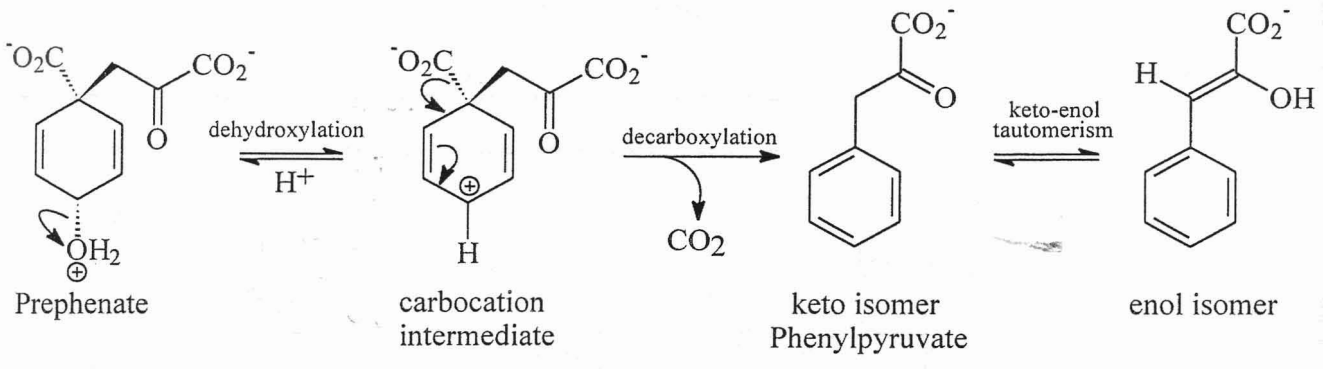
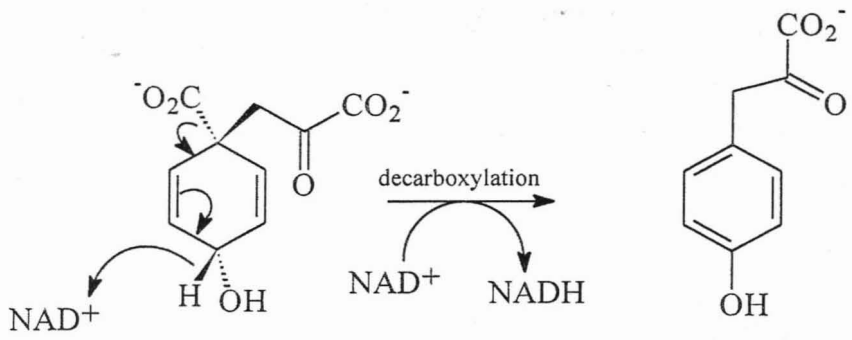


Figure 1.2.10: Step-wise mechanism of Phenylpyruvate formation[32]

p-Hydroxyphenylpyruvate (step 7, figure 1.2.8) is formed from prephenate only in the presence of prephenate dehydrogenase with NAD^+ cofactor. Mechanistic studies using labelling experiments have found the reaction to be a concerted one. Dehydrogenation occurs first and is then followed by decarboxylation[32]. The mechanism for this reaction is illustrated in figure 1.2.11.

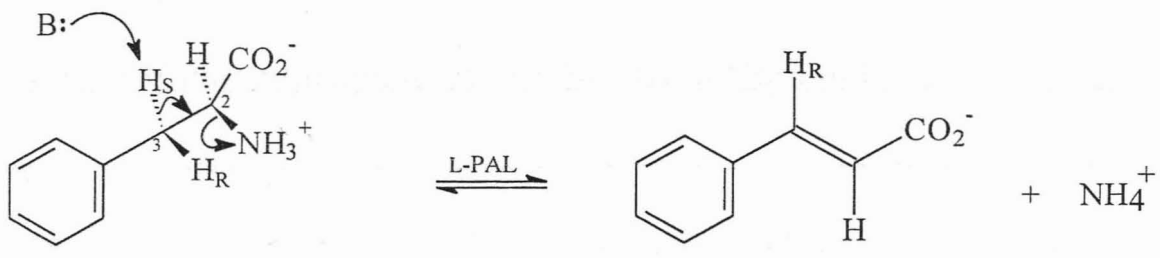
Phenylalanine undergoes reversible deamination in the presence of the enzyme L-phenylalanine ammonia-lyase (PAL), forming *trans*-cinnamic acid with the elimination of ammonia via an E_2 mechanism[36]. This stereospecific deamination occurs via a concerted *anti*-mechanism as it involves loss of the 3-*pro-S* proton of L-phenylalanine. The *trans*-planar conformation is most favourable for such α,β-eliminations[16, 17]. Figure 1.2.12 illustrates this process.



Prephenate

p-Hydroxyphenyl pyruvate

Figure 1.2.11: Concerted mechanism for *p*-Hydroxyphenylpyruvate formation[32]



L-Phenylalanine

trans-Cinnamate

Figure 1.2.12: Cinnamate production via L-Phenylalanine deamination[16, 17, 26]

Cinnamic acid is the precursor of a wide range of phenylpropanes which are very important phenolic metabolites in higher plants. A range of phenylpropane derivatives were isolated in this work. The biosynthesis of these phenolics is illustrated in figure 1.2.13. The multifunctional oxygenases which make use of molecular oxygen, catalyse the hydroxylation of cinnamic acid and its derivatives. This type of hydroxylation is unique as it occurs by the NIH shift which involves the migration and not the loss of the hydrogen atom being replaced by the hydroxyl group[36, 37]. The NIH shift was discovered and proved using labelling experiments, where phenylalanine and cinnamic acid were tritiated at the 4-position. This proton migration was discovered at the National Institute of Health (NIH), from which this process gets its name[17, 18, 37].

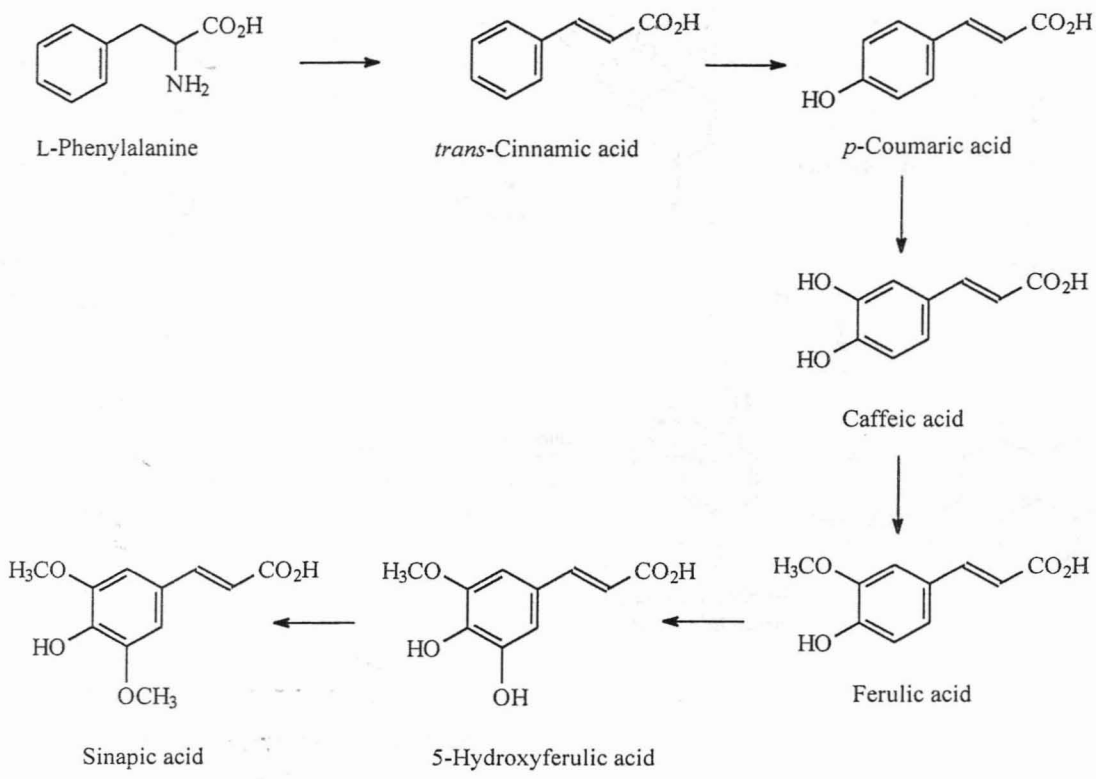


Figure 1.2.13: Phenylpropane derivatives from Cinnamic acid precursor[17, 18]

An illustration of such a proton shift is evident in the oxidation of phenylalanine to tyrosine making use of molecular oxygen and phenylalanine 4-monooxygenase, which itself contains Fe^{2+} ions, and requires NADPH and tetrahydrobiopterin (THB) cofactors. The NIH shift mechanism for phenylalanine is illustrated in figure 1.2.14. THB forms a peroxide which can oxidise phenylalanine. The new hydroxylated THB loses water forming dihydrobiopterin (DHB) and the arene oxide (epoxide), of phenylalanine. Epoxide ring opening accompanied by a 1,2-tritium shift is followed by aromatisation which, in this case, results in the tyrosine product[38].

The cinnamic acid derivatives are used as precursors in the biosynthesis of benzoic acid derivatives, lignins, coumarins, tannins, quinones and flavonoids. The biosynthesis of coumarins shall be further elaborated.

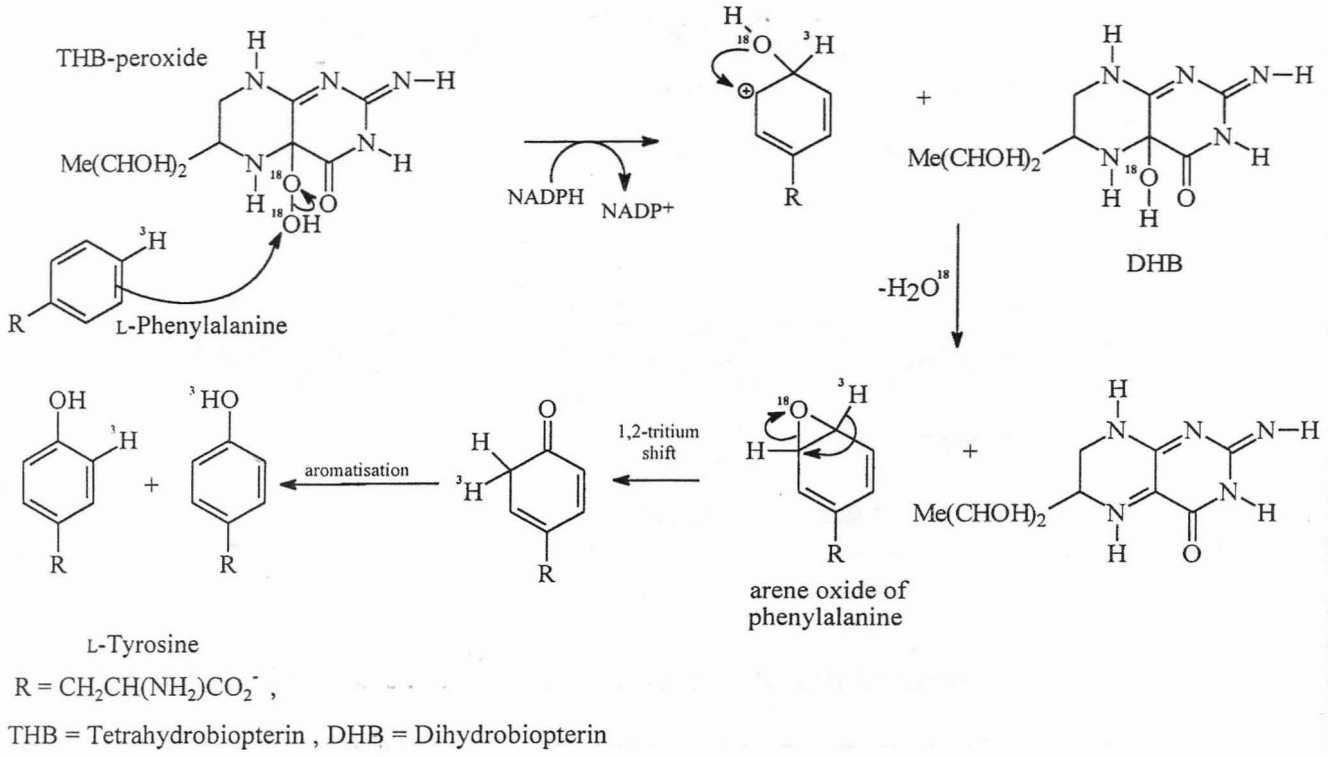
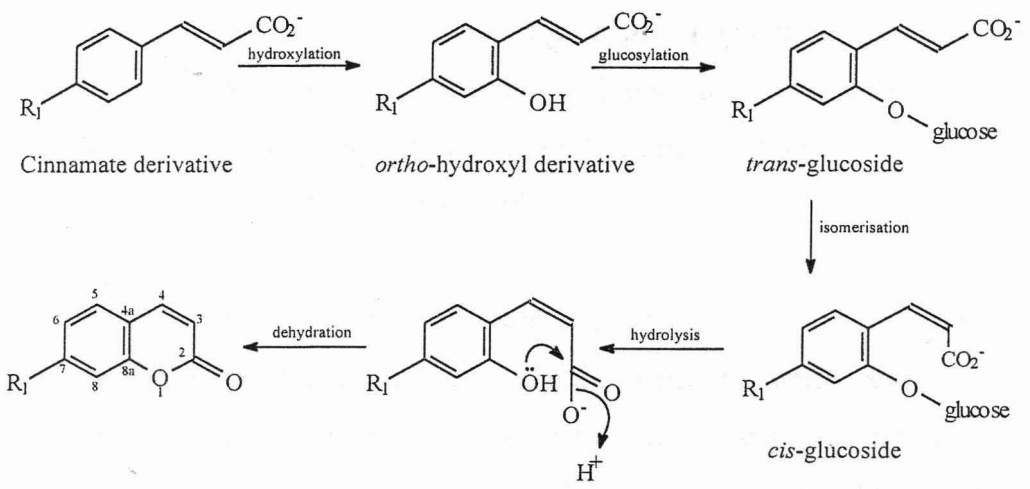


Figure 1.2.14: NIH-shift mechanism[38]

The Coumarins

Coumarins are δ-lactones or more specifically α–benzopyrones. They can be divided into four main classes, namely simple coumarins (C_6 - C_3 nucleus), furanocoumarins, pyranocoumarins and arylcoumarins[28, 39]. Furano- and pyranocoumarins can occur in linear and angular conformations, where the former are more common. Arylcoumarins are phenol substituted either at C-3 or C-4 and have a mixed biogenesis, making use of both the shikimate and acetate-malonate pathways[40]. In higher plants, the shikimate pathway predominates in coumarin production, while in microbials, coumarins have been found to be acetate derived via polyketide intermediates, (e.g. aflatoxins)[39, 41].

The biosynthesis of simple coumarins from cinnamic acid derivatives begins by *ortho*-hydroxylation, a reaction catalysed by phenolase and involving the NIH shift. The *ortho*-hydroxyl group can undergo glucosylation, allowing not only the *trans*-, but the *cis*-isomer to exist as well. Hydrolysis of the sugar via enzymatic cleavage of the glycoside, is followed by spontaneous cyclisation (lactone formation), resulting in the final coumarin product[17, 18, 40]. The biosynthesis of simple coumarins is illustrated in figure 1.2.15.



	Precursor	Product
$R_1 = H$	Cinnamate	Coumarin
$R_1 = OH$	*p* -Coumarate	Umbelliferone

Figure 1.2.15: Biosynthetic route to coumarin production[17, 18, 20, 40]

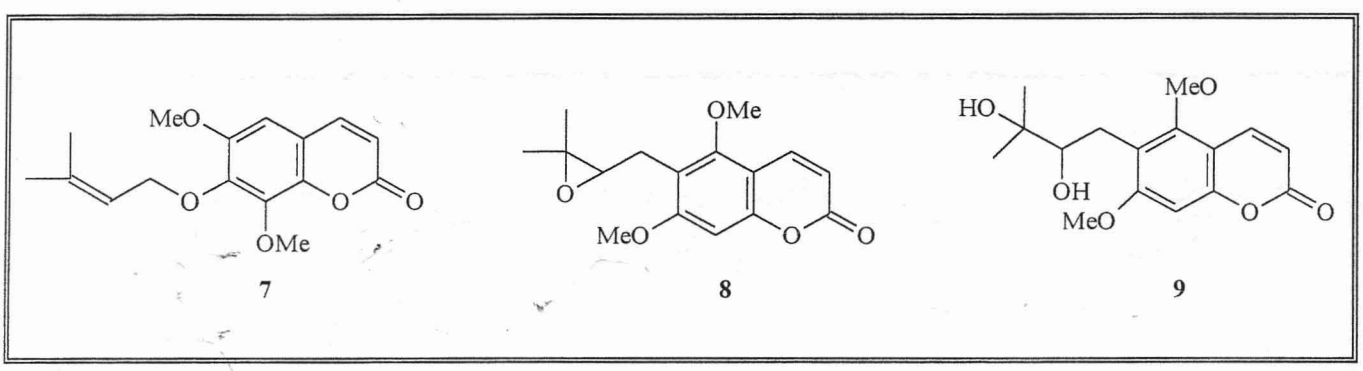
Coumarin itself is the simplest known compound of this group, and is produced directly from cinnamic acid. It was first isolated by Vogel over a century ago from *Dipteryx (Coumarouna) odorata*, (the tonka bean) and it is a colourless crystalline material with a pleasant odor [39, 40]. Although coumarin gives its name to this group of compounds and is widely distributed, it is itself atypical of the group as it is not oxygenated at position seven[42, 43]. Of the many naturally occurring coumarins which have been isolated, the majority contain an oxygen substituent in the seven position.

Umbelliferone is the simplest of the common 7-oxygenated coumarins in which both *para*-followed by *ortho*-hydroxylation have occurred in cinnamic acid. Figure 1.2.15 illustrates the biosynthesis of coumarin and the 7-hydroxycoumarin which is regarded as the parent compound of other 7-oxygenated coumarins[41, 42]. It must be noted that the biosynthetic pathway of such coumarins are similar to those which are not 7-oxygenated, but in the former *p*-hydroxylation precedes *o*-hydroxylation, followed by lactone formation[28]. Once *o*-hydroxylation occurs, it is followed directly by spontaneous lactone formation.

Many phenolic metabolites, are modified with isoprenoid chains, of 1, 2 or 3 units, which can be attached to oxygen or carbon atoms of the phenolic nucleus. Such isoprenoid residues arise from

isopentyl pyrophosphate (IPP), involved in the biosynthesis of steroids and terpenoids. Such derivatives are found to occur in both higher plants and microorganisms and are particularly common in both α- and γ-benzopyrones, including both coumarins and chromones respectively[44]. In coumarins, both C- and O-prenylations are known to occur, leading to numerous polyisoprenoid derivatives which can, in turn, be further modified.

Two general types of isoprenoid derivatives occur namely αα-dimethylallyl and γγ-dimethylallyl derivatives, where the latter are far more common owing to steric reasons. They involve γγ-dimethylallyl pyrophosphate units reacting with the appropriate sites. Such C- and O-prenylations for the γγ-derivatives are illustrated in figure 1.2.16. The double bond of the isoprenoid residue can be further modified by oxygenation to the isopentenyl epoxide which can in turn hydrolyse to an isopentane diol. Such epoxide and diol modifications are common in coumarin derivatives. Examples of the former include puberulin (**7**) and aculeatin (**8**) and the latter, toddalolactone (**9**)[44].



7 **8** **9**

A very common modification of such isoprenoid side chains is cyclisation, resulting either in fused furanyl or chromane rings and thus furano- and pyranocoumarins respectively[42]. The pyranocoumarins, although rare, shall be elaborated on as such compounds were isolated from *Cedrelopsis grevei*. They are 2',2'-dimethylchromene derivatives which can be derived from both C- and O-isoprenoid derivatives. In the former, oxidation of the phenolate anion is followed by benzylic proton loss resulting in an *ortho*-methylenequinone intermediate which then cyclises to form the 2',2'-dimethylchromene derivative[14, 39]. On the other hand, direct cyclisation can occur in the presence of NADPH, resulting in the 2',2'-dimethylchroman derivative[44]. The mechanisms for both such cylisations are illustrated in figure 1.2.17. Furthermore, pyranocoumarins can, like furanocoumarins, also be formed by acid catalysed ring closure of

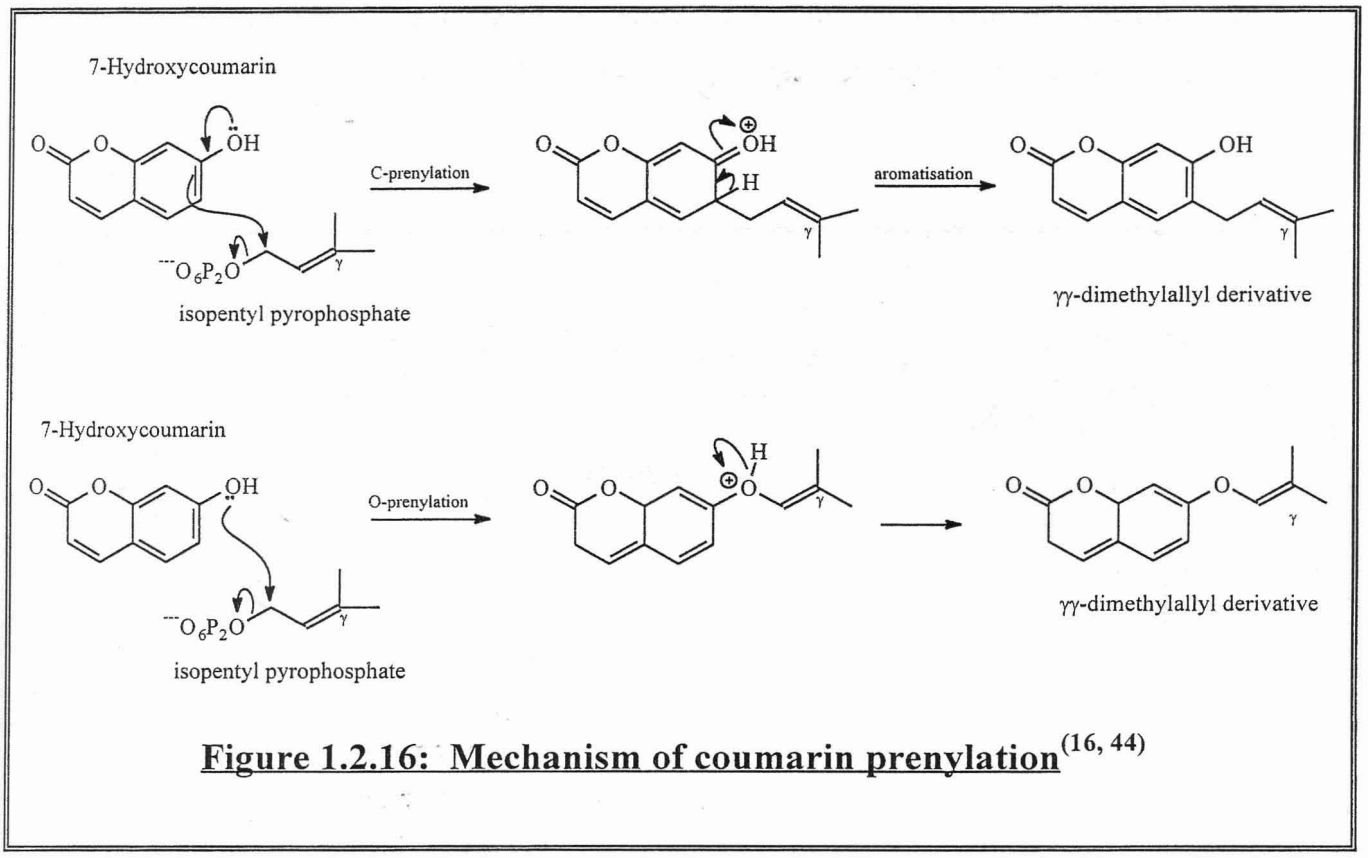


Figure 1.2.16: Mechanism of coumarin prenylation[16, 44]

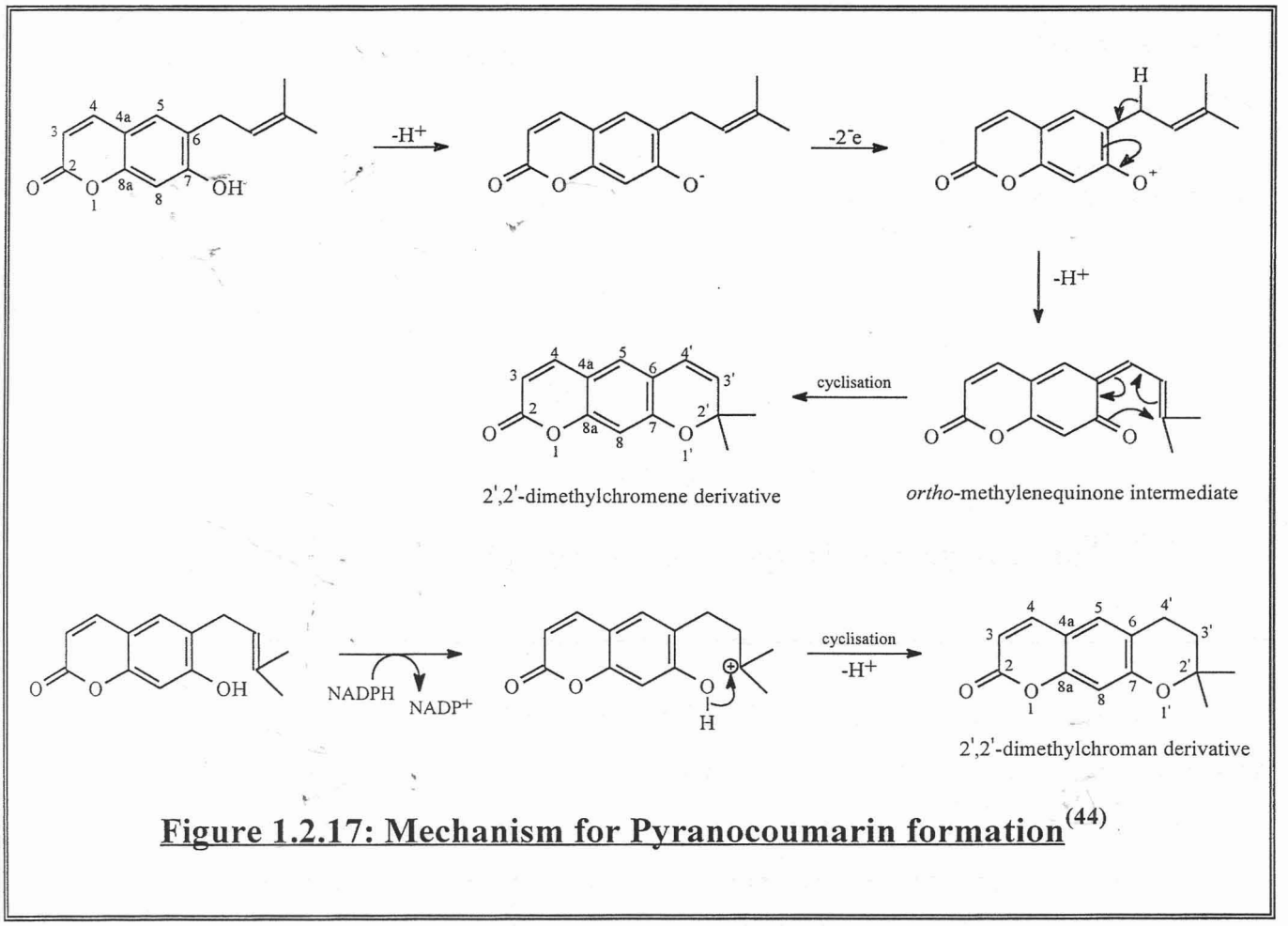


Figure 1.2.17: Mechanism for Pyranocoumarin formation[44]

γγ-dimethylallyl epoxides as illustrated in figure 1.2.18 for xanthyletin and seselin. Almost all pyranocoumarins are 2',2'-dimethylchromene derivatives, or more specifically, chromeno-α-pyrones.

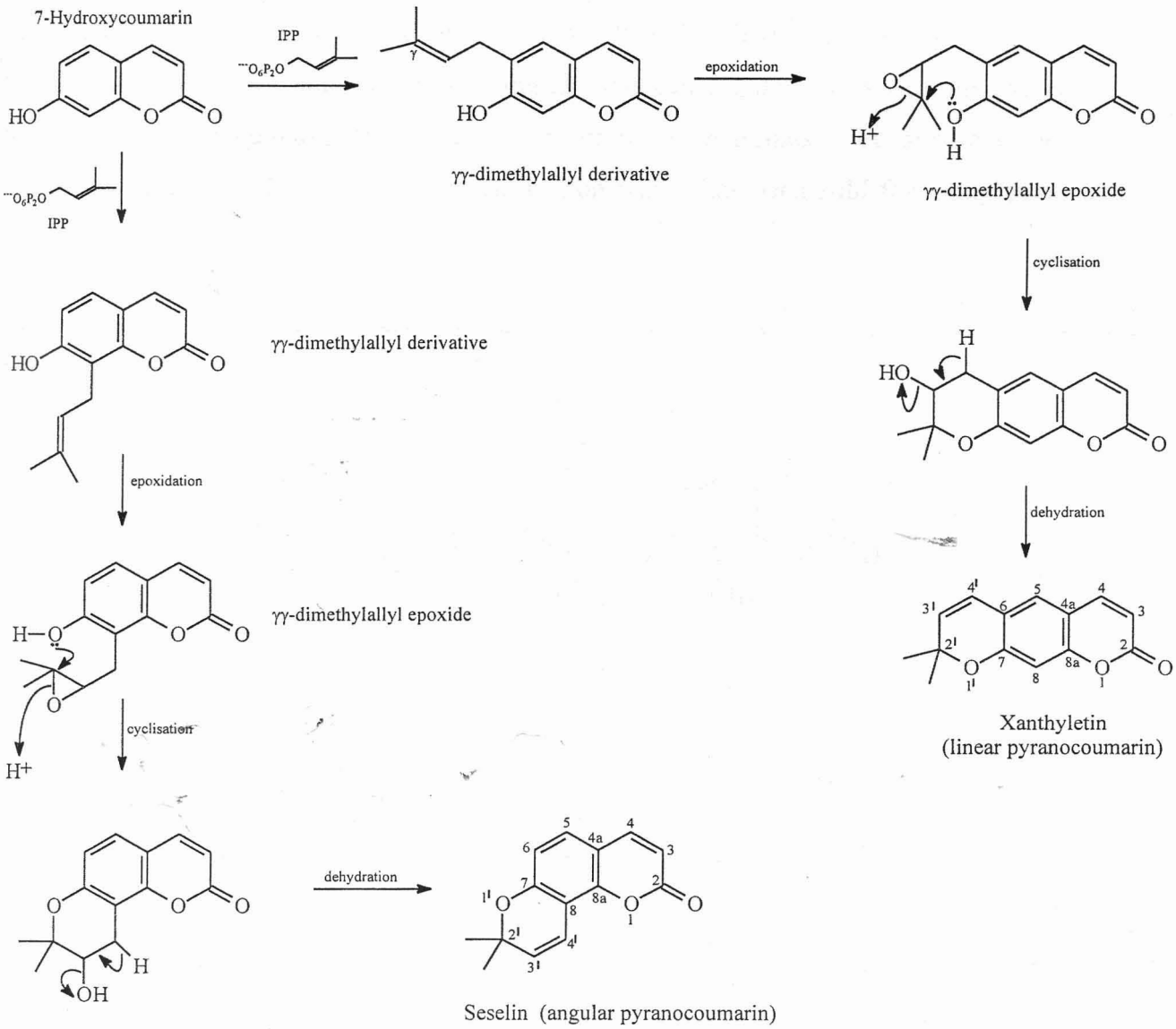
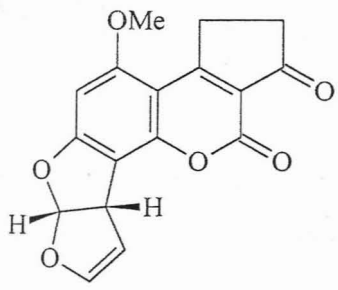


Figure 1.2.18: Pyranocoumarin formation via epoxide intermediate[16, 39, 41, 44]

All except two natural coumarins have been isolated from botanical sources. They are widespread in Angiosperms but rare in Gymnosperms and lower plants. Isolations have been especially successful in Apiaceae (previously Umbelliferae), Rutaceae, Fabaceae (previously Leguminosae), Orchidaceae and Asteraceae (previously Compositae) families, where coumarins have been reported to be found in all parts of the plant including roots, leaves, fruit, flowers, bark and heartwood[39, 42].

Biological Activity

Coumarins are physiologically active compounds having varied effects on living cells. Aflatoxins, metabolites of *Asperigillus flavus*, are intense liver poisons and among the most potent known carcinogens[40]. Aflotoxin B_1 (**10**) is such an example. Novobiocin (steptonivicin) (**11**) is a useful antibiotic, and dicoumarol (**12**) and other 4-hydroxycoumarins have anticoagulant activity[40]. The 3-phenylcoumarins have estrogenic activity. Psoralen (**13**) and its derivatives (simple linear furanocoumarins), have skin photosensitising abilities causing dermatitis with the exposure of skin to light[40, 45]. Coumarin (**14**) itself is a toxin to mammals. A synthetic derivative of 4-hydroxycoumarin (**15**) is an effective rat poison which has been sold for many years under the commercial name, Warfarin[45].



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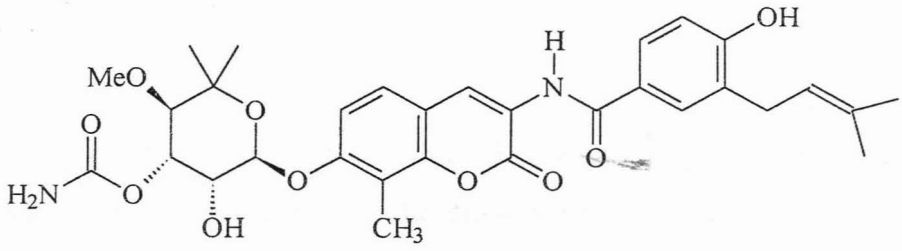

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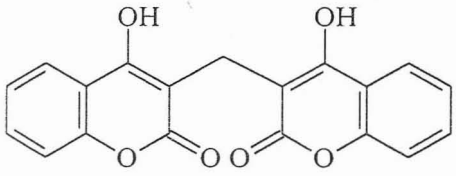

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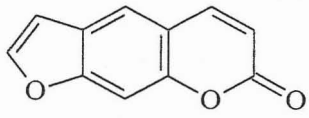

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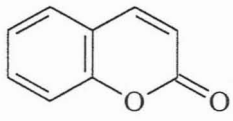

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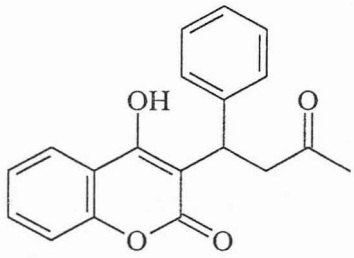

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1.3 THE ACETATE-MALONATE PATHWAY

Aromatic compounds are formed either by the shikimate or the acetate-malonate pathways, where the latter seems to dominate in microorganisms and fungi, and the former in higher plants[46]. The major products of the acetate-malonate pathway are fatty acids and their derivatives, and, to a lesser extent, various plant aromatics produced via polyketide intermediates. For this reason, the acetate-malonate pathway is also commonly referred to as the polyketide pathway. Compounds produced by this pathway include primary metabolites as well as various secondary metabolites which are more unusual compounds of varied structure and have restricted distribution. Such secondary metabolites are referred to directly as polyketides and are found in microorganisms and higher plants. They include simple and complex phenols, chromones, quinones, xanthones, flavonoids and numerous mycotoxins. Although very diverse, such compounds are linked by their common biogenetic origin[47, 48, 49]. The biosynthesis of chromones shall be elaborated on, as they were isolated in this work.

All living organisms contain an acetyl-CoA metabolic pool which is continuously depleted and replenished by various catabolic and anabolic processes[46]. The basic metabolic pool structure is illustrated in figure 1.3.1. Acetyl-CoA can be produced by fatty acid degradation, pyruvic acid decarboxylation as well as by certain amino acid degradations and all these processes occur in the cell[50]. During these catabolic processes, biochemical energy is released and stored in the form of ATP. ATP can then be used in anabolic metabolism resulting in an array of primary and secondary products. From the scheme outlined in figure 1.3.1 it may be seen that an array of secondary metabolites with very diverse structures are formed from acetic acid precursors.

In living organisms, acetic acid is activated as its thioester with coenzyme A forming acetyl-CoA, the structure of which is shown in figure 1.3.2[51]. Acetyl-CoA is a very reactive thioester with a unique chemical nature allowing it to take part in a wide variety of reactions. It exhibits both electrophilic and nucleophilic characteristics as illustrated in figure 1.3.3. The former results from the retention of the carbonyl group owing to the electronegative sulphur-atom preventing resonance stability around the thioester linkage. The carbonyl group can thus react in acyl-transfer reactions in which coenzyme A is released. On the other hand, the α-carbon atom displays nucleophilic properties, allowing carboxylation with CO_2-biotinylenzyme to give malonyl-CoA, a vital polyketide precursor, as well as condensation with oxaloacetate or acetoacetate-CoA to form

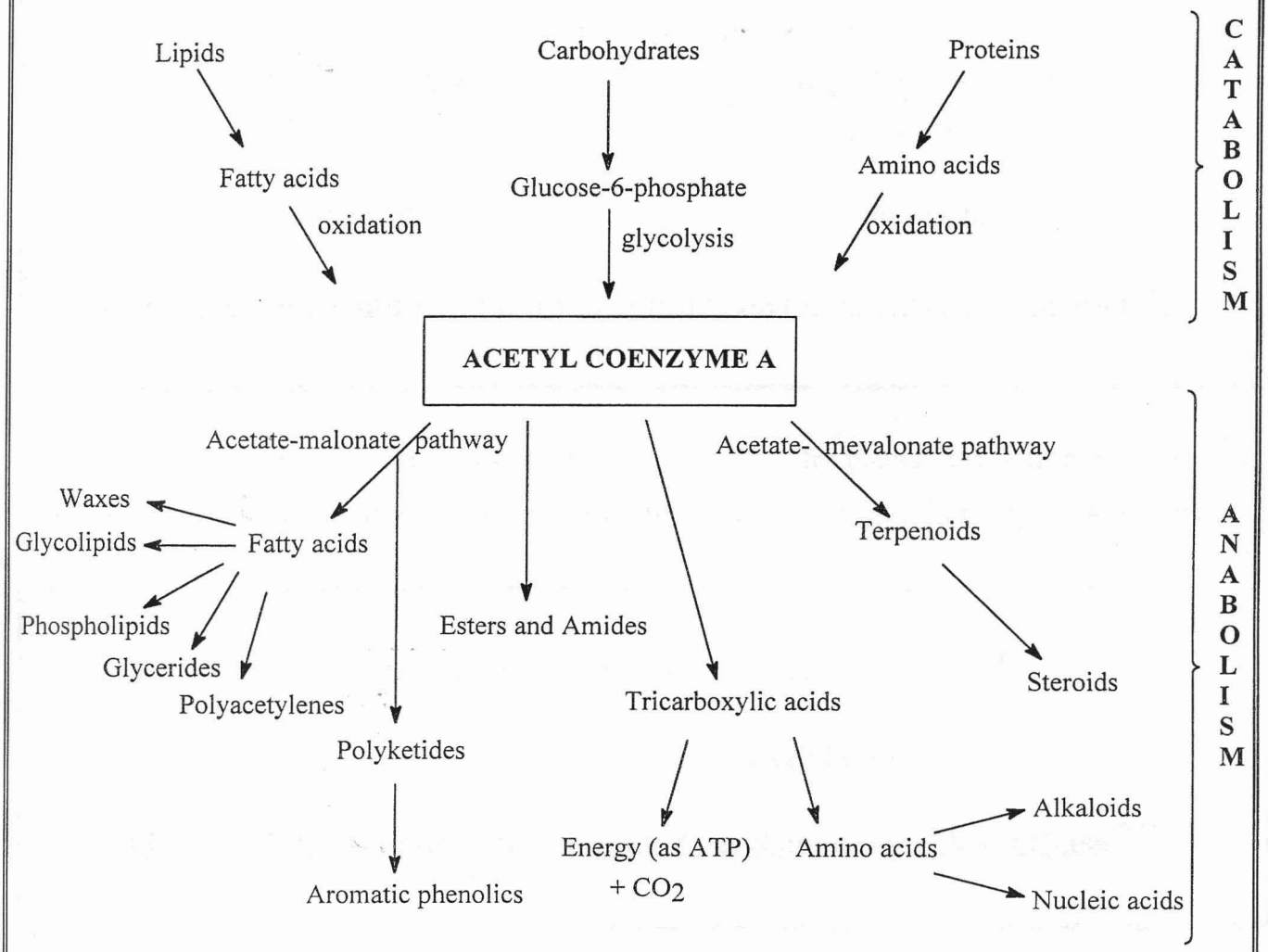


Figure 1.3.1: Role of Acetyl coenzyme A in metabolism[46, 52]

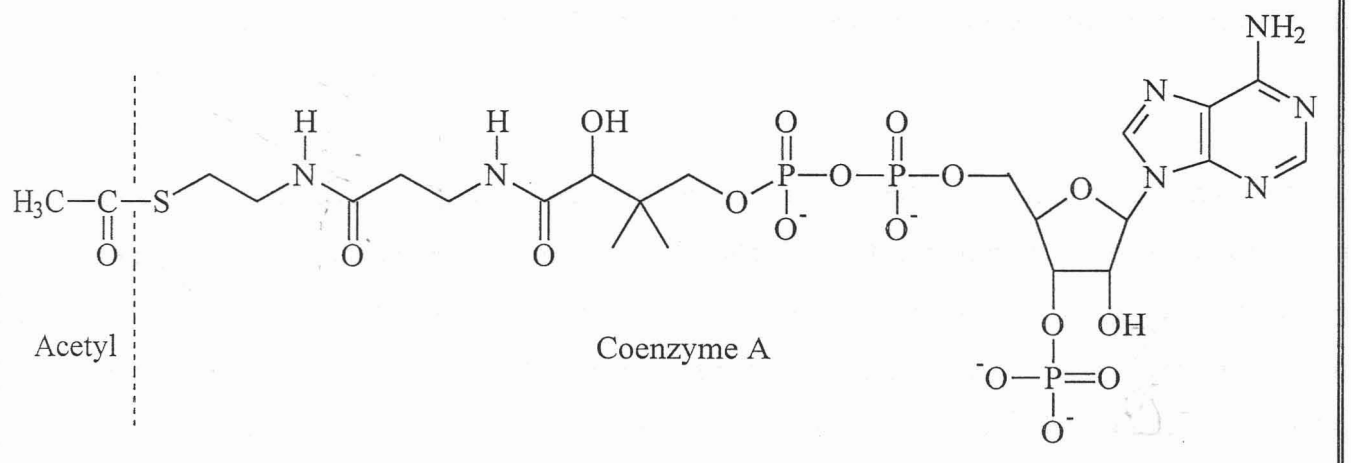


Figure 1.3.2: Acetyl coenzyme A[49, 52]

Figure 1.3.3: Electrophilic and nucleophilic Acetyl coenzyme A properties [52]

citrate or hydroxymethylglutaryl-CoA respectively[50, 52]. In plants, the enzyme acetyl-CoA synthase catalyses the formation of the thiol ester from acetate, as shown in figure 1.3.4 below.

Figure 1.3.4: Acetate activation by Acetyl coenzyme A synthase [46]

The Acetate Hypothesis and Polyketides

The acetate-malonate pathway, results in the joining together of linear chains of repeating acetate units known as polyketides. These metabolites contain within them, the $[-(CH_2-CO)-]_n$ unit which was recognised by Collie as early as 1905[49]. Only after 50 years did Birch independantly bring forward the acetate hypothesis, when realising that many secondary metabolites are formed with repeated CH_2-CO units based on acetate[49, 53].

Polyketide biogenesis involves acetyl-CoA starter units, which condense linearly with malonyl-CoA units via a head-to-tail linkage with elimination of water[51]. This process results in the formation of the poly-β-keto-acyl-CoA chain. Malonyl- CoA is formed by acetyl-CoA carboxylation resulting in α–carbon activation of the latter, which can then induce Claisen condensation via the formation of the malonyl carbanion. This carboxylation reaction is mediated by ATP with biotin as the CO_2 carrier, where the latter is bound to the ε–amino group of the lysine residue of the enzyme via its carboxyl group. The unstable N'-carboxybiotin intermediate forms in this reversible reaction of

biotin carboxylation in the presence of ATP. Stereochemical labelling investigations show *pro-R*-hydrogen loss from the coenzyme A species and further replacement by carbon dioxide with retention of configuration[1, 50, 53]. The mechanism of this carboxylation process and for the Claisen-type condensation are illustrated in figures 1.3.5 and 1.3.6 respectively, where the latter acetylation process proceeds with configurational inversion[1]. The chain extension unit in both cases is malonyl-CoA. The incorporation of only one acetyl-CoA unit, the starter unit and numerous malonyl-CoA units has been proven by labelling experiments[54].

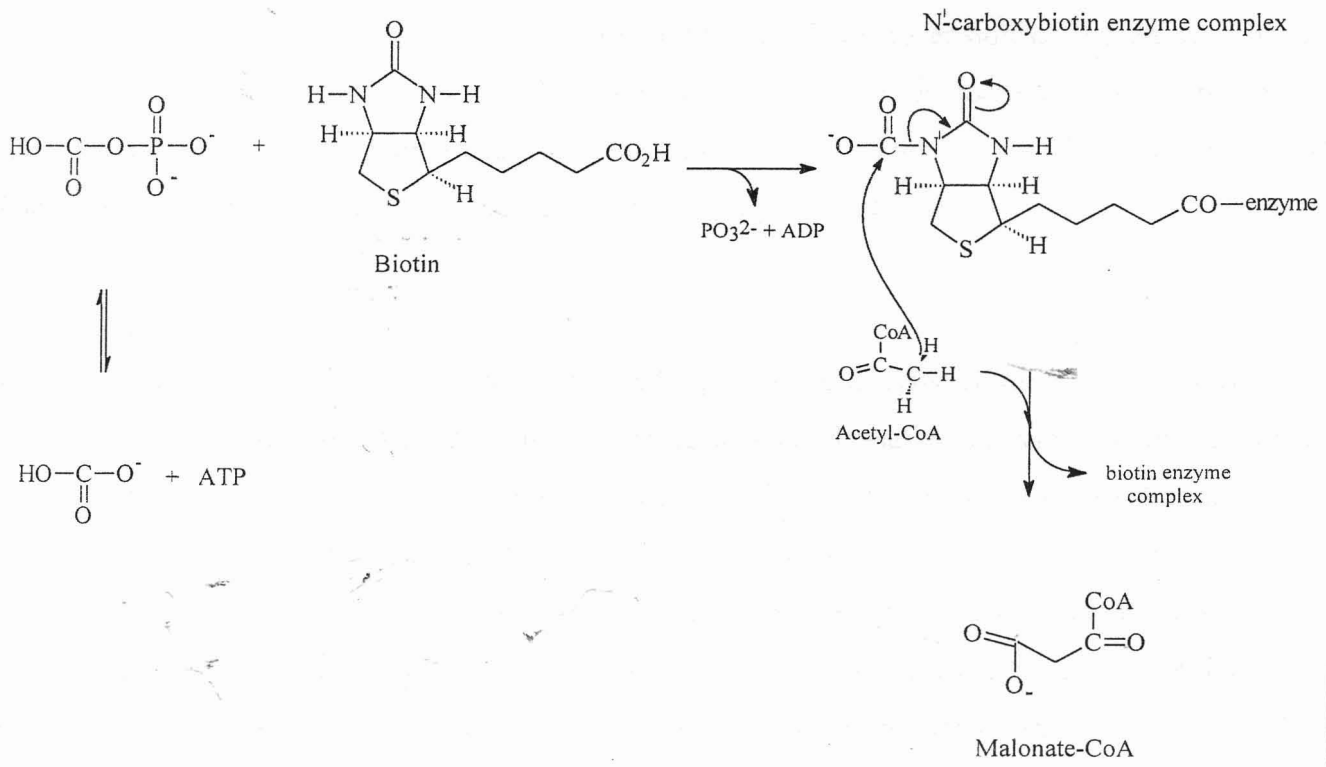


Figure 1.3.5: Mechanism of Acetyl Coenzyme A carboxylation[50]

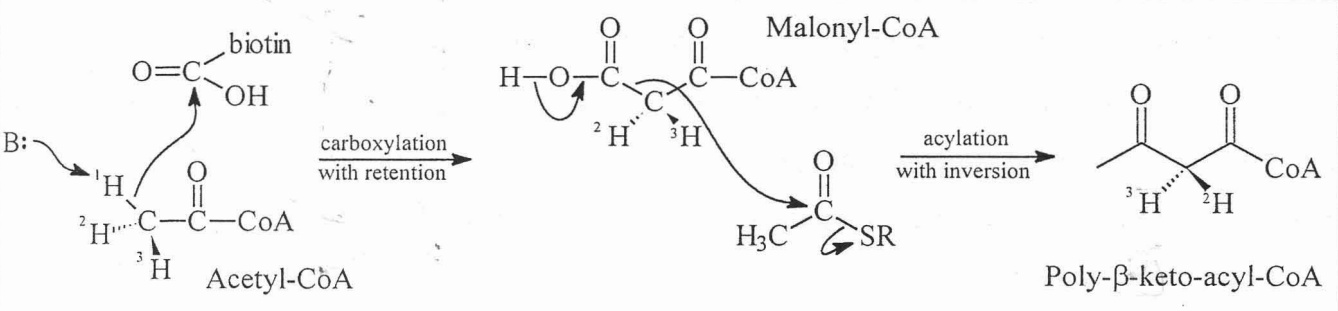


Figure 1.3.6: Claisen type condensation of Acetyl- and Malonyl-CoA units.[49, 50]

The polyketide intermediate is thus a poly-β-ketoacid which is very reactive as it contains active methylene groups (nucleophilic), as well as carbonyl groups (electrophilic) and is thus prone to cyclisation. It must, therefore, be kept stable prior to cyclisation in order to permit chain extension. This is achieved by the intermediate remaining enzyme bound in the enol form throughout the chain growth stage. Both the starter acetyl-CoA unit and the malonyl-CoA units are bound, prior to condensation, to enzyme thiol groups[46, 49, 53]. The enolate anions bind to appropriately situated amino acid sites and chelation with metal ions, as well as hydrogen bonding of oxygen functions of the polyketide with the enzyme also assist in stabilisation[50]. The C_2 unit condensation is, as mentioned previously, of the Claisen type, where a thiol bound acyl group condenses with a thiol bound malonyl unit on the same multienzyme complex. Chain extension of the enzyme bound complex is illustrated in figure 1.3.7. Thus, complete growth prior to cyclisation is ensured by the multicomplex enzymes which catalyse such processes. This theory of an enzyme bound intermediate is also supported by the fact that free polyketomethylene derivatives have not been detected[48].

Once the required chain length has been achieved, appropriate enzyme folding brings the relevant chain sites into close proximity inducing intramolecular condensation reactions which terminate into various phenolic products. These condensation reactions, illustrated in figure 1.3.8, are of the Claisen and aldol type, where only the latter results in double bond formation prior to enolisation, in the presence of dehydratase[48, 49]. Proton removal results in methylene group activation producing carbanions or enolates and carbonyl group polarisation provides their carbocation character[50]. Lactonisation and etherification type cyclisations can also occur and are illustrated in figure 1.3.8. Once cyclisation occurs the product is liberated from the enzyme. Polyketide chains which have the potential for polycyclic ring formation will rarely undergo single condensations only. Intermolecular condensation reactions between two polyketide chains is also rare, but does occur[54].

The oxygen bridges are formed during the condensation process either by lactonisation, (condensation between carboxylic and hydroxyl functions), or by etherification, (condensation between two hydroxyl functions). The former results in α-pyrone systems, producing coumarin and isocoumarin type products, and the latter in γ-pyrone systems producing chromones. The latter pathway is far more common in higher plants while the former occurs readily in microorganisms[54]. The mechanism for such cyclisation processes is illustrated in figure 1.3.9.

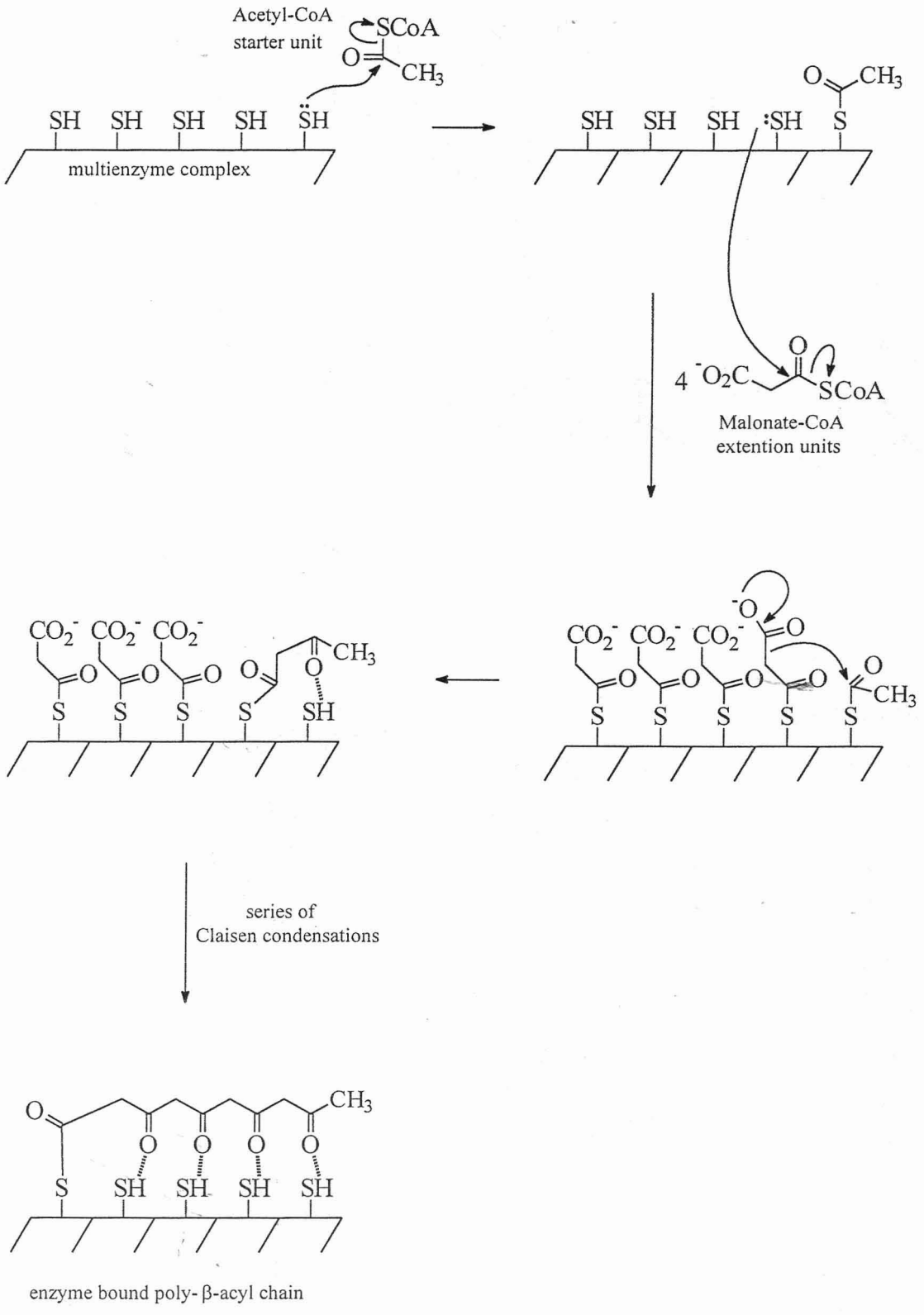


Figure 1.3.7: Chain extension on multienzyme complex[46, 49, 54]

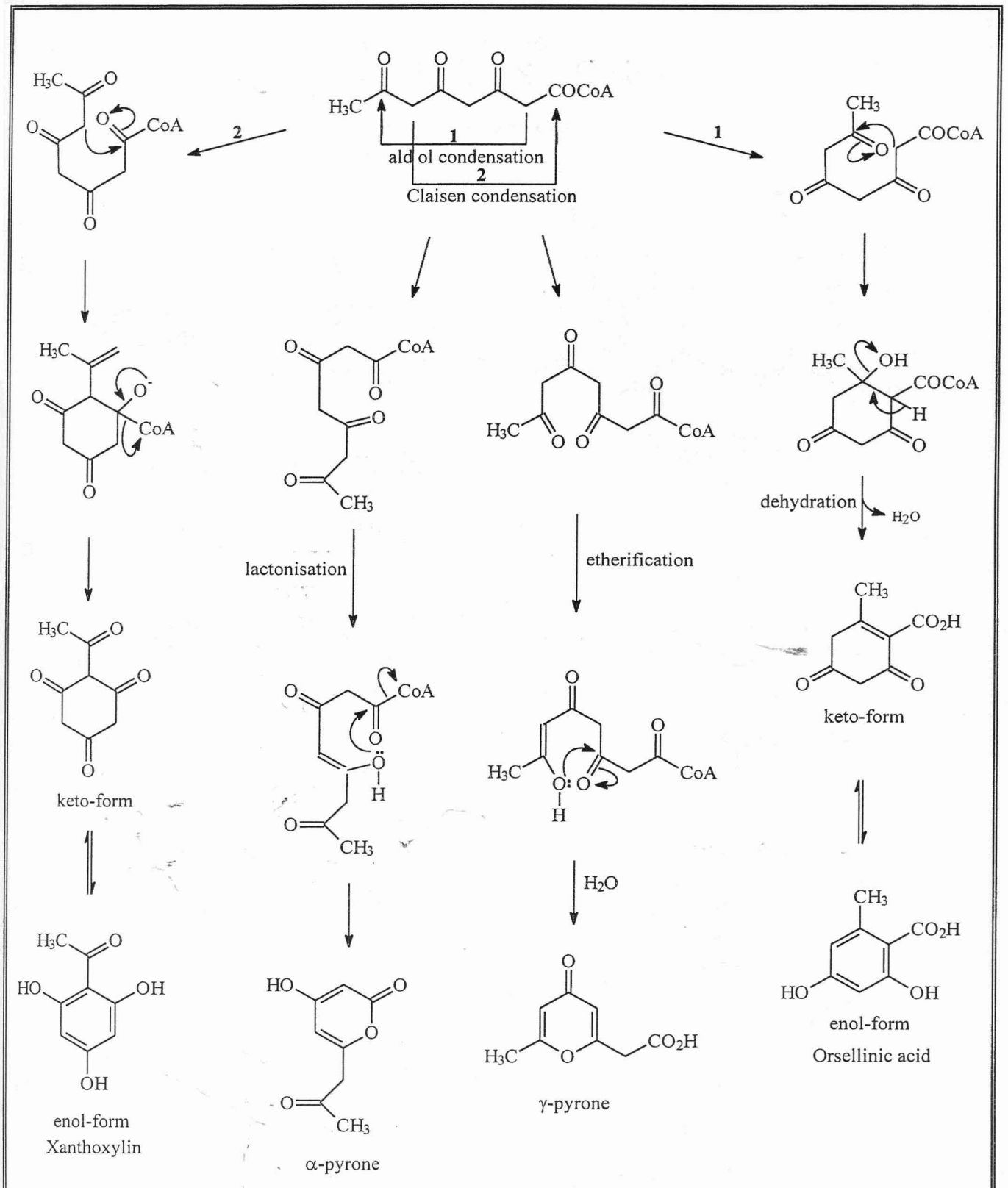


Figure 1.3.8: Tetraketide cyclisations to simple phenols and pyrones[50, 54]

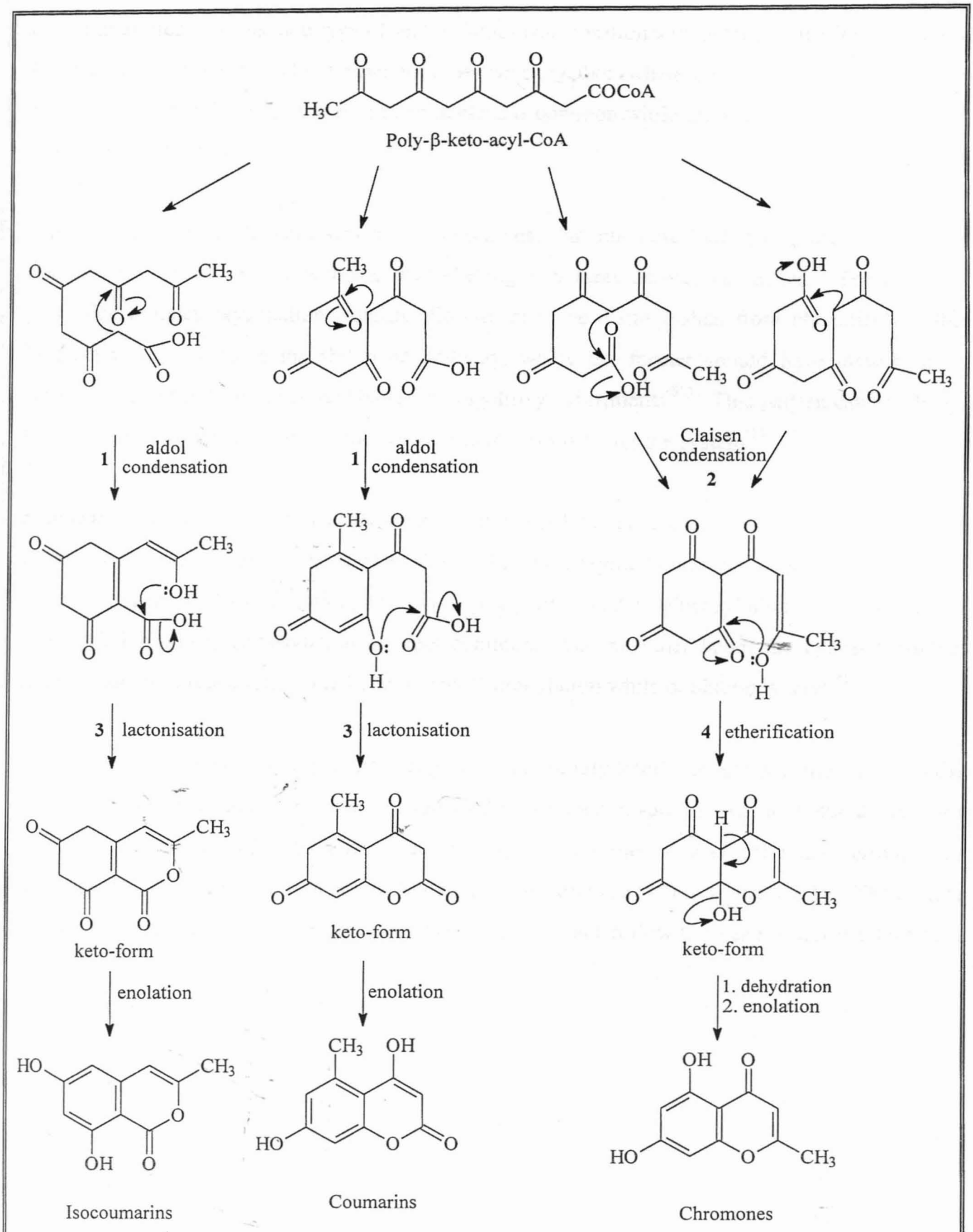


Figure 1.3.9: Chromone, Coumarin and Isocoumarin formation[47, 54]

It is common for condensation types 1 and 3, (aldol condensation and lactone formation) and 2 and 4, (Claisen condensation and etherification), to occur together, where the latter is more common in multi-ring systems[54]. Thus the chromone nucleus is common while the coumarin nucleus is a rare product of this pathway.

It is evident, from both these sets of condensations, that phenolic hydroxyls occur in alternate positions (ie: every second carbon), thus making structural derivations easier. These typical polyketide product oxygenation patterns allow them to be distinguished from phenolics of other biogenetic origin such as the shikimate pathway, where the former should have *meta*-hydroxy substituents, while the latter should have *ortho*-hydroxy substituents[50]. This pattern cannot always be relied on as oxygenative and reductive modifications may alter the system[51, 53].

Secondary modification reactions can occur during the polyketide intermediate stage as well as after cyclisation. Labelling experiments have shown that the enzyme bound polyketide intermediate is prone to oxidation, O-methylation, O- and C-prenylation and O-glucosylation, while reduction, C-methylation and C-glycosylation are less common. On the other hand, the cyclised product readily undergoes reduction, C-methylation and C-prenylation while oxidation is rare[54].

The acetate hypothesis thus allows the grouping of seemingly totally unrelated compounds, on the basis of biogenetic origin. It has also been of vital importance in various structural elucidations, for example, with the natural chromone, eleutherinol, whose structure was previously erroneously determined as (**16**). Eleutherinol is now known to have structure (**17**) in figure 1.3.10. The acetate hypothesis immediately rules out structure (**16**) as it does not follow the head-to-tail condensation rule[47, 49, 50].

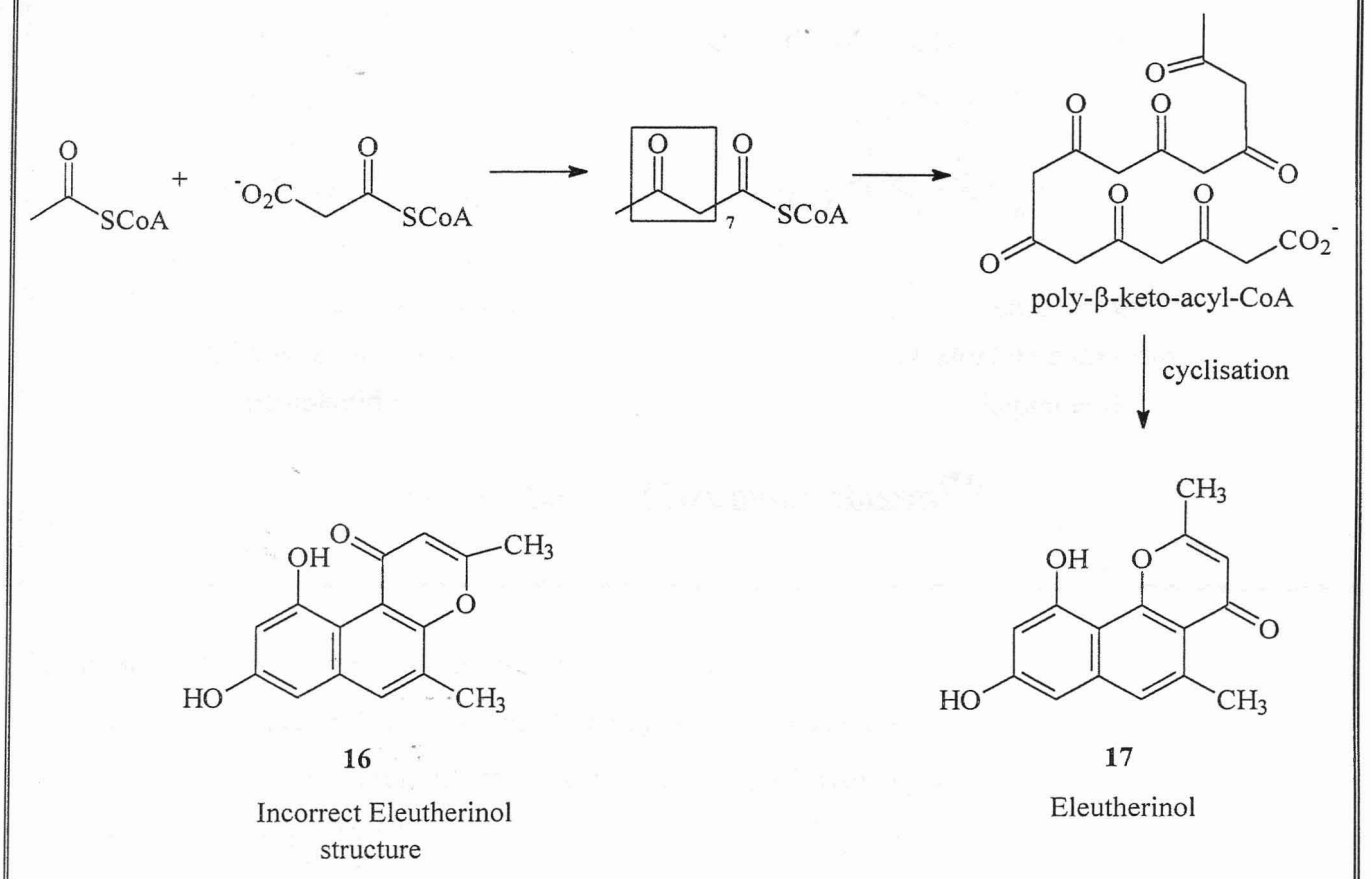
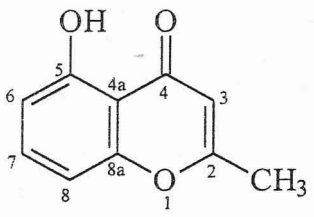


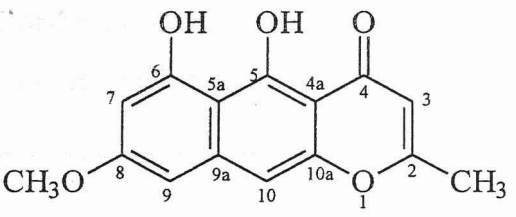
Figure 1.3.10: Biosynthesis of Eleutherinol[47, 49]

Chromones

Polyketides are divided into classes depending on chain length determined by the number of C_2 units present; triketides are considered to be the smallest molecule size. Tetra-, penta-, hepta- and octaketides are very common in nature, while tri-, hexa-, nona- and decaketides are rare[50]. Chromones, which all contain a γ-benzopyrone nucleus, fall under the penta- (simple chromones) and heptaketide (propanochromones) classes and are thus formed from the starter acetyl-CoA unit condensing either with four or six malonyl-CoA units respectively. Figure 1.3.11 illustrates examples of these most common chromone classes although others are known. The 2-methyl substituent and the 5,7-oxygenated positions are very common in natural chromones. This 2-methyl group originates from the starter acetate unit and acts as a marker essential in distinguishing between chromones and coumarins.



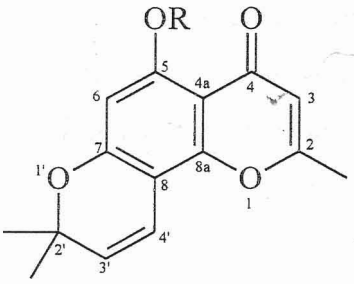
5-Hydroxy-2-methylchromone
(Daldimo coucentria)
pentaketide



Rubrofusarin
(Fusarium culmorum)
heptaketide

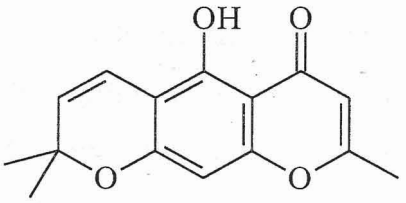
Figure 1.3.11: Chromone classes[54]

Heteroannulated chromones are formed in both linear and angular forms via the same mechanisms illustrated for isoprenoid cyclisation in coumarins. These are particularly common chromone natural products and include the widespread 5,7-dihydroxy-2-methylchromone derivatives illustrated in figure 1.3.12[55].



R=Me Methylallopteroxylin
or Perforatin A

R=H Allopteroxylin



Spatheliachromen

Figure 1.3.12: Chromone isoprenoid derivatives[55]

Cyclisation of the isoprenoid residue can also occur in a slightly different mode to produce a third, 7-membered, oxepin ring. Such chromones occur in nature, but are very rare. They have been isolated only from *Cedrelopsis grevei* and *Ptaeroxylon obliquum* of the Ptaeroxylaceae family[56]. The presence of such oxepin ring chromones in both these species, resulted in their

removal from the Meliaceae family, as no other species in the Meliaceae has been shown to produce such compounds[57]. Chromones are thus very important chemotaxonomic markers. The biosynthesis of ptaeroxylin, an oxepin ring chromone, is illustrated in figure 1.3.13 below.

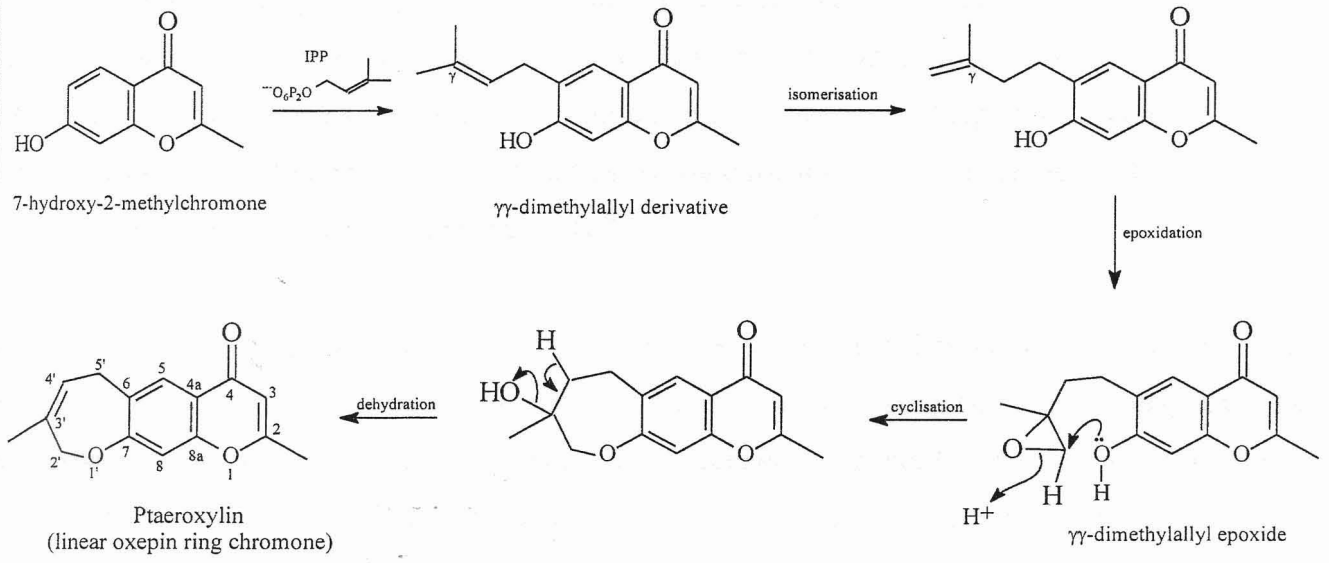
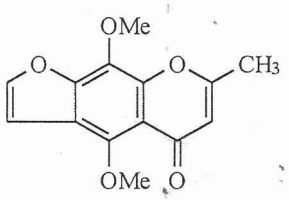


Figure 1.3.13: Biosynthesis of Oxepin ring Chromones

Biological Activity

Several plants used in folk medicine have active chromone ingredients, making these compounds pharmacologically interesting. The alkoxychromones, such as khellin (**18**), are important antispasmodics as well as a pain relievers, where their primary action lies in vasodilation[58]. 2-Methylchromone (**19**) possesses excellent vitamin K activity thus promoting blood coagulation. It also acts as a muscle relaxant and has vasodilatory effects on coronary blood vessels. 2-Ethylchromone (**20**) has insecticidal activity against body lice while 3-ethylchromone (**21**) has some activity as a coronary dilator[59].



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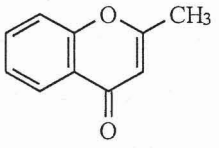

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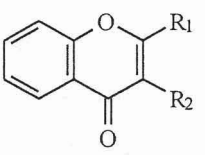


20 $R_1 = CH_2CH_3$; $R_2 = H$
21 $R_1 = H$; $R_2 = CH_2CH_3$

1.4 THE ACETATE-MEVALONATE PATHWAY

Terpenoids are secondary metabolites of very diverse structure and of wide and abundant distribution in the plant kingdom. All terpenoids are related by their common biogenetic origin and formal isoprene make-up. They are also commonly referred to as isoprenoids or isopentanoids and include all steroids. Several thousand terpenoids have been isolated from many genera of higher plants and microorganisms, as well as from animals. The term "terpenoid", is used for all compounds built up of isoprene units, regardless of functional groups. Terpenes on the other hand refer to hydrocarbon isoprene compounds only[60].

Terpenoids are classified according to the number of isoprene units they contain. Thus far, seven basic classes can be identified and include hemi-, mono-, sesqui-, di-, sester-, tri- and tetraterpenoids or carotenoids. Isoprene is not the biogenetic terpenoid precursor, and it is rare in nature. It was long thought not to occur naturally at all, but it has recently been isolated from higher plants[61, 62]. Many nonterpenoids contain isoprene residues owing to the high reactivity of this C_5 alkylating agent. Such compounds are referred to as hemiterpenoids, and include the pyranocoumarins and pyranochromones discussed in the previous subchapters[63, 64]. The biosynthesis of both tetra- and pentacyclic triterpenoids shall be elaborated on, as will that of limonoids (modified tetracyclic triterpenoids), as these are the compounds of interest in this work.

In the late nineteenth century, Wallach[63] was the first to notice that many compounds, especially essential oils or terpenes, were produced from common branched C_5 units. The isoprene rule was later forwarded by Ruzicka in Zurich in the 1920s while studying higher terpenoids[65]. It states that the terpene or terpenoid skeleton is composed of varying multiples of isoprene units, arranged in a 1,4-linkage or head-to-tail manner[65, 66]. This rule was found not to be strictly obeyed by all terpene natural products (eg: lanosterol) and was thus later re-examined to include these exceptions.

Only with the isolation of (+)-mevalonic acid (MVA), by Folkers *et al.* in 1956, did terpenoid biosynthesis become properly understood. By carrying out feeding experiments, labelled MVA was found to be the most effective precursor of sterol production[63, 67]. Further elucidation of the structure of the real C_5 terpenoid precursor unit followed in 1959 by Cornforth who characterised two active forms of isoprene, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which were both found to arise from MVA[66]. Acetic acid, as its acetyl-CoA derivative,

is the main MVA carbon source, where a minor route incorporating L-leucine as precursor has also been identified[66]. The terpenoid skeleton can thus be regarded as being acetate based. MVA is utilised only in terpenoid production, and in no other metabolic pathway.

Isoprenoid synthesis has four mechanistic phases:

- Mevalonate synthesis from thiol esters,
- IPP and DMAPP production,
- polyisoprenoid chain formation and
- cyclisation and optional rearrangement.

Mevalonate synthesis

Acetyl-CoA, in the presence of acetoacetyl-CoA thiolase, condenses to form acetoacetyl-CoA. Further condensation of the latter with enzyme bound acetyl-CoA, (via enzyme active site sulphhydryl group), yields the C_6 intermediate, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This aldol condensation between the acetyl-CoA methyl group and acetoacetyl-CoA carbonyl group is irreversible and occurs in a branched fashion in the presence of HMG-CoA synthase with CoA elimination. The reaction proceeds with inversion of configuration[63]. HMG-CoA reductase, in the presence of two NADPH molecules, then mediates hydride transfers from NADPH to (*S*)-HMG-CoA, via a two-step reduction mechanism[63, 68]. The irreversible reduction occurs at the thiolester group and results in (*R*)-(+)-mevalonate, which is then employed, in its only metabolic use, as the isoprenoid precursor[67]. Only (*R*)-MVA is utilised in metabolic processes, while (*S*)-MVA, (not produced in this case), is metabolically inert[66]. Mevalonate biosynthesis from L-leucine involves a biotin dependent carboxylation of senecioyl-CoA. The carbon atom which is added, is lost on mevalonate formation[68]. The mechanism involved in the formation of (*R*)-mevalonate via acetyl-CoA and L-leucine are illustrated in figure 1.4.1.

IPP and DMAPP production

Successive mevalonate phosphorylations occur via esterification of the primary hydroxyl group in the presence of ATP, producing 5-phosphomevalonate (mevalonate-5-P) and 5-pyrophosphomevalonate (mevalonate-5-PP), respectively. The first phosphorylation is mediated by mevalonate kinase and the second by phosphomevalonate kinase. The loss of the C-1 carboxyl group in mevalonate-5-PP yields the main building molecule in terpene synthesis, Δ^3-isopentenyl pyrophosphate (IPP), in the presence of ATP and pyrophosphomevalonate decarboxylase and with

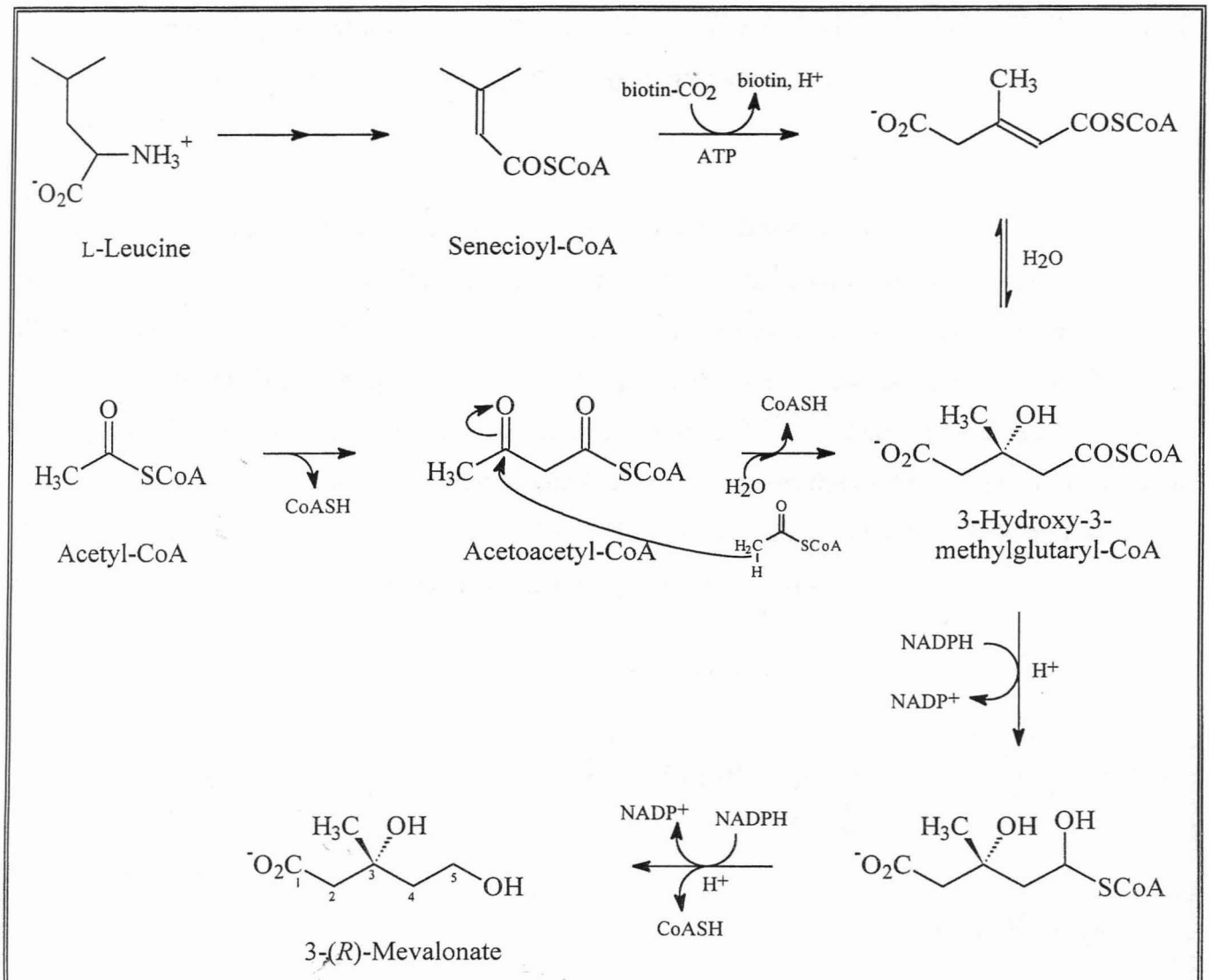


Figure 1.4.1: Mechanistic synthesis of 3-(*R*)-Mevalonate[63, 66, 68]

the release of ADP, phosphoric acid (inorganic phosphate, P^i) and CO_2. This decarboxylation-dehydration reaction involves a tertiary phosphate intermediate. The inability to isolate this intermediate favours a concerted mechanism for the above process, involving a *trans*-elimination of the carboxyl and hydroxyl groups in the presence of ATP[61, 66, 67].

Mevalonate has three prochiral methylenes at C-2, C-4 and C-5. Stereospecific labelling experiments have proven the mevalonate-5-PP decarboxylation to occur via a *trans*-elimination of the carboxyl and tertiary hydroxyl groups. This results in the IPP *pro-R*-hydrogen becoming *cis*-orientated in relation to the C-3 methyl group[63, 67]. Thus, decarboxylation is an E_2 elimination process occuring with antiperiplanar leaving groups[66]. At this point it is important to mention the

change in numbering nomenclature on IPP formation. The C-5 and C-4 atoms in mevalonate become C-1 and C-2 in IPP respectively, with inversion of configuration at C-2 as is expected for such reactions[67].

The Δ^3 IPP formed, isomerises via a 1,3-allylic shift to DMAPP, where the latter possesses electrophilic and the former, nucleophilic properties. This isomerisation occurs in the presence of IPP isomerase and is one of the few reversible reactions in terpenoid synthesis and favours DMAPP production. It involves the removal of the 4-*pro*-*S*-hydrogen of mevalonate, or the 2-*pro*-*R*-hydrogen of IPP via the *re*-face in the presence of isomerase[66, 67, 69]. Thus there is retention of the 2-*pro*-*S*-hydrogen in the latter. The mevalonate C-2 thus produces the olefinic methylene group of IPP and the *trans*-methyl group of DMAPP. The mechanism involved in IPP and DMAPP production from 3-(*R*)-mevalonate is illustrated in figure 1.4.2 below.

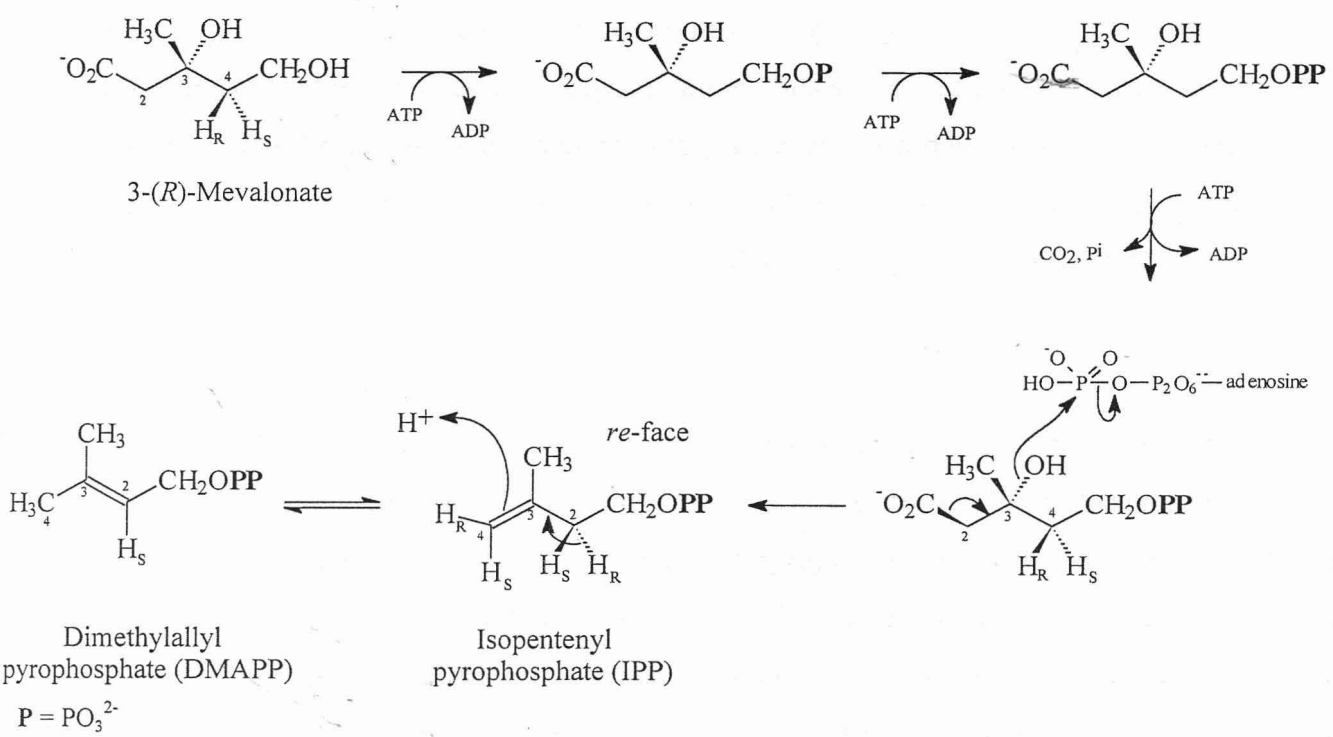


Figure 1.4.2: IPP and DMAPP formation[63, 66, 67, 69]

Polyisoprene chain formation

DMAPP acts as the starter unit and IPP as the chain extension unit in terpenoid biosynthesis, and condensation occurs via a head-to-tail mechanism[65, 68]. Owing to its allylic phosphate group,

DMAPP acts as a very reactive alkylating agent as phosphate is an excellent leaving group[63]. Mechanistically, prenylation reactions involve the ionisation of the allylic pyrophosphate of DMAPP, followed by condensation with IPP from the *si*-face, and 2-*pro*-*R*-hydrogen loss from the adduct. The first condensation of IPP with DMAPP is catalysed by geranyltransferase and occurs with pyrophosphate loss from DMAPP and proton loss from the adduct, producing a C_{10} compound, geranyl pyrophosphate (GPP), as illustrated in figure 1.4.3. GPP possesses the reactive allyl pyrophosphate and, in the presence of farnesyltransferase, can condense with a second IPP molecule in the same way, resulting in the C_{15} unit, farnesyl pyrophosphate (FPP). Both the above transferase reactions involve proton removal, where the 2-*pro*-*R* and 6-*pro*-*R*-hydrogens are lost respectively in the same fashion as for IPP isomerisation. With the formation of the new carbon-carbon bond, inversion of configuration occurs at the allylic carbon atom, C-5, of DMAPP, as expected for such S_N2 displacement reactions[66, 67]. Thus a new 2,3-(*E*)-double bond forms with each condensation.

FPP can then condense with another IPP molecule forming geranylgeranyl pyrophosphate (GGPP). Figure 1.4.3 illustrates these processes. Polymerisation ends at various points, namely the C_{10} ,C_{15} and C_{20} stages. The linear products produced are precursors to mono-, sesqui- and diterpenoids respectively. The C_{30} (triterpenoids) and C_{40} compounds (carotenoids) are formed via a tail-to-tail linkage of two FPP or two GGPP units, producing squalene and phytoene respectively.

Tetracyclic Triterpenoids

Squalene itself was first isolated from shark liver (*Squalus*), but was later found to be universal[69]. It is the C_{30} parent compound of all triterpenoids in which two all-*trans* FPP molecules condense in a tail-to-tail manner[60]. Biosynthesis of the presqualene pyrophosphate (PSPP) intermediate occurs by initial alkylation of the 2,3-double bond of one FPP unit by another, with inversion at C-1 in the former. 1-*pro*-*S*-Hydrogen elimination and further dephosphorylation results in the cyclopropane unit of presqualene which then undergoes ring expansion and configurational inversion at C-4 yielding a cyclobutyl system with a second pyrophosphate elimination. The cyclobutyl carbonium ion then collapses in the presence of squalene synthetase and NADPH with 4-*pro*-*S*-hydride transfer from the latter resulting in the linear all-*trans* squalene (the mechanistic detail involved is illustrated in figure 1.4.4)[63, 61].

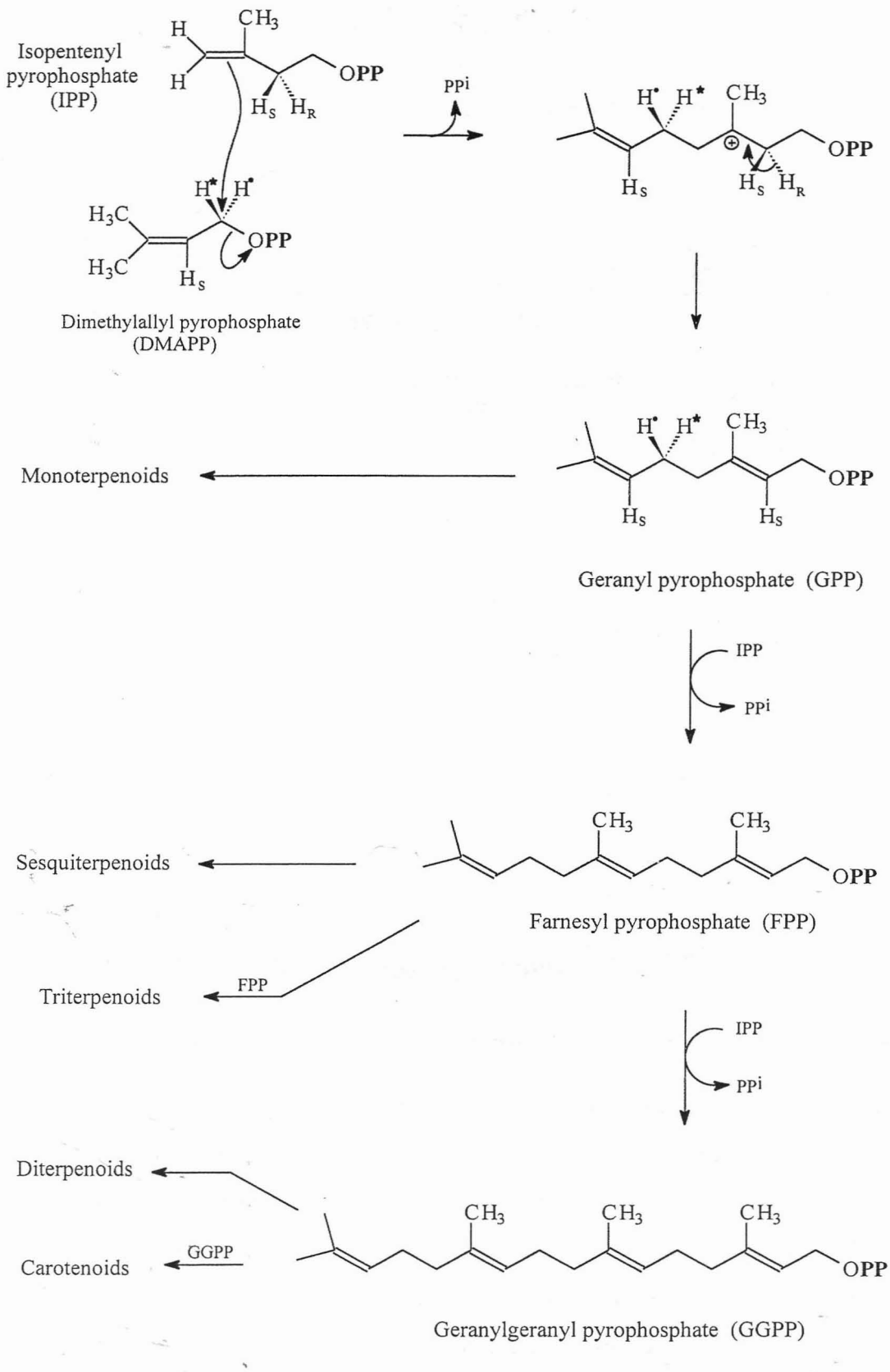


Figure 1.4.3: Formation of the various Terpenoid classes [63, 67, 68]

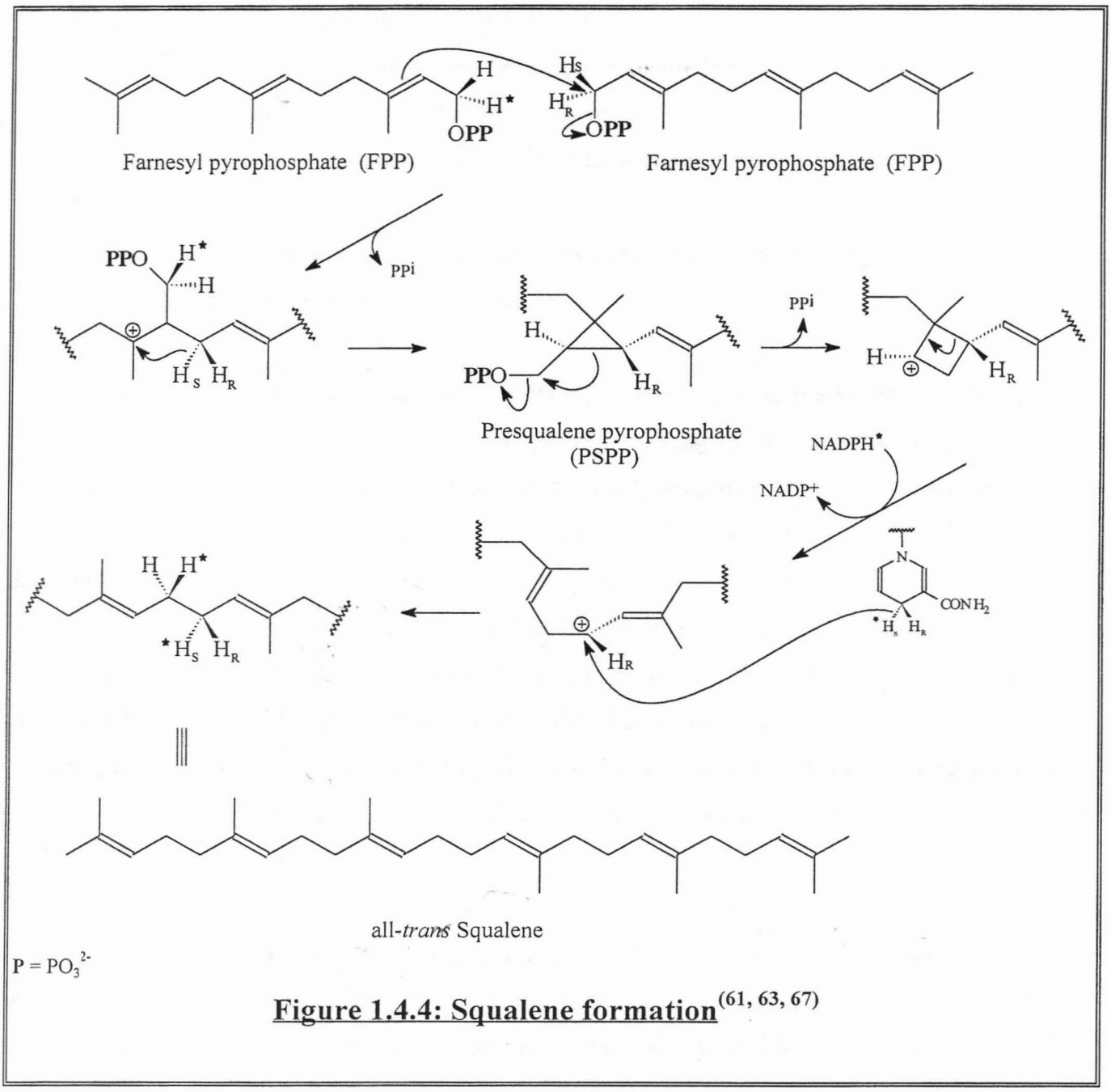


Figure 1.4.4: Squalene formation[61, 63, 67]

Before cyclisation, squalene is epoxidised forming the 2,3-epoxysqualene derivative in the presence of squalene epoxidase (a mono-oxygenase), molecular oxygen and NADPH. Proton attack on the epoxide ring initiates cyclisation via a carbocation intermediate, thus initiation always occurs at the tertiary carbon of the terminal double bond of squalene[66]. A series of concerted cationic cyclisations then occurs via the generation of allylic cations acting as intramolecular alkylating agents. This process is stereospecifically mediated by the cyclases. The C-20 carbocation resulting allows Wagner-Meerwein type reactions (1,2-shifts), to occur. All the 1,2-shifts described occur as a concerted series where each group is antiparallel to the successive and preceding one.

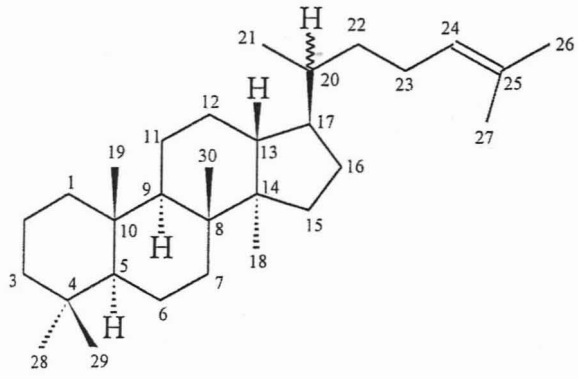
Three basic steps are involved in squalene cyclisation:

- For every cyclisation process, the squalene molecule is folded on the enzyme surface in a series of chair and / or boat like conformations.
- Cyclisation involves planar *trans*-addition to olefinic double bonds via a concerted mechanism.
- On carbocation formation, a series of Wagner-Meerwein rearrangements and hydride shifts follow when sterically favoured.

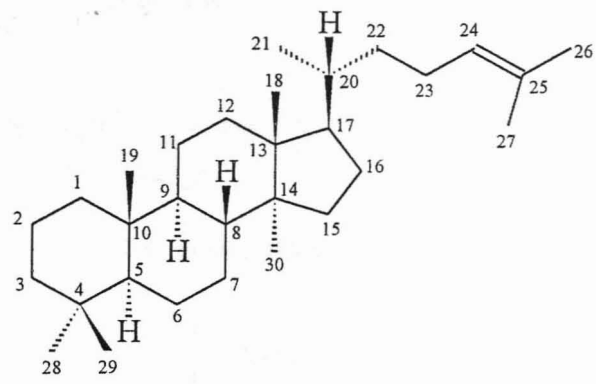
Tritepenoids have few skeletal variations even though numerous squalene folding orientations are possible. This lack of skeletal diversity is partially owing to the fact that squalene, unlike lower molecular weight terpenoid precursors, is not phosphorylated thus minimising cyclisation modes through lack of a good leaving group[66]. Generally, rings A, B and C are six-membered and if a C-3 hydroxyl group is present, it is almost always equatorial[63]. The six carbon-carbon double bonds allow for multiple cyclisations resulting in two main triterpenoid classes, tetracyclics, (including sterols), and pentacyclics, where the latter are more widely distributed. No mono- or dicyclic triterpenoids are found, while some tricyclics have been isolated[60]. These latter compounds have undergone only partial cyclisation and no Wagner-Meerwein rearrangements as simultaneous cyclisations began at both terminal squalene points leaving the potential central rings unclosed[64].

Tetracyclic terpenoids have four rings where rings A, B and C are six-membered and ring D is five membered. This latter occurrence is owing to the Markovnikov mechanism of double bond attack on the electrophilic C-13. However, ring C is formed by anti-Markovnikov attack. The thermodynamically favoured Markovnikov mechanism dominates in the absence of enzymes[66]. Triterpenoid formation has, via labelling experiments, been proven to result from cyclisation of the 3-(*S*)-squalene-2,3-epoxide isomer[65]. Ring-A forms first via a S_N2 mechanism involving the Δ^6-double bond, and is followed by a series of carbocationic intermediates resulting in further ring formations. Methyl groups do not in any way participate in cyclisation[70]. The two basic tetracyclic skeletons are the dammarane and lanostane systems, illustrated in figure 1.4.5.

The dammaranediols, euphol and tirucallol are all produced from the same cationic intermediate, where the all-*trans* squalene precursor is aligned in a chair-chair-chair-boat orientation. The former diols undergo no Wagner-Meerwein shifts as direct stabilisation occurs in the presence of water



Dammarane (20*R* or 20*S*)



Lanostane

Figure 1.4.5: The common Dammarane and Lanostane skeletons[63,64, 66]

resulting in the C-20 hydroxyl, as illustrated in figure 1.4.6. The latter two systems do undergo skeletal rearrangements. Their biosynthesis involves loss of the 9α-proton resulting in Δ^8-double bond formation. The carbocation formed at C-20 initiates the following Wagner-Meerwein 1,2-shifts to occur in the order described: H(17α → 20), H(13β → 17β), CH_3(14α → 13α) and CH_3(8β → 14β)[63, 66]. The mechanisms involved in the formation of such triterpenoids are also illustrated in figure 1.4.6. The secondary modifications involved in the production of the limonoid type triterpenoids occur with extensive degradation involving side chain transformation into a furanyl ring as well as extensive oxidation of the ring systems. In this work, limonoids are of importance and will thus be elaborated on at a later stage.

Pentacyclic Triterpenoids

In higher plants, triterpenoids can be pentacyclic and, as for tetracyclics, are formed from the all-*trans* squalene precursor, where the epoxy-squalene chair-chair-chair-boat orientated intermediate is most common. The cationic intermediate is of the same type as for euphol and tirucallol formation. In the biosynthesis of such five-ring systems, carbocation stabilisation occurs only after ring-D expansion to a six membered ring, and side chain cyclisation to a five or six membered ring. Stabilisation then occurs by deprotonation, followed by optional Wagner-Meerwein rearrangement[69]. This results in products of the lupeol and β-amyrin (oleane) type skeletons, as illustrated in figure 1.4.7. The latter compound is of importance in this work.

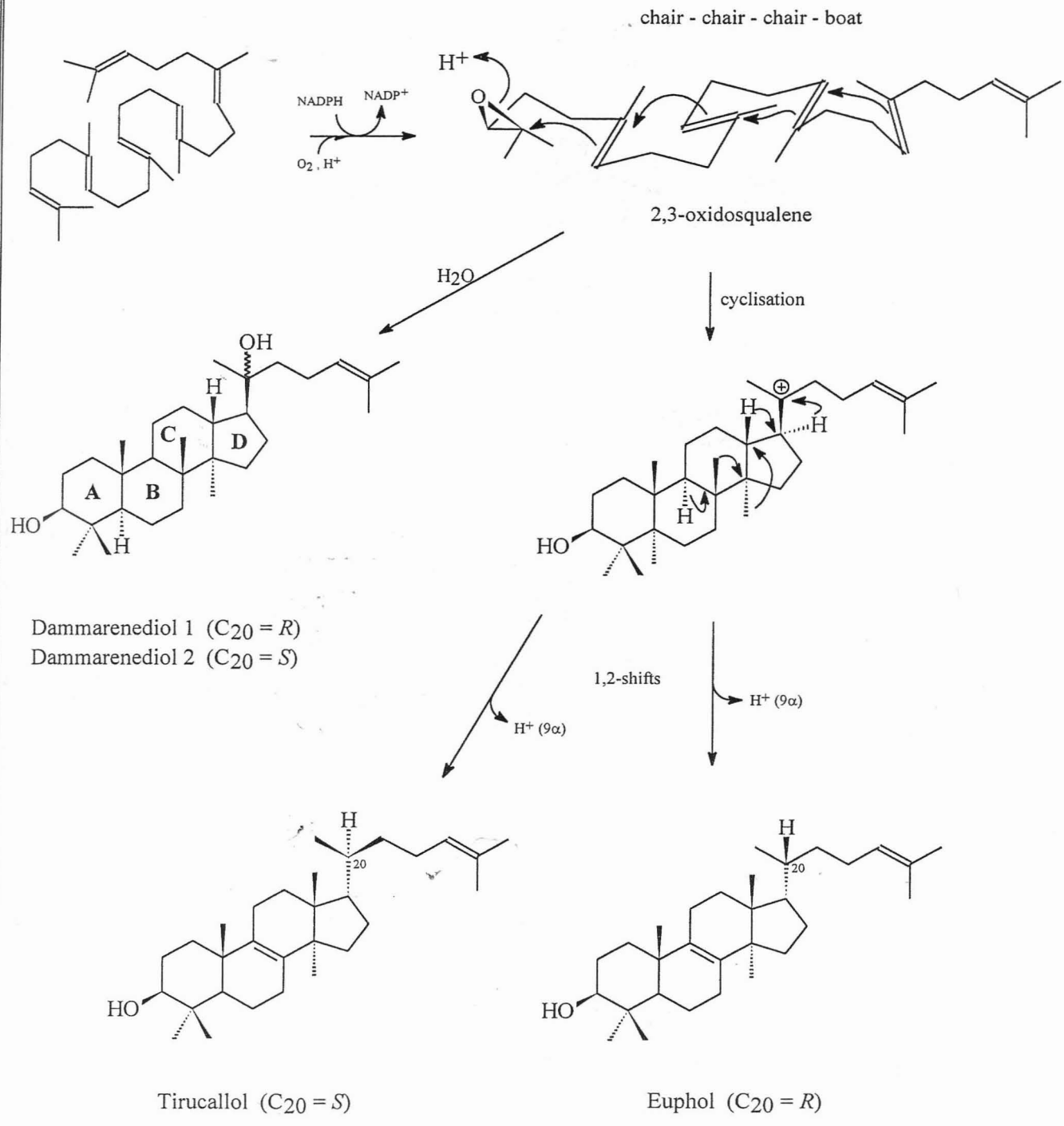


Figure 1.4.6: Euphol, Tirucallol and Dammarenediol formation[63, 66]

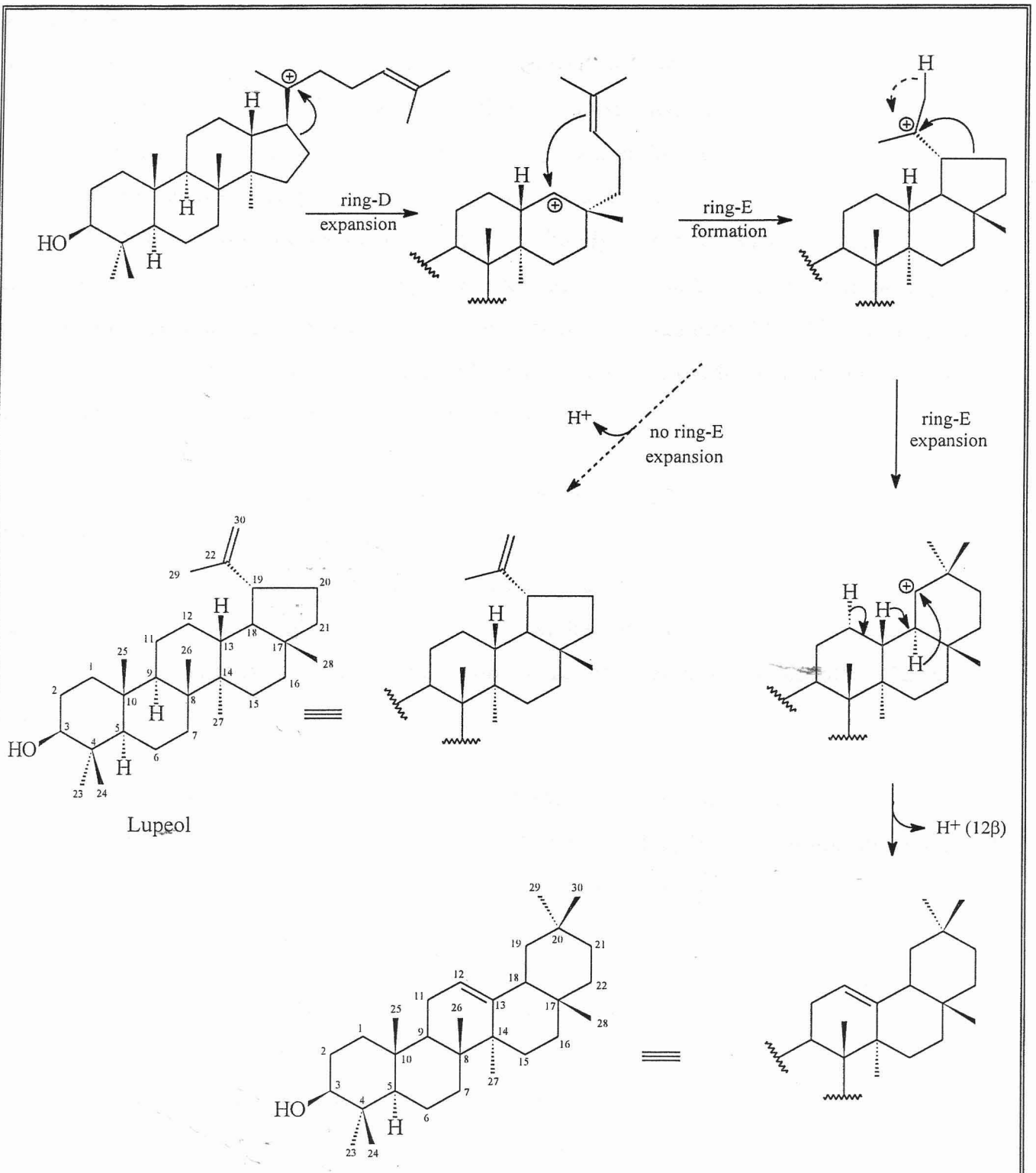


Figure 1.4.7: Lupeol and β-Amyrin biosynthesis[62, 63, 64, 66]

Most pentacyclic triterpenoids in plants have a C-3 hydroxyl with a β-configuration. Very rare cases exhibit the α-configuration or the absence this C-3 hydroxyl altogether[64].

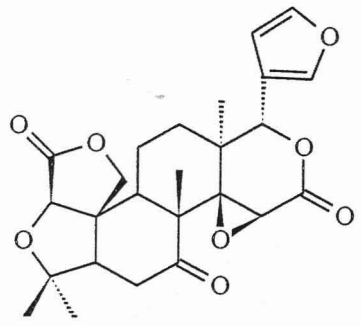
Limonoids

The families Rutaceae, Meliaceae and Cneoraceae, as well as many Simaroubaceae species are well known for containing limonoids, a group of highly oxidised terpenoids. Limonoids are altered tetracyclic triterpenoids characterised by side chain cyclisation into a C-17α furanyl ring with loss of four carbon atoms. They are thus composed of a C_{22} , 4,4,8-trimethyl-17-furanylsteroid nucleus[71]. It is common for such compounds to be highly oxidised. Such modifications include oxidative opening of one or more rings, as well as further lactone formation or alternative recyclisation. Limonoid biogenetic precursors are known as protolimonoids. They contain highly oxidised, uncyclised or partially cyclised C_8 side chains which are the furanyl ring precursors[72, 73]. These compounds have been found to occur with limonoids.

Limonoids were given their name from the compound limonin (**22**). Although limonin was not the first limonoid to be isolated, it was the first to be structurally elucidated. Limonin was isolated from *Citrus* fruits (Rutaceae) and its structure was elucidated in 1960 by Arigoni *et al.*. Gedunin (**23**), was the first limonoid isolated from the Meliaceae (1950s), from *Entandrophragma angolense*, a West African tree[73], and these compounds are therefore occasionally referred to as meliacins.

Limonoids are subdivided into various groups depending on which of the four rings in the triterpene nucleus have undergone oxidation. If protolimonoids are included, 10 such groups are recognised, where group 4 is subdivided into three classes[72]. These groups are listed below.

Group 1. Protolimonoids
Group 2. Havanensin Group (All rings intact)
Group 3. Gedunin Group (Ring D opened)
Group 4a. Limonoids with Rings B and D opened
Group 4b. Mexicanolide Group (Ring B recyclised)
Group 4c. Phragmalin Group (Ring B recyclised and Ring A bridged)
Group 5. Methyl Ivorensate Group (Rings A, B and D opened)
Group 6. Obacunol Group (Rings A and D opened)
Group 7. Nimbin Group (Ring C opened)
Group 8. Toonafolin Group (Ring B opened)
Group 9. Evodulone Group (Ring A opened)
Group 10. Prieurianin Group (Rings A and B opened)



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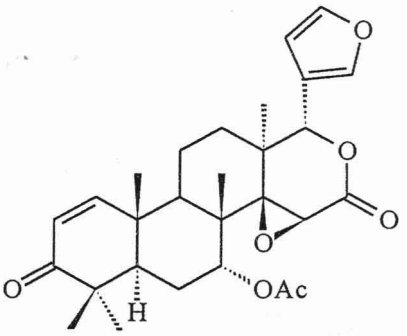

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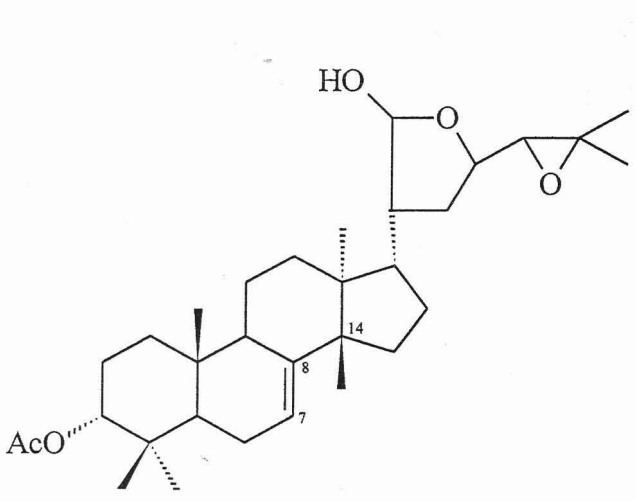
Only the biosynthesis of protolimonoids and Group 4c limonoids, which are of importance in this study, shall be discussed. The biogenetic pathways proposed for the secondary modifications leading to the above groups are mostly hypothetical as very little tracer work has been done owing to the fact that limonoids are normally extracted from timber. The pathways which follow are based mainly on the relationships between isolated compounds as well as synthetic evidence. Only *Citrus* limonoid biosynthesis has been proven by feeding experiments[74].

Limonoids are thought to be produced from Δ^7-tirucallol [C-20(*S*)] or Δ^7-euphol [C-20(*R*)] precursors. This is deduced from the fact that most protolimonoids isolated have a 20α-H configuration, while few with a 20β-H are found. Only *Melia* has been found to produce protolimonoids of the latter configuration[75]. Once this basic limonoid skeleton forms, various oxidative processes and skeletal rearrangements can occur, resulting in such varied structures, that the ten classification groups were introduced. The most common modification is D-ring oxidation to a lactone, producing the D-*seco* limonoid groups. Such oxidative changes occur in the presence of peroxidases and involve either double bond epoxidations or Baeyer-Villiger attacks on ketones followed by spontaneous rearrangements[71].

Group 1. Protolimonoids: Protolimonoids are tetracyclic triterpenoids with an intact, oxidised side chain which is often cyclised to an ether ring. Compounds of this type can be subdivided into two classes, depending on whether the *apo*-change has occurred or not. Protolimonoids with no *apo*-change have skeletons resembling that of tirucallol and euphol, thus containing a Δ^7-double bond and a β-methyl group at C-14, evident in turraeanthin (**24**). With the occurrence of the *apo*-change, there is a double bond shift to Δ^{14}, a β-methyl group occurs at C-8 and an α-hydroxyl group is found at C-7, as illustrated in grandifoliolenone (**25**). This second subgroup is referred to as the *apo* group[75].



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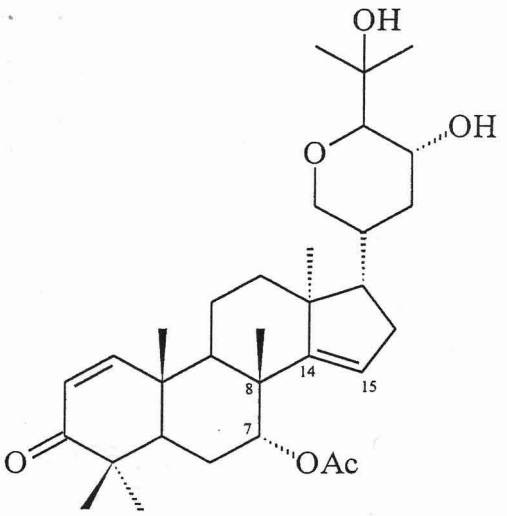

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The *apo*-change, which is mechanistically illustrated in figure 1.4.8, involves epoxidation of the Δ^7-double bond to a 7,8-epoxide. Epoxide opening then induces a Wagner-Meerwein 1,2-shift of the C-14 methyl group to C-8, with hydroxyl formation at C-7 and Δ^{14}- double bond formation. The *apo*-change thus involves the conversion of the original Δ^7-14-methyl nucleus into a 7α-hydroxy-8-methyl-Δ^{14} nucleus resulting in the 4,4,8-trimethylsteroid skeleton[72]. The fact that cyclised and uncyclised side chains occur in both groups indicates that there is no specific stage in which the *apo*-change occurs. What is known is that it occurs prior to furanyl ring formation as no compounds with a Δ^7-double bond and furanyl ring have been isolated, while many systems with a Δ^{14}-double bond, but with or without the furanyl ring, are found[75].

Side chain cyclisation occurs with loss of four carbon atoms forming the β-substituted furanyl ring at C-17α[71]. This carbon loss gives limonoids their alternative name, tetranortriterpenoids. Furanyl ring formation involves a stepwise sequence of oxidations, beginning with the tirucallol side chain forming a C-21 aldehyde, a C-23 hydroxyl group and a 24,25-epoxide. Spontaneous cyclisation occurs resulting in the turraeanthin side chain. This is followed by further oxidation yielding the C-24 ketone, which undergoes Bayer-Villager oxidative cleavage to form the C-17α furane[75]. The mechanism for these rearrangements is illustrated in figure 1.4.8.

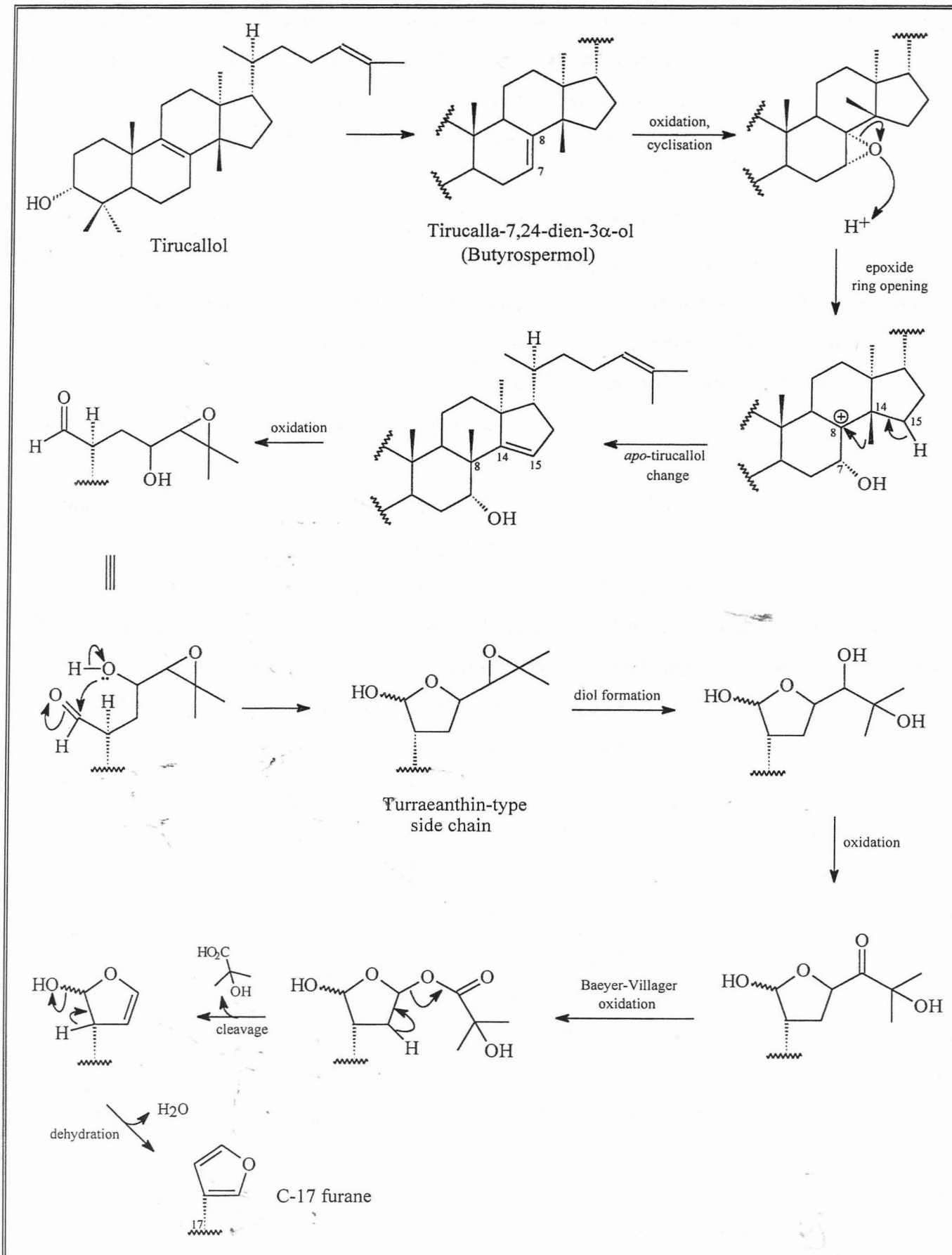


Figure 1.4.8: Biosynthesis of Limonoid nucleus[71, 75]

Group 4c. Phragmalin Group: The phragmalin group of limonoids are characterised by rings B and D being opened and recyclised, the latter to a lactone, and the former, with further oxidation to include an orthoacetate system, as well as a bridged ring A. Limonoids of this group are classified depending on the presence of a 1,8,9- or a 8,9,14-orthoacetate group.

The biosynthesis of lactone ring D occurs via an epoxyketo intermediate. Oxidation of the allylic carbon to a C-16 ketone occurs first and this is followed by Δ^{14}-double bond oxidation. Further oxidation of the Δ^{14}-double bond results in the formation of an epoxylactone ring D. This sequence of reactions cannot be interchanged and if epoxidation occurs prior to keto-formation, ring D remains intact and the lactone system does not form[75]. Such modifications, illustrated in figure 1.4.9, result in group 3, or gedunin type compounds.

Opening of ring B can then occur via Baeyer-Villiger oxidation of a 7-keto derivative of Group 3, resulting in a ring B lactone. Such ring B opened and ring D lactone systems are well represented by the andirobin group of group 4a type limonoids[75]. 1,3-Diketodiene lactone derivatives of this group are seen as group 4b and 4c precursors . Spontaneous Michael cyclisation produces a new pseudo-ring B system with double bond shift from Δ^{14} to $\Delta^{8,14}$ and retention of the 1,3-keto-groups. This yields mexicanolide of group 4b, the mexicanolide group, as illustrated in figure 1.4.9. The biosynthetic mechanism involved in such a modification is still unknown[75].

Epoxidation of the new double bond results in the 8,14α-oxides, which spontaneously form 1,8-ketal systems, with new Δ^{14}-double bond formation[75]. The mechanism would thus involve acid opening of the 8,14α-oxide with diol formation and subsequent dehydration to give the 8α-hydroxy-Δ^{14} system, as illustrated in figure 1.4.10. Spontaneous etherification then occurs, resulting in 1,8-ketal formation[75]. This ketal system is found in xyloccensin D, which was isolated, together with phragmalin triacetate, from *Xylocarpus moluccensis*[75]. Such isolations are of great importance as one can postulate biosynthetic pathways from the co-occurrence of such compounds. Xyloccensin D is presumed to be the phragmalin triacetate precursor.

It has been postulated[75] that final ring A and B modifications begin with radical formation at the C-1 hydroxy group, which can, in turn, oxidise the C-4α methyl (C-29) to a radical, as illustrated in figure 1.4.10. Attack by the latter on the C-1 keto group, which forms on 1,8-ketal decomposition, then occurs, yielding the ring A 1,29,4-bridge. This ring A bridge system is a

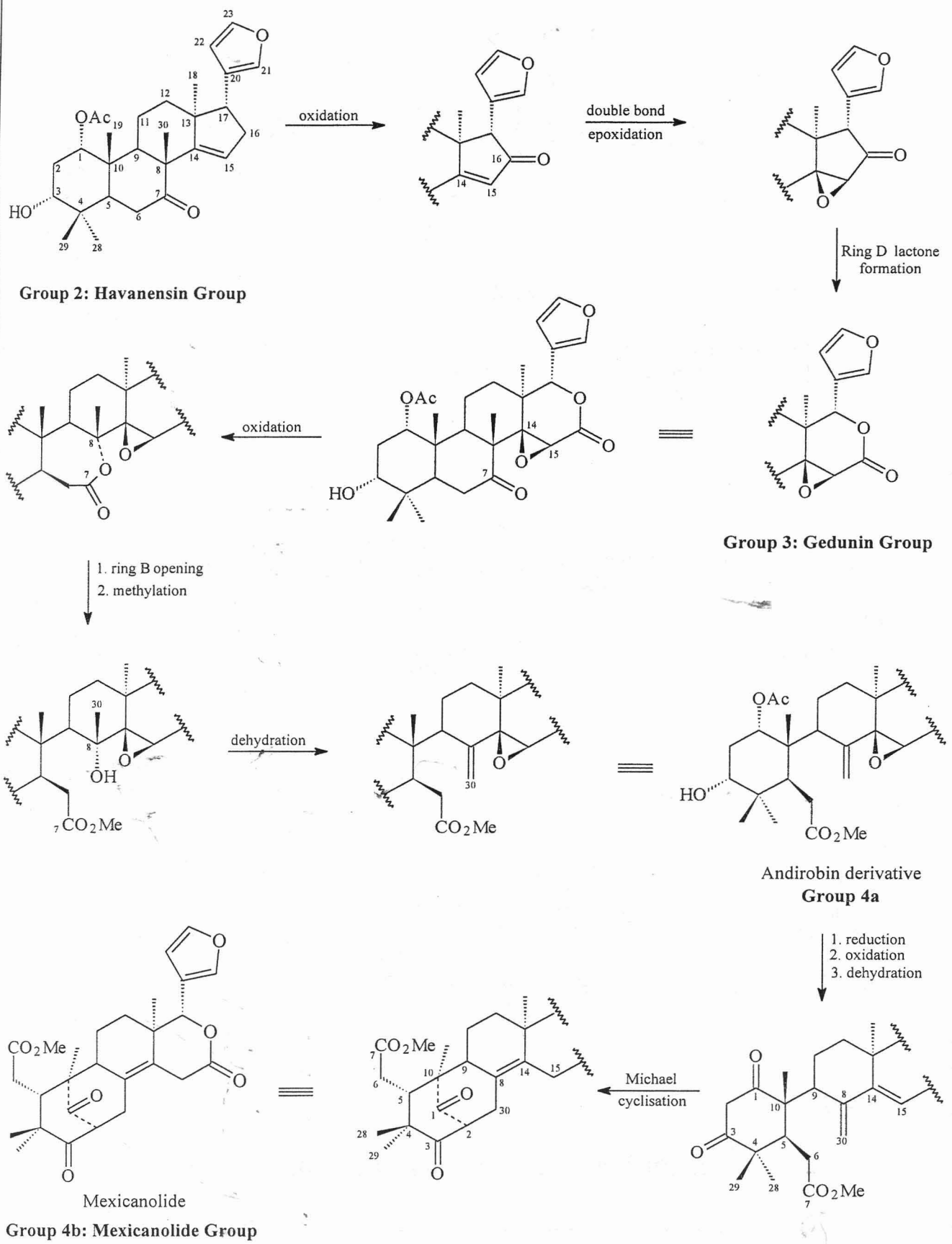


Figure 1.4.9: Biosynthesis of Phragmalin precursor group[75]

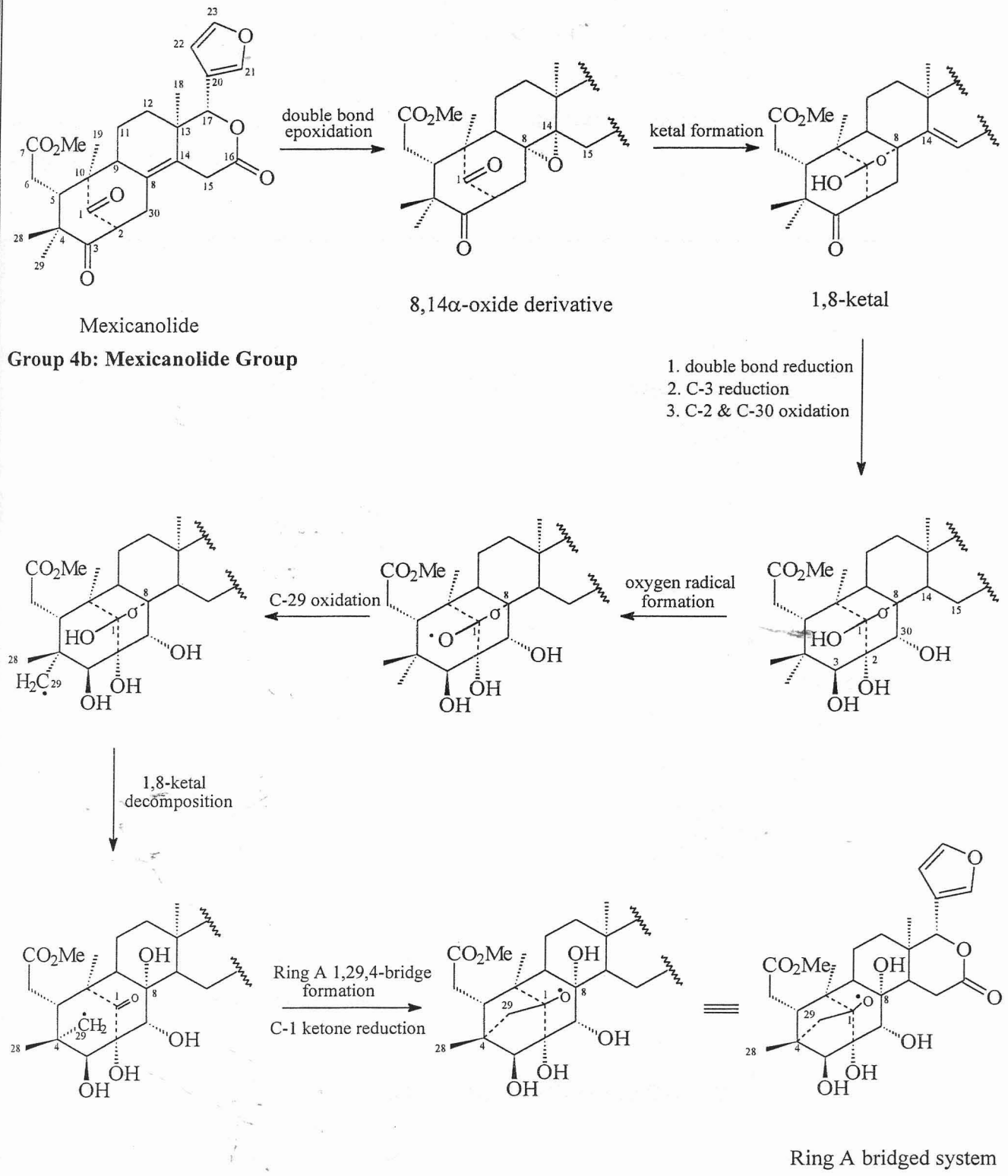


Figure 1.4.10: Formation of bridged Ring A[75]

prerequisite for such Group 4c limonoids and occurs prior to orthoacetate formation[72, 75]. Oxidation of C-9 by the C-1 oxygen radical results in a C-9 radical system which is further oxidised to a hydroxyl group. Condensation of the latter with the C-1 and C-8 hydroxyl groups already present, in the presence of acetic acid, results in orthoacetate formation. This process is illustrated in figure 1.4.11 below.

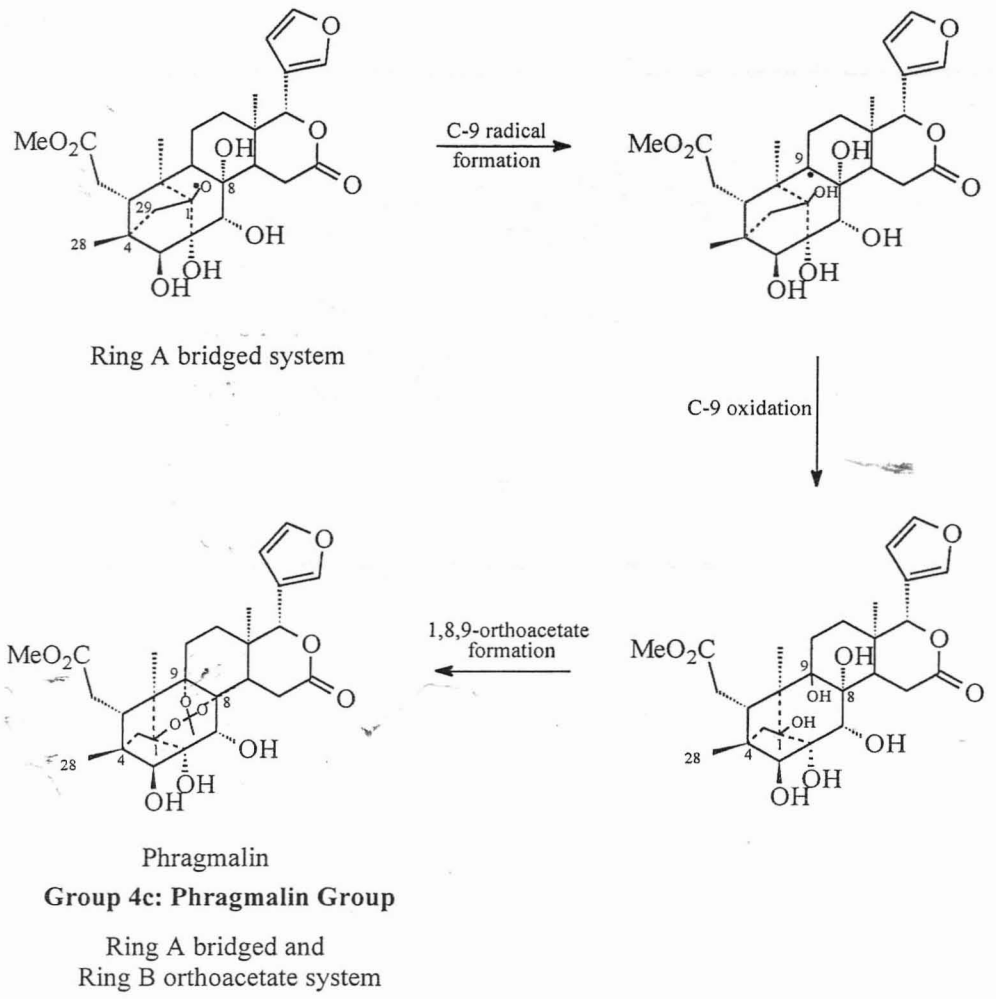
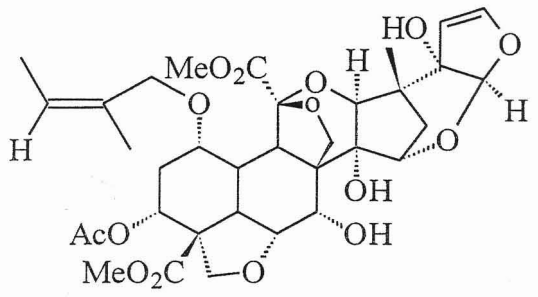


Figure 1.4.11: Phragmalin biosynthesis[75]

Biological activity

Limonoids have been found to be biologically active as insect antifeedants and possess growth regulating properties, medicinal effects such as tumor-preventative and anti-cell adhesion properties, in both humans and animals, as well as antiviral, antifungal and bactericidal activity[71, 76]. Anti-cancer activity is found to be possessed by the group 2 havanensin limonoids which have a 14,15-epoxide ring, as well as by group 10 prieurianin limonoids. Azadirachtin (**26**), isolated from *Melia azadarach*, has exceptionally good insect antifeedant properties[70].



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Chapter 2

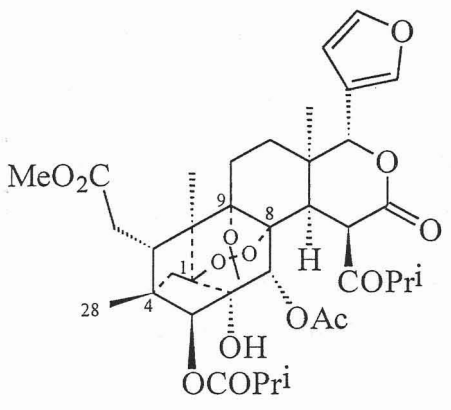
Extractives from Neobeguea mahafalensis

2. EXTRACTIVES FROM *NEOBEGUEA MAHAFALENSIS*

2.1 Introduction

Madagascan flora is comprised of some 12,000 flowering plants, 85 per cent of which occur only in Madagascar. At least seven of the 180 plant families to which the above species belong, and one quarter of the 1,600 genera, are endemic[77]. Over 1,000 orchid species are found in Madagascar and this illustrates the diversity of plant life hosted by this land. The large number of medicinal plants in Madagascar have attracted much interest. The Madagascan periwinkle (*Catharanthus roseus*), used to treat childhood leukaemia, is a well known example thereof. Katrafay (*Cedrelopsis grevei*), one of the species studied in this work, is known to relieve muscular fatigue by soaking the bark in bathwater[77].

Neobeguea mahafalensis Leroy. belongs to the *Neobeguea* genus of the Meliaceae family and is a plant unique to Madagascar. It is commonly referred to as "Handy" by the native people of Madagascar, who use it as a medicinal plant[78]. This endemic genus is thought to be related to other members of its subfamily, Swietenioideae, especially *Khaya*[72, 79]. *Neobeguea mahafalensis* has previously yielded pseudrelone A_2 (**27**), a phragmalin-type limonoid, from the wood and bark of the plant[79]. This, and other such highly oxidised limonoids occur only in advanced genera such as *Chukrasia, Entandrophragma* and *Pseudocedrela* of the Swietenioideae[79]. This would imply that *Neobeguea*, which yields limonoids of far higher complexity than the *Khaya* genus, is more advanced[79].



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The stem-bark of *N. mahafalensis* which has been analysed in this work, was collected from the dry, thorny forests of the deep south of Madagascar. Most species found in this region are unique in the world and are highly specialised in adapting to the very dry climate. They are mostly thorny, have reduced leaves and shortened branches, as well as underground water storage organs[80].

The hexane extract of the stem-bark of *N. mahafalensis* Leroy. was analysed. The major compound isolated was a limonoid, **compound I**, whose structure was found to be very similar to that of bussein A (**30**). The known pentacyclic triterpenoid, β-amyrin, **compound II,** and stigmasterol, **compound III,** were also isolated in good yield. Both these compounds were also isolated from the dichloromethane extract of this species, as well as from the hexane extract of *Cedrelopsis grevei*, the second plant in this study.

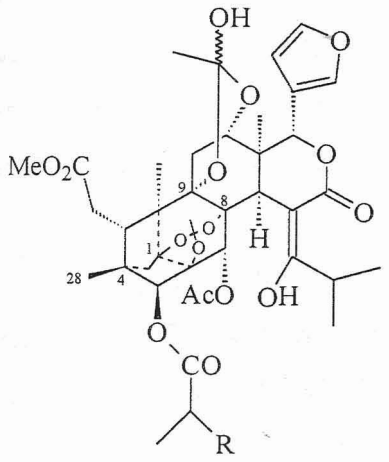
2.2 Results and Discussion

2.2.1 Structural elucidation of Compound I (Neobeguin)

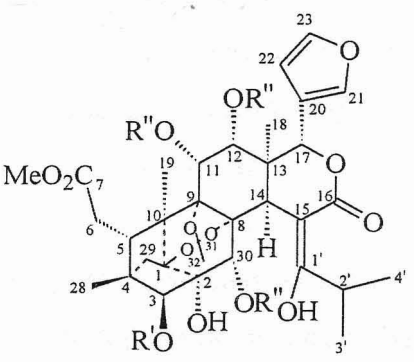
Group 4a type limonoids, contain an orthoacetate modified ring B and a bridged ring A system. The biosynthesis of such compounds is discussed in chapter 1.4. Bussein was originally isolated from *Entandrophragma bussei* and *E. caudatum*, as a mixture of bussein A and bussein B. Initially, Hanni and Tamm[81] incorrectly assigned the structures of these compounds as (**28**) and (**29**) for bussein A and B respectively, but corrected the structures some years later to (**30**) and (**31**)[82]. Other bussein derivatives have been isolated from *Chukrasia tabularis* of the genus *Chukrasia*, of the Swietenioideae subfamily of the Meliaceae family[72, 83, 84].

Compound I was isolated as a white crystalline material from the hexane extract of *N. mahafalensis*. The mass spectrum **[spectrum 1]** showed a molecular ion $[M]^+$ peak at *m/z* 714.2876 which corresponded with the molecular formula $C_{36}H_{46}O_{14}$. Other important peaks were present at *m/z* 654 ($[M-60]^+$, loss of acetic acid) and *m/z* 594 ($[M-120]^+$, loss of two acetic acid molecules), which indicated the presence of two acetyl groups in compound I. The IR spectrum **[spectrum 2]** indicated peaks at 3569 (O-H stretching), 1743 (C=O stretching) and 1638 cm^{-1} (C=C stretching).

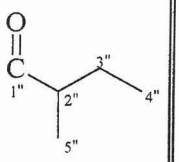
The ^1H NMR spectrum **[spectrum 3]** of compound I exhibited three one-proton resonances at δ_H7.48, 7.32 and 6.38 ascribable to the three protons of a β-substituted furanyl ring typical of a



28 R = Et
29 R = Me

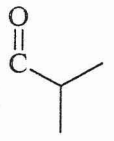


30 R' =

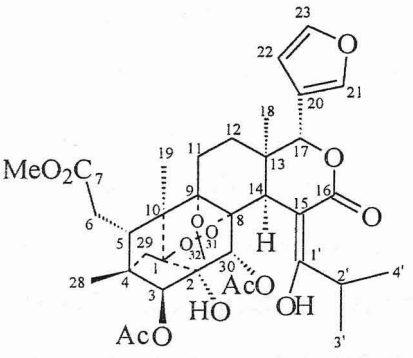


R" = COMe

31 R' =



R" = COMe



Compound I (Neobeguin)

limonoid. The stronger coupling seen between δ_H6.38 and 7.32 in the COSY NMR spectrum **[spectrum 6a]** allowed allocation of these peaks to H-22 and H-23 respectively, while the resonance at δ_H7.48 was seen to be coupled only weakly with H-22 and H-23 and was thus assigned to H-21. The HETCOR NMR spectrum **[spectrum 7]** indicated that resonances ascribable to C-23, C-21 and C-22 appeared at δ_C142.7, 141.1 and 110.0 respectively. The resonance at δ_C122.3 in the ^{13}C NMR spectrum **[spectrum 4]** was assigned to C-20 by comparison with literature values[83].

A methoxy group three-proton singlet at δ_H3.68 was characteristic of ring B opened limonoids such as those of groups 4a, 4b and 4c (Chapter 1.4). The former andirobin type group 4a limonoids were excluded on the basis of their possessing a terminal methylene group with H_{30A} and H_{30B} appearing as one-proton resonances at about δ_H4.8 and δ_H5.0[85]. These were absent in the ^{1}H NMR spectrum **[spectrum 3]** of compound I . The mexicanolide type group 4b compounds commonly possess a $\Delta^{8,14}$ or $\Delta^{14,15}$ double bond system. Double bonds terminating at C-14 in such limonoids exhibit 17-H resonating at δ_H5.2[86], and this resonance was again absent in the ^{1}H NMR spectrum

[**spectrum 3**] of compound I. The group 4c phragmalin type limonoids have ring B opened and recyclised into a pseudo-ring B with an orthoacetate system, a bridged ring A system and a ring D lactone system. The NMR spectra of compound I provided evidence for the presence of this type of limonoid. Further comparison between the NMR spectra of compound I and bussein A (**30**) (group 4c limonoid)[83], showed these two compounds to be very similar.

The methoxy group three-proton resonance at δ_H3.68 correlated with a resonance at δ_C50.1 in the HETCOR NMR spectrum [**spectrum 7**]. The COSY NMR spectrum [**spectrum 6b**] illustrated coupling between H-5 and 2H-6, where the double doublet at δ_H3.00 (H-5) coupled separately with both the non-equivalent protons at C-6, seen as double doublets at δ_H2.45 and 2.23. These 2H-6 protons were not equivalent owing to their restricted rotation due to the bulky surrounding groups. The methine resonance at δ_C37.2 correlated with the H-5 resonance at δ_H3.00 in the HETCOR NMR spectrum [**spectrum 7**] of compound I and was thus assigned to C-5. The 2H-6 signals correlated with the methylene carbon resonance at δ_C33.9 allowing its allocation to C-6. By comparison with bussein A (**30**) data[83], the quaternary carbon resonance at δ_C172.7 was allocated to C-7. This was confirmed by the DELAYED HETCOR NMR spectrum [**spectrum 8**] which indicated correlation of the C-7 resonance with the methoxy group proton resonance at δ_H3.68.

The deshielded, C-32 methyl group appeared as a three proton singlet at δ_H1.56 and, in accordance with literature, was typical for such an orthoacetate methyl group proton resonance[83, 84]. The HETCOR NMR spectrum [**spectrum 7**] indicated that C-32 appeared at δ_C20.8. The orthoacetate carbon, C-31, appeared at δ_C118.7 and the three quaternary C-O carbon resonances at δ_C84.3, 80.7 and 85.1 were assigned to the ring B C-1, C-8 and C-9 carbon atoms respectively, by comparison with bussein A (**30**) data[83].

Coupling between the 2H-29 protons of the 4,29,1-ring A bridge was evident in the COSY NMR spectrum [**spectrum 6b**] of compound I. These two non-equivalent protons coupled with each other resulting in two distinctive doublets at δ_H1.81 and 1.89. The HETCOR NMR spectrum [**spectrum 7**] showed that the 2H-29 resonances correlated with the methylene resonance at δ_C39.7 which was thus allocated to C-29. This signal correlated with the three proton methyl group resonance at δ_H0.94 in the DELAYED HETCOR NMR spectrum [**spectrum 8**], thus allowing allocation of the latter to positions of the 28-methyl group.

The resonance at δ_H5.84 was allocated to the H-17β proton, typical of limonoids with a ring D lactone system[83]. The HETCOR NMR spectrum **[spectrum 7]** allowed assignment of the resonance at δ_C70.9 to C-17. A singlet at δ_H2.65, equivalent to one proton, was assigned to H-14 on the basis that the COSY NMR spectrum **[spectrum 6b]** showed that this proton was not coupled, as well as by comparison with bussein A (**30**) data[83]. The methine resonance at δ_C44.9 was allocated to C-14. Resonances at δ_C169.4 and 91.1 were assigned to the C-16 quaternary carbonyl carbon and to C-15 respectively[83]. The DELAYED HETCOR NMR spectrum **[spectrum 8]** indicated the correlation of the C-15 (δ_C91.1) and the C-8 (δ_C80.7) resonances with that of H-14 (δ_H2.65) thus confirming their assignments.

The C-15 side chain in bussein A (**30**) was assigned to compound I as well. The reason for this assignment was due to the presence of two, three-proton doublets at δ_H1.24 and 1.11, characteristic of 3H-3' and 3H-4'. The COSY NMR spectrum **[spectrum 6b]** of compound I confirmed this, as the two methyl group proton resonances at δ_H1.11 and 1.24 were both seen to be coupled with a multiplet at δ_H2.94 which could be allocated to H-2'. This multiplet corresponded to a resonance at δ_C30.1 in the HETCOR NMR spectrum **[spectrum 7]** and was assigned to C-2', while the 4' and 3' methyl groups occurred at δ_C18.4 and 20.6. The quaternary resonance at δ_C182.9 was assigned to C-1'[82].

The remaining two, one-proton singlets at δ_H5.33 and 4.85 in the ^{1}H NMR spectrum **[spectrum 3]** of compound I were assigned to H-30 and H-3 by comparison with NMR data for bussein A (**30**)[83]. These resonances occurred downfield as acetate substitution occurred at these positions. Only two acetyl groups were present in compound I as only two acetyl methyl group proton resonances were present at δ_H2.26 and 1.96 in the ^{1}H NMR spectrum **[spectrum 3]**. The COSY NMR spectrum **[spectrum 6a]** of compound I showed no evidence of the presence of the 2-methylpropionate substituent present at C-3 in bussein A (**30**), as no appropriate coupled system was evident. The fact that the H-3 and H-30 resonances were each completely uncoupled, confirmed the quaternary nature of C-2. The methine resonance ascribable to C-2 occured at δ_C78.0. The hydroxyl group present at this position in bussein A (**30**)[83] was thus also assigned to compound I. The protons of the hydroxy group at C-2 and C-1' could not be detected in the ^{1}H NMR spectrum **[spectrum 3]** of compound I, even on addition of D_2O. The molecular formula required the presence of these groups and the chemical shifts of C-2, C-15 and C-1' agreed with literature values for hydroxy groups at these positions[83].

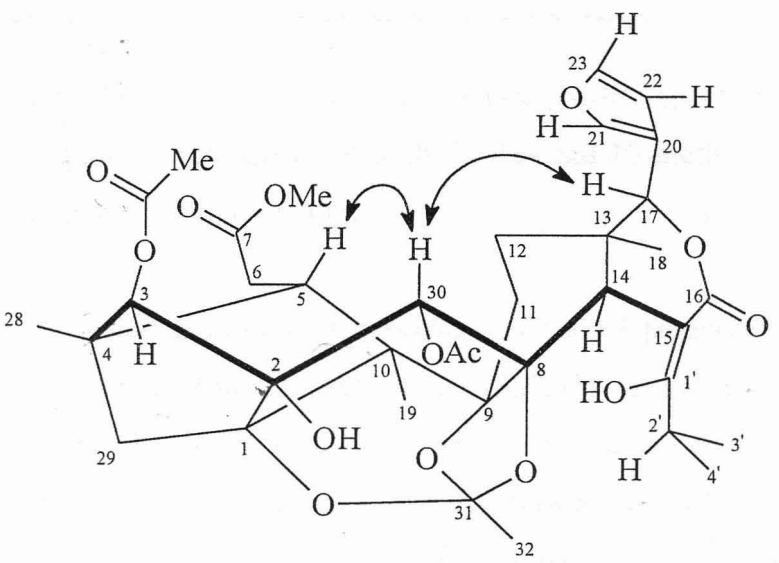
The COSY NMR spectrum **[spectrum 6b]** showed that four protons in the region δ_H1.0 to 2.1 were coupled to each other. The HETCOR NMR spectrum **[spectrum 7]** showed these protons belonged to two methylene groups. Thus two neighbouring methylene groups were present and the corresponding ^{13}C NMR resonances occurred at δ_C24.5 and 31.2. The two downfield protons at δ_H2.02 and 1.85 were attached to the carbon atom resonating at δ_C24.5 and the upfield protons at δ_H 1.48 and 1.18, to the δ_C31.2 carbon resonance. The two methylene carbons were placed at C-11 and C-12 but it was not possible to ascertain which was which, as the NOE experiments were unsuccessful owing to these resonances being obscured by other resonances.

Thus, unlike Bussein A (**30**), compound I was unsubstituted at positions C-11 and C-12. The two downfield singlets at δ_H5.65 and 4.67 allocated to H-11 and H-12 in bussein A (**30**)[83], were not present in the ^1H NMR spectrum of compound I. The three remaining methyl singlets in the ^1H NMR spectrum **[spectrum 3]** of compound I at δ_H1.21, 1.14 and 0.94 were allocated to the three skeletal methyl groups at C-18, C-19 and C-28. Specific allocation and stereochemistry of these resonances was accomplished with NOE analysis. A sharp singlet at δ_H1.59 in the ^1H NMR spectrum **[spectrum 3]** of compound I was a solvent impurity and could thus be ignored. This was confirmed when a second sample of compound I was isolated and its ^1H NMR spectrum indicated the above resonance had disappeared although the rest of the spectrum remained identical.

Stereochemical analysis of compound I

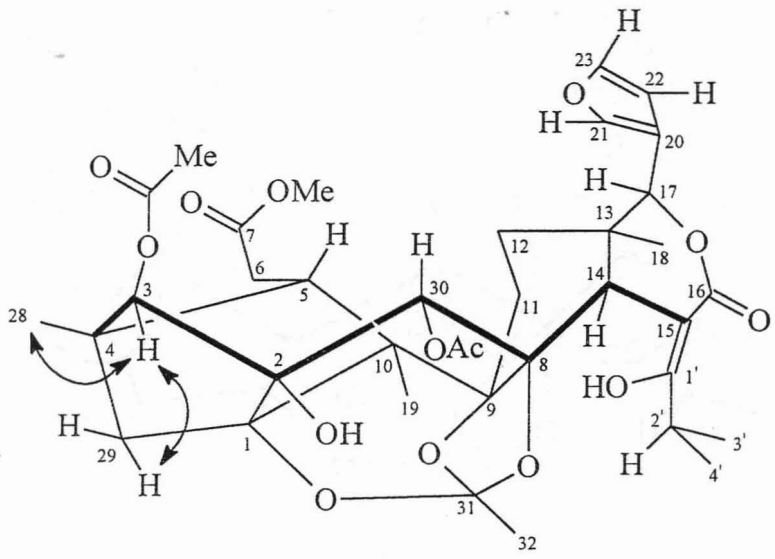
There is much inconsistency in the literature relating to the stereochemistry of group 4a compounds. On formation of the pseudo-B ring, rotation of ring A occurs around the C-9, C-10 bond in order for the C-2, C-30 bond to form. Thus, ring A undergoes stereochemical inversion, as evident in figure 1.4.10 of chapter 1.4. This results in the 19-methyl group becoming α-orientated and the 28-methyl group, β-orientated, once recyclisation of ring B occurs. In this process H-3 becomes α-orientated and H-5 becomes β-orientated. The stereochemistry of H-30 needed to be ascertained and that of H-3, H-5, the 18-, 19- and the 28-methyl groups needed confirmation. Taylor, in his paper on limonoid biosynthesis[75] indicated the stereochemistry of the oxygen substituents at the C-3, C-2 and C-30 positions to be of β–orientation in the former two cases and α-orientation in the latter. The Dictionary of Natural Products (DNP) give the α-stereochemistry for the C-2 substituent. Connolly *et al.*[87], in their paper on phragmalin derivatives, indicate a 3α-acetate and a 10β-methyl group for phragmalin triacetate but in a subsequent paper[84], indicate a 3β-acetate. Arndt[88], in his paper on phragmalin, contradicts himself in the two structures given for phragmalin, although an X-ray structure was obtained.

A model of compound I was constructed and a series of NOE experiments were performed. Compound I (**32**) is illustrated below in agreement with NOE results obtained. On irradiation of H-30 (δ_H5.33) **[spectrum 9a]** positive NOE results were obtained for H-17 (δ_H5.84) and H-5 (δ_H3.01). The H-17 proton is known to possess a β-orientation in limonoids, thus H-5 and H-30 must possess β-orientation as well. This confirms the prediction of β-stereochemistry for H-5 based on biosynthetic reasons. This is illustrated in (**32a**) below, where H-30 lies between H-5β and H-17β and is thus close enough to interact with both these protons through space. The close proximity of H-30, H-5 and H-17 was evident in the model of compound I. On irradiation of H-17β **[spectrum 9b]**, H-30β showed a positive result as H-5β was too far away from H-17β to interact. No NOE results were seen between H-17β and the 18-methyl group nor H-14 (δ_H2.65) confirming the α-orientation of both the 18-methyl group and H-14.



32a

H-3 is usually β-orientated in limonoids but the rearrangement of ring A in group 4c limonoids results in it becoming α-orientated. Irradiation of H-3 (δ_H4.85) **[spectrum 9c]** resulted in a positive NOE for one of the H-29 (δ_H1.8) protons and the most upfield methyl group proton resonance (δ_H0.94). This confirmed that H-3 must possess α-stereochemistry in order to produce the above result. The 28-methyl group must, on the basis of its biosynthesis, possess β-stereochemistry. The molecular model (**32b**) indicated the very close spatial proximity of the β 28-methyl group protons with H-3α, thus allowing allocation of the three-proton resonance at δ_H0.94 to 3H-28. This

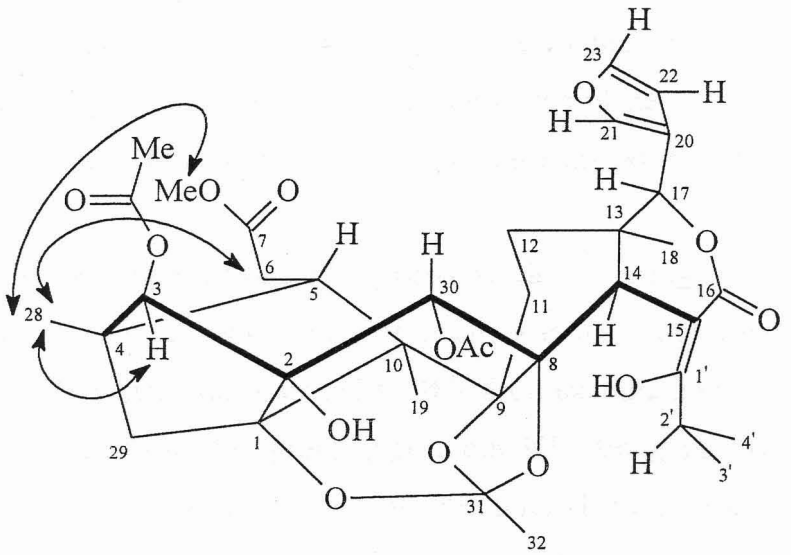


32b

allocation was confirmed by the DELAYED HETCOR NMR spectrum **[spectrum 8]**. The molecular model of compound I indicated that both the 18- and 19-methyl group protons were far too distant from H-3 to interact spatially with it.

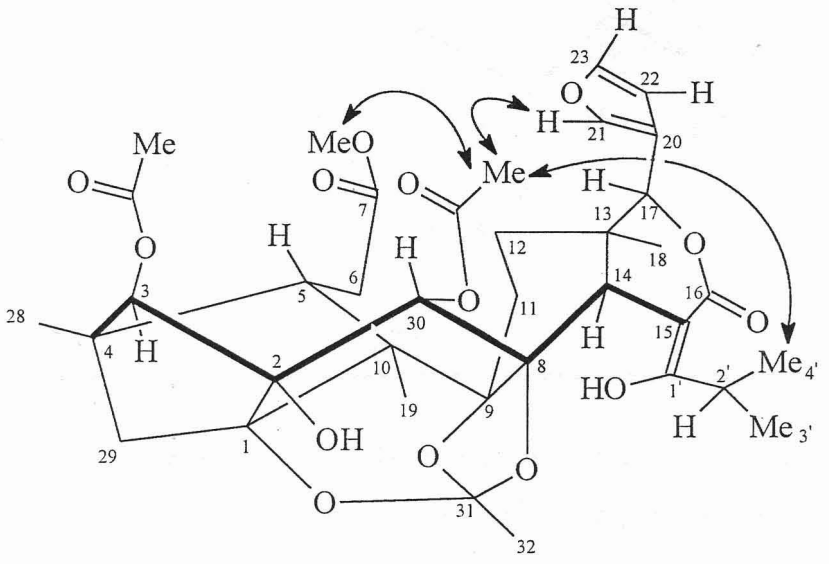
On irradiation of the 28-methyl group proton resonance at δ_H0.94 **[spectrum 9d]**, positive results were obtained for H-3α and one of the H-6 resonances (δ_H2.45), as well a weak positive result for the methoxy group protons (δ_H3.68). A molecular model of compound I (**32c**) indicated that an α-orientated C-6 carbon allowed close spatial proximity between one of the 2H-6 protons and the 28β-methyl protons. This stereochemistry was predicted on biosynthetic grounds.

Irradiation of the methyl resonance at δ_H1.56 **[spectrum 9e]** produced no results. This three-proton methyl group resonance, was known to be due to the orthoacetate methyl group protons (3H-32) on the basis of deshielding effects caused by the three orthoacetate oxygen atoms. The molecular model of compound I showed that the three H-32 protons lay below the plane of the molecule, so no NOE results were expected.



32c

On irradiation of the acetyl methyl group proton resonance at δ_H2.26 **[spectrum 9f]**, positive NOE results were seen with H-21, the methoxy group protons, as well as with the methyl group at δ_H1.11 which had previously been allocated to C-4'. Both the former groups mentioned were α-orientated substituents and the model of compound I (**32d**), showed them to be close to the C-30 acetyl group, thus confirming the suspected α-orientation of the latter.

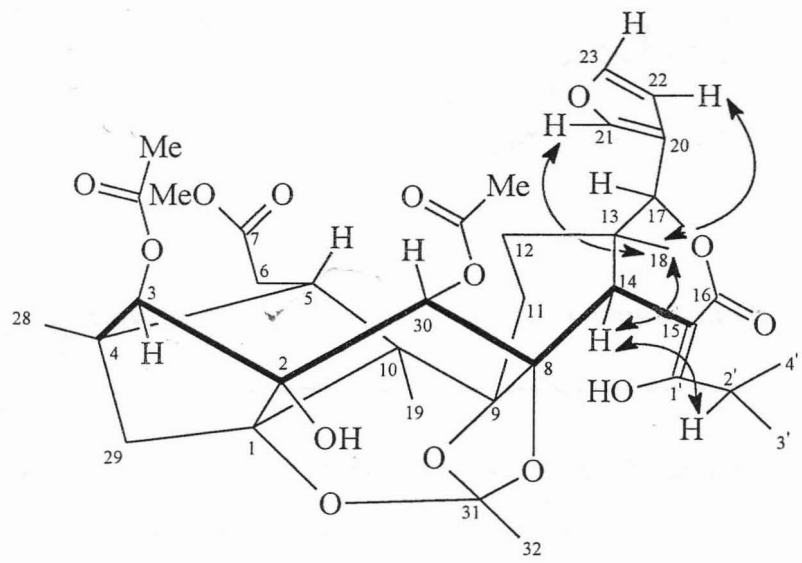


32d

Lack of NOE results of this acetyl methyl group protons with αH-14 (δ_H2.65) indicated that the distance between them was quite large. Irradiation of the acetyl methyl group proton resonance at

δ_H1.96 **[spectrum 9g]** showed no positive NOE results. This suggested that the two acetate groups possessed opposite stereochemistry. The molecular model indicated that these methyl protons belonged to the C-3 acetyl group and lay uppermost above the plane of the molecule. Thus, the distance, even from the closest H-5α, was too great to produce any positive NOE results.

On irradiation of the H-14 singlet at δ_H2.65 **[spectrum 9h]**, a strong positive NOE result was obtained for H-2' (δ_H2.94), and a weaker positive result was also found with the methyl group proton resonance at δ_H1.21 which was assigned to 3H-18 on examination of the molecular model (**32e**). On irradiation of this 18-methyl group **[spectrum 9i]**, strong positive NOE results were obtained for H-14 and H-2' as well as two of the furanyl ring protons, H-21 and H-22. This confirmed the methyl proton resonance had been correctly assigned, (as the 19-methyl group was too far away to produce such results), and also the α-orientation of H-14, as on biosynthetic grounds, both the furanyl ring and the 18-methyl group are α-orientated (**32e**).



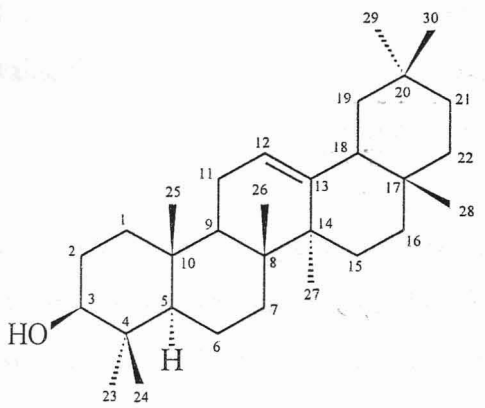
32e

The remaining methyl group resonance at δ_H1.14 could thus be assigned to the 19-methyl group. On irradiation of the C-19 methyl group protons **[spectrum 9j]**, no positive NOE results were obtained.

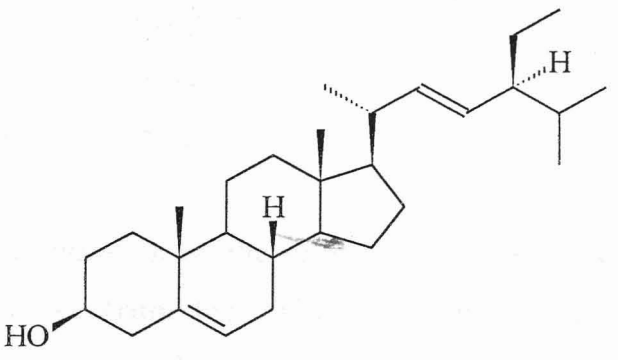
The structure of compound I (pg. 62) was assigned to neobeguin. Compounds of the bussein type which are unsubstituted at C-11 and C-12 have not been reported previously.

2.2.2 Structural elucidation of Compound II

Compound II was found as a main constituent in both *Neobeguea mahafalensis* and *Cedrelopsis grevei* and structural elucidation indicated it was the fairly common pentacyclic triterpenoid, β-amyrin. This compound was also isolated from the *Cedrelopsis grevei* species which was obtained from the wetter north-western part of Madagascar, from which various limonoids were isolated in a parallel study[89]. Stigmasterol, compound III, a common phytosterol, was also isolated from both these species. The ^{1}H NMR data **[spectrum 20]** obtained for compound III was identical to that provided by literature[90], thus allowing its identification as stigmasterol. Compound III will not be further discussed.



Compound II (β-Amyrin)



Compound III (Stigmasterol)

Compound II was isolated as a white crystalline material from both the hexane and dichloromethane extracts of both *Neobeguea mahafalensis* and *Cedrelopsis grevei* The mass spectrum **[spectrum 10]** showed a molecular ion $[M]^+$ peak at *m/z* 426.3874 which corresponded to the molecular formula $C_{30}H_{50}O$ which indicated the presence of six double bond equivalents in the compound. The IR spectrum **[spectrum 11]** indicated peaks at 3300 (O-H stretching), 2950 (saturated CH stretching), 1620 (C=C stretching), 1460 (C-H deformations), 1380 (symmetrical CH_3 deformations) and 1365 cm^{-1} (geminal dimethyl group twist).

The ^{1}H NMR spectrum **[spectrum 12]** was characteristic of a triterpenoid. It showed a one-proton triplet in the olefinic region at δ_H5.16 (J = 3.6Hz) and a one-proton multiplet at δ_H3.20 ($W_{1/2}$ = 14 Hz), representing an oxymethine proton. This former signal, which correlated with the

methine resonance at δ_C121.7 in the ^{13}C NMR spectrum **[spectrum 13]**, accounted for one of the six double bond equivalents within the triterpenoid nucleus and the lack of any further peaks in this region indicated the presence of a pentacyclic nucleus. The singlets at δ_H1.11, 0.98, 0.95, 0.92, 0.85 (6H), 0.81 and 0.77 showed that compound II contained eight tertiary methyl groups.

Compound II was acetylated using acetic anhydride and pyridine. The ^1H NMR spectrum **[spectrum 17]** of the acetate differed only by the appearance of a new sharp singlet at δ_H2.03, equivalent to three protons, and the shift of the multiplet at δ_H3.20 to δ_H4.46. The quaternary carbon peak at δ_C171.0 in the ^{13}C NMR spectrum **[spectrum 18]** of the acetyl derivative of compound II, representing the carbonyl carbon of the acetate group at C-3, was not present in the ^{13}C NMR spectrum **[spectrum 13]** of compound II. The resonance at δ_H3.20 ($W_{1/2}$ = 14 Hz) was thus assigned to H-3 and the hydroxyl group at this position was β-orientated, based on the $W_{1/2}$ value for the H-3 resonance[91]. The HETCOR NMR spectrum **[spectrum 16]** indicated correlation of this resonance to the methine signal at δ_C79.0, thus allowing its assignment to C-3.

The ADEPT NMR spectrum **[spectrum 14]** allowed allocation of the primary, secondary and tertiary carbon resonances of the ^{13}C NMR spectrum **[spectrum 13]**. The ^{13}C NMR spectrum **[spectrum 13]** indicated eight methyl carbons, ten methylene carbons, five methine carbons and seven fully substituted carbon atoms in the molecule. The presence of only one double bond was confirmed by resonances at δ_C145.2 and δ_C121.8, which were assigned to C-13 and C-12 respectively. These resonances are typical for C-13 and C-12 of the Δ^{12}-oleanones[92]. All peaks could be assigned using the COSY **[spectrum 15]** and the HETCOR **[spectrum 16]** NMR spectra and by referring to literature data of β-amyrin[91].

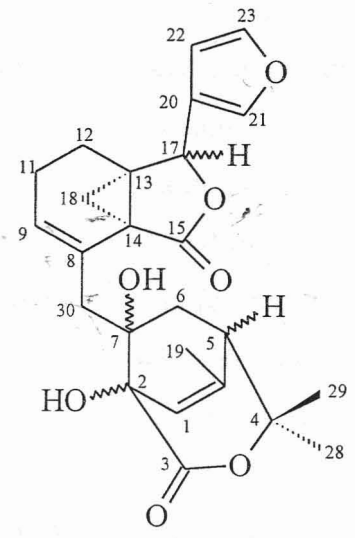
Chapter 3

Extractives from Cedrelopsis grevei

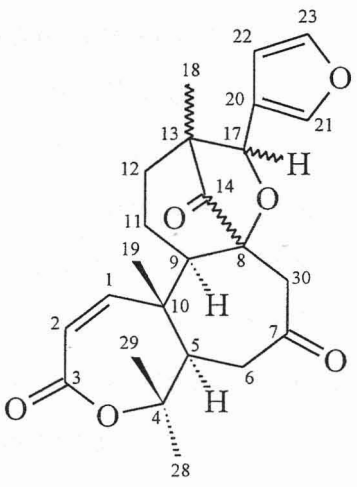
3. EXTRACTIVES FROM *CEDRELOPSIS GREVEI*

3.1 Introduction

Cedrelopsis grevei Baill. is one of seven species of the genus *Cedrelopsis* which are confined to Madagascar[93]. This species, commonly referred to as "Katrafay" by the Madagascan people[78], is said to relieve muscular fatigue when the bark is added to bath water[77]. In this investigation, the stem bark of *Cedrelopsis grevei* was obtained from the dry southern part of Madagascar and yielded a variety of chromones and coumarins, as well as the pentacyclic triterpenoid, β-amyrin. A recent study on this species obtained from the wetter north-western part of Madagascar resulted in the isolation of β-amyrin, as well as two novel limonoids, a pentanortriterpenoid, cedmilinol (**33**), and a hexanortriterpenoid, cedmiline (**34**)[89]. It was the two latter isolations which called for further investigation of *Cedrelopsis grevei*, as limonoids have not been reported from this species prior to this recent investigation.



Cedmilinol (33)



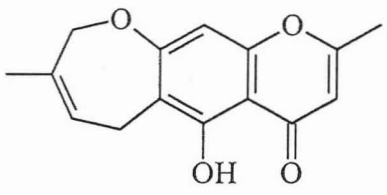
Cedmiline (34)

Examination of the chemistry of *Cedrelopsis grevei* has been of particular interest in the past thirty years, as prior inclusion of this species in the Meliaceae family has always been taxonomically questionable. This species has yielded a wide range of chromones and coumarins, but no limonoids nor protolimonoids have been isolated until now. Both these classes of compounds act as important taxonomic markers. The South African species *Ptaeroxylon obliquum* (Thung.) Radlk., commonly

known as sneezewood, nieshout or umtatin, is a member of the Ptaeroxylaceae family and of the monospecific genus *Ptaeroxylon* which is confined to Africa[92, 94, 95]. It has been found to be so chemically similar to *Cedrelopsis grevei*, that *Cedrelopsis* has been placed in the Ptaeroxylaceae family[96]. The Ptaeroxylaceae are thought to be closely related to the Sapindaceae family[73]. The fact that chromones, which are relatively rare compounds[95], occur in both these species but in neither the Sapindaceae nor Meliaceae families, supported their placement in a separate family. The Rutaceae and Meliaceae have been found to contain coumarins but not chromones[97]. The only other family which has yielded species containing both chromones and coumarins, is the Apiaceae (previously Umbelliferae), and it is thought that this family is closely related to Ptaeroxylaceae[95]. Chemotaxonomic differentiation of these two families, is based on the very unique oxepin ring chromones such as ptaeroxylin (**35**), which have only been isolated from the two above-mentioned species[94, 98]. Formation of this type of oxepin ring containing chromone was discussed previously in chapter 1.3. The recent isolation of limonoids from this species, could possibly lead to a re-examination of whether the Ptaeroxylaceae should be regarded as a separate family, or as a separate genus of the Meliaceae family.

Compounds which have previously been isolated from *Cedrelopsis grevei* include the chromones ptaeroxylin (desoxykarenin) (**35**), alloptaeroxylin (**36**), peucenin (**37**), greveichromenol (**38**), greveiglycol (**39**), alloptaeroxylin methyl ether (**40**) heteropeucenin (**41**) as well as ptaeroglycol[96,97], isolated in this investigation as **compound V**. *Ptaeroxylon obliquum* has been found to contain all these chromones, as well as the oxepin ring chromones dehydroptaeroxylin (**42**), ptaeroxylone (**43**) and ptaeroxylinol[98], isolated in this work as **compound IV**, which like ptaeroxylin, are all oxepin ring chromones[98]. These compounds are illustrated in figures 3.1 and 3.2. In this work, only two chromones were isolated from *Cedrelopsis grevei*, namely ptaeroxylinol (**compound IV**) and ptaeroglycol (**compound V**). Ptaeroglycol had been previously isolated from this species as well as from *Ptaeroxylon obliquum* whereas ptaeroxylinol has only been isolated from *Ptaeroxylon obliquum*. Such isolations are of great importance in supporting the very close taxonomic relationship between *Cedrelopsis grevei* and *Ptaeroxylon obliquum*.

Cedrelopsin was isolated by Taylor *et al.*[97] from *Cedrelopsis grevei* and is the only previously reported coumarin from this species. Cedrelopsin was isolated as **compound IX** from *Cedrelopsis grevei*, in this investigation. Numerous coumarins, including cedrelopsin (**compound IX**), have been isolated from *Ptaeroxylon obliquum*, many of which are aesculetin (**44**) derivatives[97, 99].



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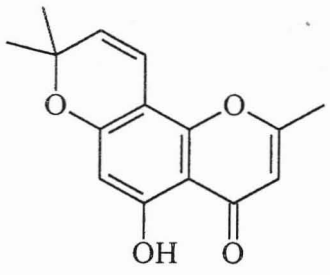

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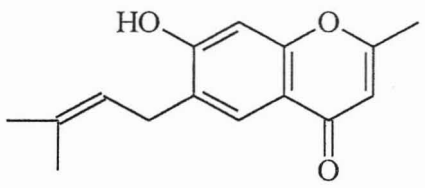

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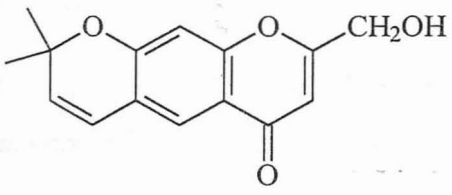

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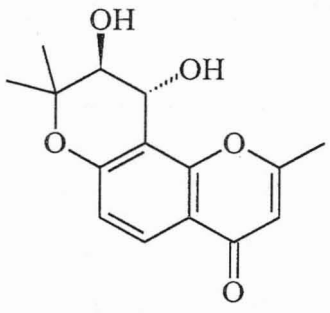

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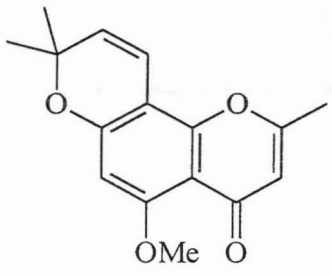

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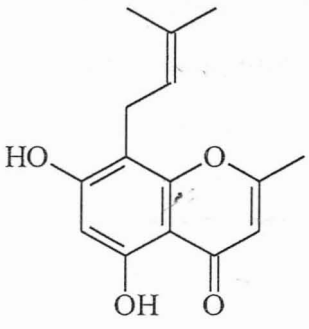

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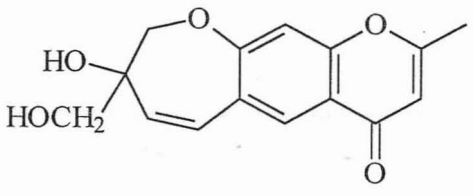
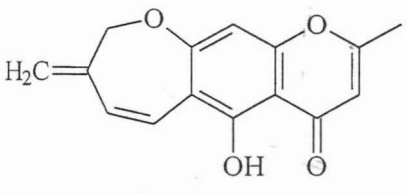


Compound V

Figure 3.1: Chromone constituents of *C. grevei* and *P. obliquum*[96, 97]



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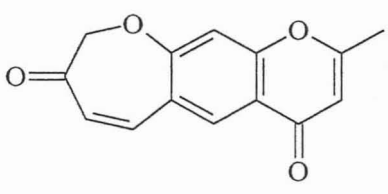

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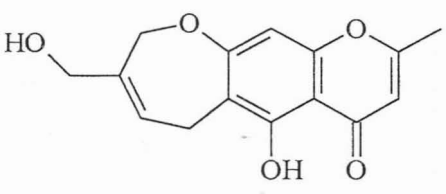
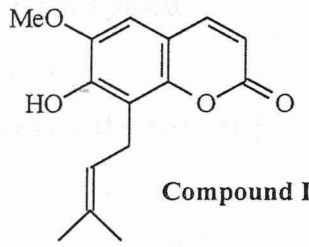
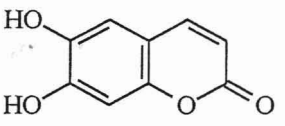


Compound IV

Figure 3.2: Chromone constituents of *P. obliquum*[98]



Compound IX



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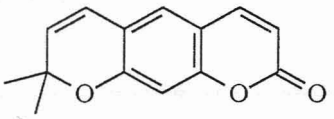

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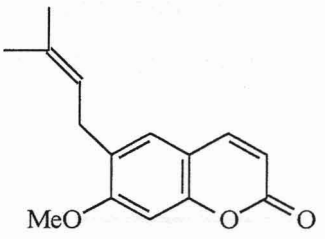

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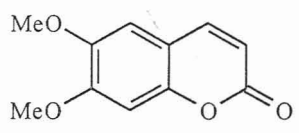
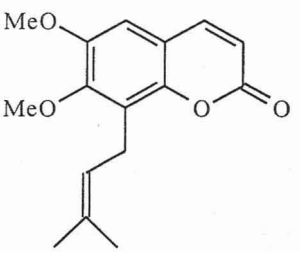
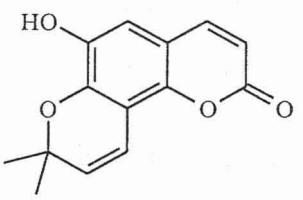
Six coumarins were isolated from *Cedrelopsis grevei* in this study. They are derivatives of coumarins such as xanthyletin (**45**) and suberosin (**46**), isolated from *Fagara* species of the Rutaceae[97]. Including cedrelopsin (**compound IX**), six coumarins were isolated from *Cedrelopsis grevei*, all of which were 6,7-dioxygenated coumarins. These included the known compounds, scoparone (**compound VI**), O-methylcedrelopsin (**compound VIII**), norbraylin (**compound X**), as well as **compound VII** and **compound XI** which have not been reported previously. No limonoids were isolated in this investigation.



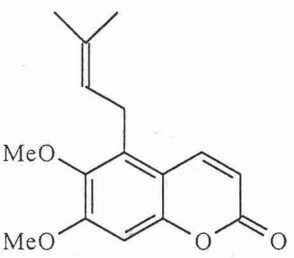
Compound VI



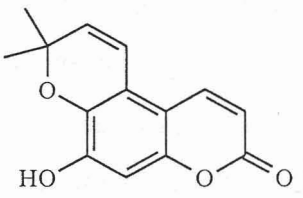
Compound VIII



Compound X



Compound VII

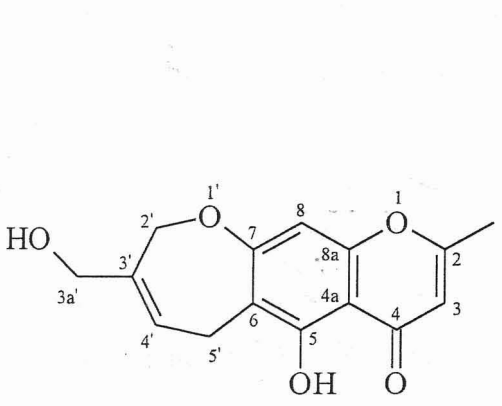


Compound XI

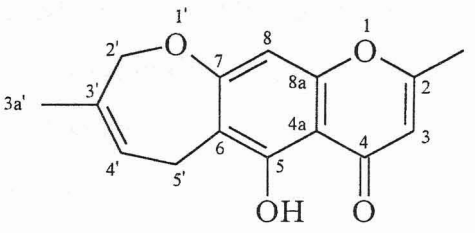
3.2 Results and Discussion

3.2.1 Structural elucidation of Compound IV

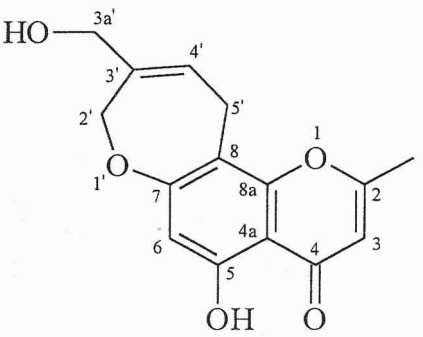
This compound was an oxepin ring-containing chromone and was found to be the known compound, ptaeroxylinol, which has been isolated previously from *Ptaeroxylon obliquum*[98], but not from *Cedrelopsis grevei*. It is a ptaeroxylin (**35**) derivative, formed by oxidation of the methyl group on the oxepin ring.



Compound IV (Ptaeroxylinol)



Ptaeroxylin (35)



Eranthin (47)

A mass spectrum could not be obtained for this compound, as the sample was misplaced while awaiting MS analysis. The IR spectrum **[spectrum 21]** indicated absorption bands at 3416 (broad band, O-H stretching), 3067 (C-H aromatic stretching), 2929 (C-H aliphatic stretching), 1652 (broad band C=O), 1610 (C=C stretching), 1166 and 1109 (C-O stretchings), 848 and 735 cm^{-1} (C-H out-of-plane deformations). The C=O stretching frequency was important in this case as it was characteristic of the γ-pyrone nucleus of chromones[100]. Coumarins, which possess α-pyrone rings, exhibit a C=O absorption band between 1700-1750 cm^{-1} [100]. The UV spectrum **[spectrum 22]** exhibited absorption maxima at 257 (log ε 4.15) and 235 nm (log ε 4.0). This again

provided evidence of a chromone nucleus, as such compounds exhibit strong absorptions between 240-250 nm (log ε 4.0), while their coumarin isomers have a minimum at this wavelength[101].

Complete NMR data for this compound was not available in literature[98]. Thus ^{1}H NMR data of ptaeroxylin (**35**)[94, 95] was used for comparison. The ^{1}H NMR spectrum **[spectrum 23]** of compound IV indicated its close similarity to ptaeroxylin (**35**). It was evident from this spectrum that compound IV possessed a 2-methyl chromone nucleus. The characteristic single, three-proton resonance at δ_H2.33 was assigned to the protons of the methyl group at C-2. The COSY NMR spectrum **[spectrum 26]** of compound IV showed coupling between this methyl group proton peak and a one-proton singlet at δ_H6.03 which was thus assigned to H-3. No other coupling was exhibited by H-3. By comparison with literature values of other oxepin ring chromones of this type, the above proton resonances were found to be indicative of a 2-methylchromone nucleus[95, 96, 98, 102, 103]. Correlation of these singlets with the methyl carbon peak at δ_C20.5 and the methine carbon peak at δ_C108.8 in the HETCOR NMR spectrum **[spectrum 27]** allowed their assignment to the methyl group attached to carbon atom C-2 and C-3 respectively. The most downfield resonance in the ^{13}C NMR spectrum **[spectrum 24]** at δ_C182.9 is typical of the C-4 carbonyl carbon of such γ–pyrone systems[102].

The presence of only one other resonance in the aromatic region of the ^{1}H NMR spectrum **[spectrum 23]** of compound IV indicated that the aryl ring of the chromone nucleus had only one proton. This one-proton resonance at δ_H6.51, could be allocated to H-6 or H-8, on the basis of the acetate hypothesis (chapter 1.3), which requires positions 5 and 7 to be oxygenated. The upfield shift of this resonance compared to the usual aryl ring proton chemical shift and is indicative of this proton being *ortho* to two oxygen substituents[104]. This was confirmed by comparison of the ^{1}H NMR chemical shift for H-8 (δ_H6.51) of ptaeroxylin[95]. The HETCOR NMR spectrum **[spectrum 27]** allowed allocation of the peak at δ_C99.3 to C-8. By studying the literature, a hydroxyl group was tentatively assigned to C-5, as such 2-methyl-5-hydroxy chromones were found to be the most common naturally occurring derivatives of this rare group of phenolics[95]. A singlet at δ_H3.47 in the ^{1}H NMR spectrum **[spectrum 23]** of compound IV was assigned to this hydroxyl group proton. The COSY NMR spectrum **[spectrum 26]** indicated this peak to be uncoupled and the HETCOR NMR spectrum **[spectrum 27]** showed no correlation of the singlet with any carbon resonances.

An olefinic triplet at δ_H5.98, and a doublet at δ_H3.55, equivalent to one and two protons respectively, were seen to be coupled together in the COSY NMR spectrum **[spectrum 26]**. This indicated the presence of the CH_2 - CH = C system present in ptaeroxylin[94, 95, 98]. The triplet was thus assigned to H-4' and the doublet to 2H-5' and the HETCOR NMR spectrum allowed assignment of the peaks at δ_C125.4 and 21.1 to C-4' and C-5' respectively.

The lack of a further methyl resonance, implied the hydroxylation of the C-3a' methyl group which was present in ptaeroxylin (**35**) at δ_H1.60[95, 98]. The two, two-proton singlets, at δ_H4.73 and 4.02 were assigned to 2H-2' and 2H-3a'. The COSY NMR spectrum **[spectrum 26]** indicated that both these resonances were coupled with each other, as well as with the H-4' and 2H-5' resonances. By comparison with NMR data for the ptaeroxylin (**35**)[94] and its isomer, eranthin (**48**)[103], the resonance at δ_H4.02 was assigned to 2H-2', and that at δ_H4.73, to 2H-3a'. The two methylene resonances in the ^{13}C NMR spectrum **[spectrum 24]** at δ_C65.7 and 71.1 were assigned to C-2' and C-3a' respectively. The quaternary carbon resonances in the ^{13}C NMR spectrum **[spectrum 24]** were assigned with reference to the ^{13}C NMR data of eranthin (**47**)[103], as no ^{13}C NMR data was available for ptaeroxylin nor ptaeroxylinol in the literature. Thus compound IV was found to be ptaeroxylinol.

An olefinic triplet at δ_H5.98, and a doublet at δ_H3.55, equivalent to one and two protons respectively, were seen to be coupled together in the COSY NMR spectrum **[spectrum 26]**. This indicated the presence of the CH_2 - CH = C system present in ptaeroxylin[94, 95, 98]. The triplet was thus assigned to H-4' and the doublet to 2H-5' and the HETCOR NMR spectrum allowed assignment of the peaks at δ_C125.4 and 21.1 to C-4' and C-5' respectively.

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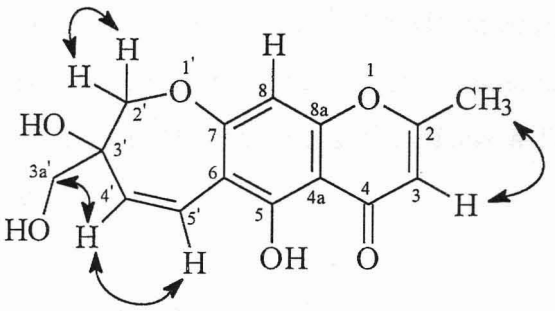
3.2.2 Structural elucidation of Compound V

This compound was identified as the known oxepin ring chromone, ptaeroglycol. Compound V was previously isolated from the heartwood of *Ptaeroxylon obliquum*, *Cedrelopsis grevei* and *Cneorum pulverulentum*[96].



Compound V (Ptaeroglycol)

Positive NOE results obtained

The mass spectrum of compound V **[spectrum 28]** indicated a molecular ion $[M]^+$ peak at *m/z* 290.0798 which corresponded to the molecular formula $C_{15}H_{14}O_6$ indicating the presence of nine double bond equivalents.

The NMR data available for this compound in the literature, was again incomplete[98]. Compound V, like compound IV, was identified as a 2-methylchromone from the ^1H NMR spectrum **[spectrum 29]**, by the characteristic downfield three-proton methyl group resonance at $\delta_H 1.95$ which correlated with the peak at $\delta_C 19.8$ in the HETCOR NMR spectrum **[spectrum 33]**. The COSY NMR spectrum **[spectrum 32]** indicated long range coupling of this methyl group proton resonance with the singlet at $\delta_H 5.98$ which was thus allocated to H-3. This one-proton singlet exhibited no other coupling in the COSY NMR spectrum **[spectrum 32]** and the HETCOR NMR allowed assignment of the methine resonance at $\delta_C 108.5$ to C-3. On irradiation of H-3 **[spectrum 34a]**, a positive NOE result was obtained with the methyl group proton resonance, thus confirming the above assignments. The ^{13}C NMR spectrum **[spectrum 30]** showed evidence of the γ-pyrone system by the presence of the characteristic downfield quaternary carbon signal at $\delta_C 182.9$ representing C-4.

The ^{1}H NMR spectrum **[spectrum 29]** of compound V exhibited an upfield aromatic one-proton singlet at δ_H6.47 which was assigned to H-8. This chemical shift indicates a proton *ortho* to two oxygen substituents[104]. This was supported by NOE experiments, where irradiation of this singlet **[spectrum 34b]** produced no NOE results. If this resonance was due to H-5, positive NOE results would have been exhibited owing to H-5' of the oxepin ring. The COSY NMR spectrum **[spectrum 32]** indicated that the resonance at δ_H6.47 was not coupled. Correlation of this resonance with the upfield resonance at δ_C98.0 in the ^{13}C NMR spectrum **[spectrum 30]** confirmed that C-8, assigned to this resonance, was next to two oxygen atoms, as such systems are known to cause an upfield shift of both ^{13}C and ^{1}H resonances[104]. Thus a 5-hydroxy-2-methylchromone nucleus was assigned to compound V.

The chromone nucleus accounted for seven of the nine double bond equivalents present in compound V. The ^{1}H NMR spectrum **[spectrum 29]** exhibited a pair of one-proton doublets in the double bond region indicating an extra double bond, thus indicating the presence of a third ring was necessary owing to the one remaining double bond equivalent. The lack of further methyl proton resonances indicated that a third pyrano ring was not present, thus the allocation of an oxepin ring system. The resonances at δ_H7.05 and 6.24 in ^{1}H NMR spectrum **[spectrum 29]** were assigned to H-5' and H-4' respectively, on the basis of NOE results. Irradiation of the doublets at δ_H7.05 (H-5') **[spectrum 34c]** showed a positive NOE result only with the doublet at δ_H6.24 (H-4'), while irradiation of the latter **[spectrum 34d]** produced positive NOE results with the resonance at δ_H7.05 (H-5') as well as with the pair of doublets at δ_H4.00 and 4.05. The COSY NMR spectrum **[spectrum 32]** indicated strong coupling of H-5' and H-4' and the HETCOR NMR spectrum **[spectrum 33]** allowed assignment of the methine peaks at δ_C118.2 and 134.4 to C-5' and C-4' respectively.

The two remaining pairs of doublets at δ_H4.29 and 4.82, and δ_H4.00 and 4.05 in the ^{1}H NMR spectrum **[spectrum 29]** of compound V were typical of non-equivalent protons of methylene groups. The COSY NMR spectrum **[spectrum 32]** showed strong coupling within each pair of doublets, but no coupling was evident between the two pairs themselves. The former pair was assigned to 2H-2' and the latter, to 2H-3a' on the basis of NOE results and in accordance with the results obtained for ptaeroxylon and with literature[95, 98]. Irradiation of the doublets at δ_H4.00 and 4.05 (2H-3a') **[spectrum 34e]** showed a positive NOE result with the resonance at δ_H6.24 (H-4') only, allowing their assignment to 2H-3a'. Irradiation of the doublet at δ_H4.29 (H-2'A)

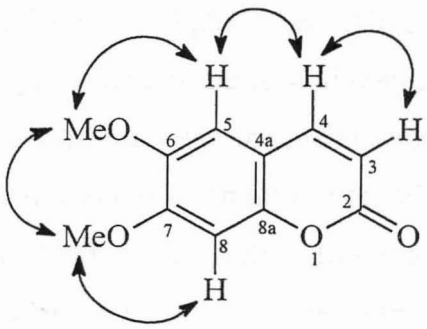
[spectrum 34f] showed positive NOE results only with the doublet at δ_H4.82 (H-2'B), and similarly, irradiation of the latter indicated positive results with the resonance at δ_H4.29 (H-2'A) only. These results confirmed the assignment of the pair of doublets at δ_H4.29 and 4.82 to 2H-2' as they were in close spatial proximity with the 3'-hydroxyl group only. The HETCOR NMR spectrum **[spectrum 33]** allowed assignment of C-2' and C-3a' to the methylene resonances at δ_C75.1 and 66.6 respectively.

Acetylation of compound V resulted in shifting of both the ring and the side chain methylene proton resonances such that they occurred as two pairs of doublets at δ_H4.92 and 4.83, and δ_H4.55 and 4.61, as seen in the ^{1}H NMR spectrum of the acetate derivative of compound V **[spectrum 35]**. The presence of two acetate methyl proton resonances at δ_H2.06 and 2.10 in this spectrum indicated that the tertiary hydroxyl group at C-3' was also acetylated.

The remaining resonances in the ^{13}C NMR spectrum **[spectrum 30]** were assigned with reference to literature of such compounds[103]. The structure of compound V was thus assigned to ptaeroglycol.

3.2.3 Structural elucidation of Compound VI

This compound was found to be the known, simple coumarin, scoparone, which has been isolated from various sources[105] (e.g. *Artemisia scoparia* and *A. capillaris*), but not from *Cedrelopsis grevei* nor *Ptaeroxylon obliquum*. It was the only simple coumarin isolated from *Cedrelopsis grevei*.



Compound VI (Scoparone)

↷ Positive NOE results obtained

The mass spectrum **[spectrum 36]** of this compound exhibited a molecular ion $[M]^+$ peak at *m/z* 206.0575, which corresponded to the molecular formula $C_{11}H_{10}O_4$. This compound thus possessed six double bond equivalents. The IR spectrum **[spectrum 37]** indicated peaks at 2966 (C-H aromatic stretching), 2855 (C-H aliphatic stretching), 1723 (C=O stretching) and 1605 cm^{-1} (C=C stretching). The carbonyl frequency was typical for α-pyrone carbonyl group stretching[100]. The UV absorption spectrum **[spectrum 38]** showed absorptions at 343 (log ε 2.3), 293 (log ε 1.8), and 232 nm (log ε 4.2), as well as a shoulder absorption at 260 nm. This spectrum was found to be typical of a 6,7-dioxygenated coumarin[106].

The ^1H NMR spectrum **[spectrum 39]** of compound VI indicated the presence of a coumarin nucleus, with an unsubstituted pyrone ring, by the characteristic doublets at δ_H7.92 and 6.30 assigned to the vicinal protons, H-4 and H-3 respectively[107]. All six double bond equivalents could be allocated to this nucleus, indicating the presence of a simple coumarin. The COSY NMR spectrum **[spectrum 40]** indicated coupling between H-4 and H-3. The NOESY NMR spectrum **[spectrum 41]** showed that, on irradiation of the resonance at δ_H6.30 (H-3), positive NOE results were obtained only with the resonance at δ_H7.92 (H-4). Irradiation of the H-4 resonance indicated positive NOE results with H-3 as well as the singlet at δ_H7.19 which was thus allocated to H-5. The

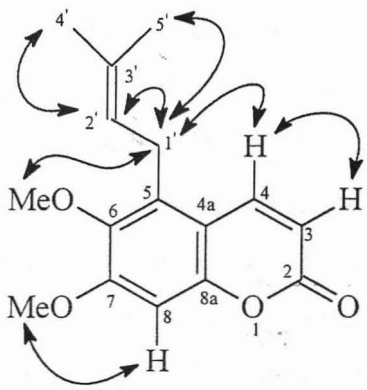
COSY NMR spectrum **[spectrum 40]** indicated that the δ_H7.19 resonance was not coupled, and, on irradiation of this signal **[spectrum 41]**, positive NOE results were obtained with H-4 as well as with the methoxy group proton resonance at δ_H3.90. This indicated that the C-6 position possessed a methoxy substituent. The HMQC NMR spectrum **[spectrum 42]** allowed allocation of the carbon resonances at δ_C146.0, 112.5 and 109.0 to C-4, C-3 and C-5 respectively.

On irradiation of the methoxy methyl proton resonance at δ_H3.90 **[spectrum 41]**, positive NOE results were obtained with the H-5 singlet (δ_H7.19), as well as with the second methoxy proton resonance at δ_H3.95, thus allowing placement of the second methoxy substituent at C-7. The COSY NMR spectrum **[spectrum 40]** indicated that both the methoxy proton signals were uncoupled and the HMQC NMR spectrum **[spectrum 42]** allowed allocation of the resonances at δ_C55.5 and 56.0 to C-6 and C-7 methoxy group carbons respectively. Irradiation of the methoxy methyl group protons at C-7 (δ_H3.95) **[spectrum 41]** produced positive NOE results with the methoxy group at C-6 (δ_H3.90) and the singlet at δ_H7.03. This allowed the latter resonance to be assigned to H-8. Further irradiation of this signal indicated a positive NOE only with the C-7 methoxy group, as expected. The COSY NMR spectrum **[spectrum 40]** indicated that this resonance was uncoupled and the HMQC NMR spectrum **[spectrum 42]** allowed assignment of the resonance at δ_C100.0 to C-8.

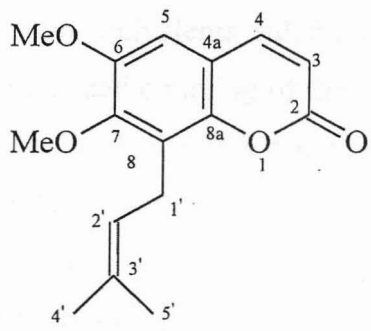
The HMBC NMR spectrum **[spectrum 43]** was used to allocate the quaternary carbon resonances of compound VI. The H-3 doublet correlated only with the resonance at δ_C162.5 which was thus assigned to C-2. This characteristic downfield carbon resonance was expected owing to the carbonyl substituent at this position. The H-8 singlet correlated with resonances at δ_C153.5 and 151.0, which with comparison to scoparone ^{13}C NMR data[102, 108], were allocated to C-7 and C-8a respectively. The H-5 resonance correlated with both the above resonances, as well as with δ_C147.0 and 146.0 where the latter was previously allocated to C-4. By referring to literature[108], δ_C147.0 was assigned to C-6. No resonance ascribable to C-4a (scoparone data[102, 108], δ_C114.9) could be detected in the HMBC NMR spectrum **[spectrum 43]** of compound VI due to the small sample size used.

3.2.4 Structural elucidation of Compound VII

This compound was found to be a 6,7 dioxygenated coumarin. A prenyl residue was present at the C-5 position, and the 6 and 7 positions were methoxylated, as illustrated below. It was thus a 5-prenyl derivative of compound VI, scoparone. According to literature, this compound has not been previously isolated. Compound VII was found to be very closely related to compound VIII, where in the latter, the prenyl residue occurred at the C-8 position.



Compound VII



Compound VIII (O-Methylcedrelopsin)

Positive NOE results obtained

The high resolution mass spectrum **[spectrum 44]** of compound VII showed a molecular ion $[M]^+$ peak at *m/z* 274.1191, which corresponded to the molecular formula $C_{16}H_{18}O_4$. This compound thus has eight double bond equivalents. The IR spectrum **[spectrum 45]** indicated peaks at 2923 (C-H aromatic stretching), 2854 (C-H aliphatic stretching), 1727 (C=O stretching), and 1605 cm^{-1} (C=C stretching), where the carbonyl frequency was typical for α-pyrone carbonyl stretching of a coumarin[100]. The UV absorption spectrum **[spectrum 46]** showed absorptions at 335 (log ε 1.9), 296 (log ε 1.9), and 232 nm (log ε 4.1), as well as a shoulder absorption at 259 nm. This spectrum, like that of compound VI, was found to possess the typical absorption pattern of a 6,7-dioxygenated coumarin[106].

The ^1H NMR spectrum **[spectrum 47]** of compound VII indicated the presence of a coumarin nucleus[107, 108]. This allowed allocation of seven of the eight double bond equivalents to the coumarin nucleus, the remaining one indicating the presence of a double bond in the side chain. The two one-proton doublets at δ_H8.00 and 6.31 were allocated to H-4 and H-3 respectively. The

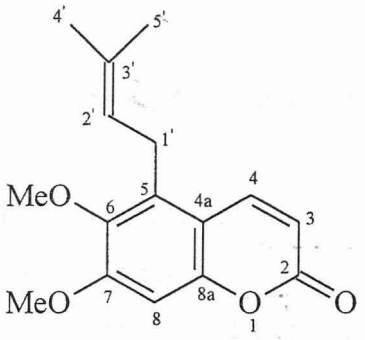
COSY NMR spectrum **[spectrum 49]** indicated coupling of these two resonances with each other only, and the HETCOR NMR spectrum **[spectrum 50]** allowed the resonances at δ_C143.3 and 113.1 to be allocated to C-4 and C-3 respectively. On irradiation of the H-3 resonance (δ_H6.31) **[spectrum 51]** a positive NOE was obtained with the resonance at δ_H8.00 (H-4) only, while irradiation of H-4 gave positive results with one resonance at δ_H6.31 (H-3) as well as the two-proton doublet at δ_H3.65. This doublet was allocated to H-1' of the 3-methylbut-2-enyl (prenyl) substituent, which was thus placed at C-5. Two non-equivalent vinyl methyl proton resonances at δ_H1.87 and 1.73 in the ^{1}H NMR spectrum **[spectrum 47]** of compound VII supported the presence of this sidechain, as did the MS which, through double bond equivalents, ruled out the presence of a third ring. The COSY NMR spectrum **[spectrum 49]** indicated coupling of the 2H-1' doublet with the one-proton alkene triplet at δ_H5.10 which was allocated to H-2' of the prenyl group. Irradiation of the 2H-1' resonance **[spectrum 51]** showed positive NOE results with H-4, H-2', the methoxy group proton resonance at δ_H3.81 and the vinyl methyl group at δ_H1.87. A methoxy group was thus placed at C-6 and the methyl resonance was assigned to 3H-5'. Irradiation of the H-2' resonance indicated positive NOE results with 2H-1' and the methyl proton resonance at δ_H1.73 which was assigned to 3H-4'. The HETCOR NMR spectrum **[spectrum 50]** allowed assignment of the resonances at δ_C25.4, 123.6, 25.9 and 18.2 to C-1', C-2', C-4' and C-5' respectively.

The fact that 2H-1' did not show positive NOE results with the one-proton singlet at δ_H6.94 indicated that this aromatic proton could not be H-6 and confirmed the placement of the methoxy group at C-6. On the basis that most natural coumarins are 7-oxygenated, this singlet was assigned to H-8. The COSY NMR spectrum **[spectrum 49]** showed that this resonance was uncoupled and the HETCOR NMR spectrum **[spectrum 51]** allowed allocation of δ_C99.7 to C-8. The NOESY NMR spectrum **[spectrum 51]**, indicated that irradiation of the resonance at δ_H6.94 showed positive NOE results only with the methoxy methyl proton resonance at δ_H3.98 which was thus allocated to C-7. The fact that only one positive result was obtained, supports the assignment of δ_H6.94 to H-8. The COSY NMR spectrum **[spectrum 49]** indicated that both the methoxy proton resonances were uncoupled, as expected, and the HETCOR NMR spectrum **[spectrum 50]** allowed assignment of the resonances at δ_C61.4 and 56.7 to the C-6 and C-7 methoxy groups carbon atoms, respectively.

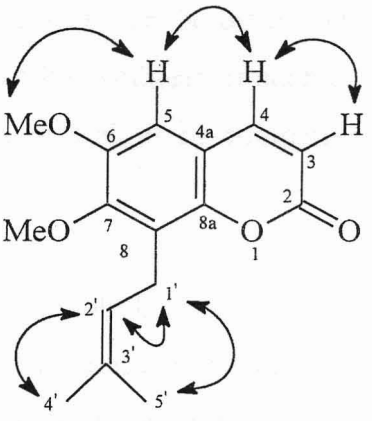
The quaternary carbon resonances in the ^{13}C NMR spectrum **[spectrum 48]**, were allocated by referring to ^{13}C NMR data of Compound VI, as well as to other literature values[102, 107, 108].

3.2.5 Structural elucidation of Compound VIII

This compound was found to be the 8-prenyl derivative of 6,7 dimethoxycoumarin (compound VI, scoparone). According to literature, this compound is the known compound, O-methylcedrelopsin which has previously been isolated from *Zanthoxylum usambarense*[109]. Compound VIII was found to be a structural isomer of compound VII.



Compound VII



Compound VIII (O-Methylcedrelopsin)

Positive NOE results obtained

The mass spectrum **[spectrum 52]** of compound VII showed a molecular ion $[M]^+$ peak at *m/z* 274.0827, which corresponded to the molecular formula $C_{16}H_{18}O_4$, indicating eight double bond equivalents. This molar mass was identical to that obtained for compound VII. The IR spectrum **[spectrum 53]** showed peaks at 2928 (C-H stretching), 1726 (C=O stretching) and 1600 cm^{-1} (C=C stretching), where the carbonyl frequency was typical for α-pyrone carbonyl group stretching[100]. The UV absorption spectrum **[spectrum 54]** indicated absorptions at 341 (log ε 1.8), 294 (log ε 2.1), and 232 nm (log ε 4.1). This spectrum, like that of compound VI and VII, was found to be typical of a 6,7-dioxygenated coumarin[106].

The NMR data available for this compound in the literature was incomplete. The ^1H NMR spectrum **[spectrum 55]** of compound VIII was similar to that of compound VII, but all the resonances had shifted slightly. The H-3 and H-4 resonances occurred at δ_H6.36 and 7.92 respectively and the COSY NMR spectrum **[spectrum 56]** indicated coupling of these two doublets with each other only. The NOESY NMR spectrum **[spectrum 57]** showed H-3 produced positive

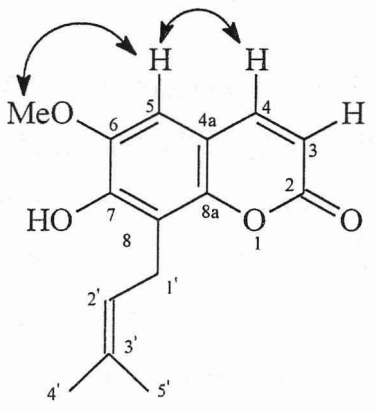
NOE results only with H-4, while irradiation of H-4 produced positive results with H-3 as well as the resonance at δ_H7.12, which was thus allocated to H-5. This result allowed the prenyl substituent to be placed at C-8, as C-6 and C-7 retained the methoxy groups present in compound VII. The H-5 resonance was shown to be uncoupled in the COSY NMR spectrum **[spectrum 56]** of compound VIII. Irradiation of H-5 produced positive NOE results with H-4 as well as with the methoxy group proton resonances δ_H3.92 which was thus assigned to the methyl protons of the methoxy group at C-6. The close proximity of the two methoxy groups in the ^{1}H NMR spectrum **[spectrum 55]** made it difficult to see whether each produced positive NOE results for the other. The second methoxy group at δ_H3.90 was placed at C-7, again on the basis of biosynthesis of such compounds, the UV spectrum being typical of a 6,7-dioxygenated coumarin, as well as by comparison to the ^{1}H NMR spectrum **[spectrum 47]** of compound VII.

The coumarin nucleus accounted for seven of the eight double bond equivalents. The remaining one indicated the presence of a prenyl group. The two non-equivalent vinylic methyl proton resonances in the ^{1}H NMR spectrum **[spectrum 55]** of compound VIII indicated that a prenyl group was present. The COSY NMR spectrum **[spectrum 56]** showed coupling between the two-proton doublet at δ_H3.58 and the olefinic triplet at δ_H5.21, which were assigned to H-1' and H-2' respectively. The COSY NMR spectrum **[spectrum 56]** indicated that both these resonances coupled with both the vinylic methyl group proton resonances as well. The NOESY NMR spectrum **[spectrum 57]** showed that irradiation of the H-1' doublet produced positive NOE results with the H-2' triplet as well as the methyl proton resonance at δ_H1.88, allowing assignment of the latter to C-5'. Irradiation of the H-2' triplet produced positive results with the H-1' doublet and the methyl proton resonance at 1.71 which was thus assigned to C-4'.

HMBC and HMQC data was unavailable, preventing any carbon assignments.

3.2.6 Structural elucidation of Compound IX

Compound IX was also found to be a 6,7-dioxygenated coumarin. It differed from the above 6,7-dimethoxycoumarins (VI - VIII) in that it was a 6-methoxy-7-hydroxy coumarin. The presence of an 8-prenyl group indicated its relation to compound VIII. This compound was found to be the known coumarin, cedrelopsin, which has been previously isolated from *Cedrelopsis grevei*[97].



Compound IX

↷ Positive NOE results obtained

The mass spectrum **[spectrum 58]** of this compound showed a molecular ion $[M]^+$ peak at *m/z* 260.1058, which corresponded to the molecular formula $C_{15}H_{16}O_4$. The eight double bond equivalents present in compound IX again indicated the presence of an uncyclised side chain. This molar mass differed by 13.9793 from that obtained for compound VIII (274.0841), implying that the former possessed one methylene group less than the latter. The IR spectrum **[spectrum 59]** showed peaks at 3352 (O-H stretching), 2922 (C-H stretching), 1707 (C=O stretching) and 1600 cm^{-1} (C=C stretching), where the carbonyl frequency was typical for α-pyrone carbonyl stretching[100]. The UV absorption spectrum **[spectrum 60]** indicated absorptions at 341 (log ε 3.5), 302 (log ε 2.3), and 231 nm (log ε 4.2), as well as a shoulder absorption at 250 nm. This spectrum, like those of compounds VI, VII and VIII, was found to be typical of a 6,7-dioxygenated coumarin[106].

No NMR data was available for this compound in literature[97]. The ^1H NMR spectrum **[spectrum 61]** indicated that the compound possessed a coumarin nucleus by the presence of the two one-proton doublets at δ_H7.89 and 6.23 ascribable to H-4 and H-3 respectively. The COSY

NMR spectrum **[spectrum 62]** indicated that these two protons were coupled with each other only. Two non-equivalent methyl group proton resonances at δ_H1.69 and 1.87 were indicative of vinylic methyl groups and were assigned to 3H-4' and 3H-5' as in compounds VI, VII and VIII. The weakness of the ^{1}H NMR spectrum **[spectrum 61]** made detection of 2H-1' and H-2' resonance assignments difficult. The COSY NMR spectrum **[spectrum 62]** clarified the coupling between the above protons and allowed assignment of resonances at δ_H3.56 and δ_H5.29 to 2H-1' and H-2' respectively.

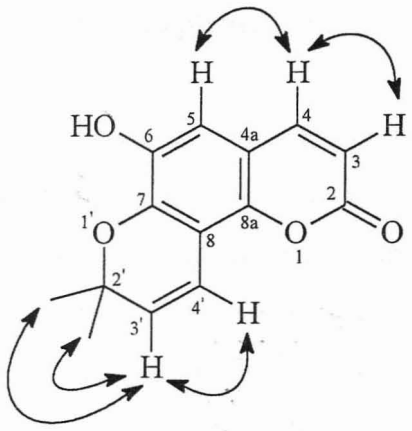
The ^{1}H NMR spectrum **[spectrum 61]** of compound IX differed from those of compounds VII **[spectrum 47]** and VIII **[spectrum 55]** in that, although the characteristic one-proton singlet was present at δ_H7.03, only one methoxy group proton resonance was evident at δ_H4.92. This difference supported the mass spectrometric results which indicated that compound IX possessed one methylene group less than compounds VIII and VII. These results, as well as those of the UV spectrum, implied that the C-6 and C-7 positions were oxygenated (UV spectrum **[spectrum 60]**), where one position was methoxy- and the other, hydroxy-substituted. This hydroxyl group resonance was not evident in the ^{1}H NMR spectrum **[spectrum 61]** of compound IX as deuteriomethanol was the solvent used in obtaining the spectra. Assignments of these groups, as well as the singlet resonance at δ_H7.03 required NOE analysis.

On irradiation of the singlet at δ_H7.03 **[spectrum 63a]**, positive NOE results were obtained with the H-4 doublet as well as the methoxy group proton singlet, allowing assignment of the singlet resonance to H-5, and the placement of the methoxy group at C-6. Irradiation of the methoxy methyl group proton resonance at δ_H3.92 **[spectrum 63b]** showed a positive NOE result with the H-5 singlet only. Thus the hydroxy group was assigned to C-7 which is almost always oxygenated in coumarins[107, 108]. The prenyl group was thus placed at C-8.

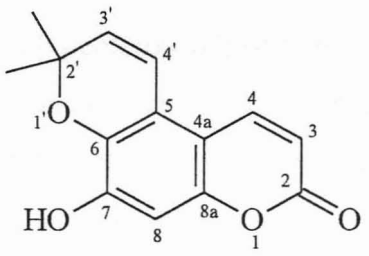
The amount of sample was too small for ^{13}C NMR analysis on the 300 MHz NMR spectrometer.

3.2.7 Structural elucidation of Compound X

This compound was found to be the previously isolated angular pyranocoumarin, norbraylin. It has been isolated from *Pitaria punctata* and *Toddalia aculeata*[110], but not from *Cedrelopsis grevei* nor *Ptaeroxylon obliquum*. Compound XI was found to be the isomeric angular pyranocoumarin of compound X.



Compound X (Norbraylin)



Compound XI

Positive NOE results obtained

The mass spectrum **[spectrum 64]** of this compound exhibited a molecular ion $[M]^+$ peak at *m/z* 244.0731, which corresponded to the molecular formula $C_{14}H_{12}O_4$. The presence of nine double bond equivalents indicated that a third ring was present in the molecule. The peak at *m/z* 229 ($[M-15]^+$) is also of importance as pyranocoumarins exhibit the characteristic loss of one of the geminal methyl groups, forming the stable benzopyrylium ion[111]. The IR spectrum **[spectrum 65]** showed peaks at 3410 (O-H stretching), 2926 (C-H stretching), and 1721 cm^{-1} (C=O stretching), where the latter frequency was typical for α-pyrone carbonyl group stretching[100]. The UV absorption spectrum **[spectrum 66]** indicated absorptions at 341 (log ε 1.8), 299 (log ε 1.7), 260 (log ε 2.5) and 231 nm (log ε 4.1).

The ^1H NMR spectrum **[spectrum 67]** of compound X was indicative of a coumarin with an unsubstituted pyrone ring[107]. This was based on the two one-proton doublets at δ_H7.81 and 6.27, assigned to H-4 and H-3 respectively. The COSY NMR spectrum **[spectrum 68]** showed that these resonances coupled only with each other and the HMQC NMR spectrum **[spectrum 70]** allowed

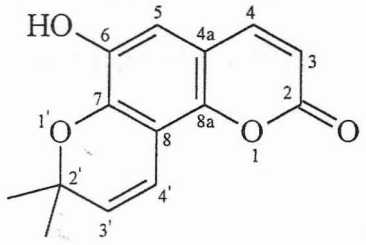
allocation of the resonances at δ_C146.5 and 113.5 to C-4 and C-3 respectively. The NOESY NMR spectrum **[spectrum 69]** indicated that on irradiation of H-3, positive NOE results were obtained only with H-4, while on irradiation of H-4, positive results were obtained with H-3 as well as the one-proton singlet at δ_H6.92. This latter resonance was thus assigned to H-5 and correlated with the resonance at δ_C112.5 in the HMQC NMR spectrum **[spectrum 70]**, which was assigned to C-5. The COSY NMR spectrum **[spectrum 68]** indicated this resonance to be uncoupled and the NOESY NMR spectrum **[spectrum 69]** showed that on irradiation, it produced positive NOE results only with H-4, implying a substituted C-6 position.

The presence of the six-proton singlet in the ^{1}H NMR spectrum **[spectrum 67]** of compound X was an indication that a pyranocoumarin was present[111]. This resonance, at δ_H1.52, was allocated to protons of the two *geminal* methyl groups at C-2', and the HMQC NMR spectrum **[spectrum 70]** allowed allocation of the resonance at δ_C27.5 to the carbon atoms of these equivalent methyl groups. The two remaining doublets at δ_H6.85 and 5.90 were respectively allocated to the olefinic protons, H-4' and H-3', of the pyran ring. This assignment was based on the positive NOE result with the doublet at δ_H5.90, obtained on irradiation of the two methyl group proton resonance **[spectrum 69a]**. Irradiation of the H-3' doublet produced positive NOE results with the doublet at δ_H6.85 (H-4') and the methyl proton resonance **[spectrum 69b]**, while irradiation of the H-4' doublet showed positive NOE results with H-3' only **[spectrum 69]**,. The resonances at δ_C114.5 and 132.5 were allocated to C-4' and C-3' respectively, with reference to the HMQC NMR spectrum **[spectrum 70]**.

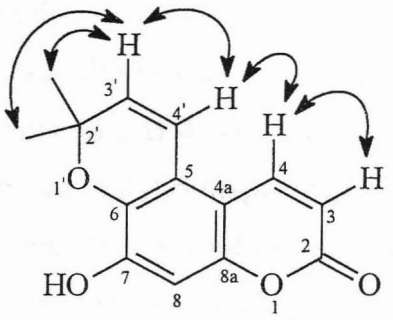
Assigning compound X as an angular pyranocoumarin was based on the fact that, on irradiation of H-4', no positive NOE result was obtained for H-5 **[spectrum 69]**. A positive result would have indicated a linear pyranocoumarin. Furthermore, the fact that no positive results were obtained between H-4' and H-4, indicated that prenylation had occurred at C-8 and further cyclisation of this group with a 7-hydroxy resulted in the angular pyranocoumarin, with the pyran ring bonded at C-7 and C-8. The lack of any further olefinic singlets in the ^{1}H NMR spectrum **[spectrum 67]** of compound X allowed allocation of a hydroxy group at C-6, which was previously assigned as a quaternary carbon. This hydroxy group was not evident in the ^{1}H NMR spectrum **[spectrum 67]** of compound X as deuteriomethanol was the solvent used in obtaining the spectra.

3.2.8 Structural elucidation of Compound XI

Compound XI was found to be an angular pyranocoumarin. Furthermore, it was the structural isomer of compound X (norbraylin). This compound has not been previously isolated.



Compound X (Norbraylin)



Compound XI

↷ Positive NOE results obtained

The mass spectrum **[spectrum 71]** of this compound exhibited a molecular ion $[M]^+$ peak at *m/z* 244.0731, which corresponded to the molecular formula $C_{14}H_{12}O_4$. The nine double bond equivalents present, indicated the presence of a three ring coumarin. This molar mass was identical to that obtained for compound X. The peak at *m/z* 229 ($[M-15]^+$) which was found in the mass spectrum of compound X, is also present here. Pyranocoumarins exhibit a characteristic loss of one of the *geminal* methyl groups, forming the stable benzopyrylium ion[111]. The IR spectrum **[spectrum 72]** showed peaks at 3374 (O-H stretching), 2927 (C-H stretching), and 1712 cm^{-1} (C=O stretching), the latter frequency being typical for the α-pyrone carbonyl stretching[100]. The UV absorption spectrum **[spectrum 73]** indicated absorptions at 316 (log ε 2.6), 275 (log ε 1.8), and 232 nm (log ε 4.1).

The ^1H NMR spectrum **[spectrum 74]** of compound XI was identical to that of compound X **[spectrum 67]**, except that the resonances had all shifted slightly. Again the two one-proton doublets at δ_H8.15 and 6.26 indicate the presence of a coumarin nucleus. These doublets were shown to couple only with each other in the COSY NMR spectrum **[spectrum 77]**, and the HETCOR NMR spectrum **[spectrum 78]** allowed assignment of the methine resonances at δ_C141.6 and 112.6 respectively. Irradiation of the H-3 doublet produced a positive NOE result with the H-4 resonance only **[spectrum 80a]**, while irradiation of H-4 indicated positive NOE results

[spectrum 80b] with H-3 as well as with the one-proton doublet at δ_H6.86. This doublet, by comparison with the ^{1}H NMR spectrum **[spectrum 67]** of compound X, was assigned to H-4' of the pyran ring, which indicated the angular form of the pyranocoumarin.

The COSY NMR spectrum **[spectrum 77]** showed coupling between the H-4' doublet and the one-proton doublet at δ_H5.97, thus assigned to H-3'. The NOE spectrum **[spectrum 80c]**, indicated that on irradiation of H-4', positive NOE results were obtained with H-4 and H-3', while irradiation of the H-3' doublet **[spectrum 80d]** produced positive results with H-4' as well as the six-proton methyl singlet at δ_H1.50. In turn, on irradiation of the resonance at δ_H1.50 **[spectrum 80e]**, positive NOE results were obtained with H-3' only. These results were all in accordance with the presence of a pyran ring system in the angular arrangement illustrated for compound XI. The HETCOR NMR spectrum **[spectrum 78]** allowed allocation of the resonances at δ_C118.0. 134.9 and 27.4 to H-4', H-3' and the two equivalent methyl groups. The DELAYED HETCOR NMR spectrum **[spectrum 79]** indicated that the resonances at δ_C134.9 and 77.7 both correlated with the methyl proton resonance, allowing allocation of the resonance at δ_C77.7 to C-2'.

The one proton singlet at δ_H6.71 in the ^{1}H NMR spectrum **[spectrum 74]** of compound XI was allocated to H-8 on the basis that coumarins are almost always oxygenated at C-7 as well as that this upfield position of an aromatic proton indicates the presence of two *ortho* oxygen substituents[104, 112]. The COSY NMR spectrum **[spectrum 77]** indicated that this singlet was uncoupled and the HETCOR NMR spectrum **[spectrum 78]** allowed allocation of the methine resonance at δ_C103.9 to C-8. Furthermore, irradiation of the resonance at δ_H6.71 **[spectrum 80f]** showed no positive NOE results, supporting its assignment to H-8. A hydroxy substituent was assigned to C-7. This was not evident in the ^{1}H NMR spectrum **[spectrum 74]** of compound XI as deuteriomethanol was used as the solvent in obtaining these spectra.

The remaining quaternary carbons were assigned with reference to the DELAYED HETCOR NMR spectrum **[spectrum 79]**, as well as other pyranocoumarin ^{13}C NMR data[108].

Chapter 4

Experimental

4. EXPERIMENTAL

4.1 Introduction

Nuclear magnetic resonance spectroscopy was performed using a Varian Gemini 300 MHz (UND) and a Bruker 400 MHz† NMR spectrometer and spectra were recorded at room temperature. Solvents used varied depending on the compounds, and included deuteriochloroform ($CDCl_3$), deuteriomethanol (CD_3OD) and deuteriopyridine (C_5D_5N). Chemical shifts (ppm) were recorded relative to TMS at $\delta = 0$ as well as

- the central line of the triplet at δ_C77.0 or the singlet at δ_H4.10 for deuteriochloroform
- the central line of the septet at δ_C49.0 or the singlets at δ_H4.10 or δ_H 3.34 for deuteriomethanol
- the central line of the triplets at δ_C149.6, δ_C135.3 and δ_C123.3 or the singlets at δ_H8,57, δ_H7.41 and δ_H7.05 for deuteriopyridine.

Infrared (IR) spectra were recorded using a Nicolet Impact 400 Fourier Transform Infrared (FT-IR) spectrophotometer. Sodium chloride plates were employed and dichloromethane utilised as a solvent for all compounds. The sample was dissolved in dichloromethane and a few drops were placed on the sodium chloride plate. Once the solvent had evaporated, the spectra were recorded. Ultraviolet (UV) absorption spectra were obtained on a Varian DMS 300 UV-visible spectrophotometer, again incorporating dichloromethane as solvent. The high resolution mass spectra were recorded at the Cape Technicon by Dr. P. Boshoff. Melting points were determined, where possible, on an Ernst Leitz Weltzlar melting point apparatus and are uncorrected. Optical rotations were measured at room temperature in chloroform using an Optical Activity AA-5 Polarimeter together with a series A2 stainless steel (4 x 200 mm) unjacketed flow tube.

Column chromatography and thin-layer chromatographic (TLC) techniques were incorporated in the isolation processes. Column chromatography involved the use of different sized columns ranging from 2 - 8cm in diameter depending on the purification stage. Initial separation with the use of 8 cm diameter columns packed with silica gel 60 (Merk Art. 7734) proved successful, where elution proceeded by gravity. Flash chromatography was performed with the use of Merck 9385 silica gel, on columns of smaller diameter as extract purification improved. The close structural similarity between many of the compounds isolated made separation difficult. Final purification processes involved open, 0.75 cm diameter pasteur pipette columns packed with Merck 9385 silica gel. Such columns, eluted under gravity with 1ml fraction collections, proved very successful in the

† - obtained by Bruker in Germany

separation of such compounds. The solvent systems used in all stages of purification involved the use of hexane, dichloromethane and ethyl acetate, in varying ratios.

Thin-layer chromatography was used in monitoring the column chromatographic separations. This was performed using 0.2 mm thick aluminium-backed silica gel (Merck Art. 5553) TLC plates. The solvent systems used in developing the plates were kept slightly more polar than those for the corresponding column chromatography, although the solvents themselves were the same. Superior separation was found to occur with compounds appearing at low R_f values as compared to those of higher R_f values. The spots on the TLC plates were visualised by spraying the developed plates with anisaldehyde spray reagent consisting of anisaldehyde, concentrated sulphuric acid and cold methanol in a 1:2:97 ratio (volume), respectively. On heating the sprayed plates, different coloured spots formed, depending on the compounds present. Prior to heating, the developed plates were also analysed under UV light (long wavelength) as many fluorescing compounds do not appear on heating, with the use of the above spray reagent.

Extraction of the plant material was carried out on finely ground, dry plant material, received in this state. Extractions were performed using a soxhlet apparatus, with refluxing hexane, dichloromethane and methanol successively, each for approximately 48 hours. The solvents were evaporated *in vacuo* after which the purification processes commenced with initial TLC chromatography.

4.2 Extractives from *Neobeguea mahafalensis*

The stem-bark of *Neobeguea mahafaliensis* Leroy. was collected in Madagascar by Dr. M. Randrianarivelojosia and a voucher specimen (No.: 004-Mj / M.Dul) was retained in the herbarium at the Botany Department of the University of Antananarivo in Madagascar. A 700 g sample of stem-bark was extracted with hexane, dichloromethane and methanol respectively. The masses of the extracts obtained after evaporation of the respective solvents were:

- hexane extract - 35.9 g
- dichloromethane extract - 44.6 g
- methanol extract - 97.6 g

Only the former two samples were analysed. A 7g sample of each extract was subjected to chromatographic separation. Both extracts were found to possess compounds II (β-amyrin) and III (stigmasterol). Compound I was isolated from the hexane extract only.

4.2.1 Physical data of Compound I

Name: *Neobeguin*

Yield: 230 mg (white cryst. MeOH) (3.89% yield)

Molecular formula: $C_{37}H_{46}O_{14}$

Mass spectrum: EIMS *m/z* 714.2876 (13%) ($C_{36}H_{46}O_{14}$, req. 714.2865), 654 $[M-60]^+$ (19%), 594 $[M-120]^+$ (5%), 583 $[M-131]^+$ (59%), 481 $[M-233]^+$ (26%), 463 $[M-251]^+$ (18%), 43 $[M-671]^+$ (100%) **[spectrum 1]**

Melting point: 228-230°C

Optical rotation: $[\alpha]_D = -51.78^\circ$ (c, 0.007242 g / ml in $CHCl_3$)

Infrared spectrum: ν_{max} (NaCl): 3569 (O-H stretching), 1743 (C=O stretching) and 1638 cm^{-1} (C=C stretching). **[spectrum 2]**

^{1}H NMR: **[spectrum 3]**

Compound I ($CDCl_3$)		**Bussein A** ($CDCl_3$)[83]	
Proton	Chemical shift, δ /ppm	Proton	Chemical shift, δ /ppm
3	4.85 *s*	3	4.99 *s*
5	3.00 *m*	5	1.95 *m*
6	2.45 and 2.23 *m*	6	2.76 *m*
11*	2.02 and 1.85 *m*	11	5.65 *d* J = 2.5Hz
12*	1.18 and 1.48 *m*	12	4.67 *d* J = 2.5Hz
14	2.65 *s*	14	3.34 *s*
17	5.84 *s*	17	5.81 *s*
21	7.48 *s*	21	7.64 *s*
22	6.38 *br s*	22	6.45 *br s*
23	7.32 *br s*	23	7.30 *br s*
29	1.81 and 1.89 *d* (J = 6.7 Hz)	29	-
30	5.33 *s*	30	5.50 *s*
32	1.56 *s*	32	1.63 *s*
2'	2.94 *m*		-
CO_2Me	3.68 *s*	CO_2Me	3.76 *s*
3-Acet-Me	1.96 *s*	Acet-Me	2.17 *s*
30-Acet-Me	2.26 *s*		2.00 *s*
			1.66 *s*
18-Me	1.21 *s*	C-Methyls	1.54 *s*
19-Me	1.14 *s*		1.28 *s*
28-Me	0.94 *s*		1.00 *s*
3'-Me	1.24 *d* (J = 6.7 Hz)		
4'-Me	1.11 *d* (J = 6.7 Hz)		
*** - resonance may be interchanged**			

^{13}C NMR: **[spectrum 4]**

Compound I ($CDCl_3$)		**Bussein A** ($CDCl_3$)[83]	
Carbon	Chemical shift, δ / pm	Carbon	Chemical shift, δ / pm
1	84.3 *s*	1	83.3 *s*
2	77.9 *s*	2	77.2 *s*
3	83.1 *d*	3	82.9 *d*
4	40.1 *s*	4	44.9 *s*
5	37.2 *d*	5	36.9 *d*
6	33.9 *t*	6	33.3 *t*
7	172.7 *s*	7	172.7 *s*
8	80.7 *s*	8	79.6 *s*
9	85.1 *s*	9	84.9 *s*
10	46.0 *s*	10	45.7 *s*
11*	24.5 *t*	11	70.0 *d*
12*	31.2 *t*	12	68.9 *d*
13	45.7 *s*	13	45.7 *s*
14	44.9 *d*	14	43.6 *d*
15	91.1 *s*	15	90.9 *s*
16	169.4 *s*	16	168.7 *s*
17	70.9 *d*	17	73.9 *s*
20	122.3 *s*	20	122.0 *s*
21	141.1 *d*	21	142.7 *d*
22	110.0 *d*	22	109.4 *d*
23	142.7 *d*	23	141.3 *d*
29	39.7 *t*	29	39.9 *t*
30	74.5 *d*	30	70.0 *d*
31	118.7 *s*	31	119.1 *s*
1'	182.9 *s*	1'	182.8 *s*
2'	30.1 *d*	2'	41.4 *d*
3'	18.4 *q*	3'	15.8 *q*
4'	20.6 *q*	4'	14.2 *q*
18	21.8 *q*	18	20.8 *q*
19	15.6 *q*	19	20.8 *q*
28	14.7 *q*	28	20.5 *q*
32	20.8 *q*	32	20.2 *q*
		11-Acet-Me	16.6 *q*
3-Acet-Me	20.6 *q*	12-Acet-Me	30.1 *q*
30-Acet-Me	21.1 *q*	30-Acet-Me	17.8 *q*
MeO	52.1 *q*	MeO	51.8 *q*
		C-11 C=O	169.1 *s*
		C-12 C=O	168.4 *s*
C-3 C=O	170.4 *s*	C-30 C=O	170.7 *s*
C-30 C=O	170.8 *s*	1''	176.1 *s*
		2''	-
		3''	26.5 *t*
		4''	18.3 *q*
		5''	19.6 *q*
* - resonances may be interchanged			

4.2.2 Physical data of Compound II

Names: β-*amyrin, 12-oleanen-3β-ol*

Yield: 105 mg (white cryst. MeOH) (1.50% yield from *Neobeguea mahafalensis*)

Molecular formula: $C_{30}H_{50}O$

Mass spectrum: EIMS *m/z* 426.3874 (8%) ($C_{30}H_{50}O$, req. 426.3861), 411 $[M-15]^+$ (2%), 218 $[M-208]^+$ (100%), 203 $[M-233]^+$ (22%), 189 $[M-237]^+$ (8%) **[spectrum 10]**

Melting point: 196-197°C (lit. value = 197-197.5°C)[113]

Optical rotation: $[\alpha]_D = +82.1°$ (c, 0.300 g / mol in $CHCl_3$; lit. value +88.4°)[113]

Infrared spectrum: ν_{max} (NaCl): 3300 (O-H stretching), 2950 (saturated CH stretching), (C-H deformations), 1380 ($-CH_3$ symmetrical deformation), 1365 cm^{-1} (gem. dimethyl group twist) **[spectrum 11]**

^{1}H NMR data: **[spectrum 12]**

Compound II ($CDCl_3$)		**β-Amyrin** ($CDCl_3$)[91]	
Proton	Chemical shift, δ /ppm	Proton	Chemical shift, δ /ppm
3	3.20 *m* 1H ($W_{1/2}$ = 14.0 Hz)	3	3.22 *m* 1H ($W_{1/2}$ = 12.0 Hz)
12	5.16 *t* 1H (J = 3.6 Hz)	12	5.18 *t* 1H (J = 4.0 Hz)
23-Me*	0.95 *s* 3H	23-Me	0.96 *s* 3H
24-Me*	0.77 *s* 3H	24-Me	0.78 *s* 3H
25-Me*	0.92 *s* 3H	25-Me	0.93 *s* 3H
26-Me*	0.98 *s* 3H	26-Me	0.99 *s* 3H
27-Me*	1.11 *s* 3H	27-Me	1.12 *s* 3H
28-Me*	0.81 *s* 3H	28-Me	0.82 *s* 3H
29-Me*	0.85 *s* 3H	29-Me	0.86 *s* 3H
30-Me*	0.85 *s* 3H	30-Me	0.86 *s* 3H
*** - resonances may be interchanged**			

^{13}C NMR data: **[spectrum 13]**

Compound II ($CDCl_3$)		**β-Amyrin** ($CDCl_3$)[91]	
Carbon	Chemical shift, δ / ppm	Carbon	Chemical shift, δ / ppm
1	38.6 *t*	1	38.7 *t*
2	27.2 *t*	2	27.3 *t*
3	79.0 *d*	3	79.0 *d*
4	39.8 *s*	4	38.8 *s*
5	55.2 *d*	5	55.3 *d*
6	18.4 *t*	6	18.5 *t*
7	32.6 *t*	7	32.8 *t*
8	38.8 *s*	8	38.7 *s*
9	47.6 *d*	9	47.7 *d*
10	37.0 *s*	10	37.6 *s*
11	23.5 *t*	11	23.6 *t*
12	121.7 *d*	12	121.8 *d*
13	145.2 *s*	13	145.1 *s*
14	41.7 *s*	14	41.8 *s*
15	26.1 *t*	15	26.2 *t*
16	26.9 *t*	16	27.0 *t*
17	32.5 *s*	17	32.5 *s*
18	47.2 *d*	18	47.3 *d*
19	46.8 *t*	19	46.9 *t*
20	31.1 *s*	20	31.1 *s*
21	34.7 *t*	21	34.8 *t*
22	37.1 *t*	22	37.2 *t*
23*	28.4 *q*	23	28.2 *q*
24*	15.6 *q*	24	15.5 *q*
25*	15.5 *q*	25	15.6 *q*
26*	16.8 *q*	26	16.9 *q*
27*	26.0 *q*	27	26.0 *q*
28*	28.1 *q*	28	28.4 *q*
29*	33.3 *q*	29	33.3 *q*
30*	23.7 *q*	30	23.7 *q*
* - resonances may be interchanged			

Acetylation of Compound II

Acetic anhydride (5 ml) was added to a solution of compound II (15 mg) in pyridine (5 ml). The mixture was warmed on the steam bath and left to stand overnight under anhydrous conditions. The excess acetic anhydride was removed by the addition and *in vacuo* removal of methanol (5 ml), after which the pyridine was removed by the addition of toluene (3 x 10 ml) and azeotropic distillation. The final traces of toluene were removed by further addition and removal of methanol (3 x 10 ml).

Yield: 14 mg (white needle-like cryst.) (93% yield)

Molecular formula: $C_{32}H_{52}O_2$

Melting point: 243-245°C (lit. value 244-245°C)[113]

Optical Rotation: $[\alpha]_D = +81.1^\circ$ (c, 0.010 g / mol in $CHCl_3$; lit. value $+81.4^\circ$)[113]

^{1}H NMR: **[spectrum 17]**

δ (ppm): 0.80 (3H *s* 3H-24*) O.85 (15H *s* 3H-28, 3H-29, 3H-30, 3H-26, 3H-25*) 2.03 (3H *s* $OCOCH_3$), 4.48 (1H m H-3α), 5.16 (1H *t* *J*= 3.7 Hz H-12)

resonances may be interchanged

4.3 Extractives from *Cedrelopsis grevei*

The stem-bark of *Cedrelopsis grevei* was collected in Madagascar by Dr. M. Randrianarivelojosia and a voucher specimen (No.: 002-Mj / M.Dul) was retained in the herbarium at the Botany Department of the University of Antananarivo in Madagascar. A 1051.7 g sample of stem-bark was extracted with hexane, dichloromethane and methanol respectively. The masses of the extracts obtained after evaporation of the respective solvents were:

- hexane extract - 35.8 g
- dichloromethane extract - 43.4 g
- methanol extract - 108.9 g

Only the hexane extract of this species was investigated. A 7.5 g sample of the crude hexane extract was examined. Compounds II and III which were isolated from the hexane and dichloromethane extracts of the stem-bark of *Neobeguea mahafalensis*, were also isolated from the hexane extract of *Cedrelopsis grevei*. Two oxepin ring chromones, compound IV (ptaeroxylon) and compound V (ptaeroglycol) were also isolated, where the former had been previously identified from this species. A further six compounds were isolated from this extract, where all were found to be coumarins. These included the known compounds, scoparone (compound VI), O-methylcedrelopsin (compound VIII), cedrelopsin (compound IX) and norbraylin (compound X), as well as compound VI and compound XI which had not been previously isolated.

4.3.1 Physical data of Compound IV

Name: *Ptaeroxylinol; 6,9-Dihydro-5-hydroxy-8-hydroxymethyl-2-methyl-4H-pyrano-[3,2-h][1]benzopexin-4-one; Pteroxylinol*

Yield: 31mg (0.41% yield)

Molecular formula: $C_{15}H_{14}O_5$ (A mass spectrum was not obtained for this compound as the sample was misplaced while awaiting MS analysis in Cape Town).

Infrared spectrum: ν_{max} (NaCl): 3416 (broad band, O-H stretching), 3067 (C-H aromatic stretching), 2929 (C-H aliphatic stretching), 1652 (broad band, C=O and C=C stretching), 1166 and 1109 (C-O stretchings), 848 and 735cm^{-1} (C-H out-of-plane deformations) **[spectrum 21]**

UV spectrum: λ_{max} : 257 (log ε 4.15) and 235 (log ε 4.0) nm **[spectrum 22]**

^{1}H NMR: **[spectrum 23]**

^{13}C NMR data: **[spectrum 24]**

Compound IV (Ptaeroxylinol) ($CDCl_3$)			
Carbon	Chemical shift, δ / ppm	Proton	Chemical shift, δ / ppm
2	158.2 *s*		
3	108.8 *d*	3	6.03 *s* 1H
4	182.9 *s*		
4a	115.8 *s*		
5	164.7 *s*		
6	106.7 *s*		
7	167.2 *s*		
8	99.3 *d*	8	6.51 *s* 1H
8a	155.9 *s*		
2'	65.7 *t*	2'	4.02 *s* 2H
3'	138.0 *s*		
4'	125.4 *d*	4'	5.98 *t* 1H
5'	21.1 *t*	5'	3.55 *d* 2H (J = 5.5 Hz)
3a'	71.1 *t*	3a'	4.73 *s* 2H
CH_3	20.5 *q*	CH_3	2.33 *s* 3H
		C-5 OH	3.47 *s*

4.3.2 Physical data of Compound V

Names: *Ptaeroglycol; 8,9-Dihydro-5,8-dihydroxy-8-(hydroxymethyl)-2-methyl-4H-pyrano[3,2-h][1]benzopexin-4-one*

Yield: 26 mg (pale yellow cryst.) (0.35% yield)

Molecular formula: $C_{15}H_{14}O_6$

Mass spectrum: EIMS *m/z* 290.0798 (17%) ($C_{15}H_{14}O_6$, req. 290.0790), 259 $[M-31]^+$ (100%), 243 $[M-47]^+$ (48%), 231 $[M-59]^+$ (19%), 203 $[M-87]^+$ (13%) **[spectrum 28]**

Melting point: 227-229°C (lit. value 234°C)[98, 114]

^{1}H NMR: **[spectrum 29]**

^{13}C NMR: **[spectrum 30]**

Compound V (C_5D_5N)			
Carbon	Chemical shift, δ / ppm	Proton	Chemical shift, δ / ppm
2	167.9 *s*		
3	108.5 *d*	3	5.98 *s* 1H
4	182.8 *s*		
4a	105.9 *s*		
5	156.4 *s*		
6	110.9 *s*		
7	164.8 *s*		
8	98.0 *d*	8	6.47 *s* 1H
8a	160.9 *s*		
2'	66.6 *t*	2'	4.00 & 4.05 *d* 2H (J = 16 Hz)
3'	138.0 *s*		
4'	134.4 *d*	4'	6.24 *d* 1H (J = 12.5 Hz)
5'	118.2 *t*	5'	7.05 *d* 1H (J = 12.5 Hz)
3a'	75.1 *t*	3a'	4.29 & 4.82 *d* 2H (J =11 Hz)
CH_3	19.8 *q*	CH_3	1.95 *s* 3H

Acetylation of Compound V

Compound V (10 mg) was acetylated in the same manner as compound II (β-amyrin)

Yield: 8 mg (yellow crystals) (80 % yield)

Molecular formula: $C_{32}H_{52}O_2$

^{1}H NMR: **[spectrum 35]**

δ (ppm): 1.95 (3H *s* CH_3), 2.06 (3H *s* $OCOCH_3$), 2.10 (3H *s* $OCOCH_3$), 4.55 (2H *d* H-2'), 4.61 (2H *d* H-2'), 4.83 (2H *d* 3a'), 4.92 (2H *d* 3a'), 6.12 (1H *s* H-3), 6.12 (1H *d* H-4'), 6.59 (1H *s* H-8) and 7.15 (1H *d* H-5')

4.3.3 Physical data of Compound VI

Names: *6,7-Dimethoxy-2H-1-benzopyran-2-one, 6,7-Dimethoxycoumarin, Scoparone, Escoparone, Scoparin*

Yield: 13 mg (yellow gum) (0.17% yield)

Molecular formula: $C_{11}H_{10}O_4$

Mass spectrum: EIMS *m/z* 206.0575 (100%) ($C_{11}H_{10}O_4$, req. 206.0579), 191 $[M-15]^+$ (34%), 178 $[M-28]^+$ (11%), 163 $[M-43]^+$ (24%) **[spectrum 36]**

Infrared spectrum: ν_{max} (NaCl): 2966 (C-H aromatic stretching), 2855 (C-H aliphatic stretching), and 1723 cm^{-1} (C=O stretching) **[spectrum 37]**

UV spectrum: λ_{max} : 343 (log ε 2.3), 293 (log ε 1.8), 260 and 232 nm (log ε 4.2) **[spectrum 38]**

^{1}H NMR: **[spectrum 39]**

^{13}C NMR:

Compound VI (CD_3OD)		**Scoparone** ($CDCl_3$)[102, 107]	
Proton	Chemical shift, δ / ppm	Proton	Chemical shift, δ / ppm
3	6.30 *d* (*J* = 9.5 Hz)	3	6.25 *d* (*J* = 9.5 Hz)
4	7.92 *d* (*J* = 9.5 Hz)	4	7.58 *d* (*J* = 9.5 Hz)
5	7.19 s	5	7.20 s
8	7.03 s	8	6.82 s
C-6 OMe	3.90 s	C-6 OMe	3.89 s
C-7 OMe	3.95 s	C-7 OMe	3.92 s

Compound VI (CD_3OD)		**Scoparone** ($CDCl_3$)[102, 108]	
Carbon	Chemical shift, δ / ppm	Carbon	Chemical shift, δ / ppm
2	162.5 *s*	2	160.2 *s*
3	112.5 *d*	3	114.9 *d*
4	146.0 *d*	4	143.5 *d*
4a	-	4a	114.9 *s*
5	109.0 *d*	5	113.6 *d*
6	147.0 *s*	6	148.8 *s*
7	153.5 *s*	7	149.4 *s*
8	100.0 *d*	8	104.5 *d*
8a	151.0 *s*	8a	147.5 *s*
C-6 OMe	55.5 *q*	C-6 OMe	-
C-7 OMe	56.0 *q*	C-7 OMe	-

4.3.4 Physical data of Compound VII

Names: *6,7-Dimethoxy-5-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one,*
6,7-dimethoxy-5- prenylcoumarin

Yield: 12 mg (yellow gum) (0.16% yield)

Molecular formula: $C_{16}H_{18}O_4$

Mass spectrum: EIMS *m/z* 274.1191 (25%) ($C_{16}H_{18}O_4$, req. 274.1205), 244 $[M-30]^+$ (8%), 233 $[M-41]^+$ (17%), 205 $[M-69]^+$ (12%), 167 $[M-107]^+$ (45%), 149 $[M-125]^+$ (100%) **[spectrum 44]**

Infrared spectrum: ν_{max} (NaCl): 2923 (C-H aromatic stretching), 2854 (C-H aliphatic stretching), and 1727 cm^{-1} (C=O stretching) **[spectrum 45]**

UV spectrum: λ_{max}: 335 (log ε 1.9), 296 (log ε 1.9), 259 and 232 nm (log ε 4.1) **[spectrum 46]**

^{1}H NMR: **[spectrum 47]**

^{13}C NMR: **[spectrum 48]**

Compound VII (CD_3OD)			
Proton	Chemical shift, δ / ppm	Carbon	Chemical shift, δ / ppm
		2	163.5 *s*
3	6.31 *d* (*J* = 9.5 Hz)	3	113.1 *d*
4	8.00 *d* (*J* = 9.5 Hz)	4	143.3 *d*
		4a	112.2 *s*
		5	133.9 *s*
		6	154.0 *s*
		7	158.1 *s*
8	6.94 *s*	8	99.7 *d*
		8a	145.3 *s*
1'	3.65 *d* (*J* = 6.5 Hz)	1'	25.4 *t*
2'	5.10 *t*	2'	123.6 *d*
		3'	133.7 *s*
4'	1.73 *s*	4'	25.9 *q*
5'	1.87 *s*	5'	18.2 *q*
C-6 OMe	3.81 *s*	C-6 OMe	61.4 *q*
C-7 OMe	3.98 *s*	C-7 OMe	56.7 *q*

4.3.5 Physical data of Compound VIII

Names: *O-Methylcedrelopsin, 6,7-dimethoxy-8-prenylcoumarin*

Yield: 10 mg (yellow gum) (0.13% yield)

Molecular formula: $C_{16}H_{18}O_4$

Mass spectrum: EIMS *m/z* 274.0827 (100%) ($C_{16}H_{18}O_4$, req. 274.1205), 259 $[M-15]^+$ (24%), 243 $[M-31]^+$ (31%), 149 $[M-125]^+$ (43%) **[spectrum 52]**

Infrared spectrum: ν_{max} (NaCl): 2928 (C-H stretching), and 1726 cm^{-1} (C=O stretching) **[spectrum 53]**

UV spectrum: λ_{max}: 341 (log ε 1.8), 294 (log ε 2.1), and 232 nm (log ε 4.1) **[spectrum 54]**

^{1}H NMR: **[spectrum 55]**

Compound VIII (CD_3OD)	
Proton	Chemical shift, δ / ppm
3	6.36 *d* (*J* = 9.5 Hz)
4	7.92 *d* (*J* = 9.5 Hz)
5	7.12 *s*
1'	3.58 *d* (*J* = 6.5 Hz)
2'	5.21 *t*
4'	1.71 *s*
5'	1.88 *s*
C-6 OMe	3.92 *s*
C-7 OMe	3.90 *s*

4.3.6 Physical data of Compound IX

Names: *Cedrelopsin, 7-Hydroxy-6-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one, 8-Dimethylallyl-7-hydroxy-6-methylcoumarin, Cneorumcoumarin A*

Yield: 5 mg (yellow gum) (0.07% yield)

Molecular formula: $C_{15}H_{16}O_4$

Mass spectrum: EIMS *m/z* 260.1058 (90%) ($C_{15}H_{16}O_4$, req. 260.1048), 245 $[M-15]^+$ (10%), 205 $[M-55]^+$ (70%), 204 $[M-56]^+$ (100%) **[spectrum 58]**

Infrared spectrum: ν_{max} (NaCl): 3352 (O-H stretching), 2922 (C-H stretching), and 1707 cm^{-1} (C=O stretching) **[spectrum 59]**

UV spectrum: λ_{max}: 341 (log ε 3.5), 302 (log ε 2.3), 250 and 231 nm (log ε 4..2) **[spectrum 60]**

^{1}H NMR: **[spectrum 61]**

Compound IX (CD_3OD)	
Proton	Chemical shift, δ / ppm
3	6.23 *d* (*J* = 9.5 Hz)
4	7.89 *d* (*J* = 9.5 Hz)
5	7.03 *s*
1'	3.56 *d* (*J* = 6.5 Hz)
2'	5.29 *t*
4'	1.70 *s*
5'	1.87 *s*
C-6 OMe	4.92 *s*

4.3.7 Physical data of Compound X

Names: *6-Hydroxy-8,8-dimethyl-2H,8H-benzo[1,2-b:3,4-b]dipyran-2-one, Norbraylin*

Yield: 3 mg (yellow gum) (0.04% yield)

Molecular formula: $C_{14}H_{12}O_4$

Mass spectrum: EIMS *m/z* 244.0731 (28%) ($C_{14}H_{12}O_4$, req. 244.0735), 229 $[M15]^+$ (100%), 201 $[M-43]^+$ (10%), 149 $[M-95]^+$ (25%) **[spectrum 64]**

Infrared spectrum: ν_{max} (NaCl): 3410 (O-H stretching), 2926 (C-H stretching), and 1721 cm^{-1} (C=O stretching) **[spectrum 65]**

UV spectrum: λ_{max}: 341 (log ε 1.8), 299 (log ε 1.7), 260 (log ε 2.5) and 231 nm (log ε 4.1) **[spectrum 66]**

^{1}H NMR: **[spectrum 67]**

^{13}C NMR:

Compound X (CD_3OD)			
Proton	Chemical shift, δ / ppm	Carbon	Chemical shift, δ / ppm
3	6.27 *d* (*J* = 9.5 Hz)	3	113.5 *d*
4	7.81 *d* (*J* = 9.5 Hz)	4	146.5 *d*
5	6.92 *s*	5	111.2 *d*
3'	5.90 *d* (*J* = 10 Hz)	3'	132.5 *d*
4'	6.85 *d* (*J* = 10 Hz)	4'	114.5 *d*
2'-Me	1.52 *s*	2'-Me	25.4 *q*
2'-Me	1.52 *s*	2'-Me	27.5 *q*

4.3.8 Physical data of Compound XI

Names: *Cedrelopsinol*

Yield: 13 mg (yellow gum) (0.17% yield)

Molecular formula: $C_{14}H_{12}O_4$

Mass spectrum: EIMS *m/z* 244.0731 (26%) ($C_{14}H_{12}O_4$, req. 244.0735), 229 $[M-15]^+$ (100%), 201 $[M-43]^+$ (10%), 100 $[M-144]^+$ (1%) **[spectrum 71]**

Infrared spectrum: ν_{max} (NaCl): 3374 (O-H stretching), 2927 (C-H stretching), and 1712 cm^{-1} (C=O stretching) **[spectrum 72]**

UV spectrum: λ_{max}: 316 (log ε 2.6), 275 (log ε 1.8), and 232 nm (log ε 4.1) **[spectrum 73]**

^{1}H NMR: **[spectrum 74]**

^{13}C NMR: **[spectrum 75]**

Compound XI (CD_3OD)			
Proton	Chemical shift, δ / ppm	Carbon	Chemical shift, δ / ppm
		2	163.8 *s*
3	6.26 *d* (*J* = 9.7 Hz)	3	112.6 *d*
4	8.15 *d* (*J* = 9.7 Hz)	4	141.6 *d*
		4a	108.6 *s*
		5	118.9 *s*
		6	139.2 *s*
		7	151.3 *s*
8	6.71 *s*	8	103.9 *d*
		8a	151.8 *s*
		2'	77.7 *s*
3'	5.97 *d* (*J* = 10 Hz)	3'	134.8 *d*
4'	6.86 *d* (*J* = 10 Hz)	4'	118.0 *d*
2'-Me	1.49 *s*	2'-Me	27.4 *q*
2'-Me	1.49 *s*	2'-Me	27.4 *q*

Comparison of ^{1}H NMR data of compounds VI - XI:

^{1}H	Compound VI	Compound VII	Compound VIII	Compound IX	Compound X	Compound XI
3	6.30 *d* (J = 9.5 Hz)	6.31 *d* (J = 9.5 Hz)	6.36 *d* (J = 9.5 Hz)	6.23 *d* (J = 9.5 Hz)	6.27 *d* (J = 9.5 Hz)	6.26 *d* (J = 9.7 Hz)
4	7.92 *d* (J = 9.5 Hz)	8.00 *d* (J = 9.5 Hz)	7.92 *d* (J = 9.5 Hz)	7.89 *d* (J = 9.5 Hz)	7.81 *d* (J = 9.5 Hz)	8.15 *d* (J = 9.7 Hz)
5	7.19 *s*	-	7.12 *s*	7.03 *s*	6.92 *s*	-
8	7.03 *s*	6.94 *s*	-	-	-	6.71
C-6 OMe	3.90 *s*	3.81 *s*	3.92 *s*	4.92 *s*	-	-
C-7 OMe	3.95 *s*	3.98 *s*	3.90 *s*	-	-	-
1'	-	3.65 *d* (J = 6.5 Hz)	3.58 *d* (J = 6.5 Hz)	3.56 *d* (J = 6.5 Hz)	-	-
2'	-	5.10 *t*	5.21 *t*	5.29 *t*	-	-
3'	-	-	-	-	5.90 *d* (J = 10 Hz)	5.97 *d* (J = 10 Hz)
4'	-	1.73 *s*	1.71 *s*	1.70 *s*	6.85 *d* (J = 10 Hz)	6.86 *d* (J = 10 Hz)
5'	-	1.87 *s*	1.88 *s*	1.87 *s*	-	-
2'- Me	-	-	-	-	1.52 *s*	1.49 *s*
2'- Me	-	-	-	-	1.52 *s*	1.49 *s*

Comparison of ^{1}H NMR data of compounds VI - XI:

^{1}H	Compound VI	Compound VII	Compound VIII	Compound IX	Compound X	Compound XI
3	6.30 *d* (J = 9.5 Hz)	6.31 *d* (J = 9.5 Hz)	6.36 *d* (J = 9.5 Hz)	6.23 *d* (J = 9.5 Hz)	6.27 *d* (J = 9.5 Hz)	6.26 *d* (J = 9.7 Hz)
4	7.92 *d* (J = 9.5 Hz)	8.00 *d* (J = 9.5 Hz)	7.92 *d* (J = 9.5 Hz)	7.89 *d* (J = 9.5 Hz)	7.81 *d* (J = 9.5 Hz)	8.15 *d* (J = 9.7 Hz)
5	7.19 *s*	-	7.12 *s*	7.03 *s*	6.92 *s*	-
8	7.03 *s*	6.94 *s*	-	-	-	6.71
C-6 OMe	3.90 *s*	3.81 *s*	3.92 *s*	4.92 *s*	-	-
C-7 OMe	3.95 *s*	3.98 *s*	3.90 *s*	-	-	-
1'	-	3.65 *d* (J = 6.5 Hz)	3.58 *d* (J = 6.5 Hz)	3.56 *d* (J = 6.5 Hz)	-	-
2'	-	5.10 *t*	5.21 *t*	5.29 *t*	-	-
3'	-	-	-	-	5.90 *d* (J = 10 Hz)	5.97 *d* (J = 10 Hz)
4'	-	1.73 *s*	1.71 *s*	1.70 *s*	6.85 *d* (J = 10 Hz)	6.86 *d* (J = 10 Hz)
5'	-	1.87 *s*	1.88 *s*	1.87 *s*	-	-
2'- Me	-	-	-	-	1.52 *s*	1.49 *s*
2'- Me	-	-	-	-	1.52 *s*	1.49 *s*

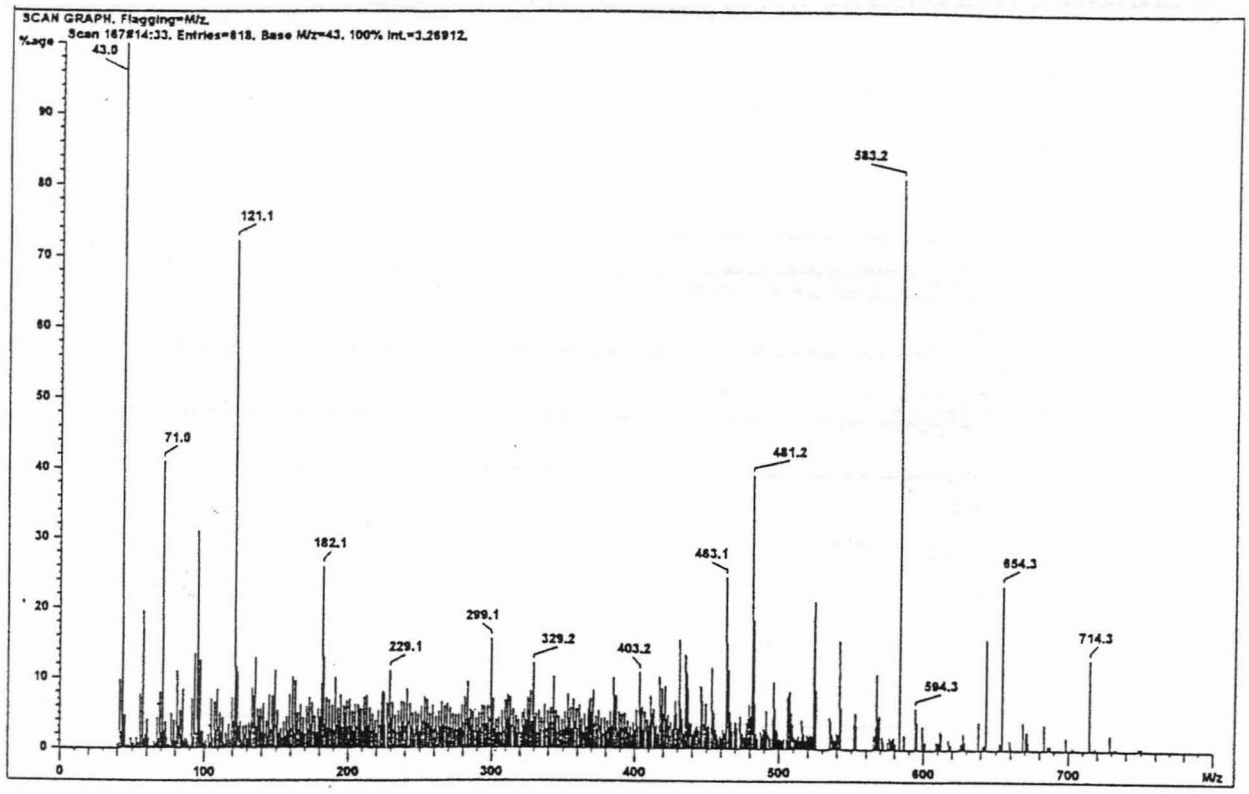
Appendix A

List of Spectra

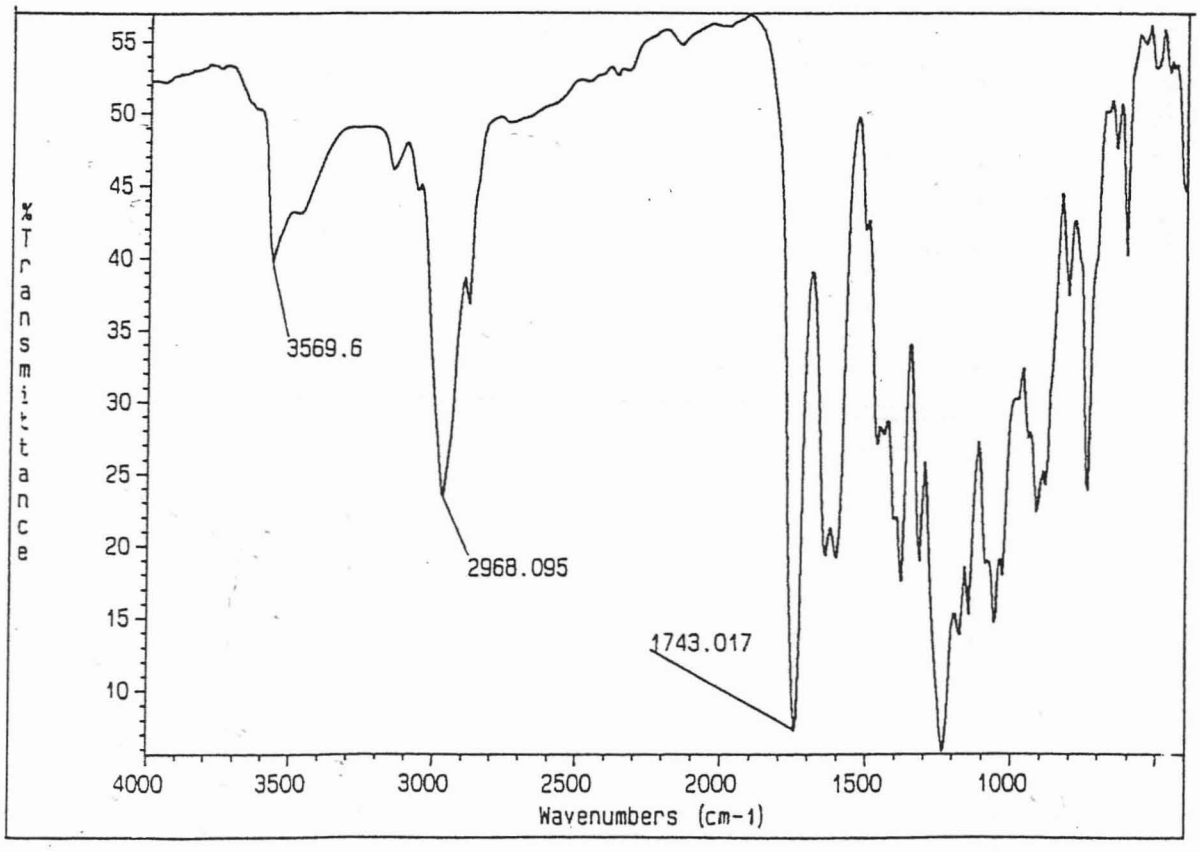
†- Bruker 400 MHz NMR Spectrophotometer

†- Bruker 400 MHz NMR Spectrophotometer

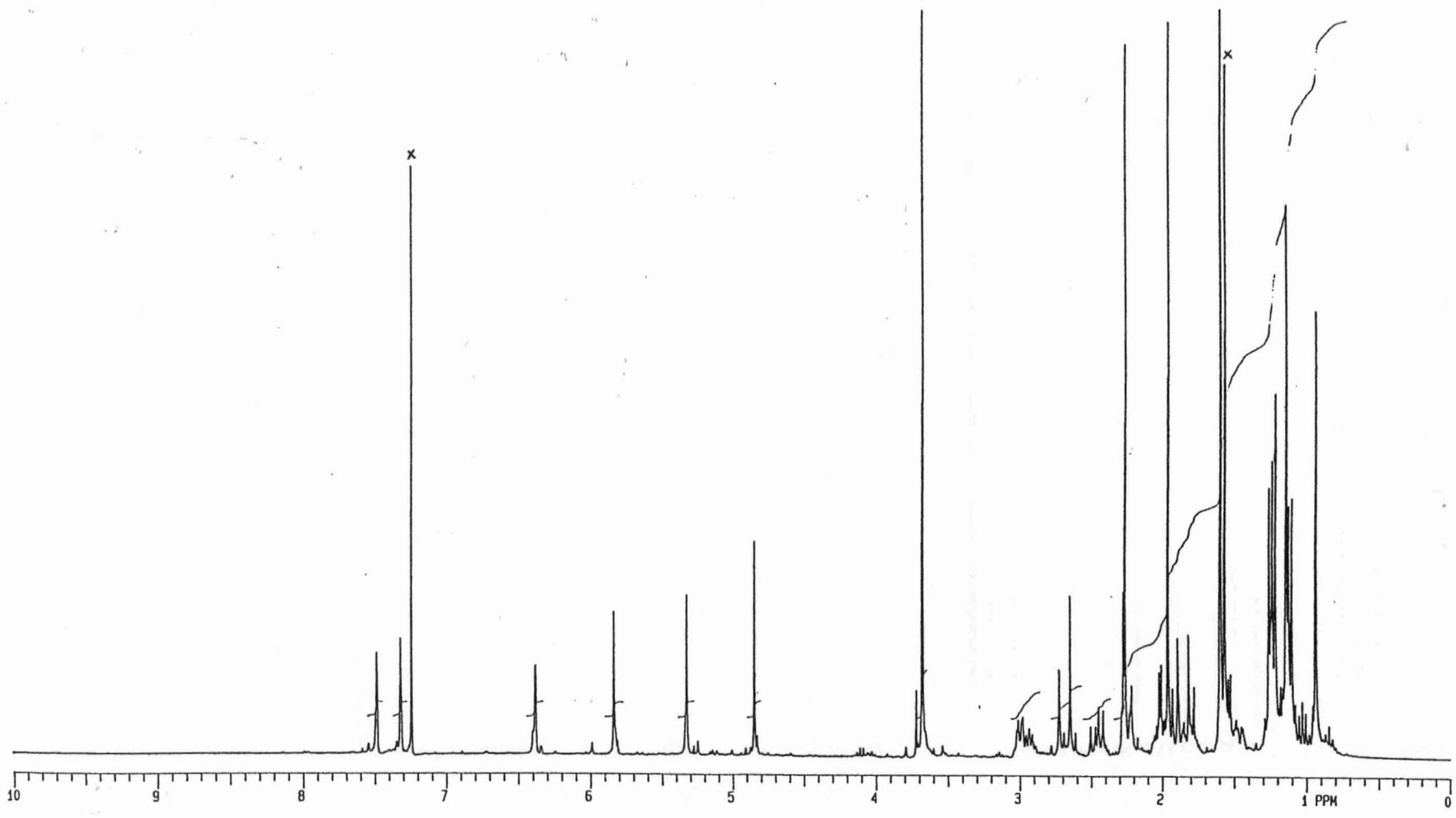
- Bruker 400 MHz NMR Spectrophotometer



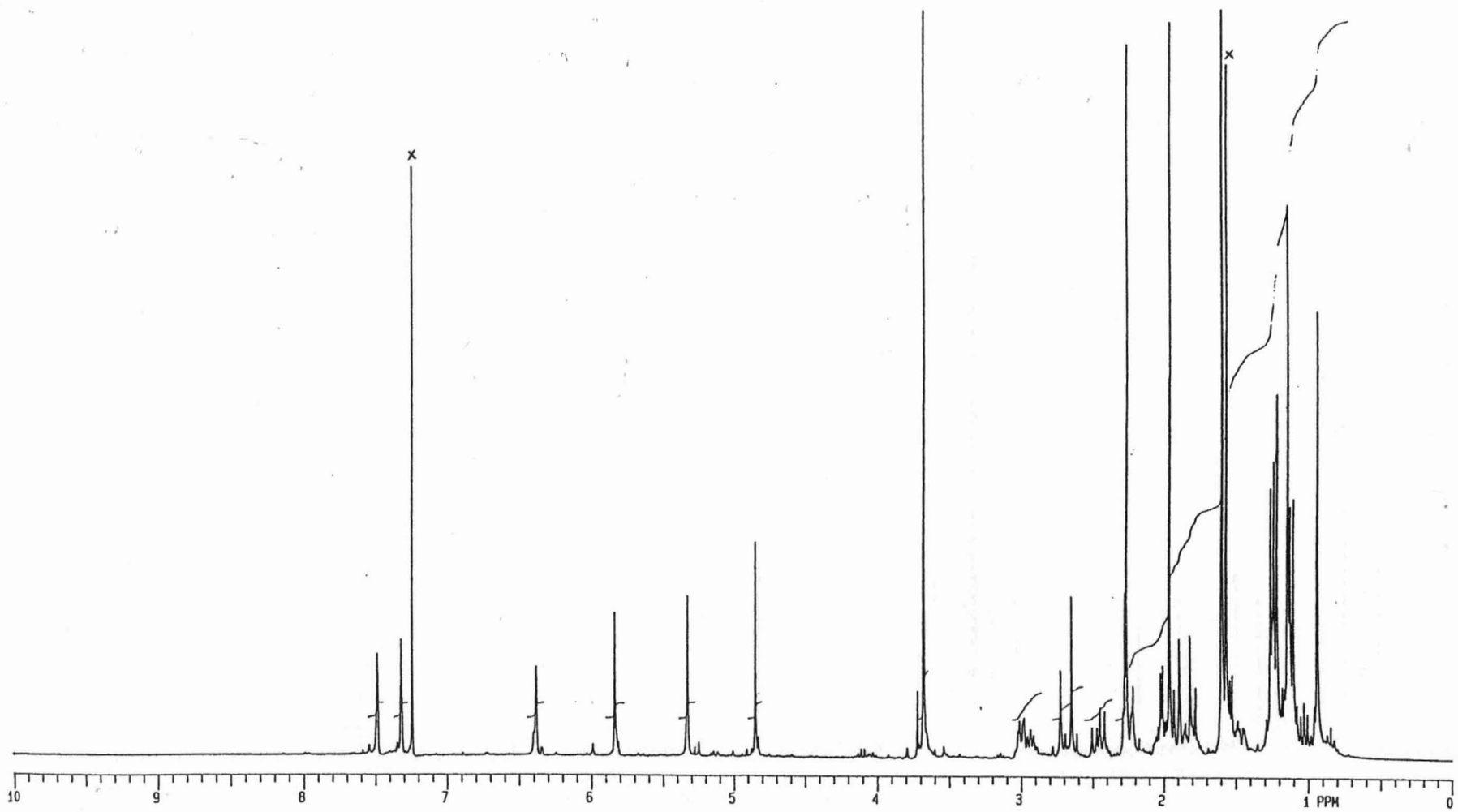
Spectrum 1: Mass spectrum of Compound I



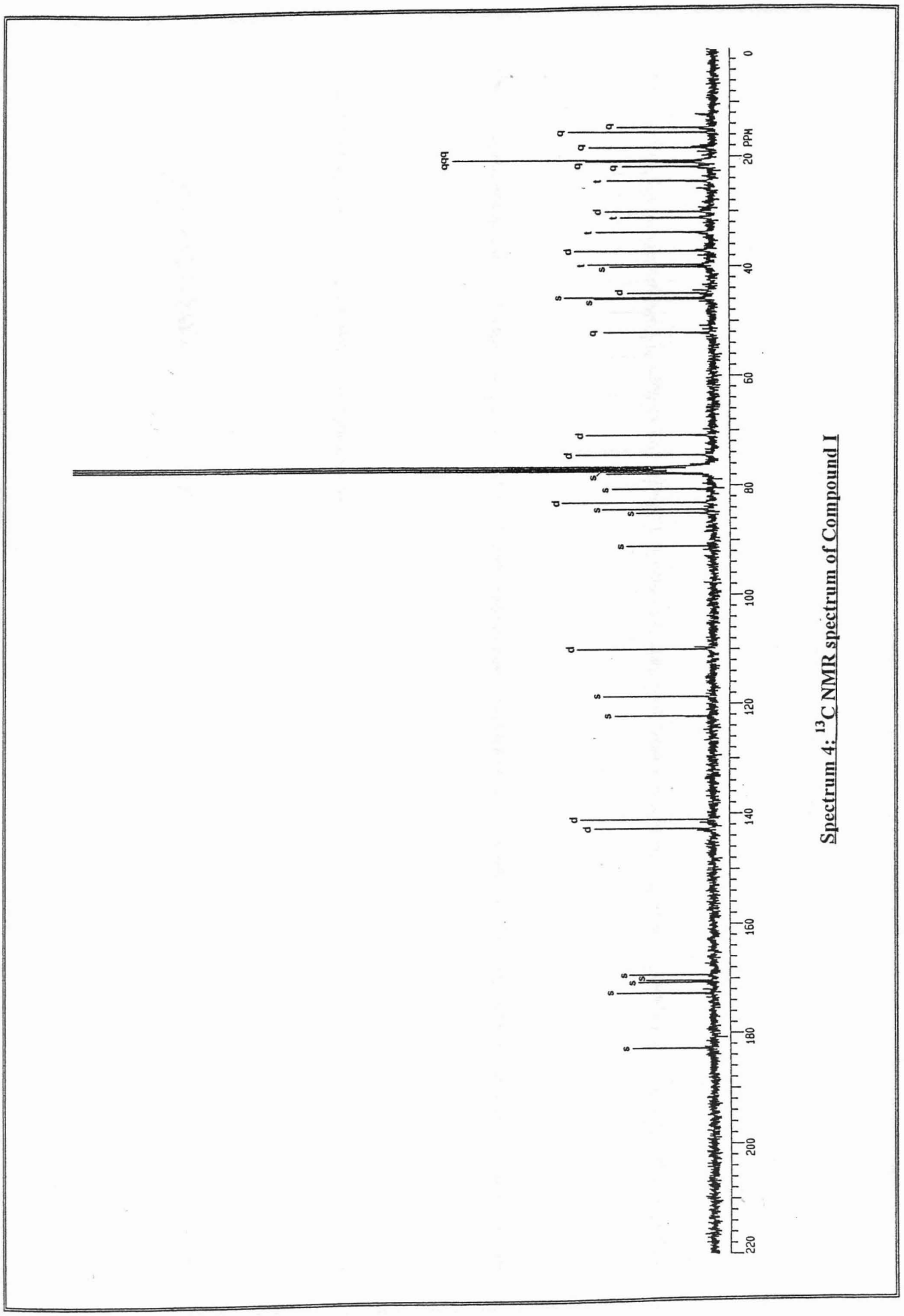
Spectrum 2: IR spectrum of Compound I



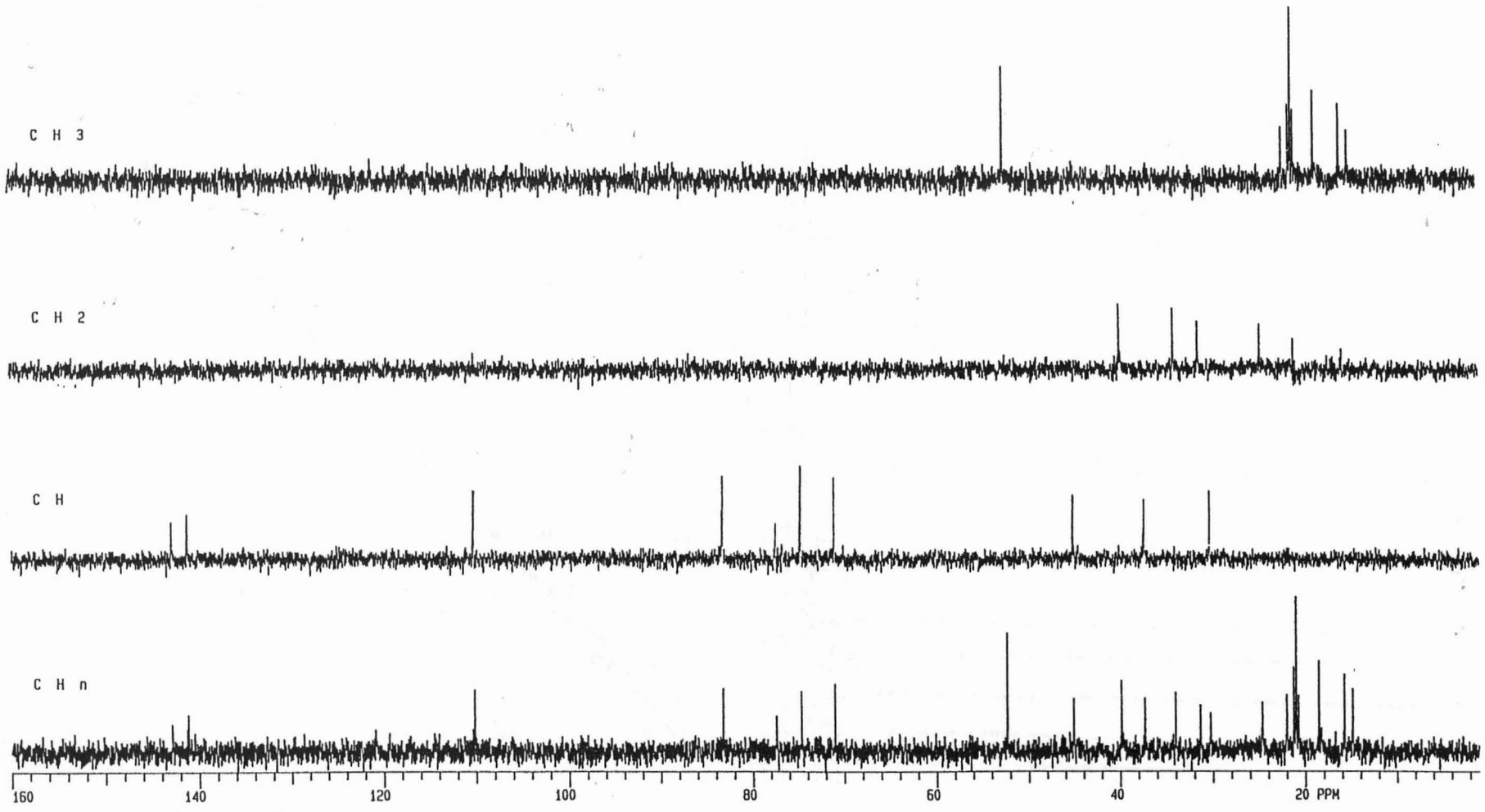
Spectrum 3: ^{1}H NMR spectrum of Compound I



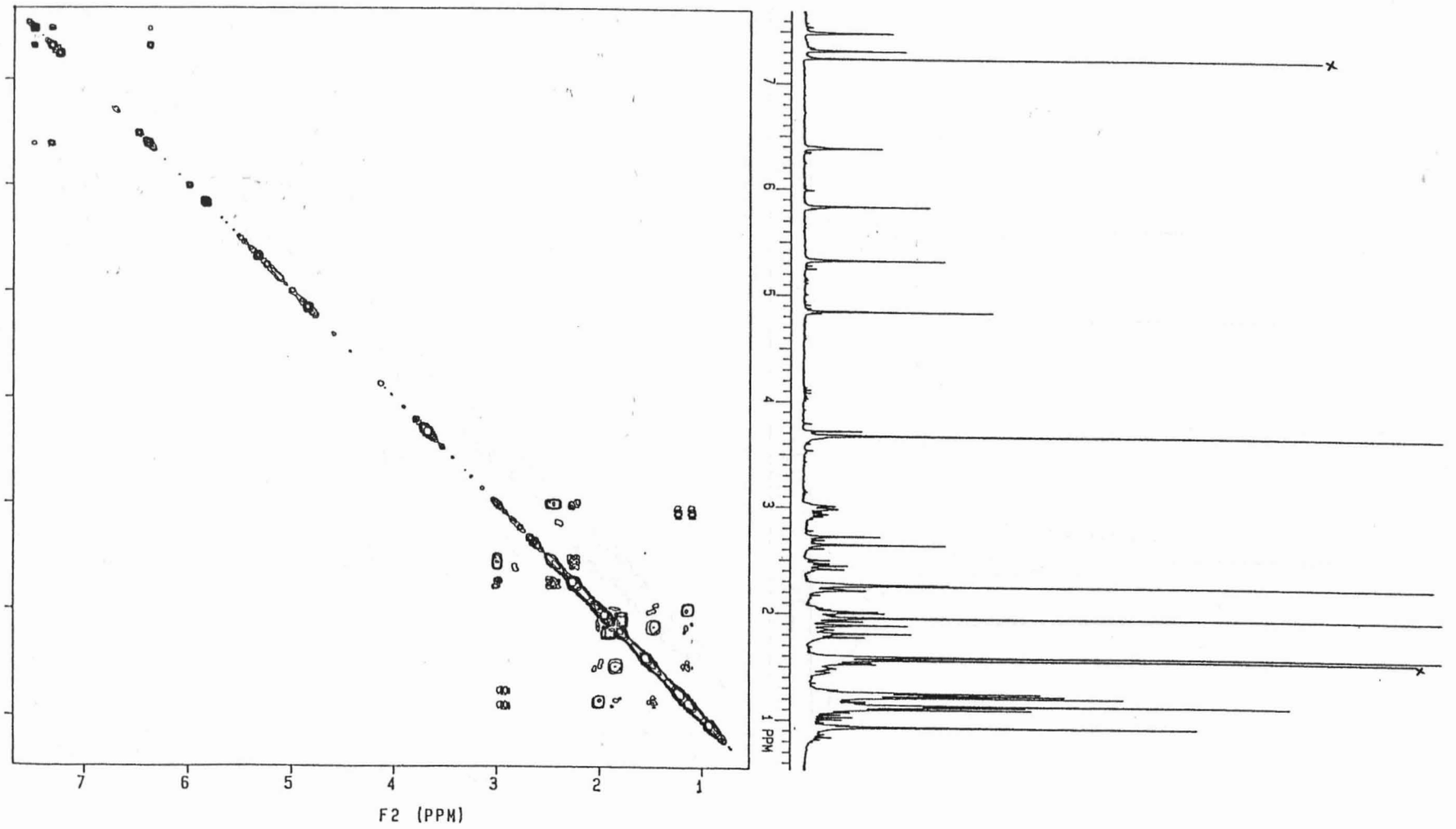
Spectrum 3: ^{1}H NMR spectrum of Compound I



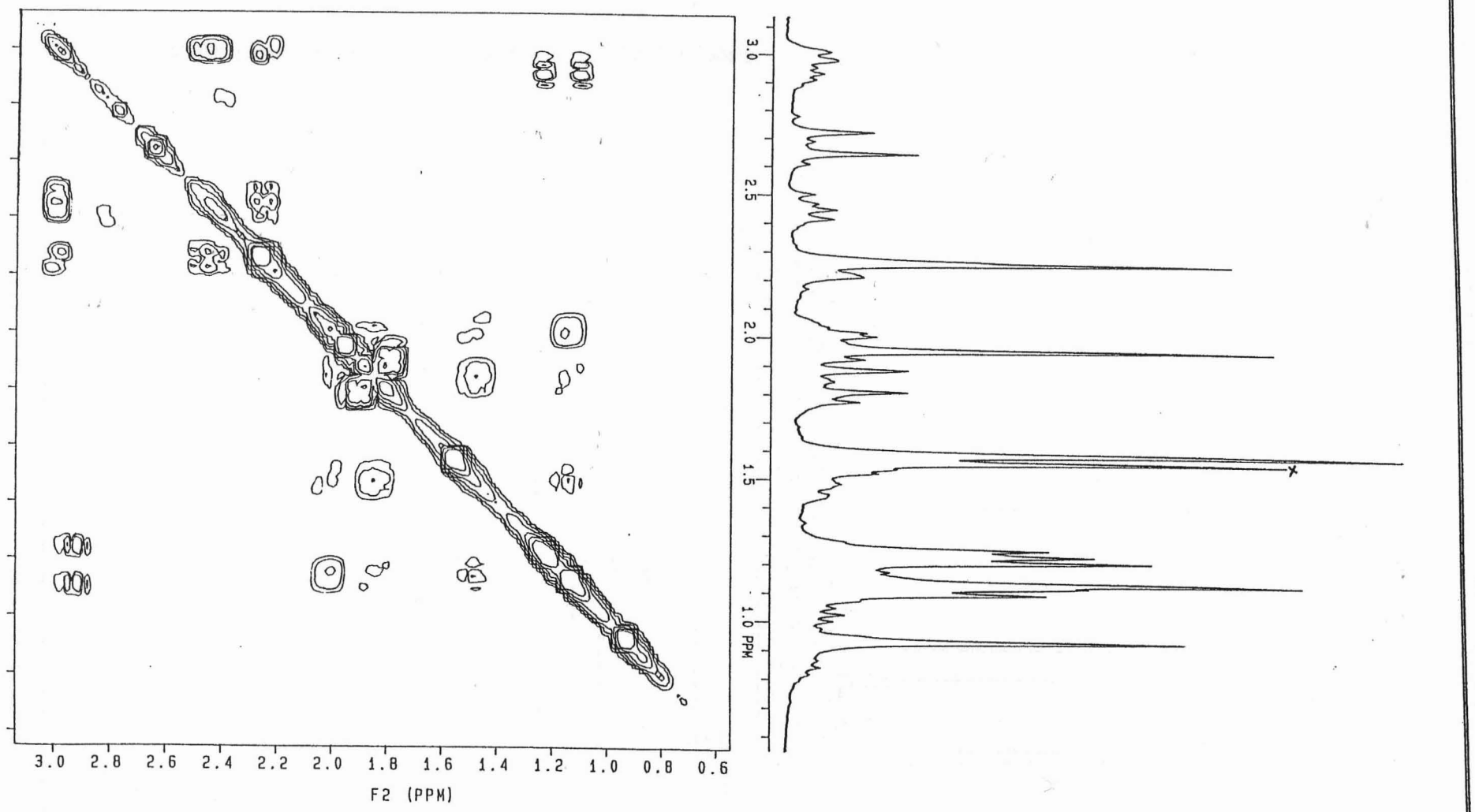
Spectrum 4: ^{13}C NMR spectrum of Compound I



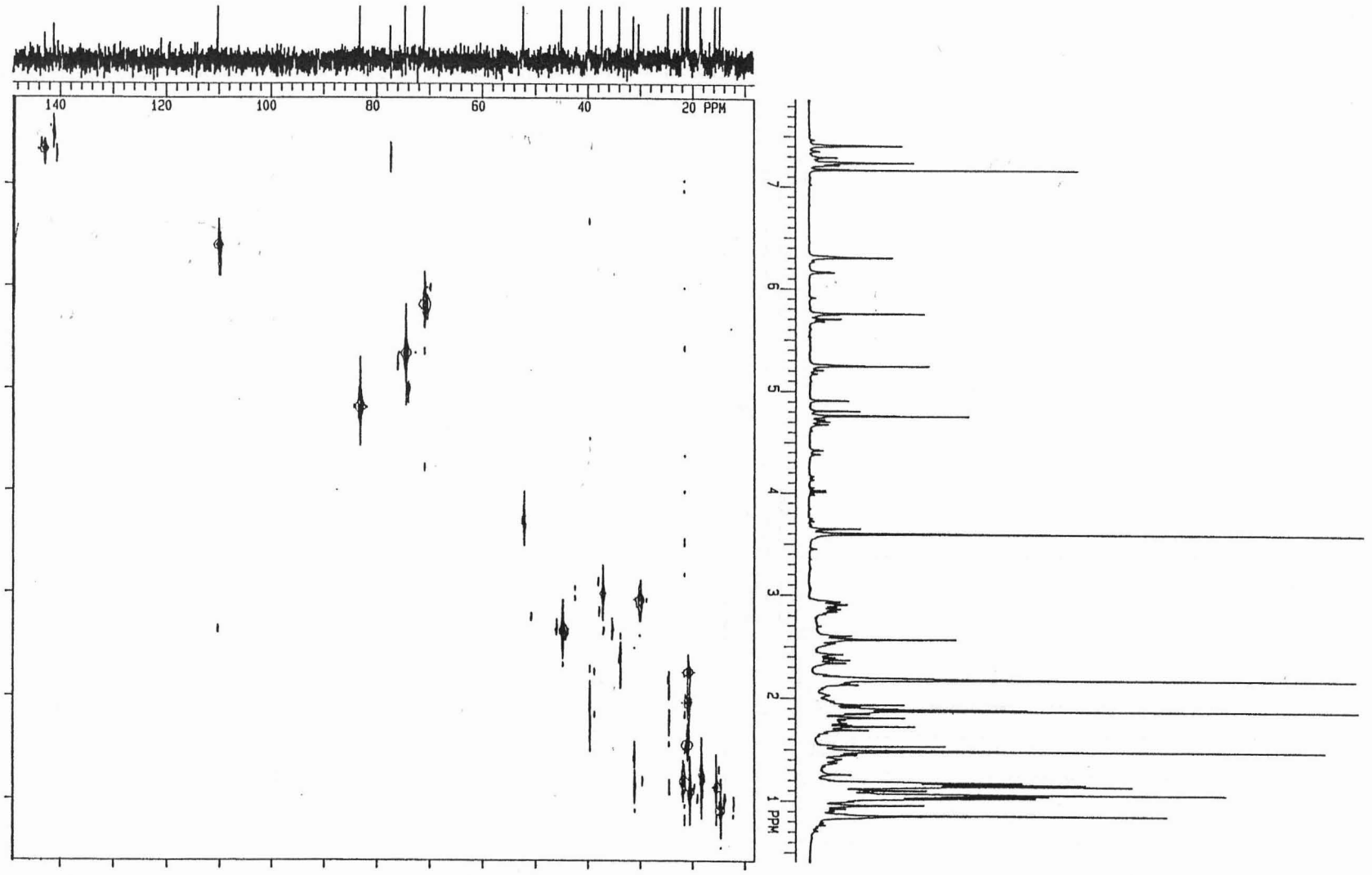
Spectrum 5: ADEPT NMR spectrum of Compound I



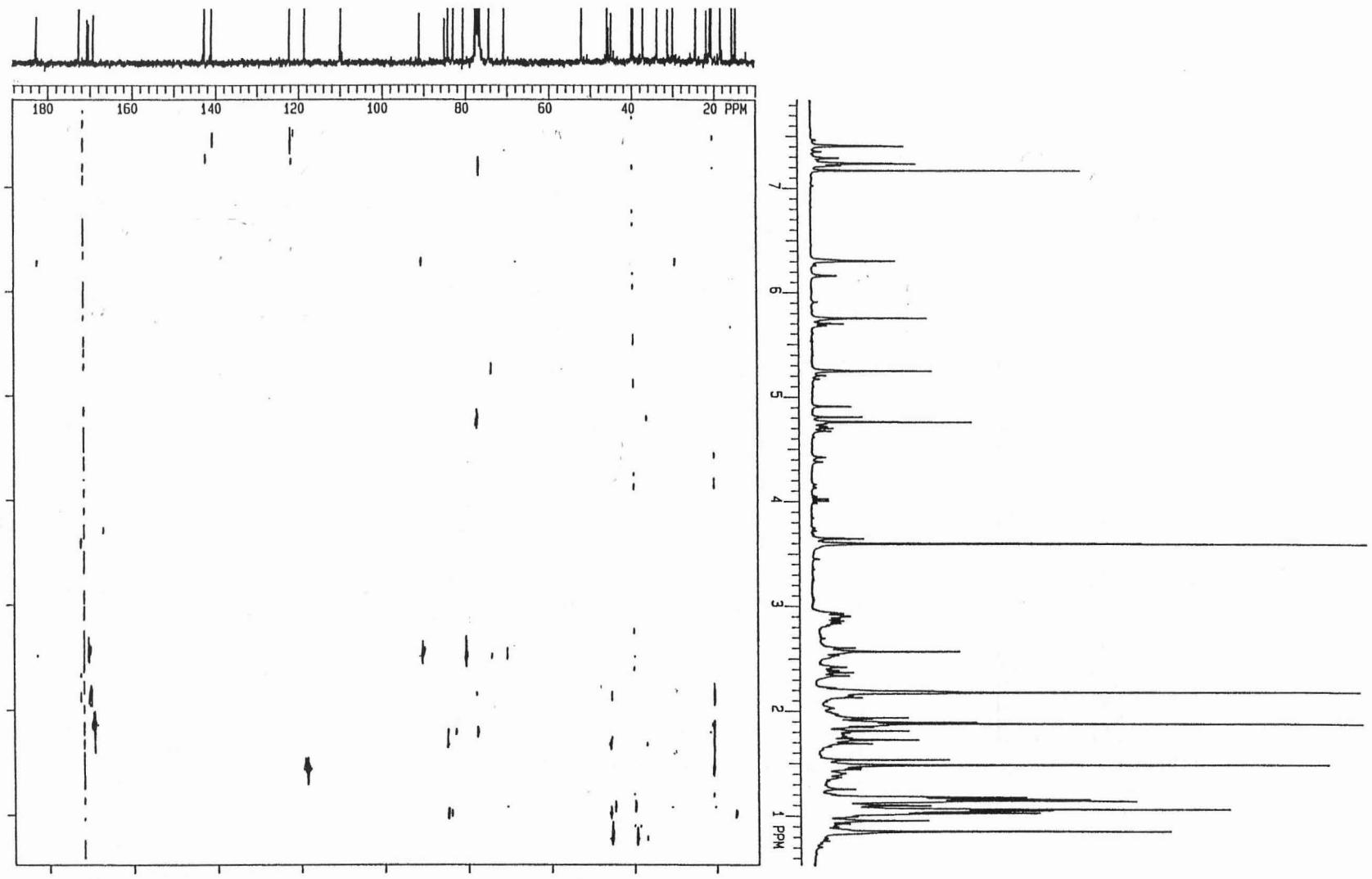
Spectrum 6a: COSY NMR spectrum of Compound I



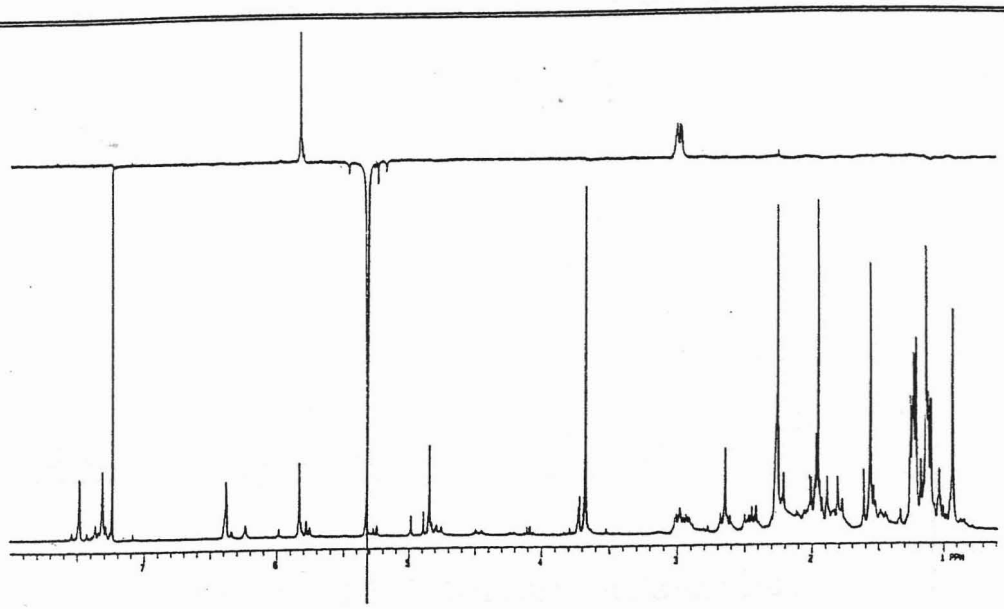
Spectrum 6b: COSY NMR spectrum of Compound I



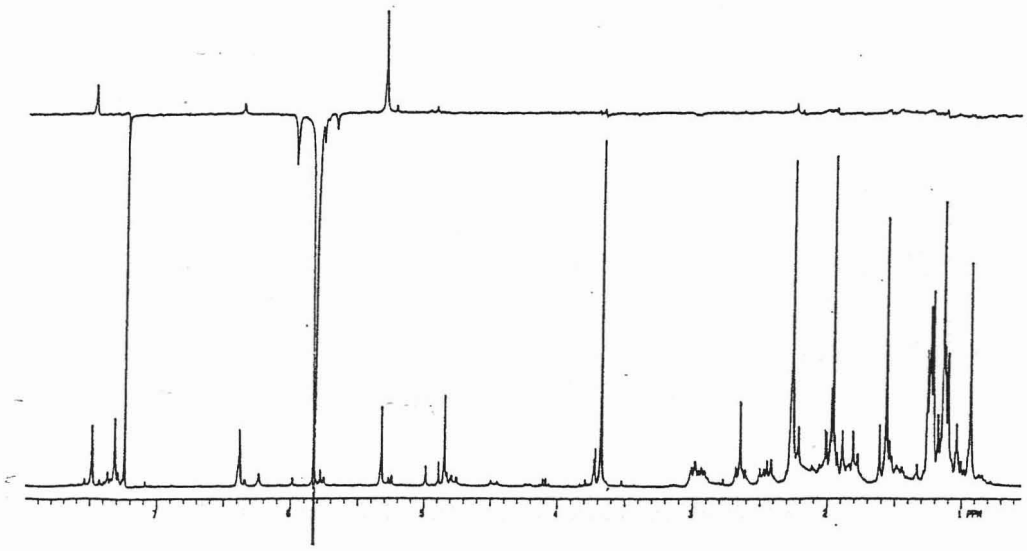
Spectrum 7: HETCOR NMR spectrum of Compound I



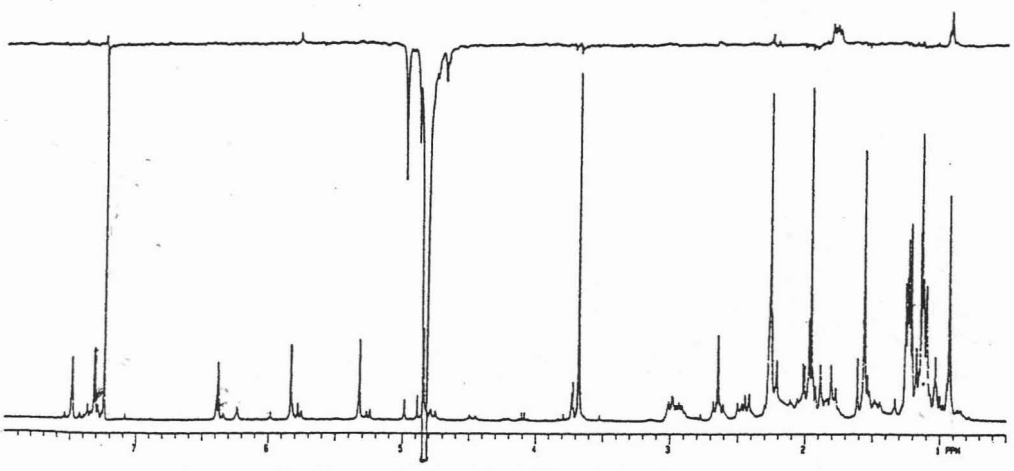
Spectrum 8: DELAYED HETCOR NMR spectrum of Compound I



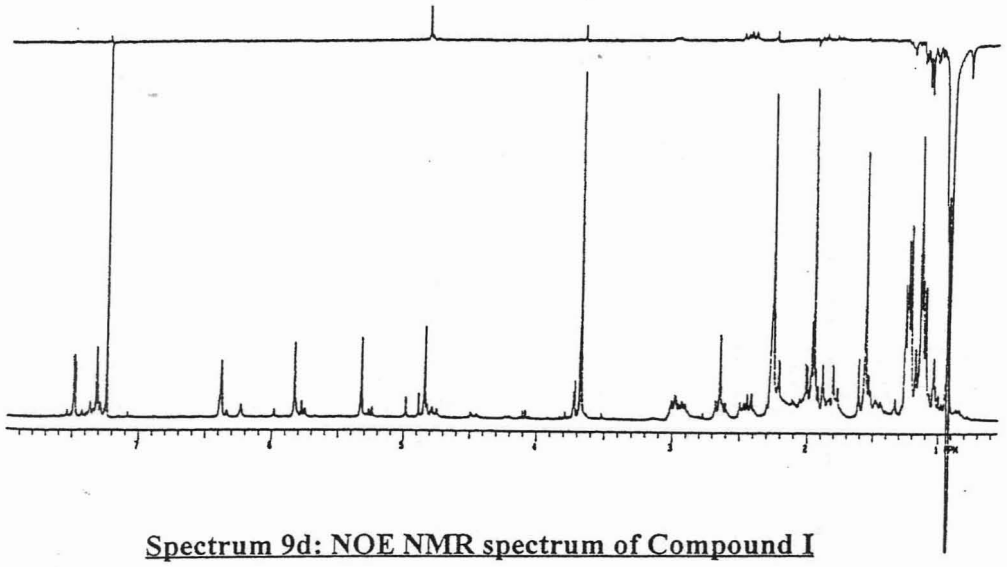
Spectrum 9a: NOE NMR spectrum of Compound I



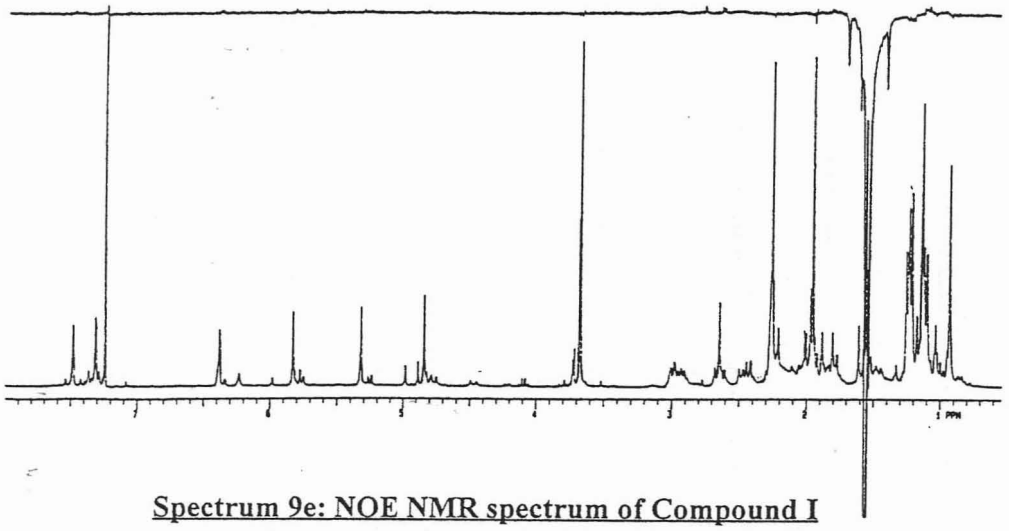
Spectrum 9b: NOE NMR spectrum of Compound I



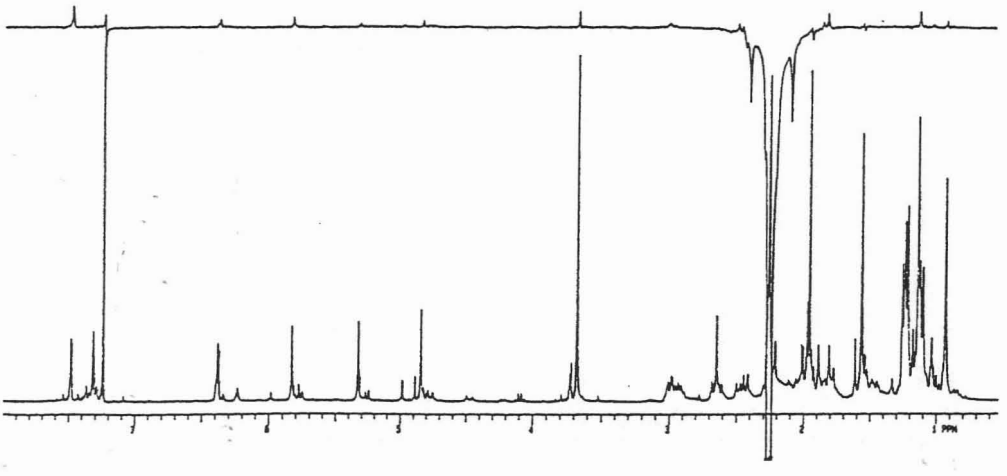
Spectrum 9c: NOE NMR spectrum of Compound I



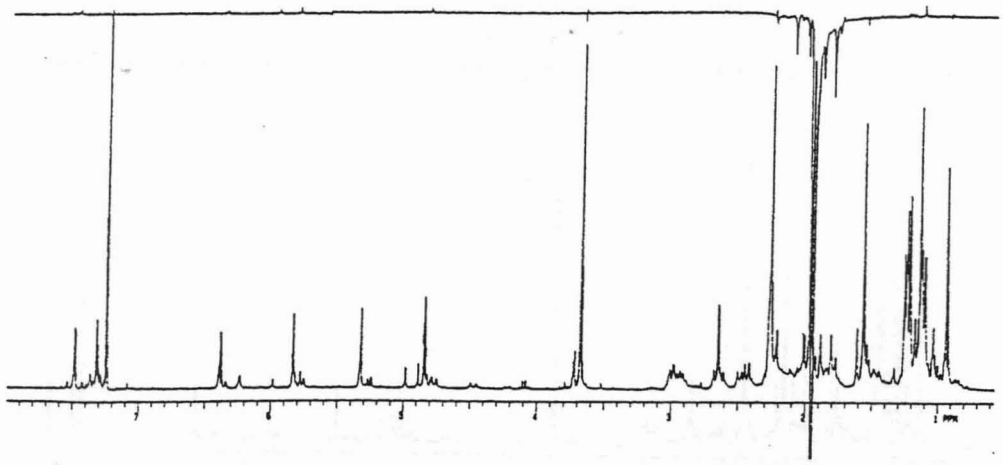
Spectrum 9d: NOE NMR spectrum of Compound I



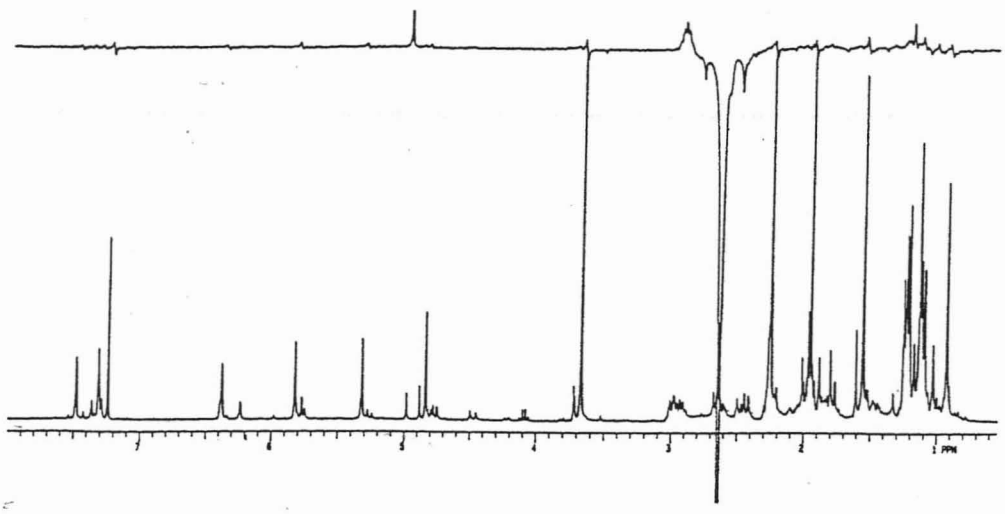
Spectrum 9e: NOE NMR spectrum of Compound I



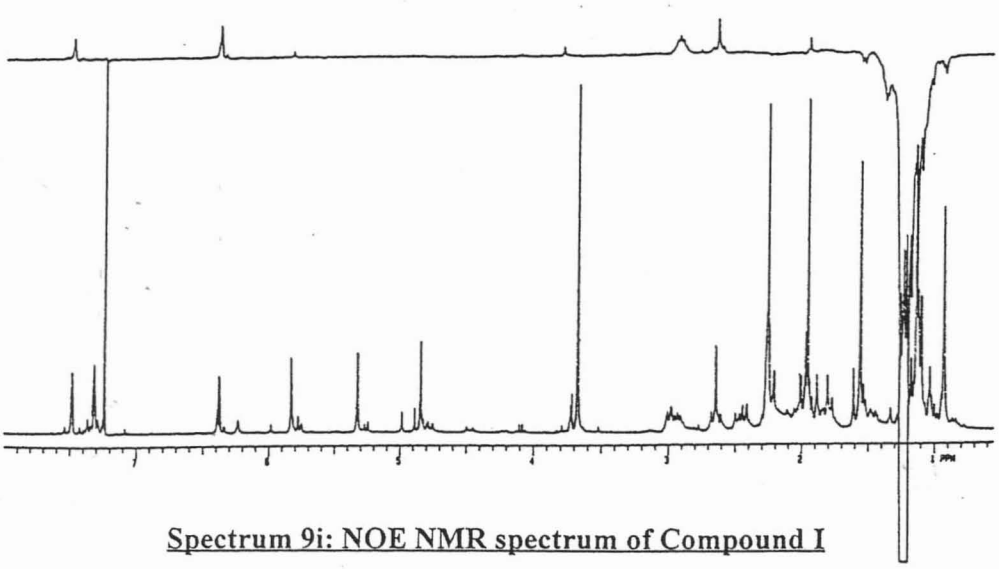
Spectrum 9f: NOE NMR spectrum of Compound I



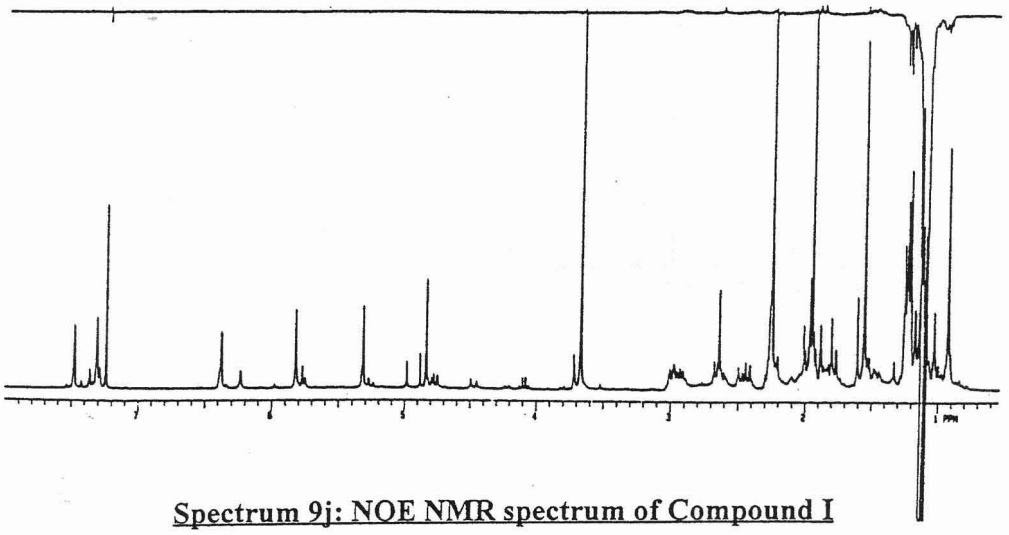
Spectrum 9g: NOE NMR spectrum of Compound I



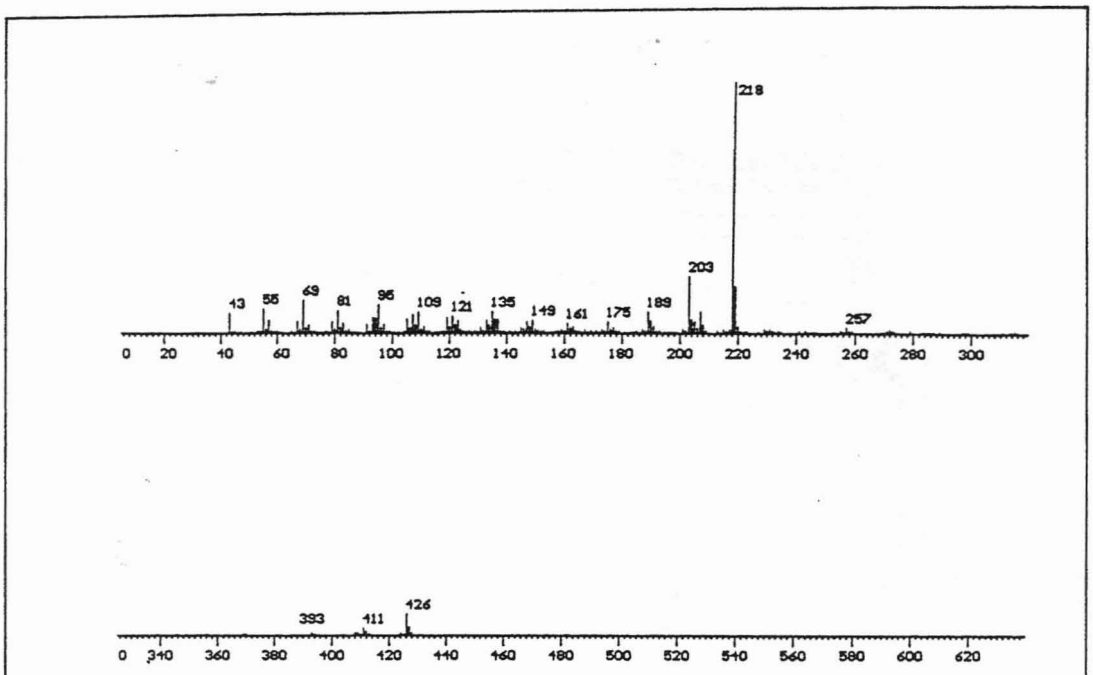
Spectrum 9h: NOE NMR spectrum of Compound I



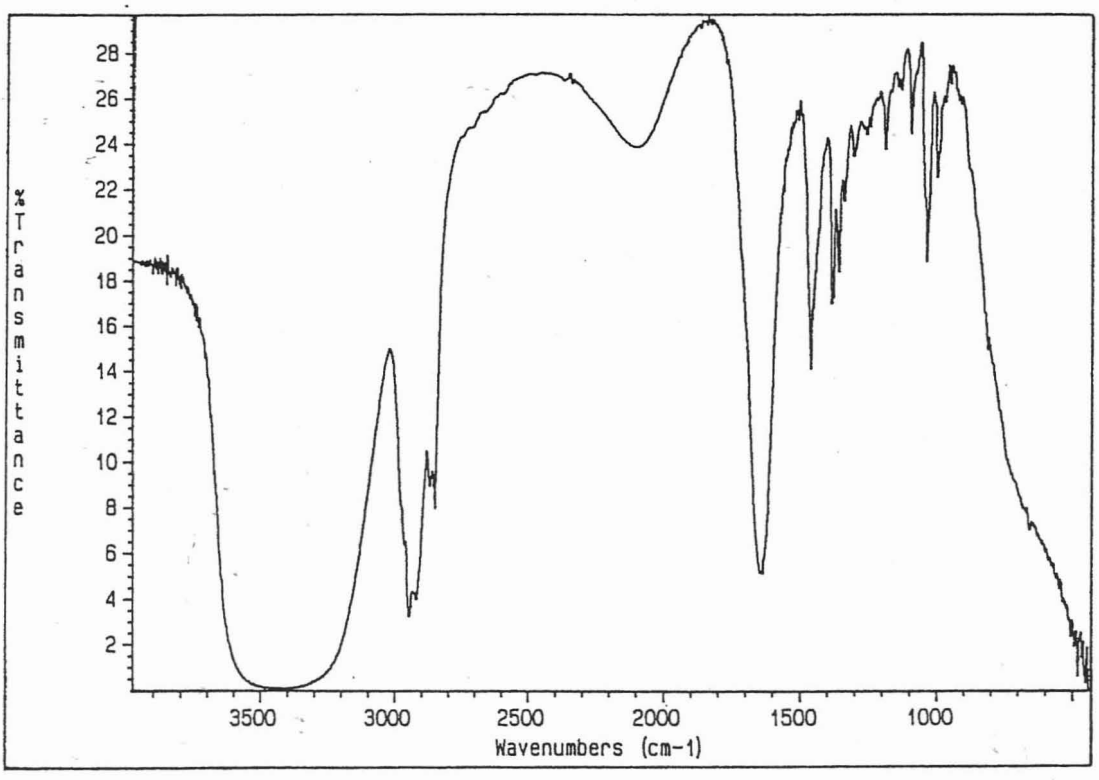
Spectrum 9i: NOE NMR spectrum of Compound I



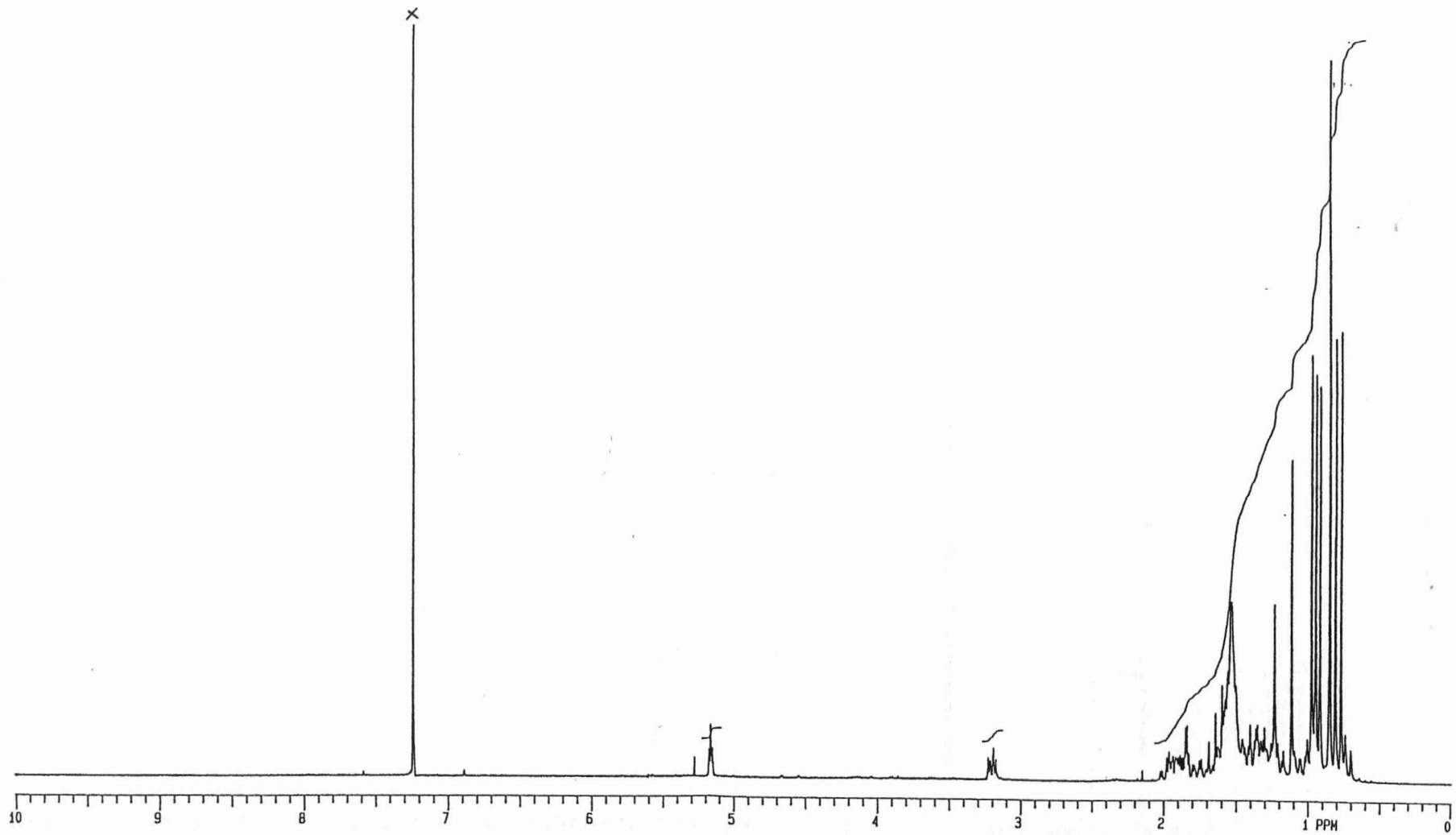
Spectrum 9j: NOE NMR spectrum of Compound I



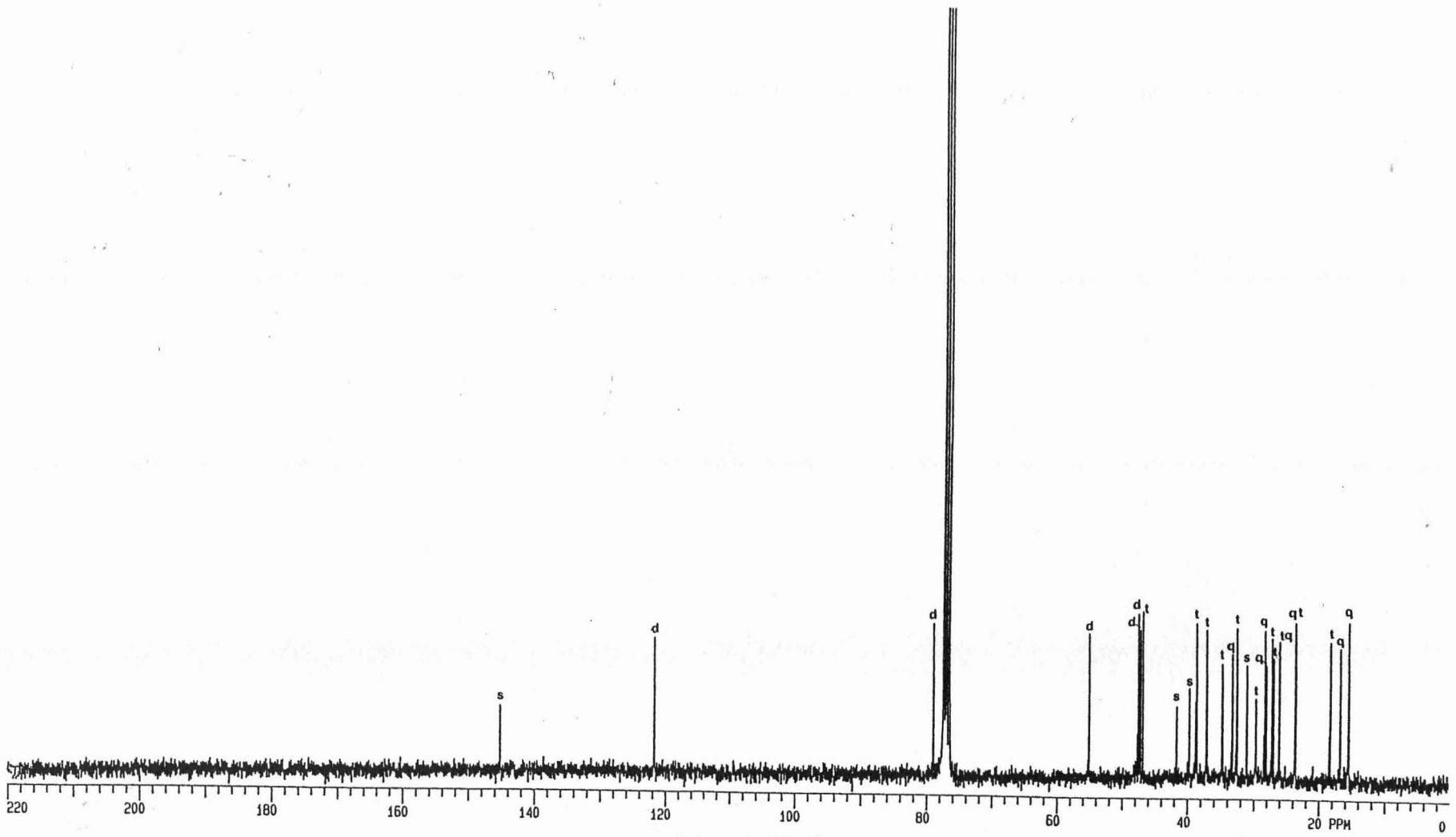
Spectrum 10: Mass spectrum of Compound II



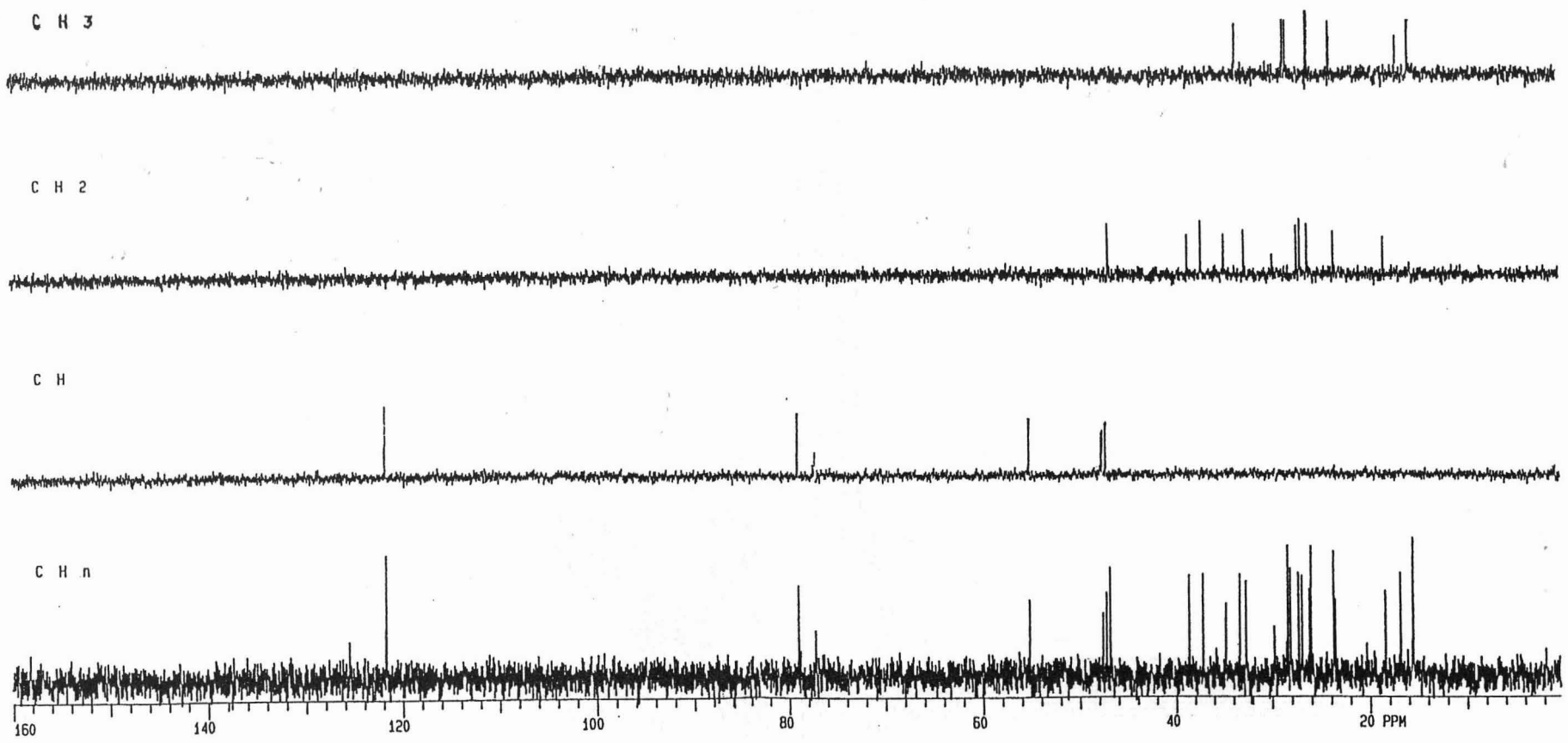
Spectrum 11: IR spectrum of Compound II



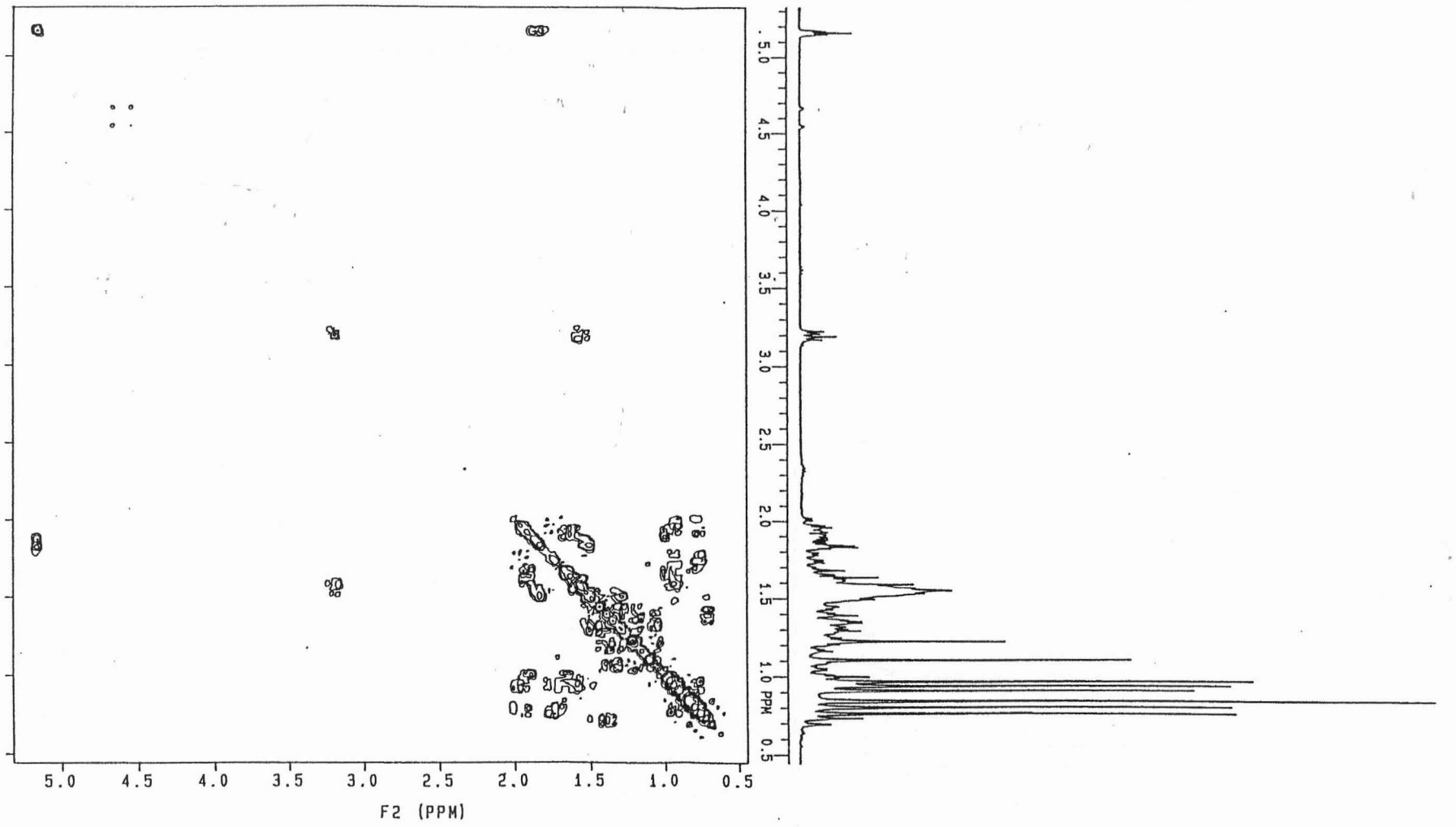
Spectrum 12; ^{1}H NMR spectrum of Compound II



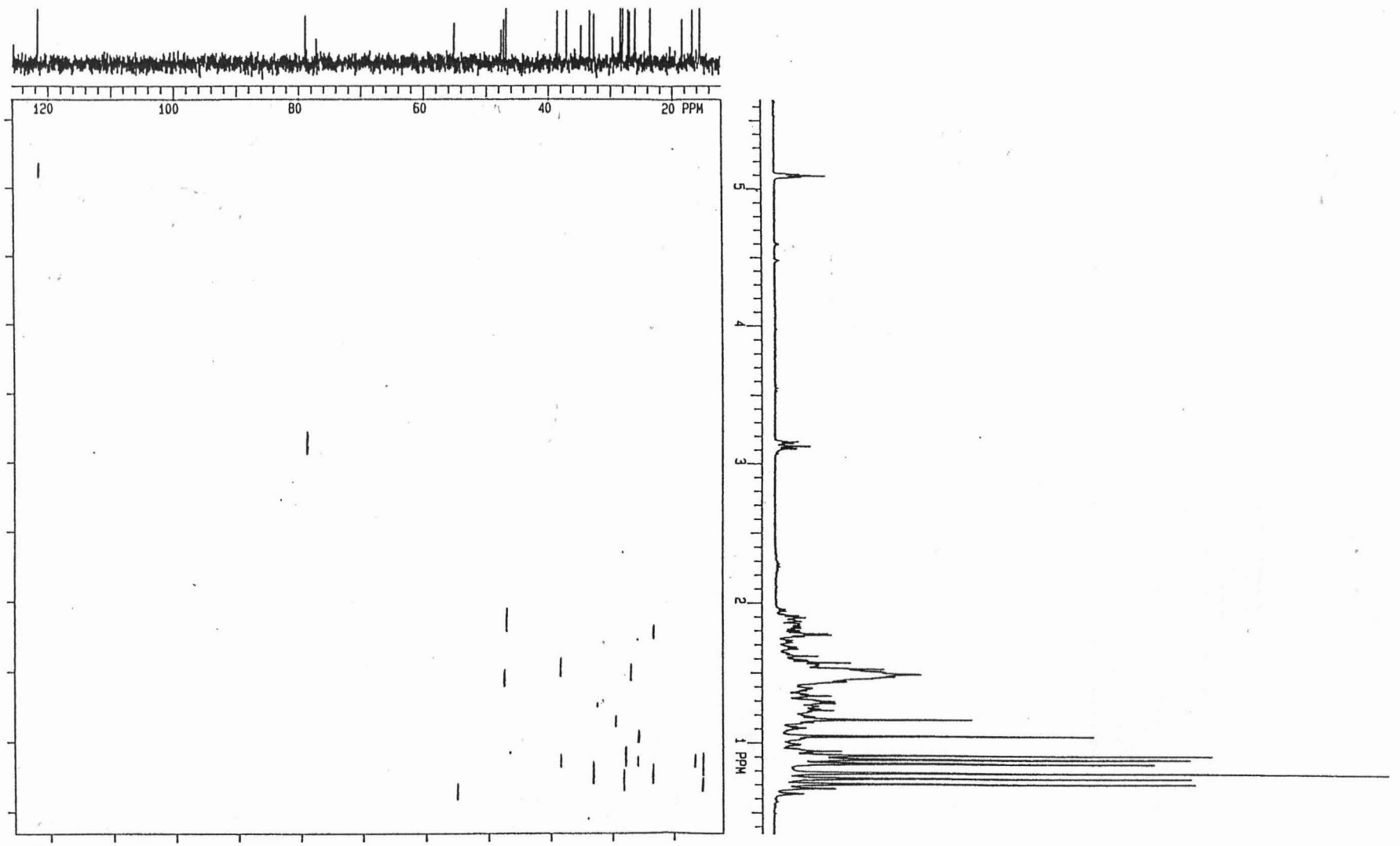
Spectrum 13: ^{13}C NMR spectrum of Compound II



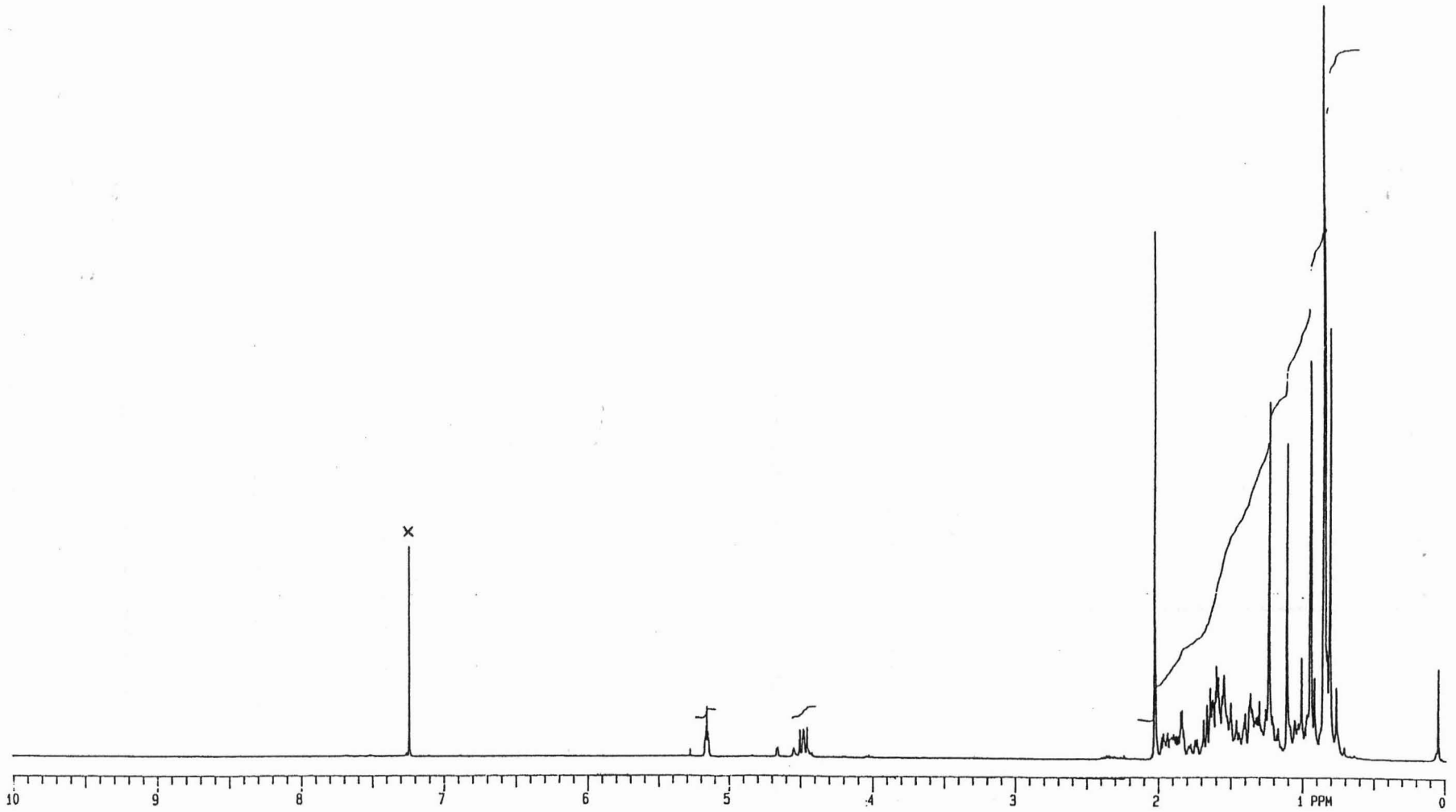
Spectrum 14: ADEPT NMR spectrum of Compound II



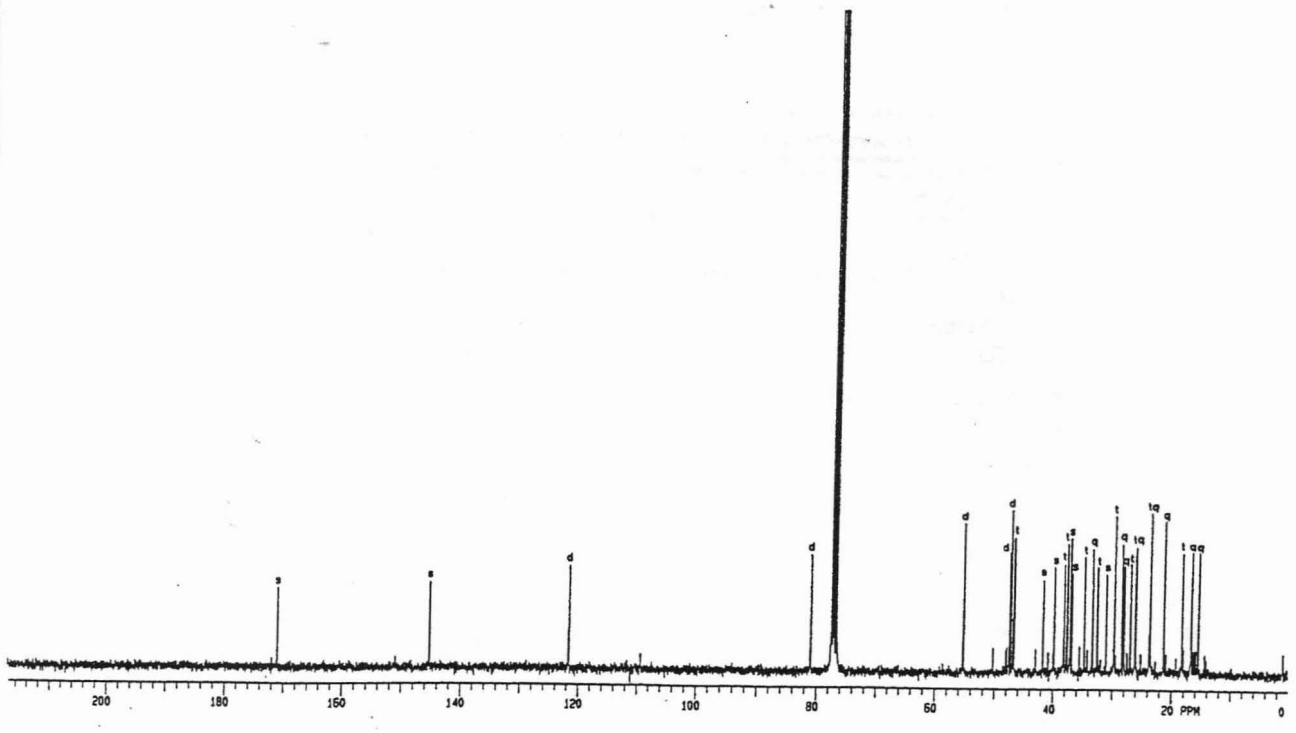
Spectrum 15: COSY NMR spectrum of Compound II



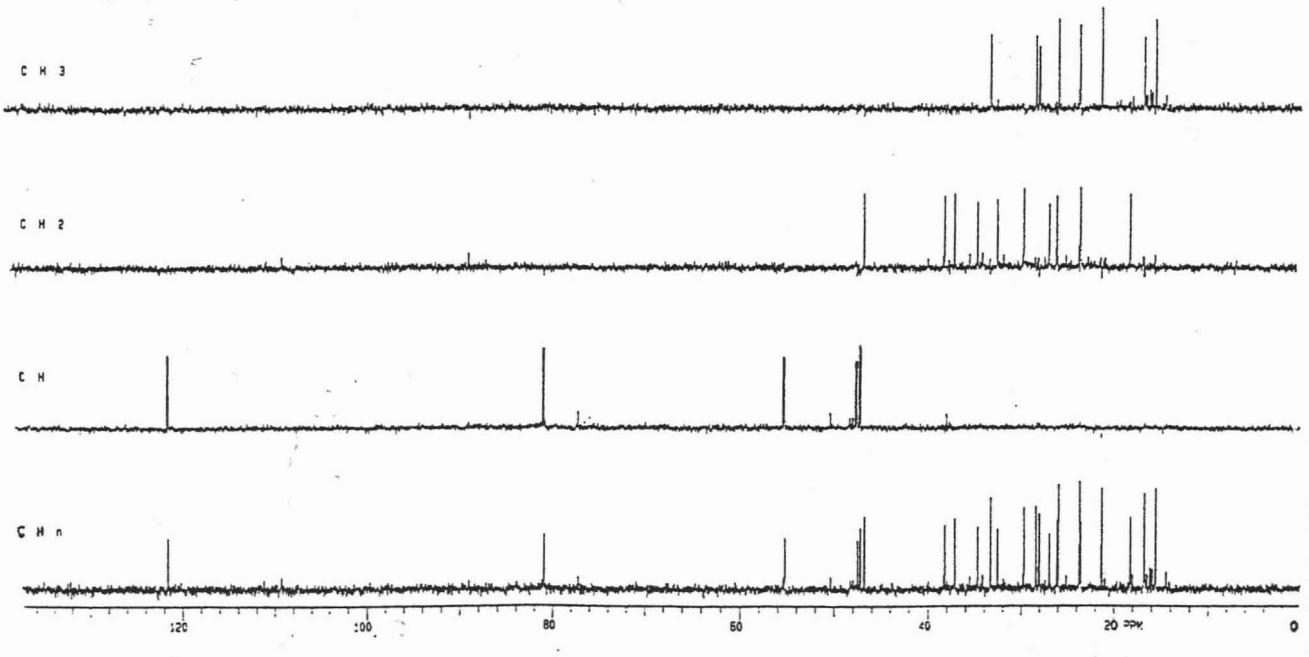
Spectrum 16: HETCOR NMR spectrum of Compound II



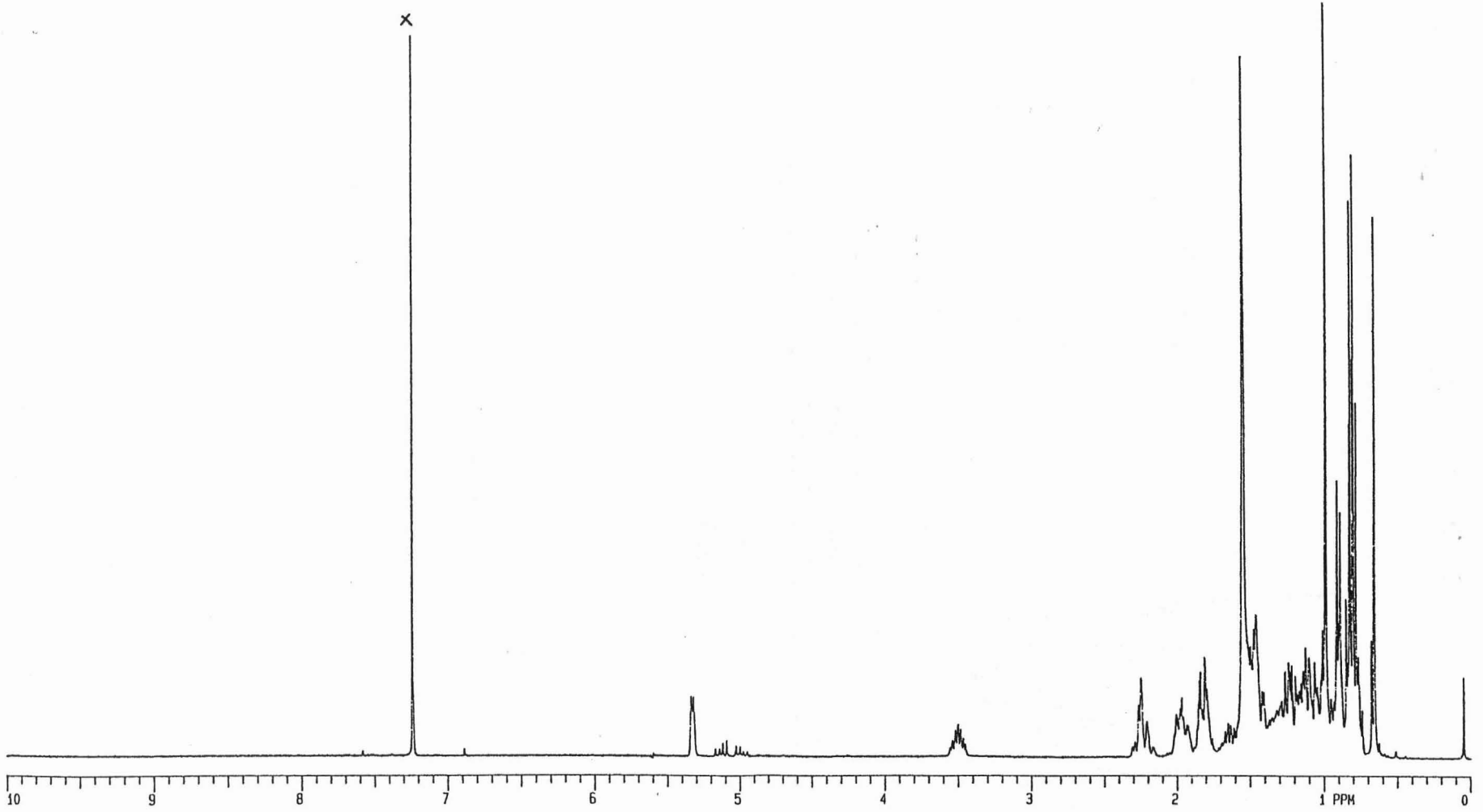
Spectrum 17: ^{1}H NMR spectrum of Compound II - Acetate



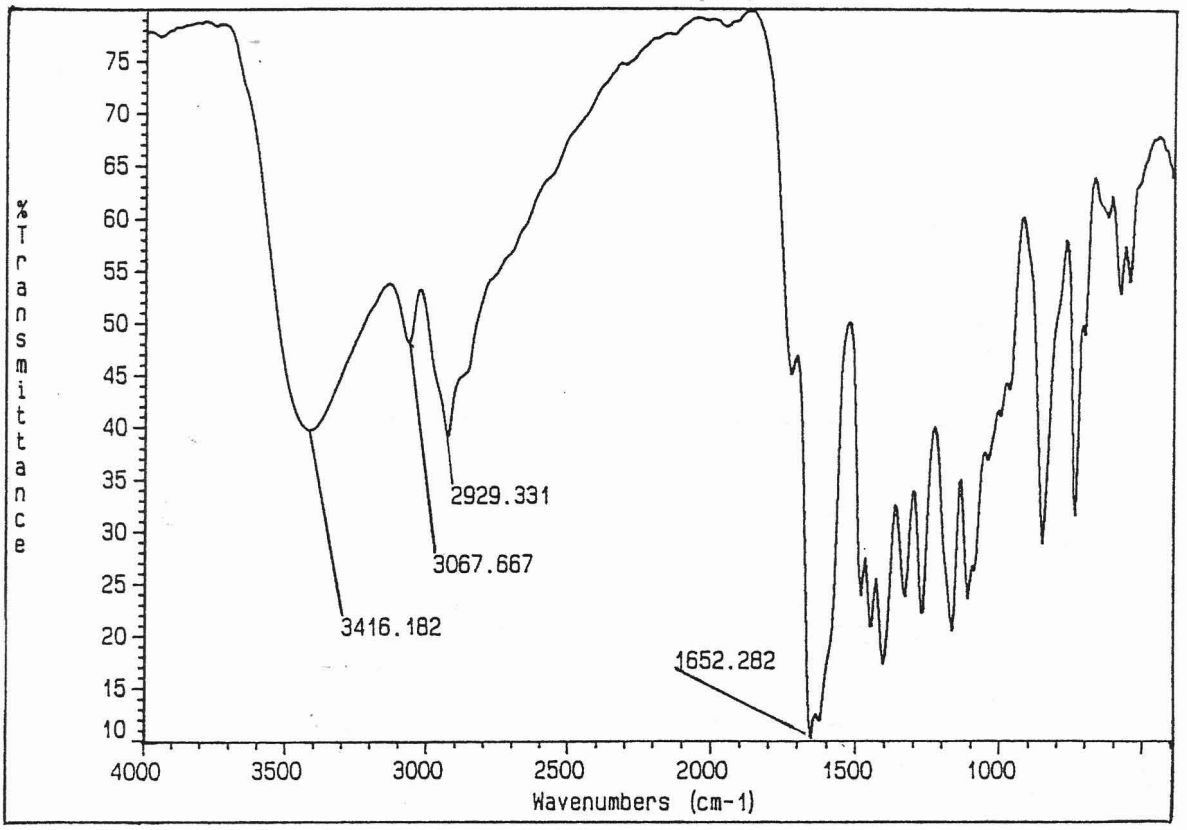
Spectrum 18: ^{13}C NMR spectrum of Compound II - Acetate



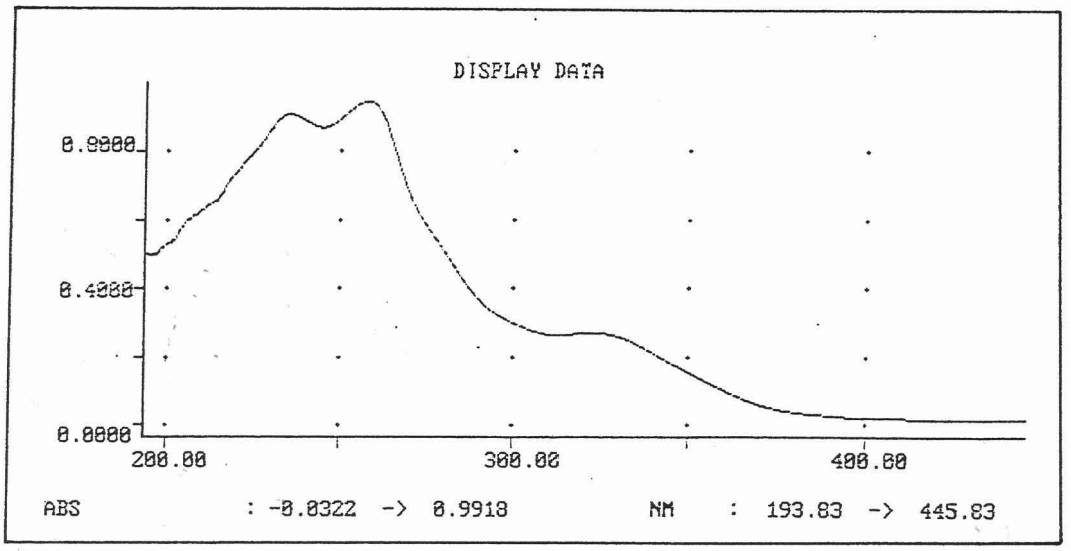
Spectrum 19: ADEPT NMR spectrum of Compound II - Acetate



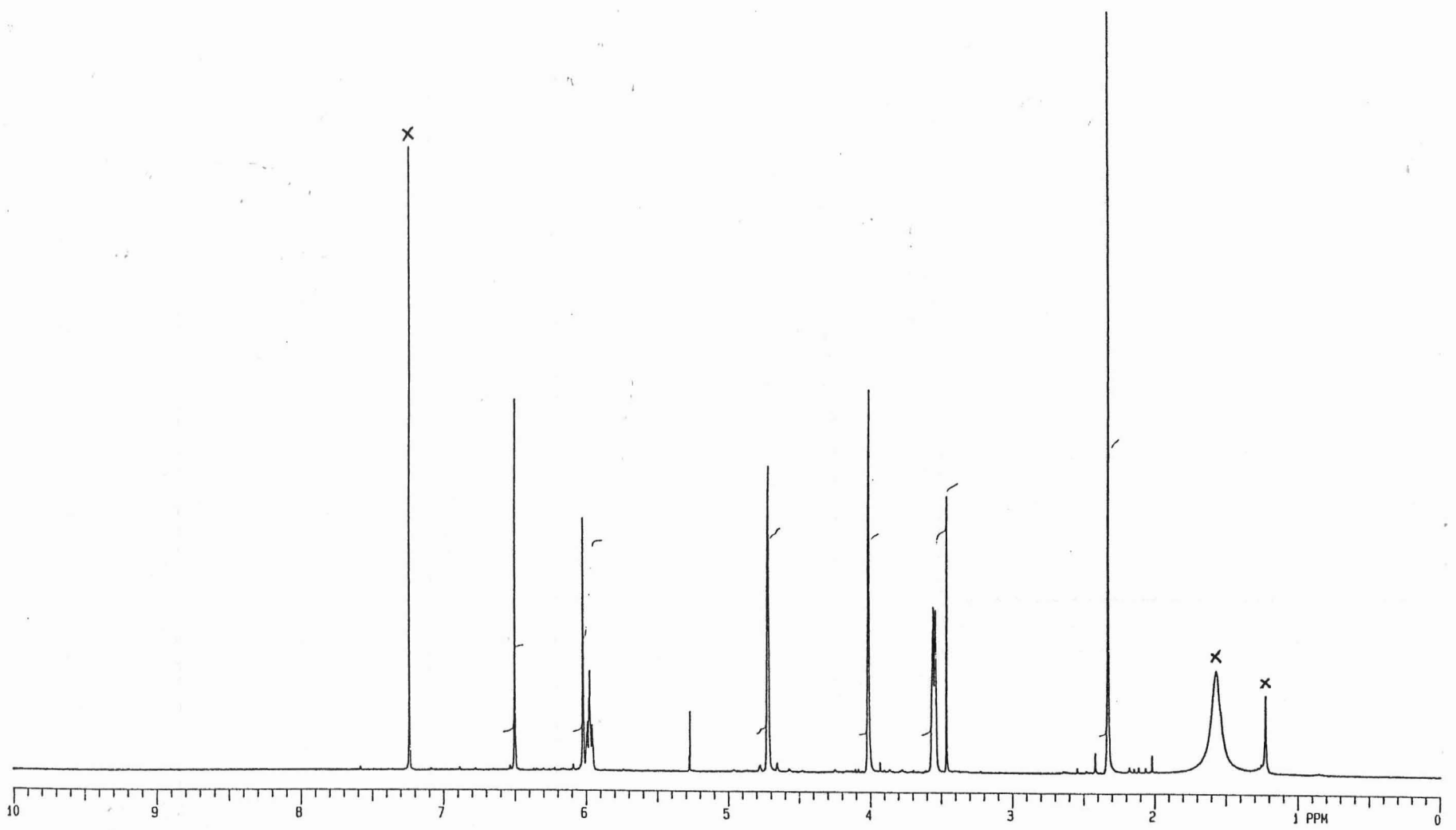
Spectrum 20: ^{1}H NMR spectrum of Compound III



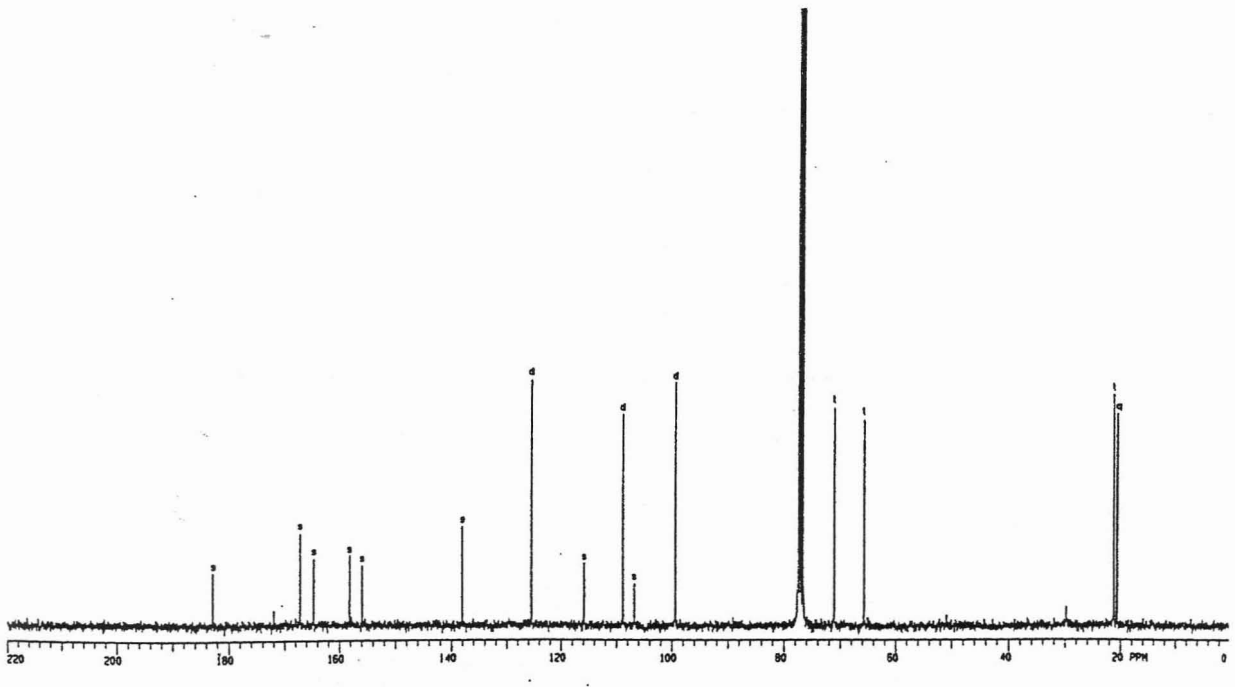
Spectrum 21: IR spectrum of Compound IV



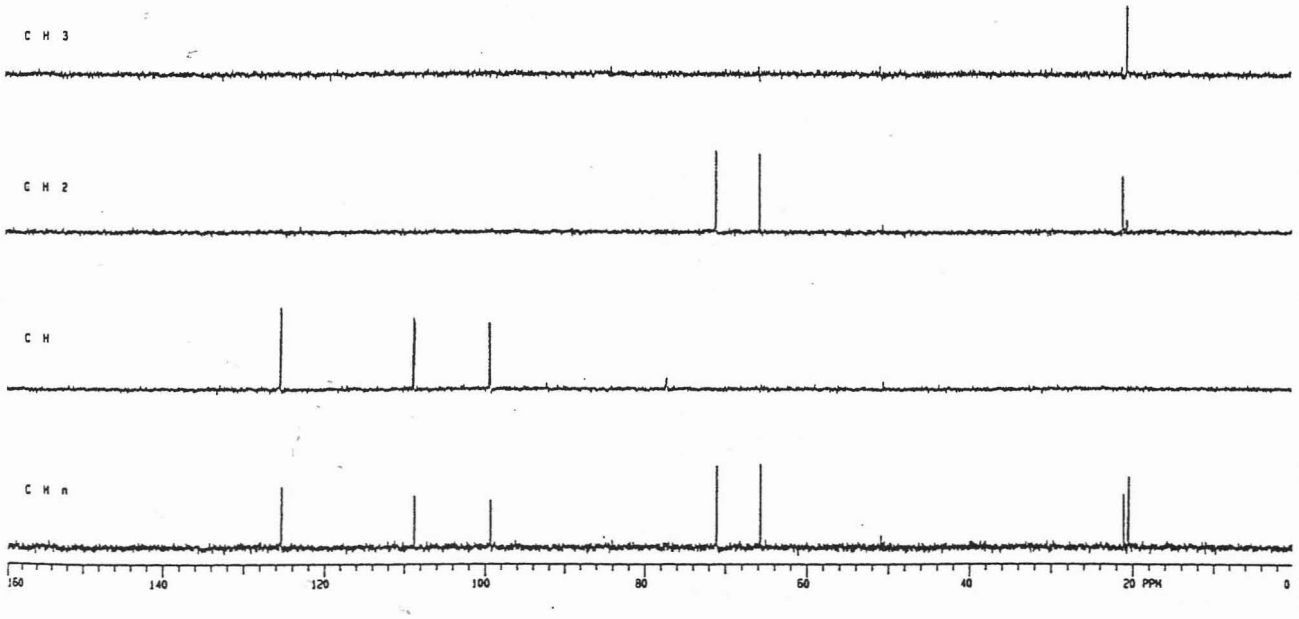
Spectrum 22: UV spectrum of Compound IV



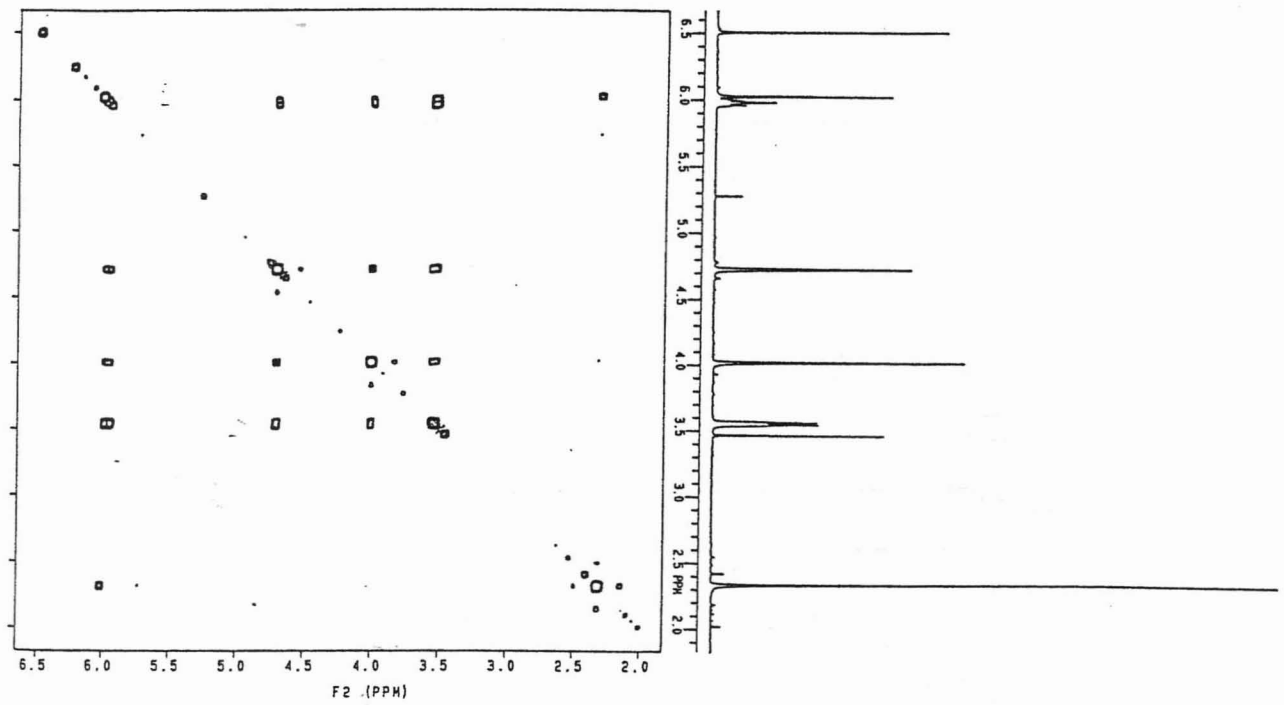
Spectrum 23: ^{1}H NMR spectrum of Compound IV



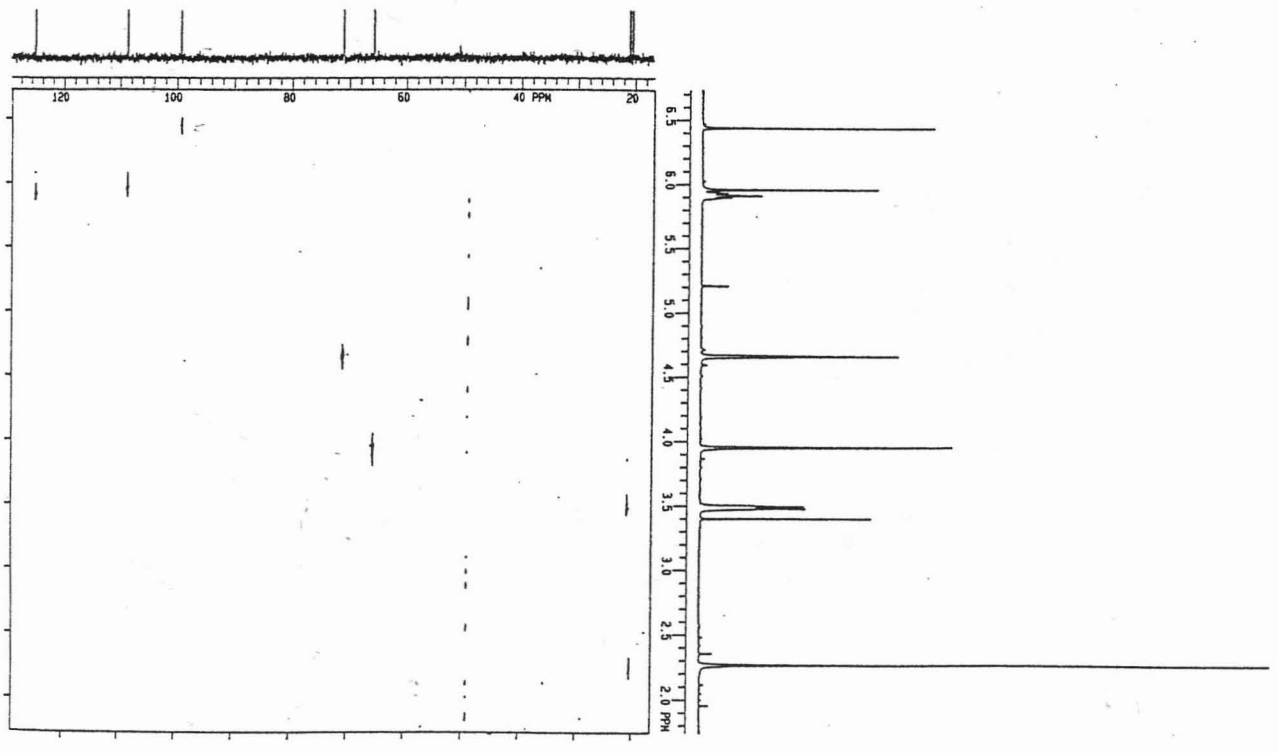
Spectrum 24: ^{13}C NMR spectrum of Compound IV



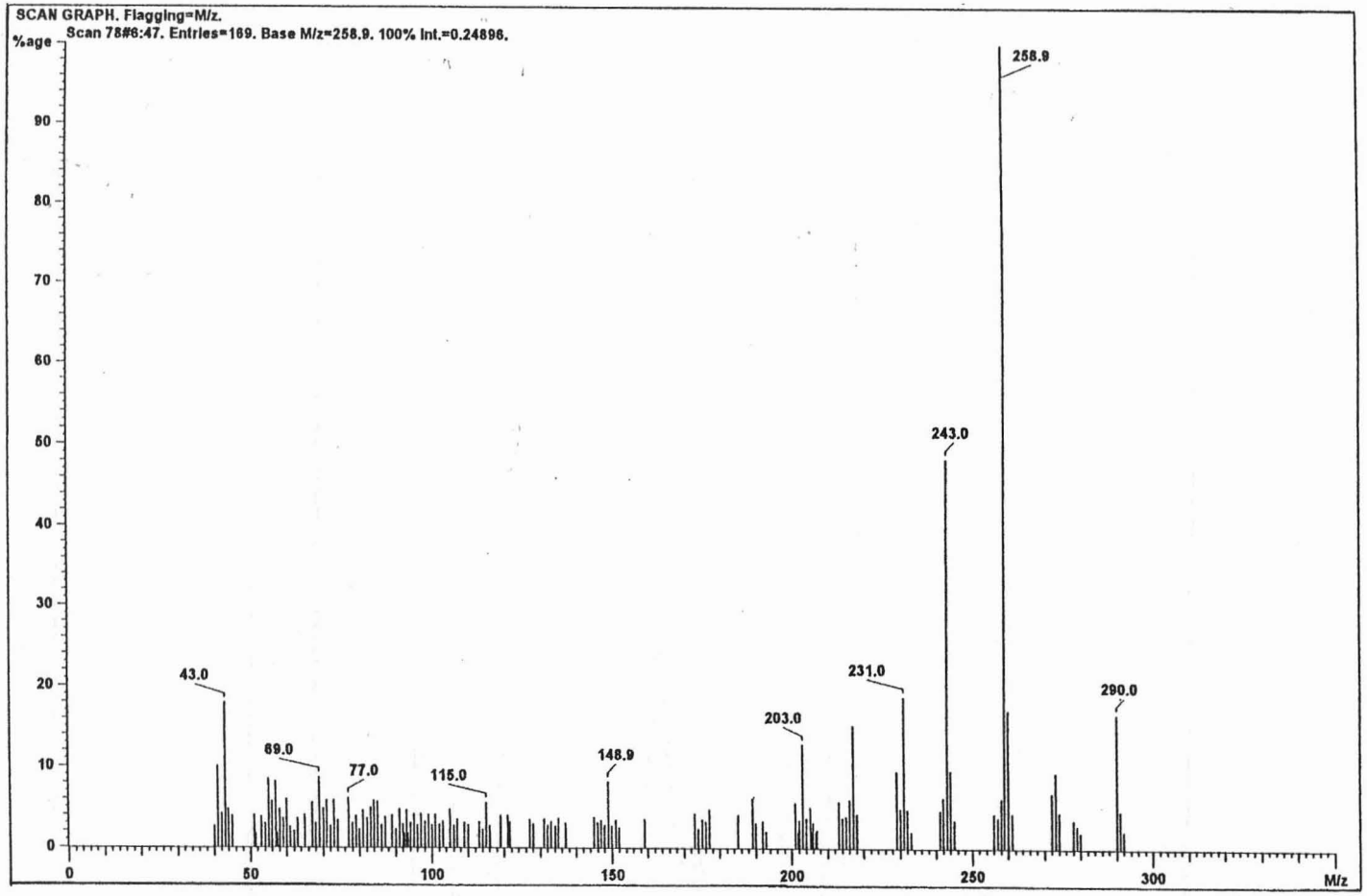
Spectrum 25: ADEPT NMR spectrum of Compound IV



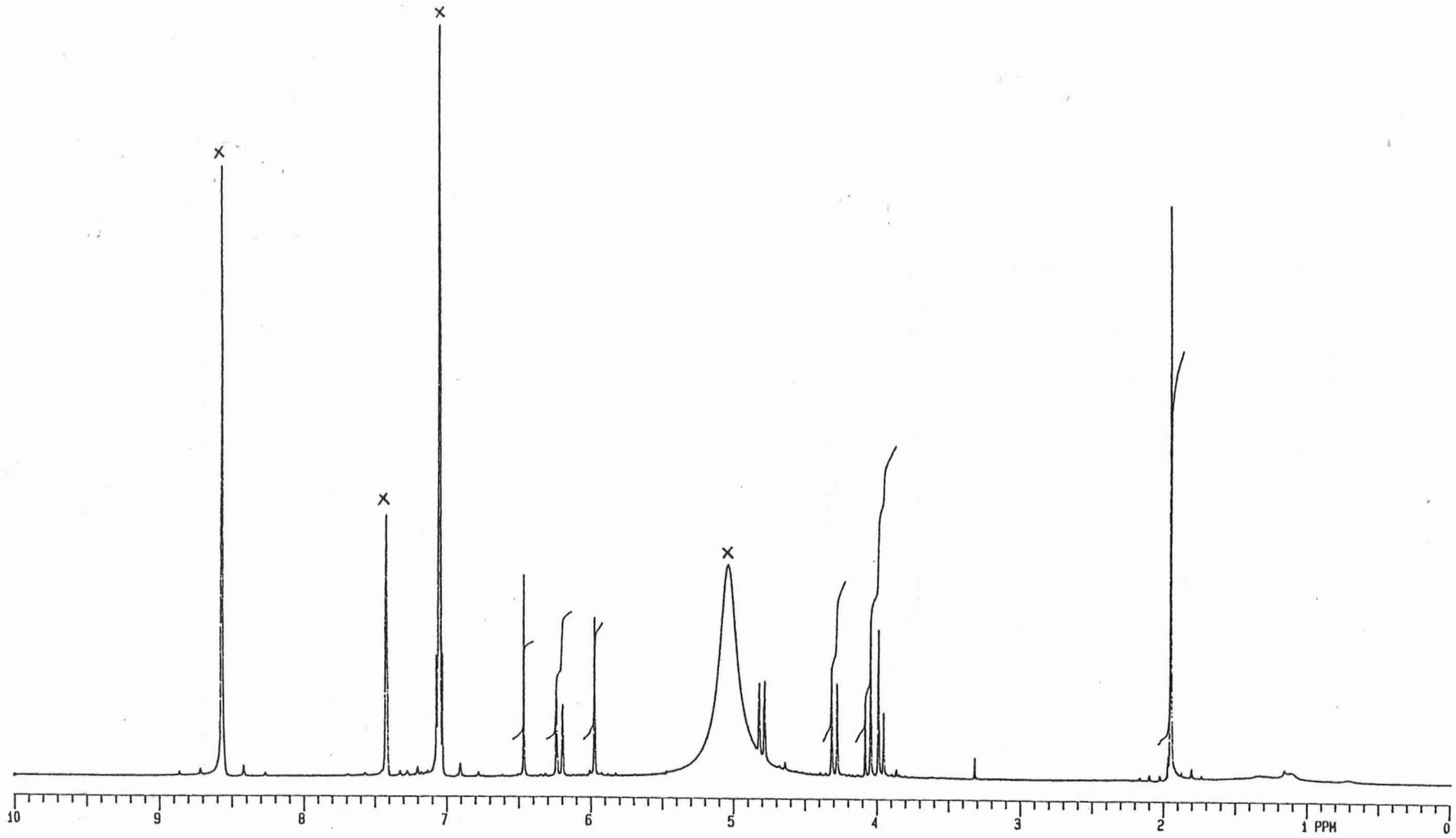
Spectrum 26: COSY NMR spectrum of Compound IV



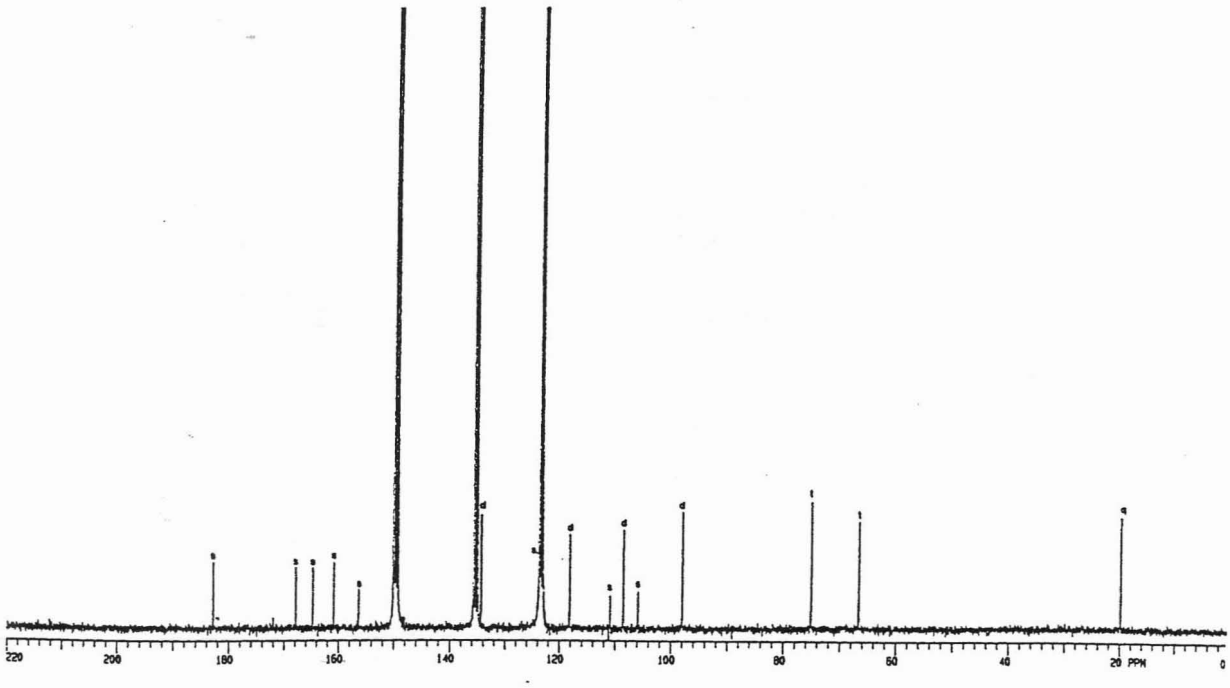
Spectrum 27: HETCOR NMR spectrum of Compound IV



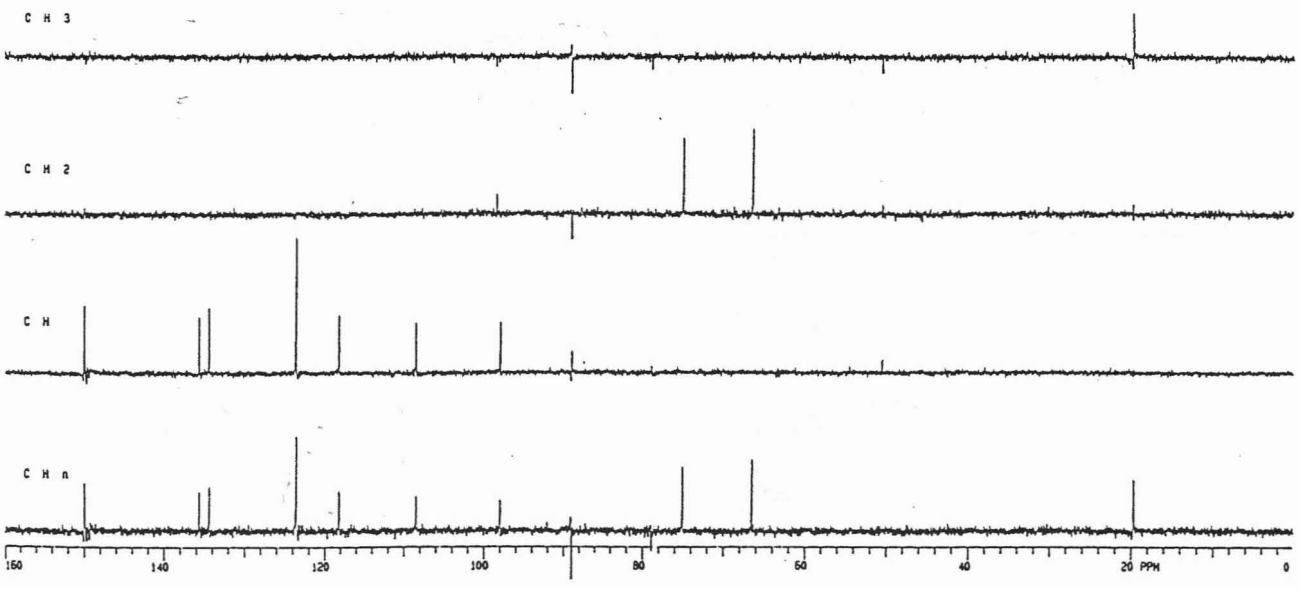
Spectrum 28: Mass spectrum of Compound V



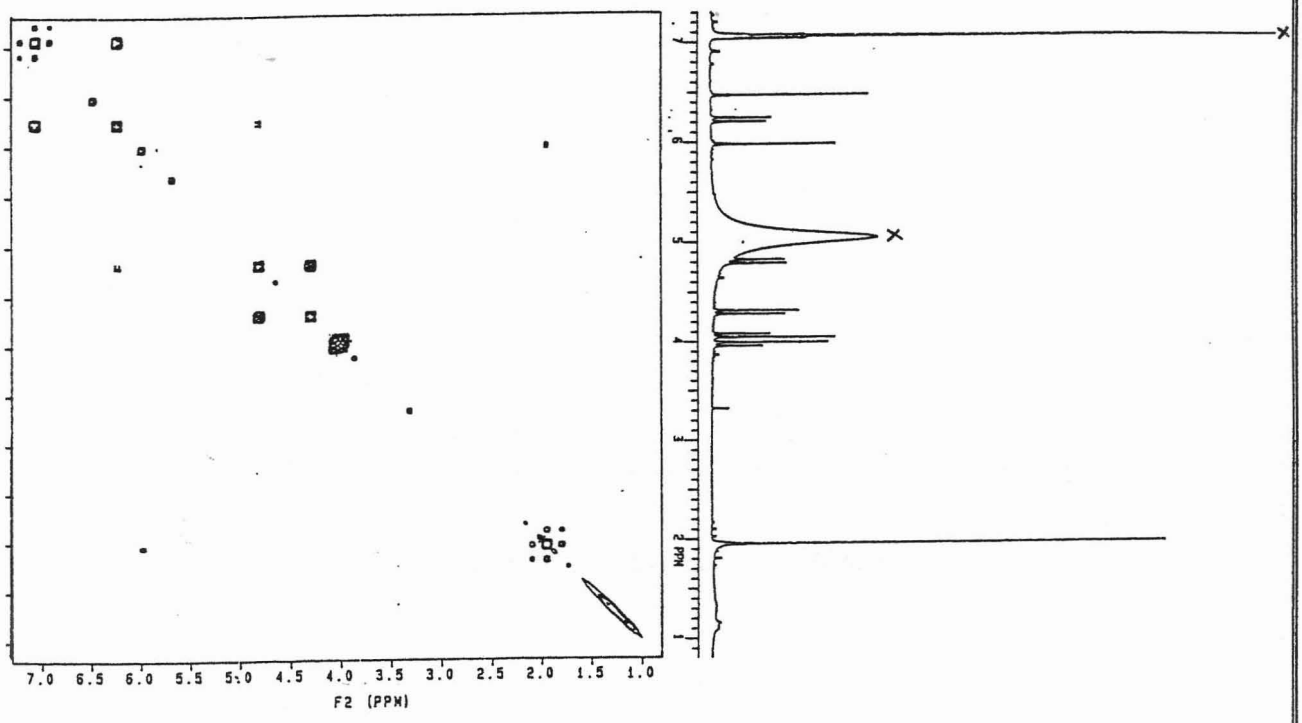
Spectrum 29: ^{1}H NMR spectrum of Compound V



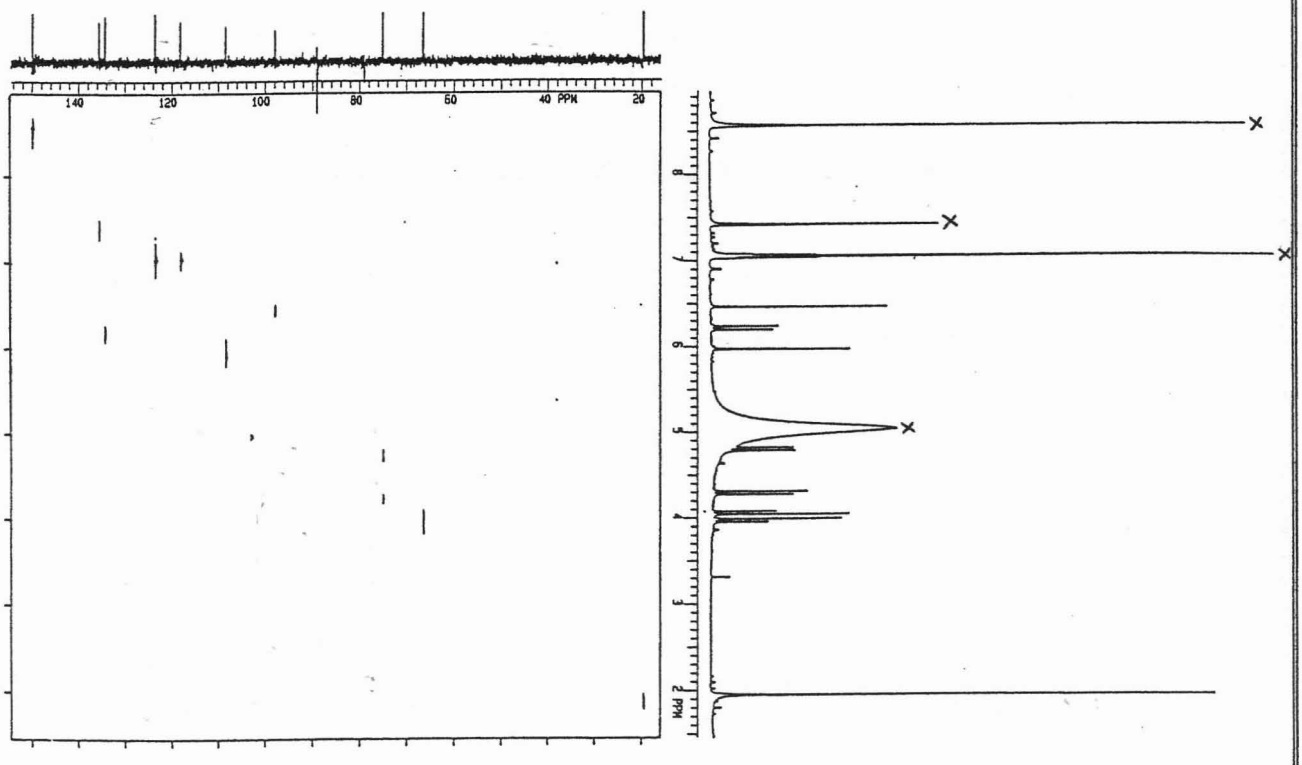
Spectrum 30: ^{13}C NMR spectrum of Compound V



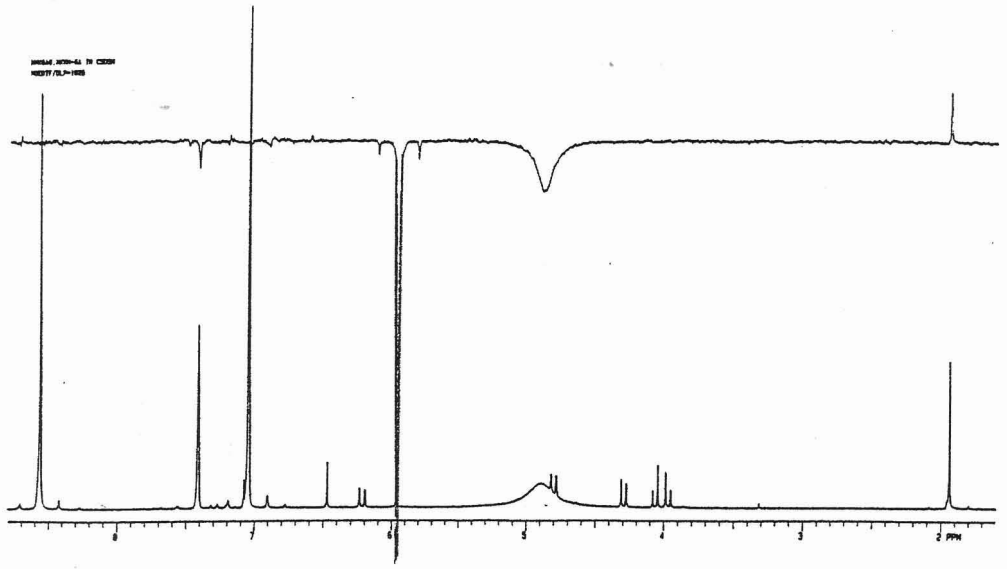
Spectrum 31: ADEPT NMR spectrum of Compound V



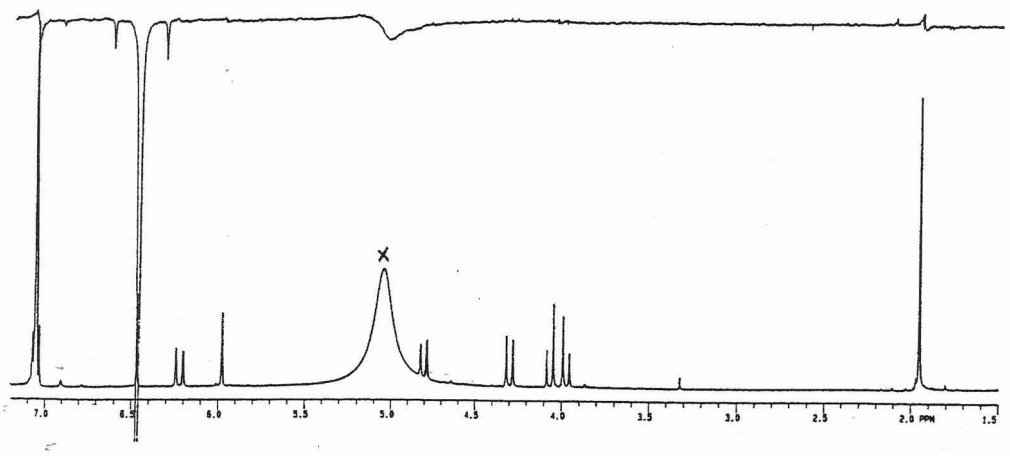
Spectrum 32: COSY NMR spectrum of Compound V



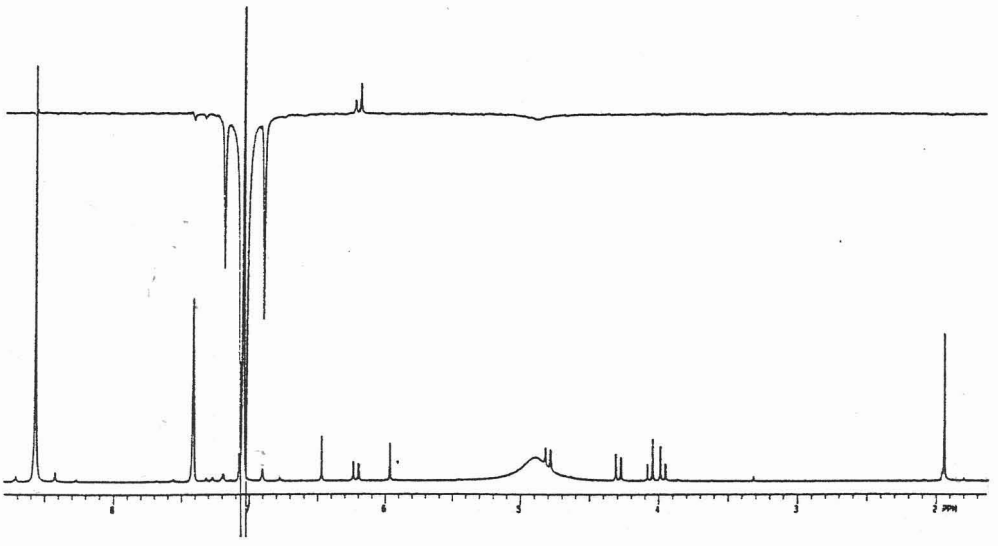
Spectrum 33: HETCOR NMR spectrum of Compound V



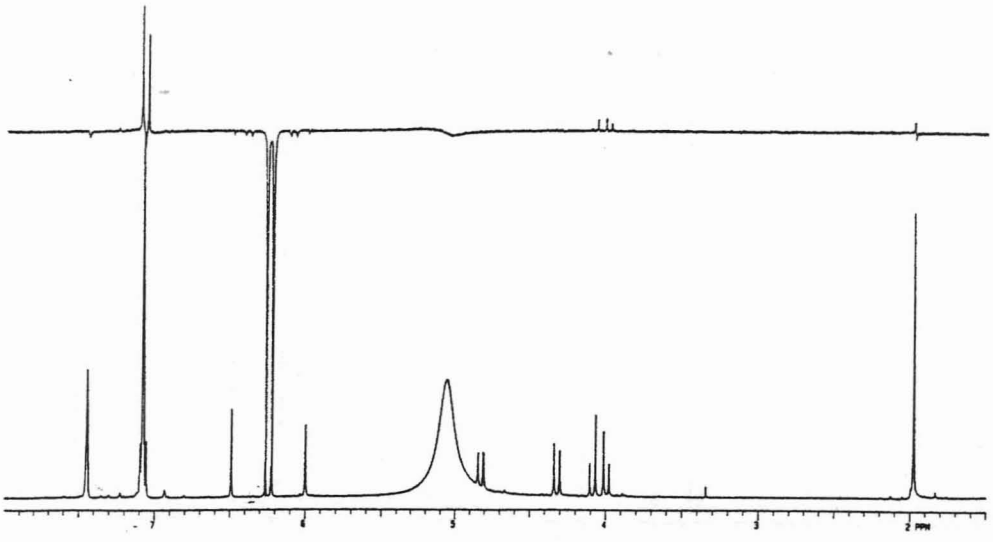
Spectrum 34a: NOE NMR spectrum of Compound V



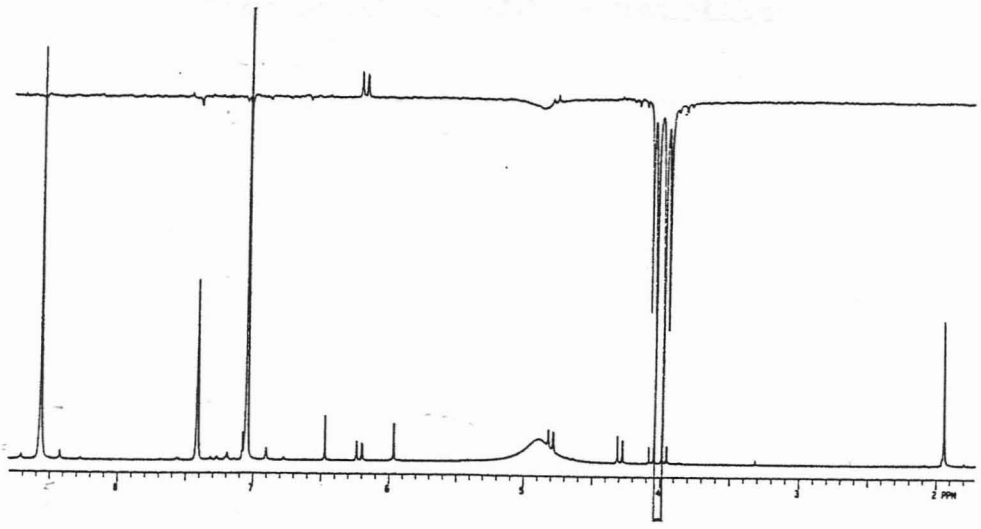
Spectrum 34b: NOE NMR spectrum of Compound V



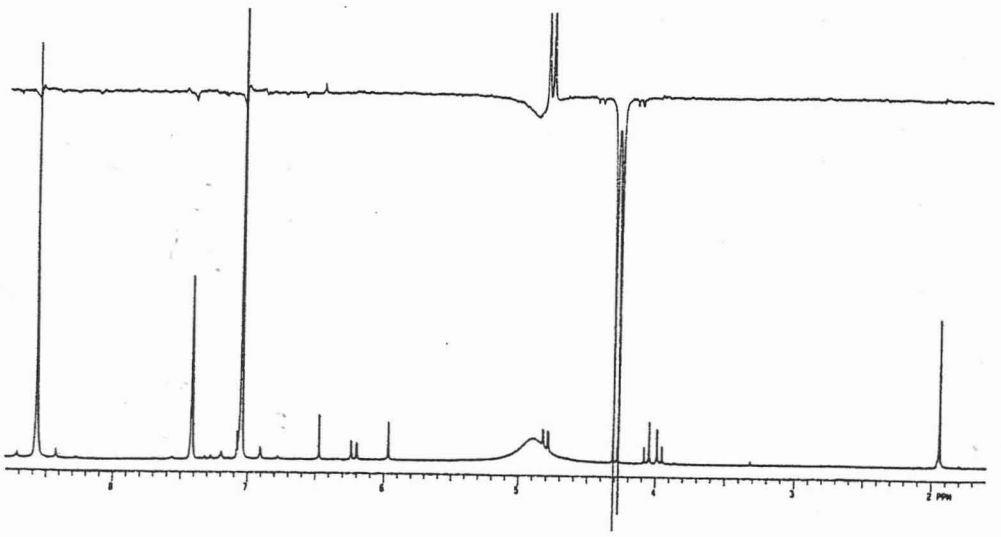
Spectrum 34c: NOE NMR spectrum of Compound V



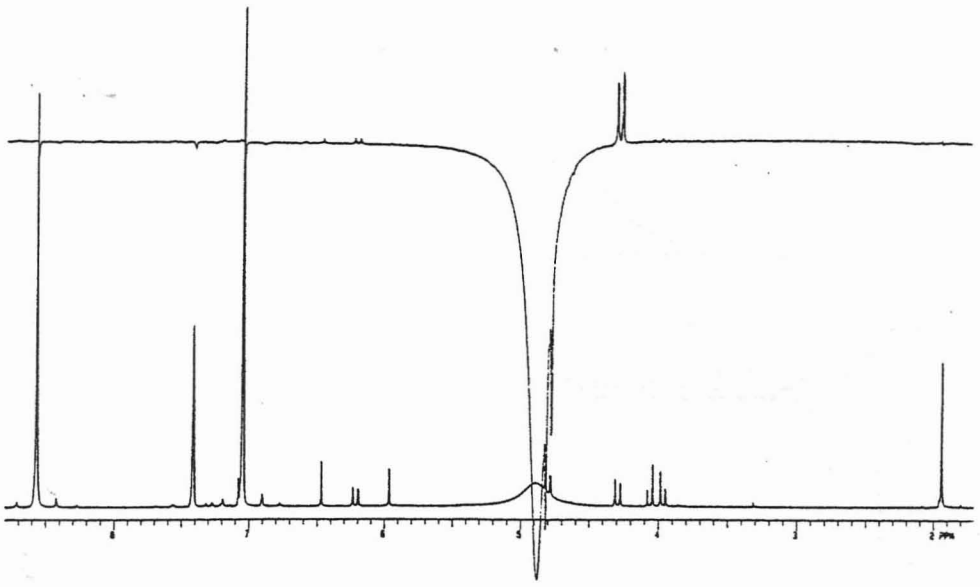
Spectrum 34d: NOE NMR spectrum of Compound V



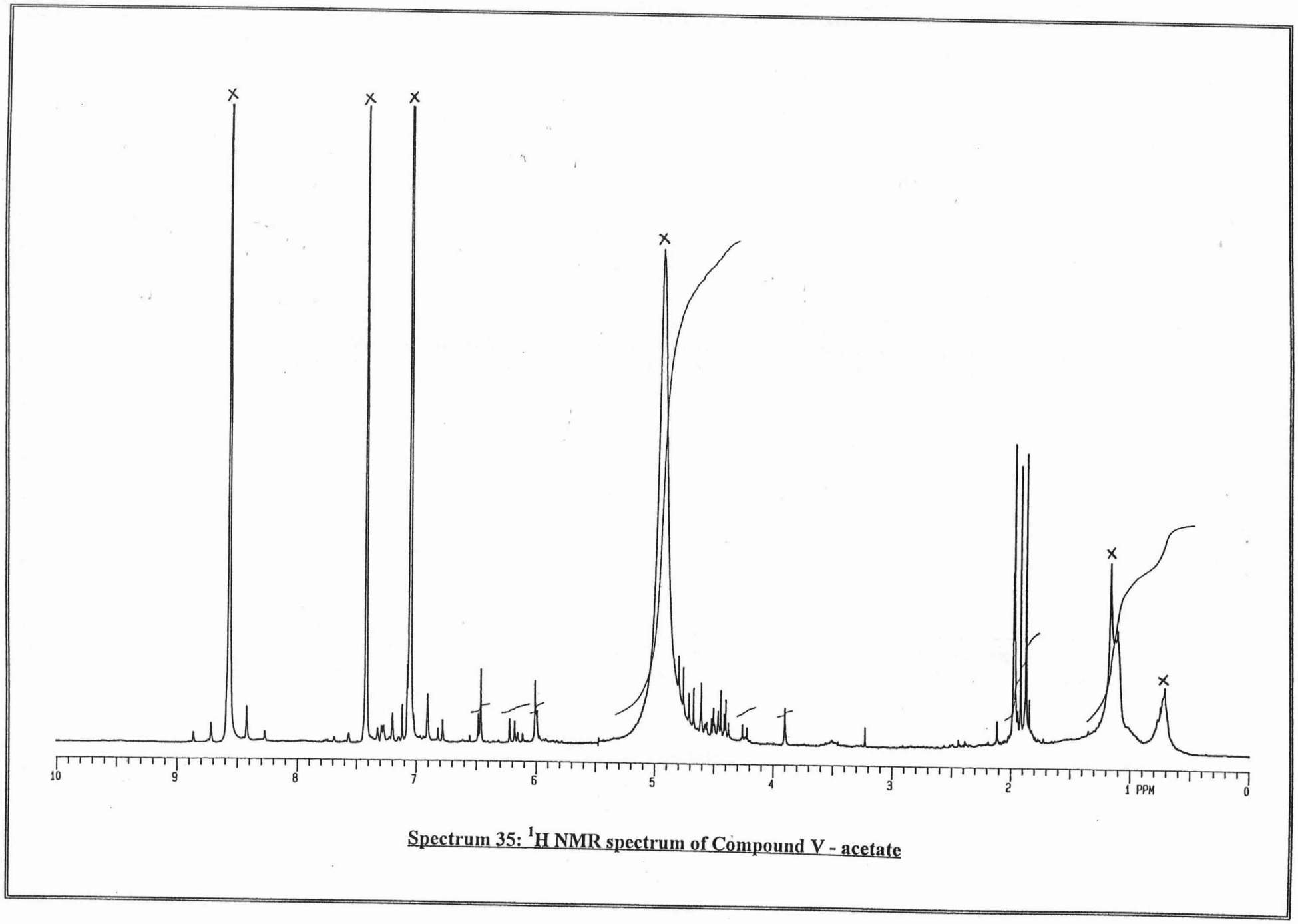
Spectrum 34e: NOE NMR spectrum of Compound V



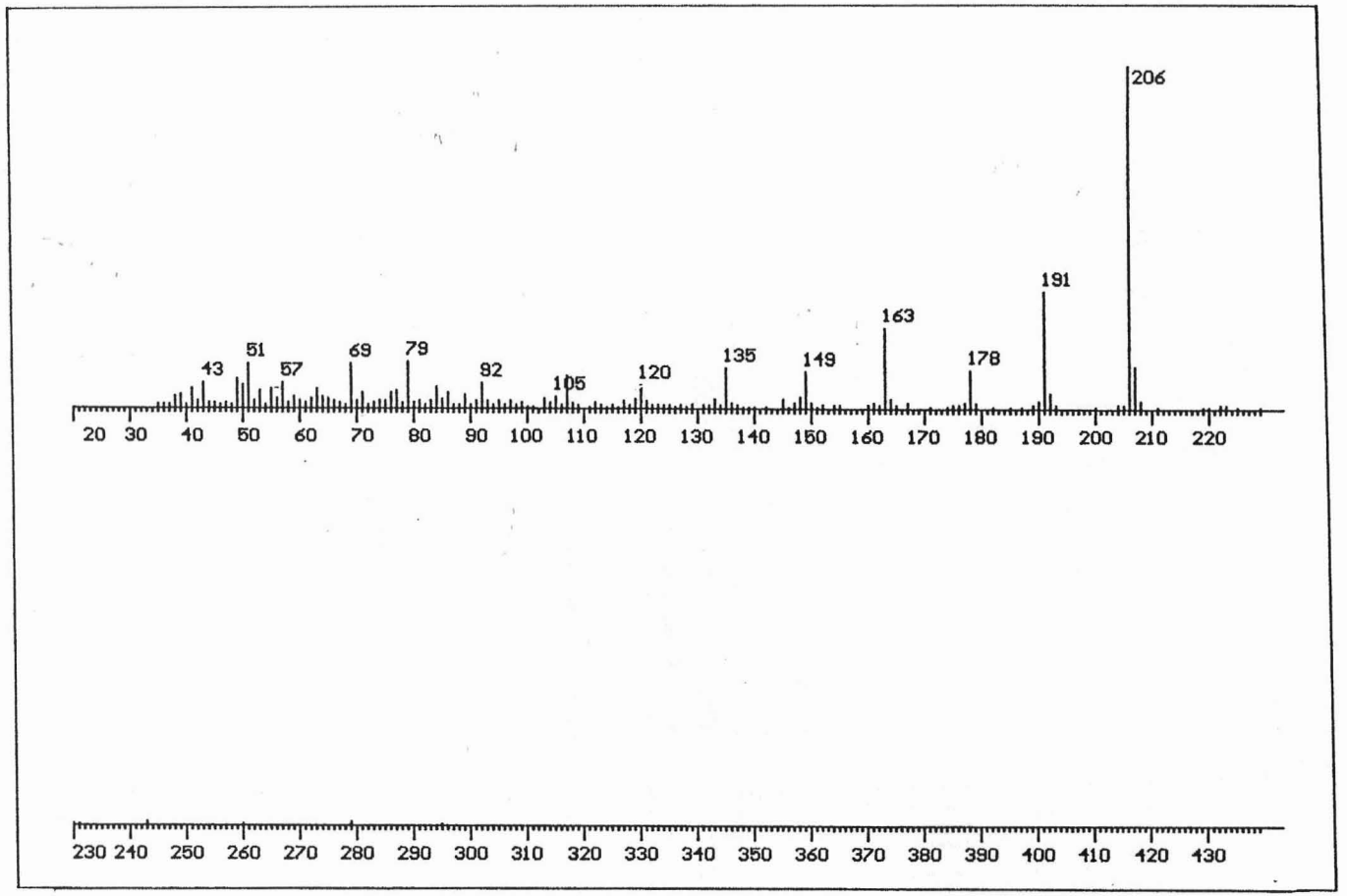
Spectrum 34f: NOE NMR spectrum of Compound V



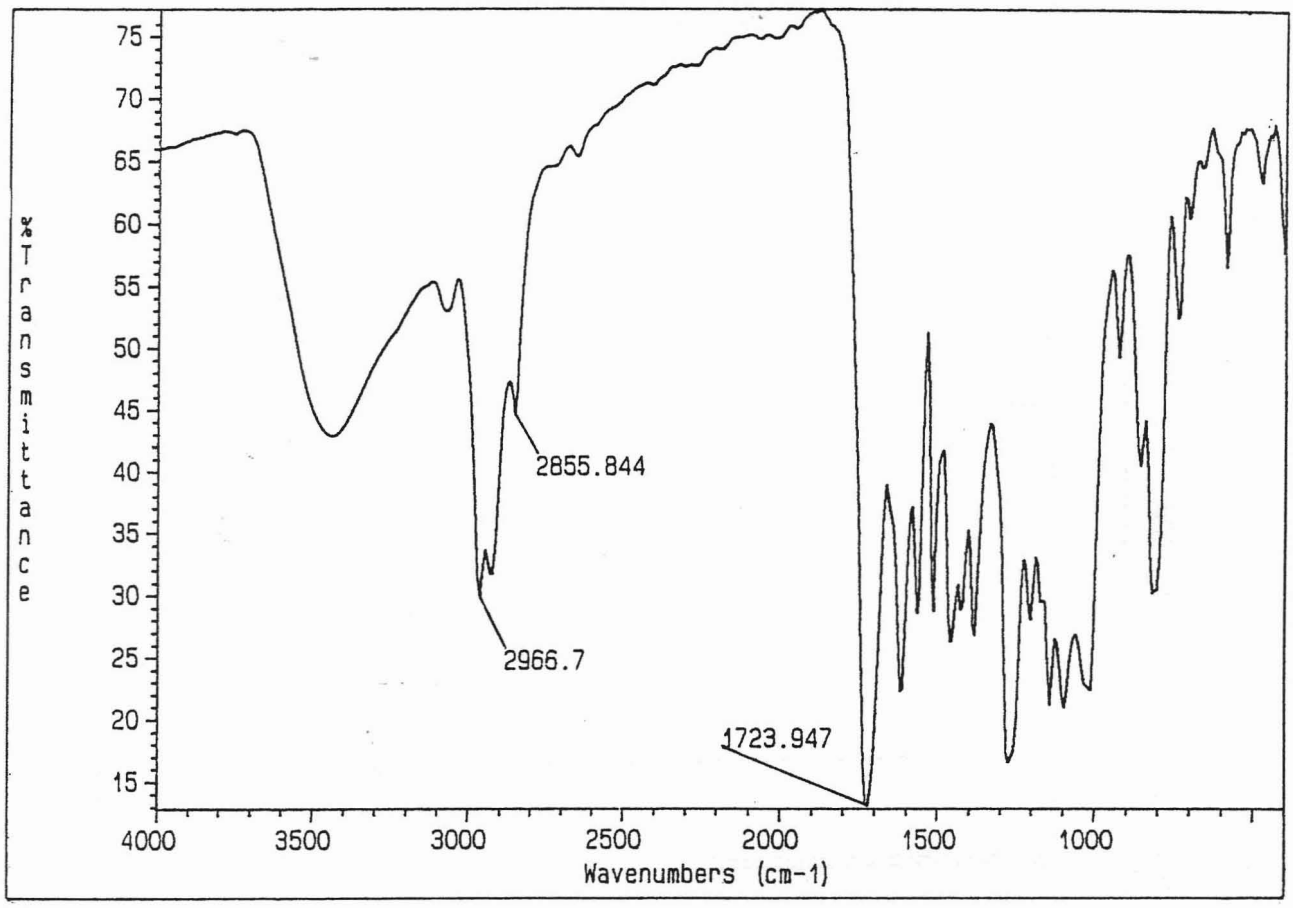
Spectrum 34g: NOE NMR spectrum of Compound V



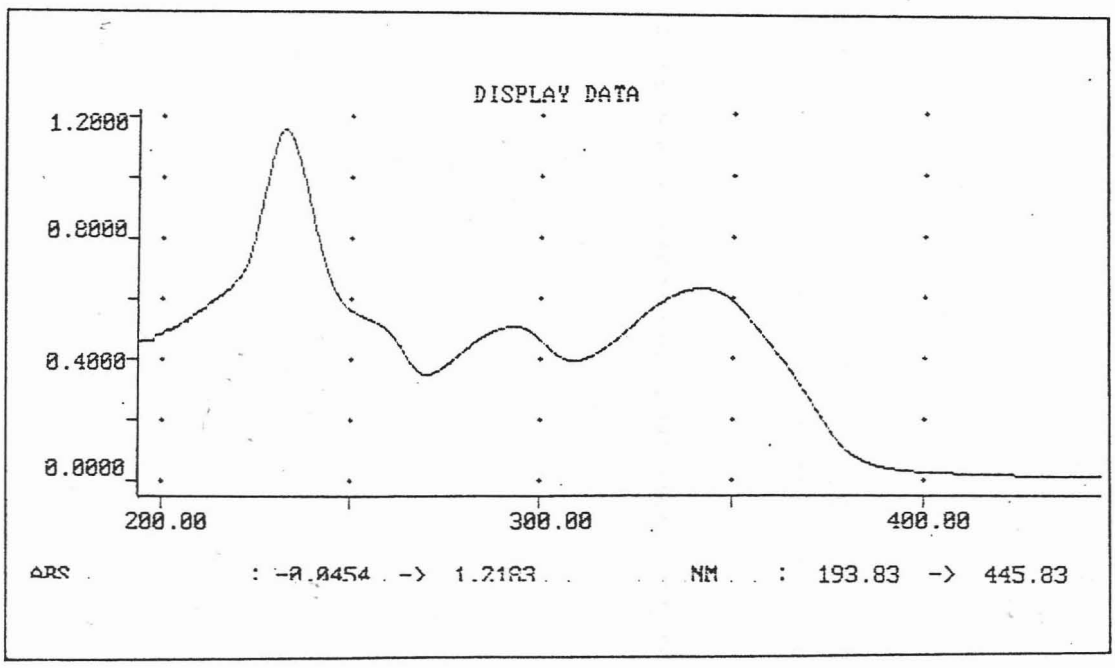
Spectrum 35: ^{1}H NMR spectrum of Compound V - acetate



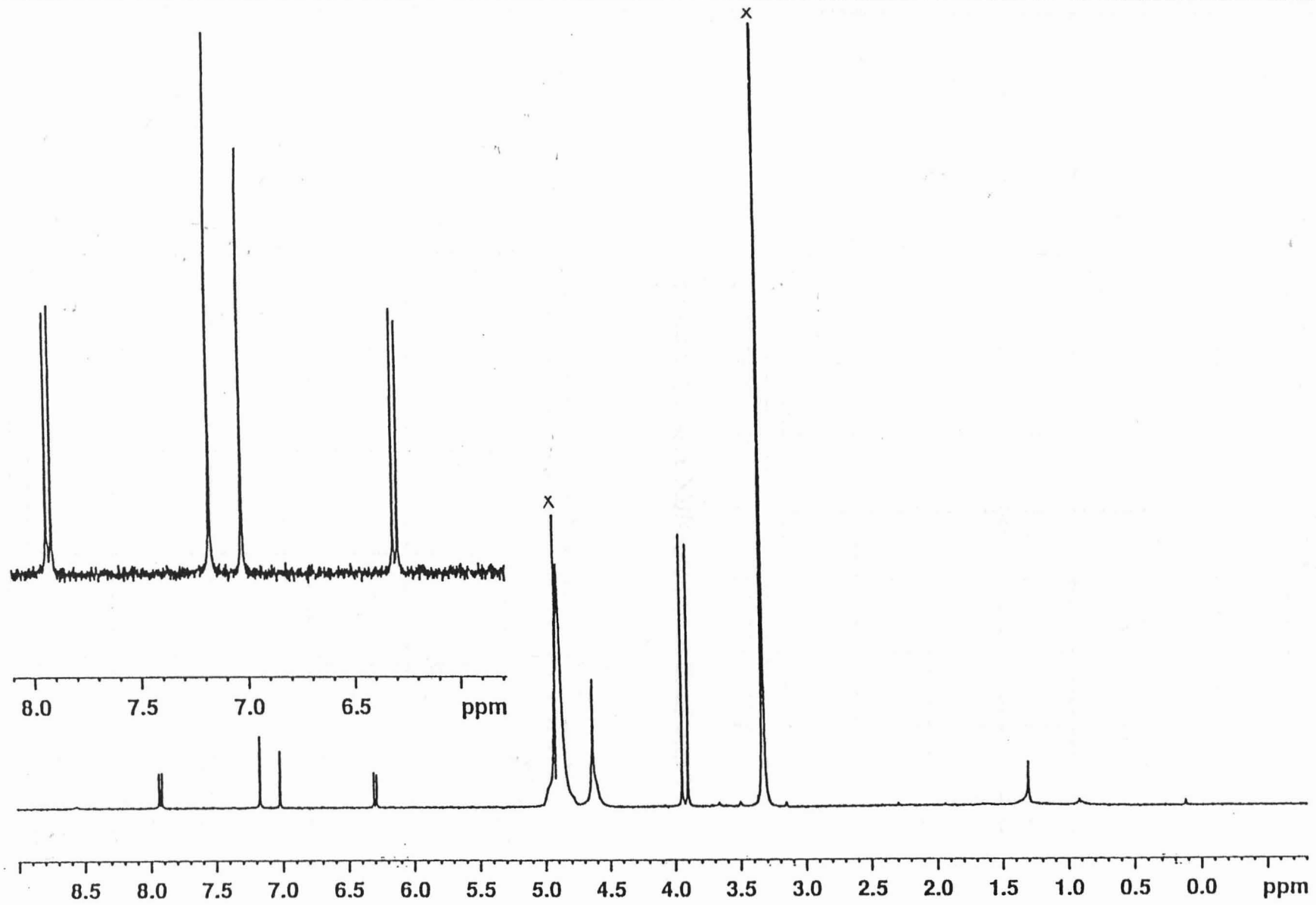
Spectrum 36: Mass spectrum of Compound VI



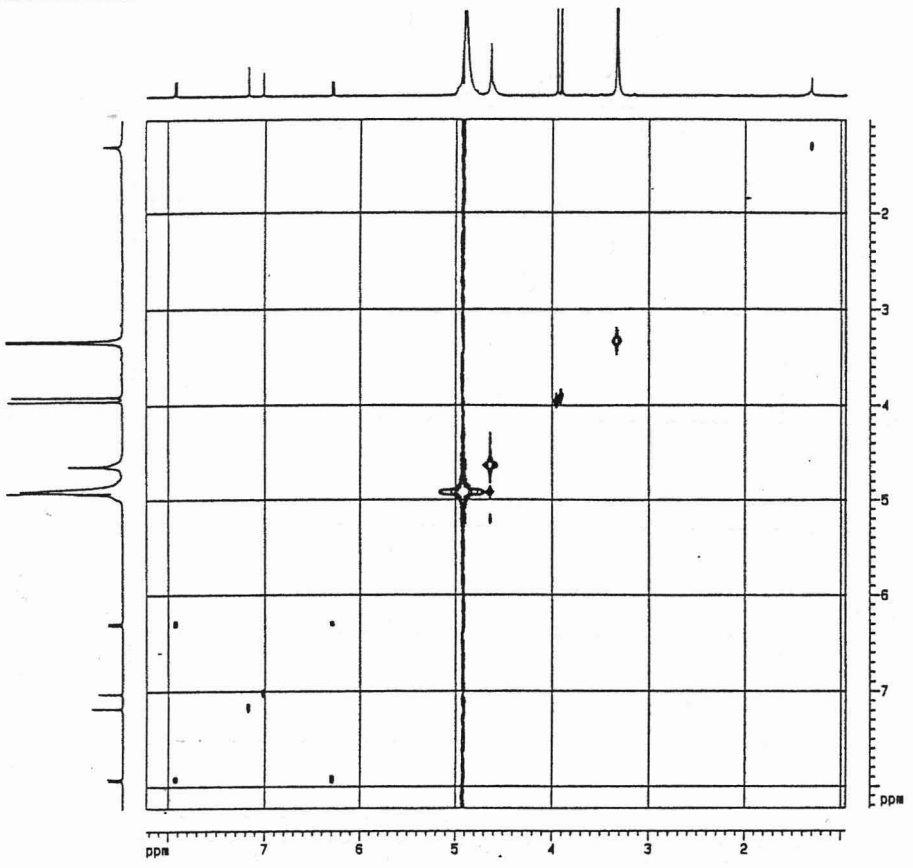
Spectrum 37: IR spectrum of Compound VI



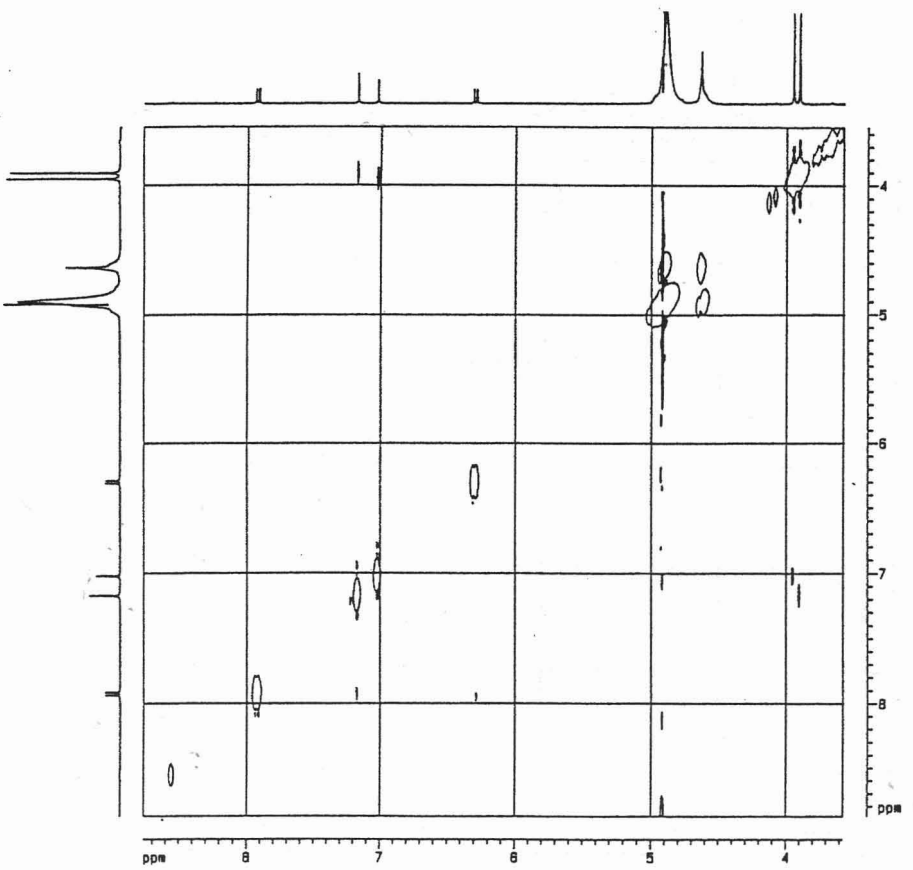
Spectrum 38: UV spectrum of Compound VI



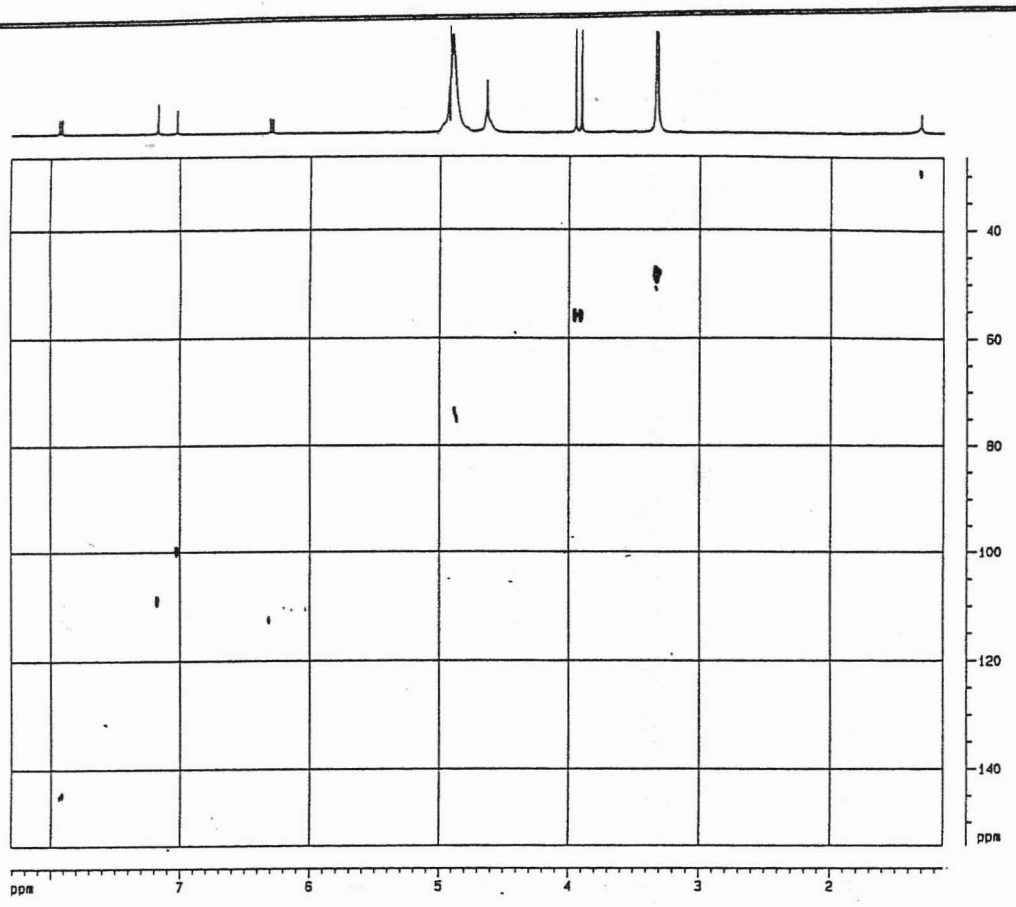
Spectrum 39: ^{1}H NMR spectrum of Compound VI



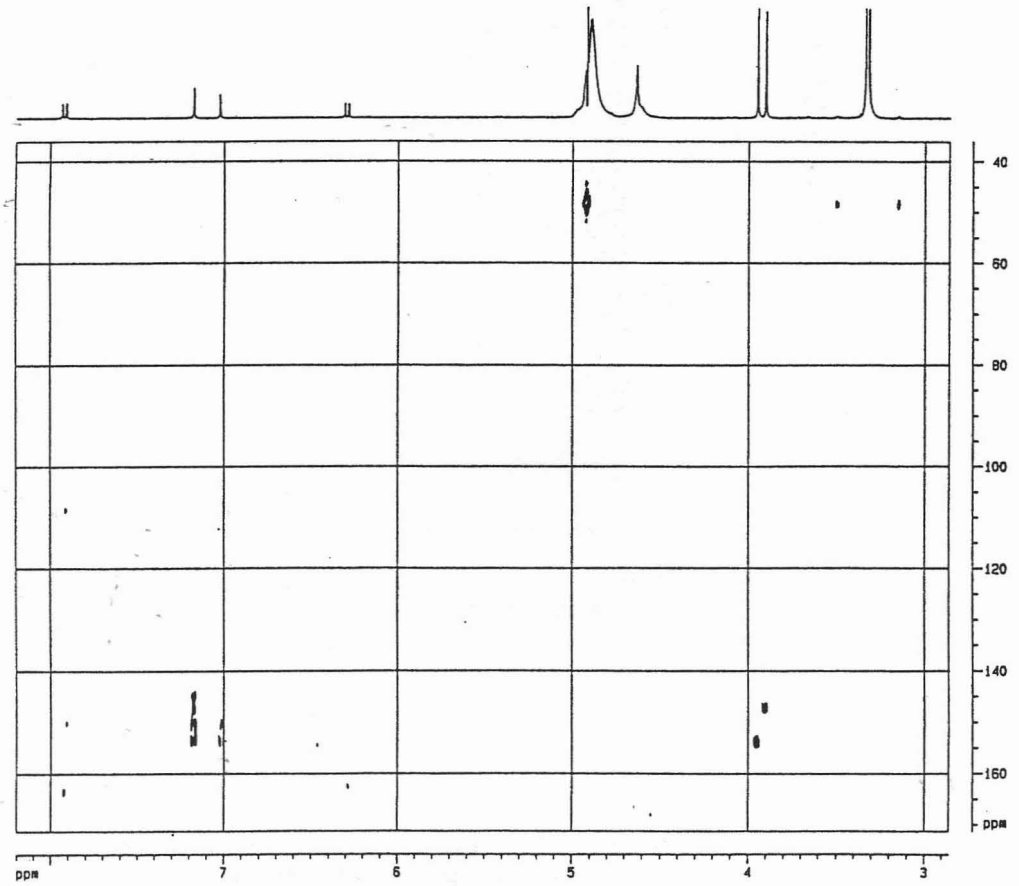
Spectrum 40: COSY NMR spectrum of Compound VI



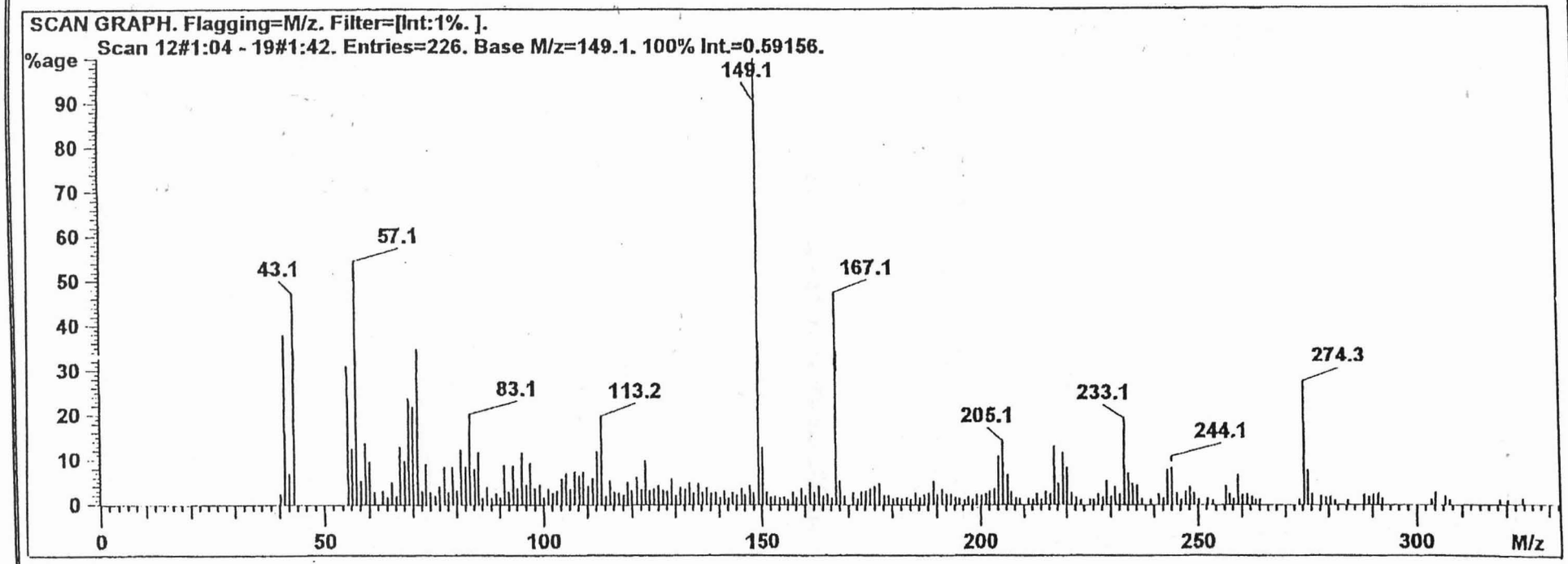
Spectrum 41: NOESY NMR spectrum of Compound VI



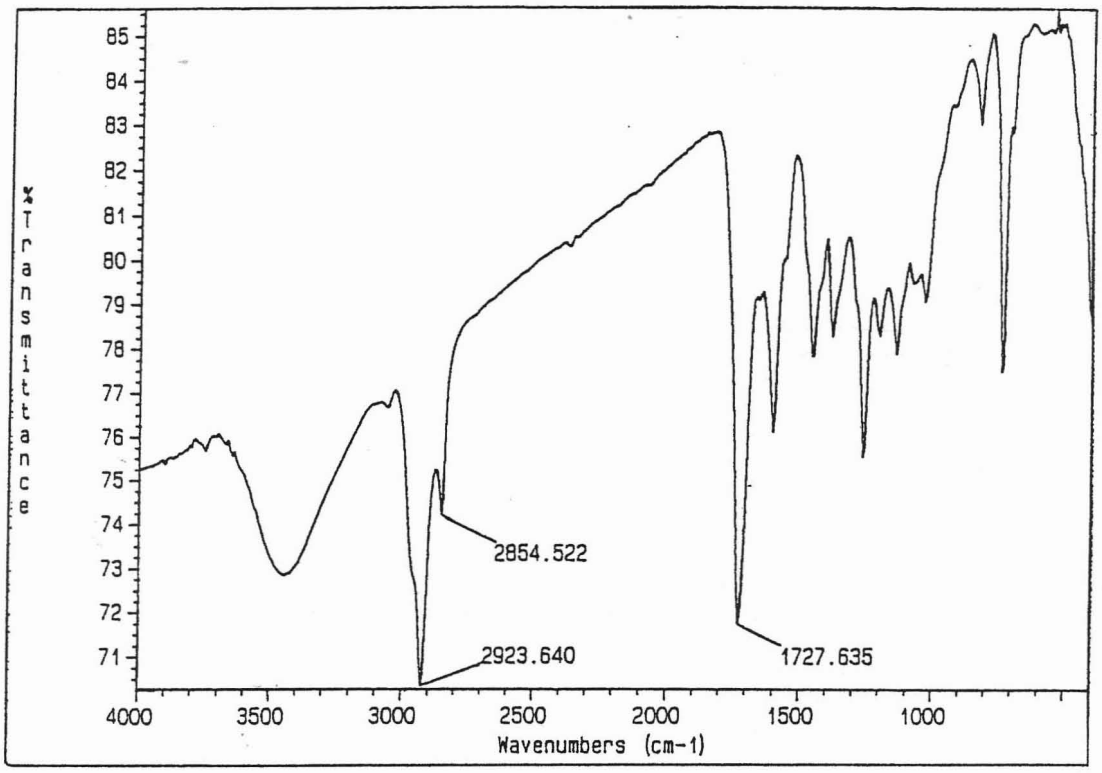
Spectrum 42: HMQC NMR spectrum of Compound VI



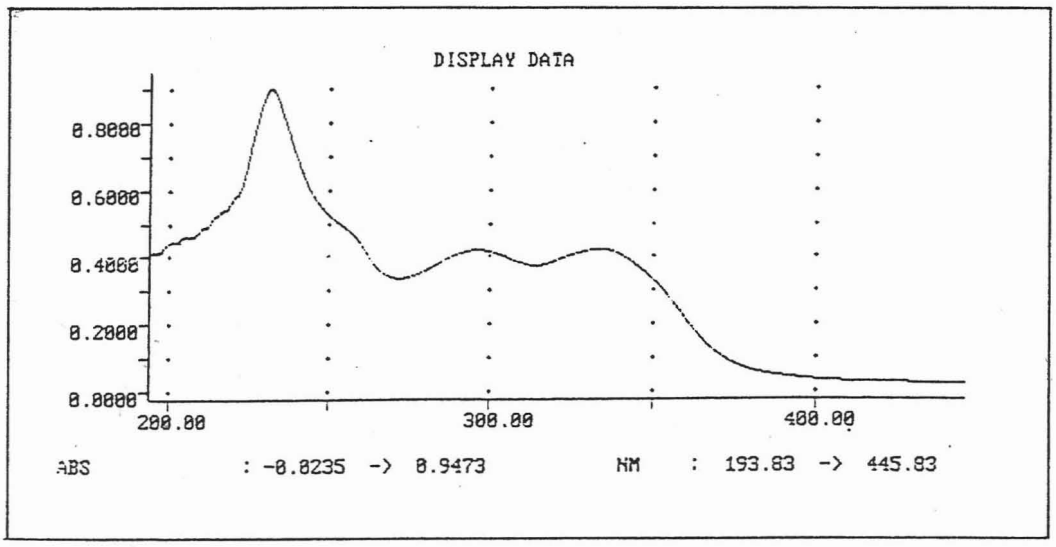
Spectrum 43: HMBC NMR spectrum of Compound VI



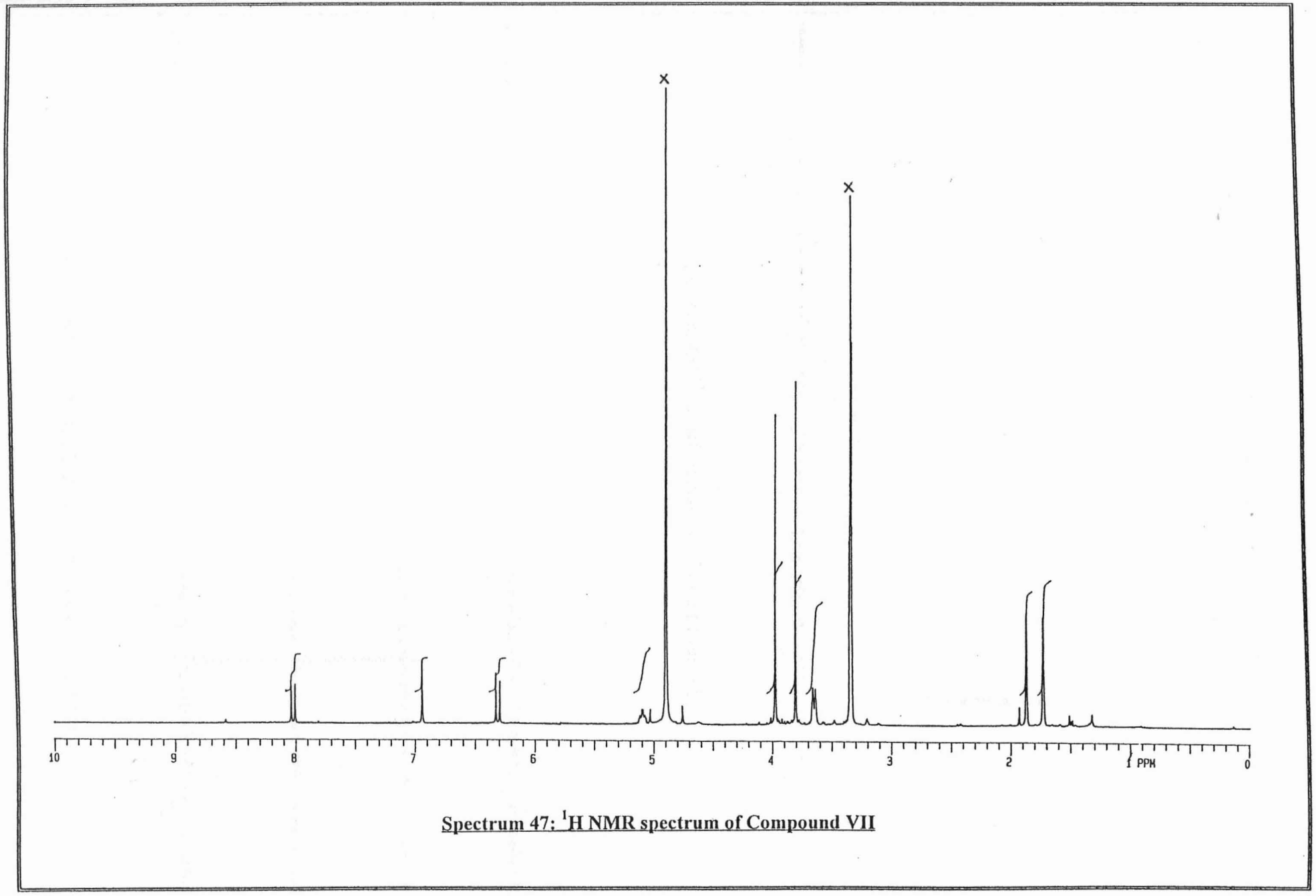
Spectrum 44: Mass spectrum of Compound VII



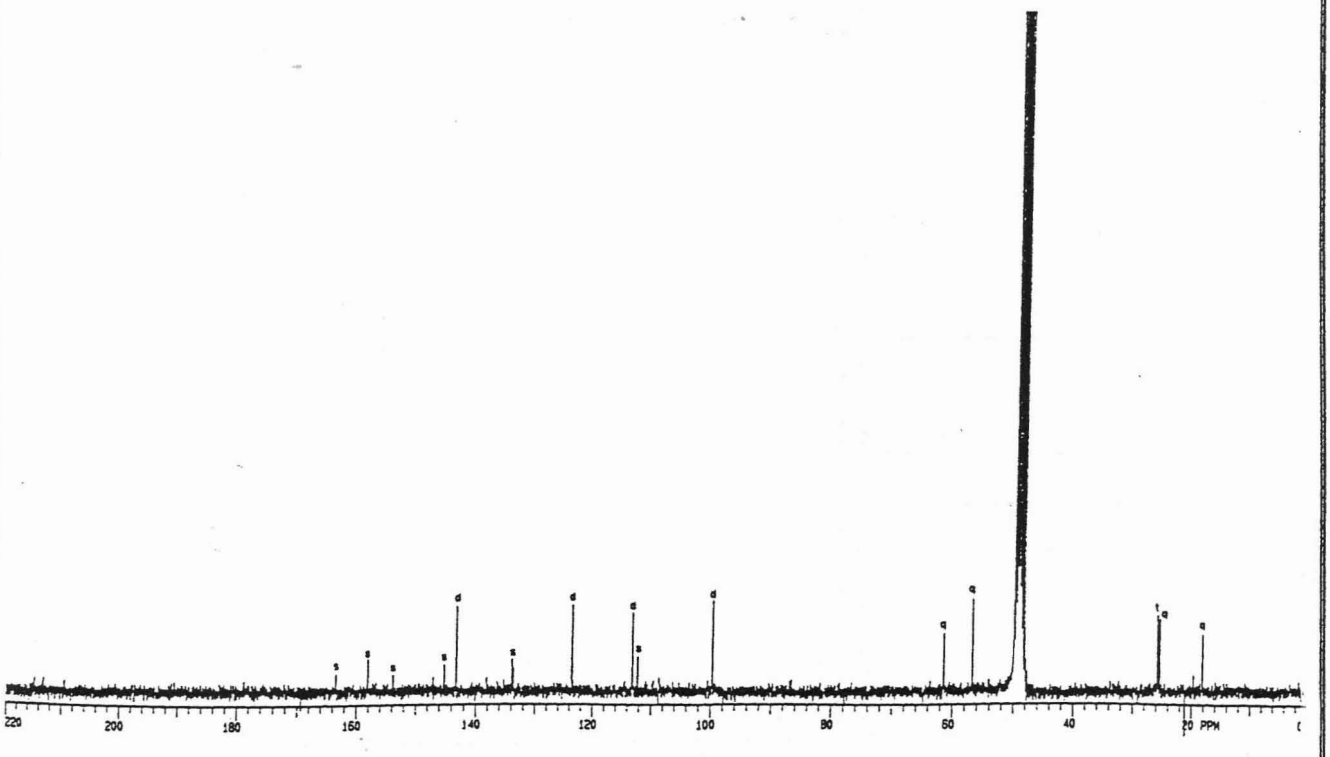
Spectrum 45: IR spectrum of Compound VII



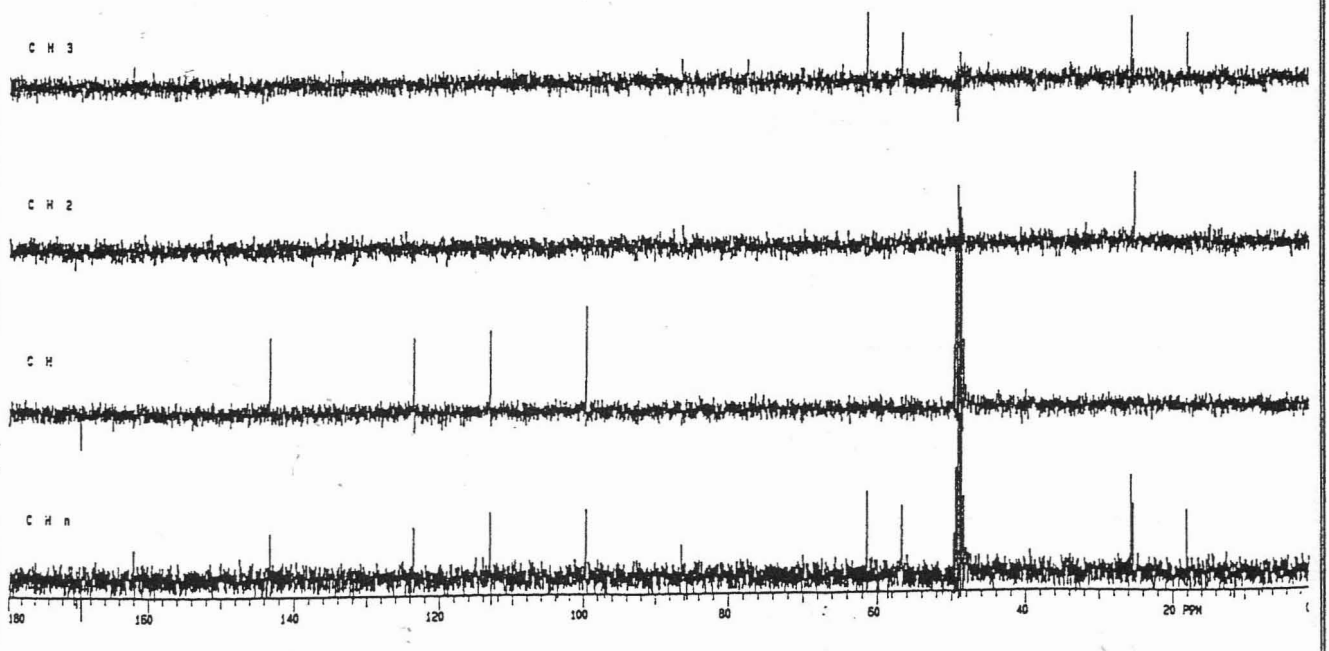
Spectrum 46: UV spectrum of Compound VII



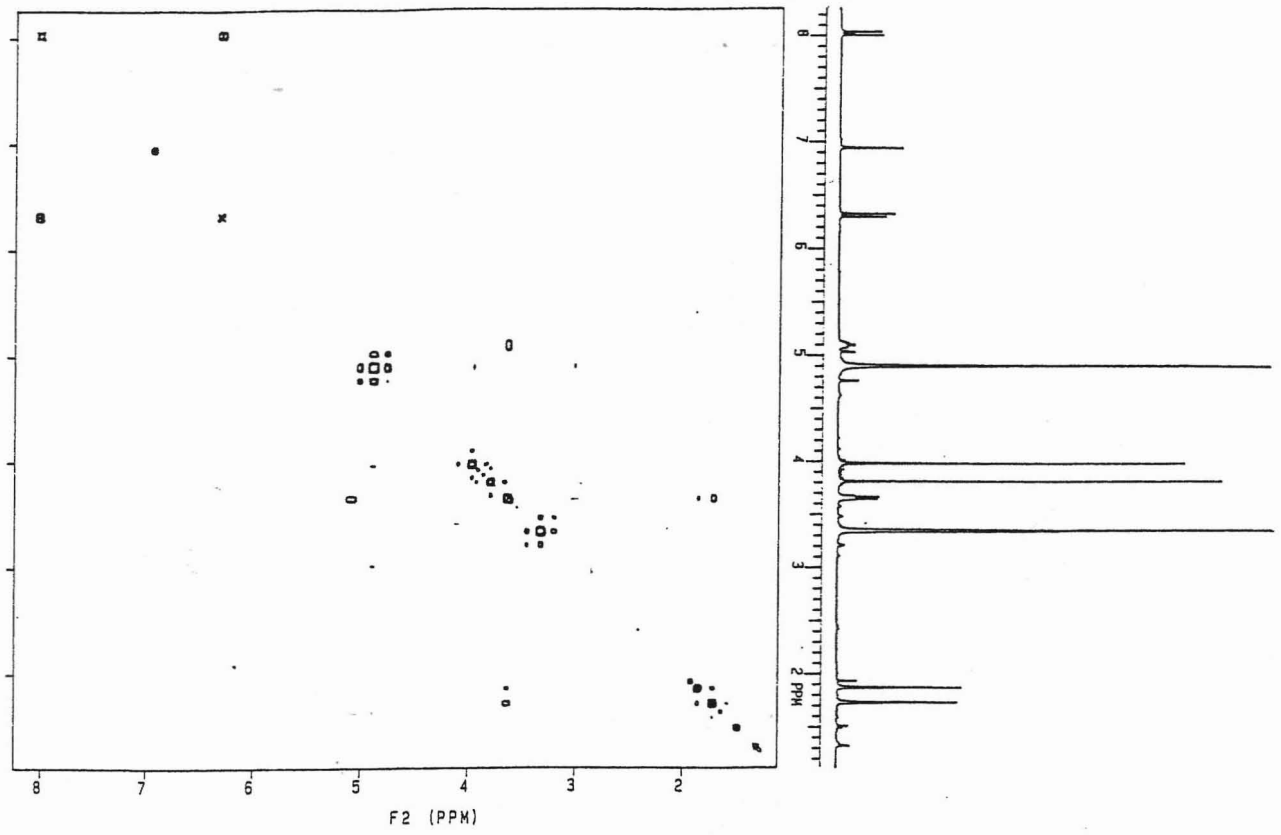
Spectrum 47: ^{1}H NMR spectrum of Compound VII



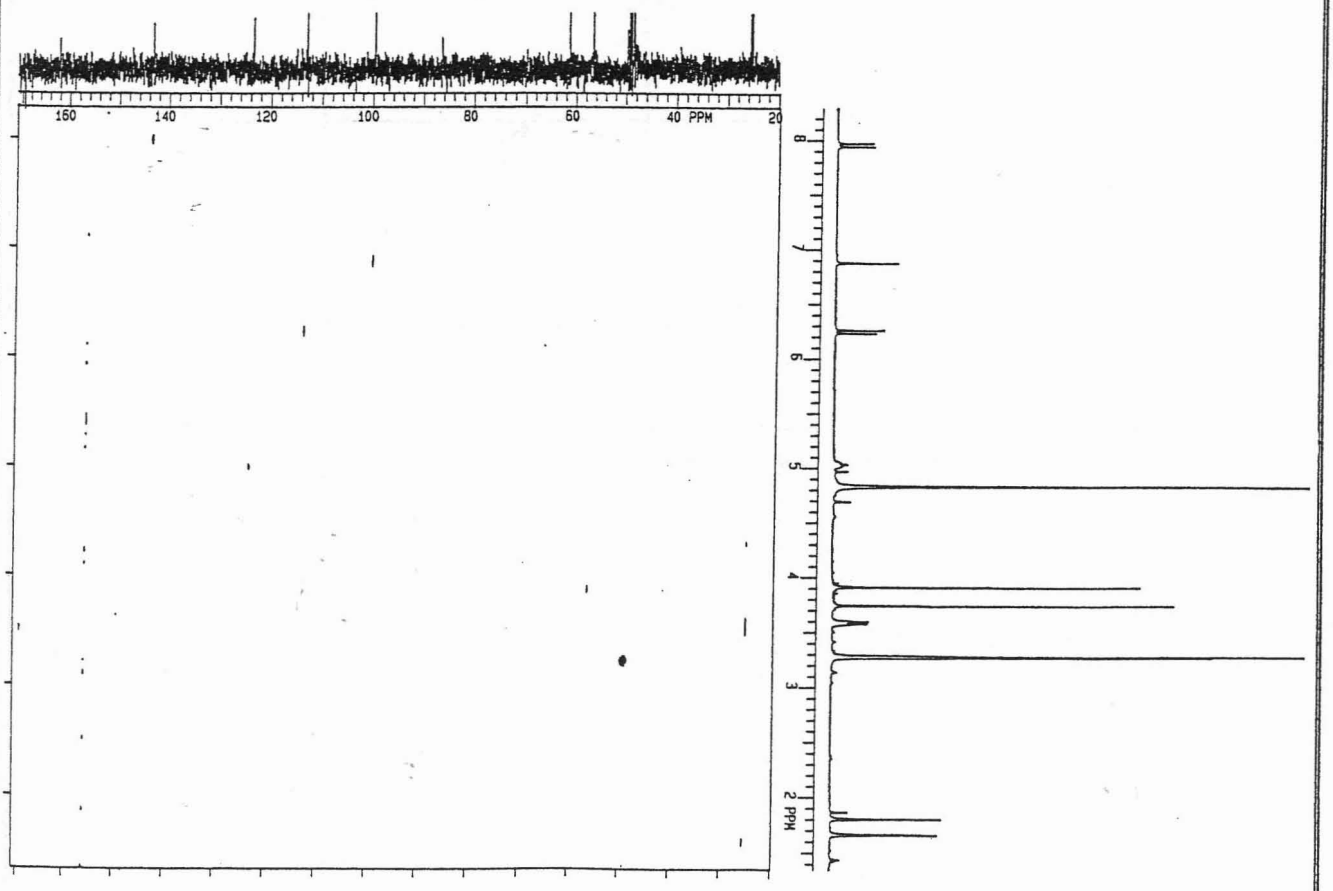
Spectrum 48: ^{13}C NMR spectrum of Compound VII



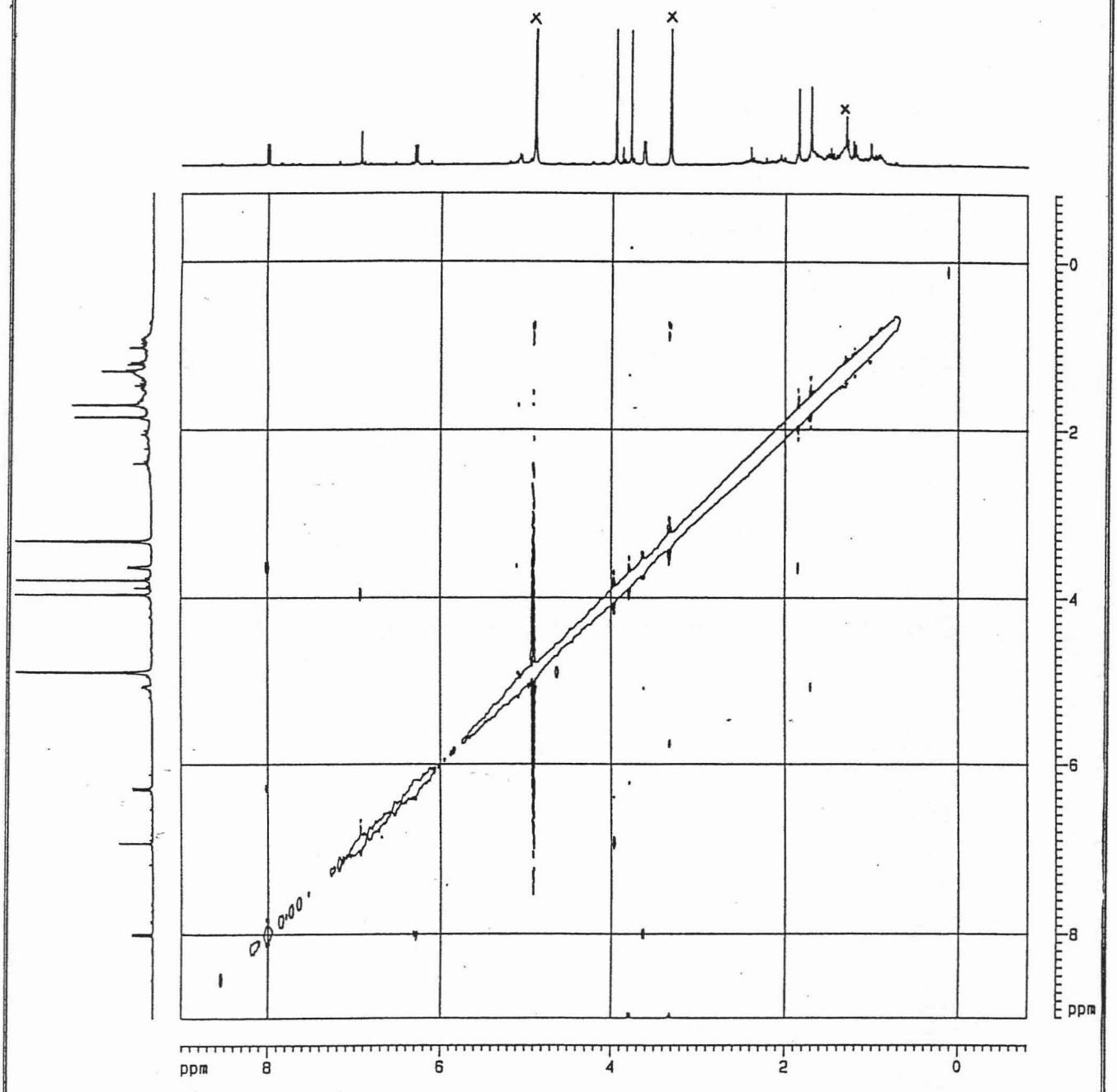
Spectrum 48a: ADEPT NMR spectrum of Compound VII



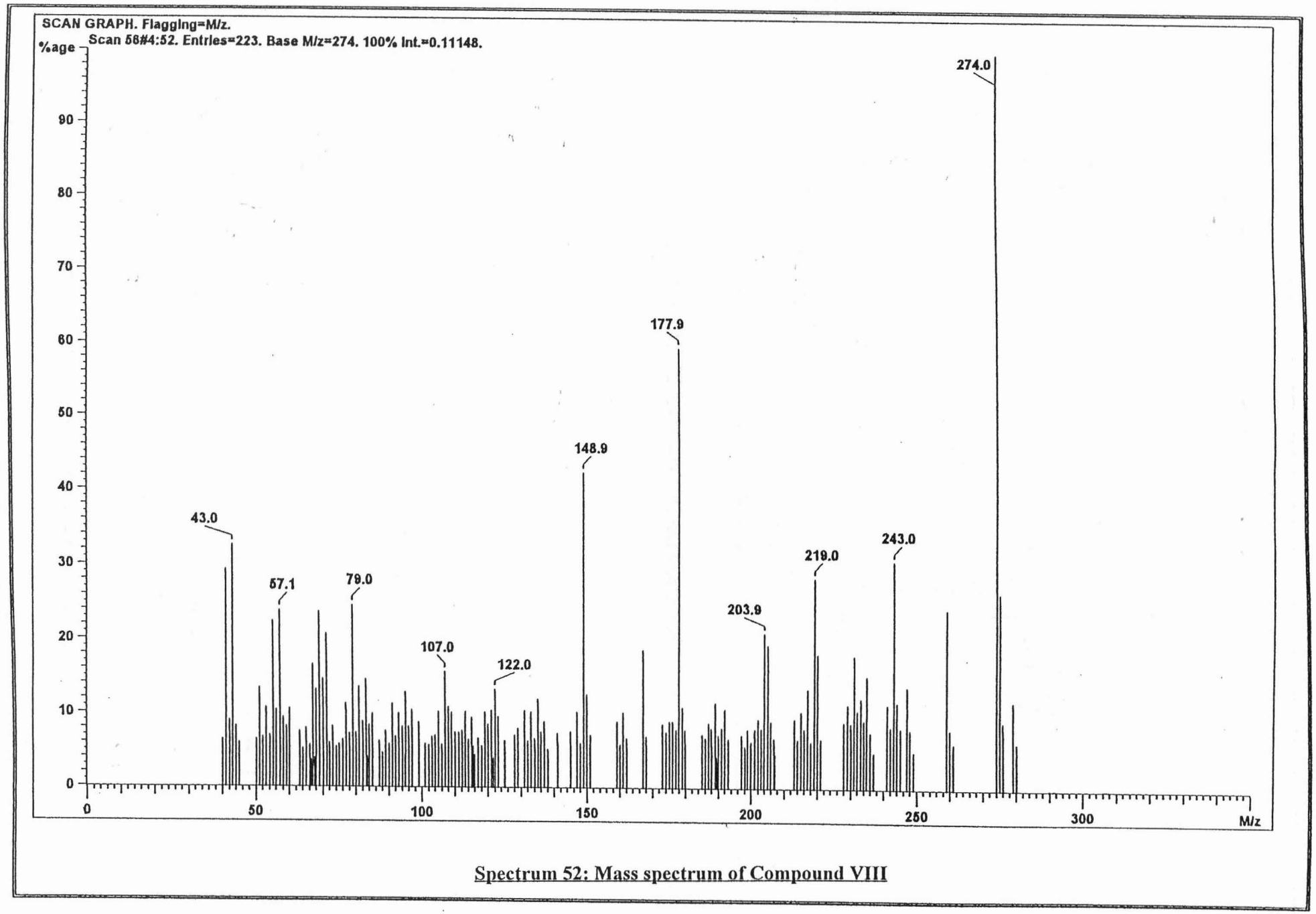
Spectrum 49: COSY NMR spectrum of Compound VII



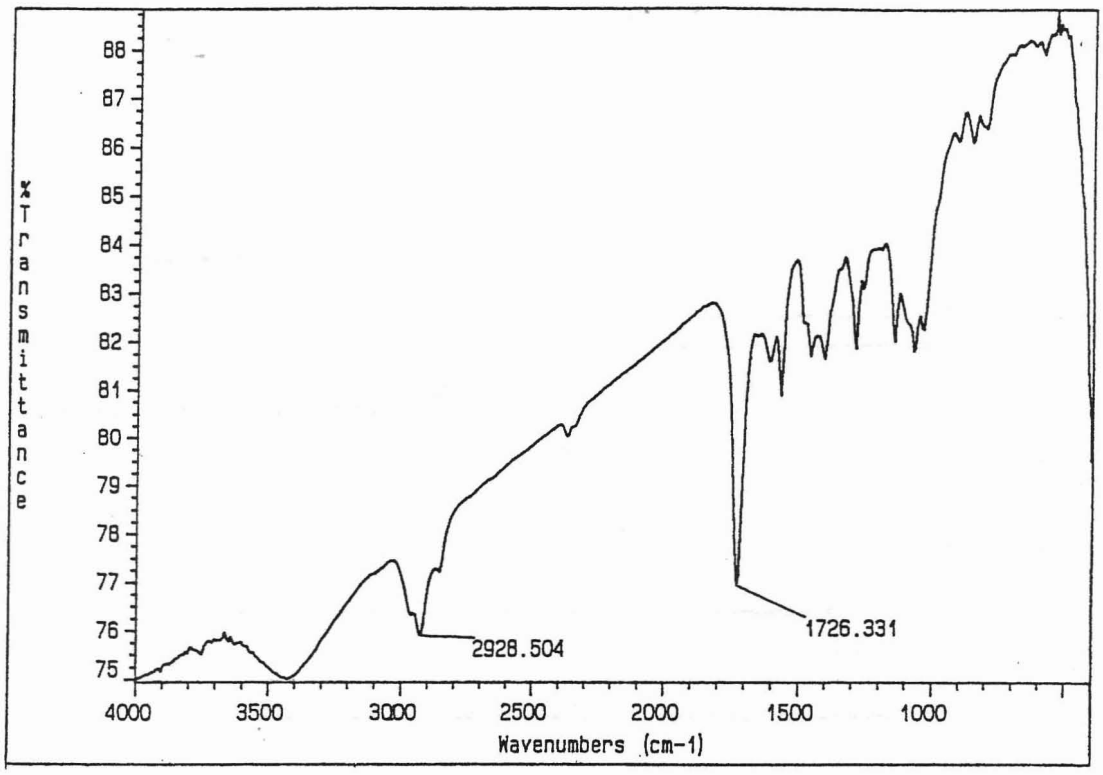
Spectrum 50: HETCOR NMR spectrum of Compound VII



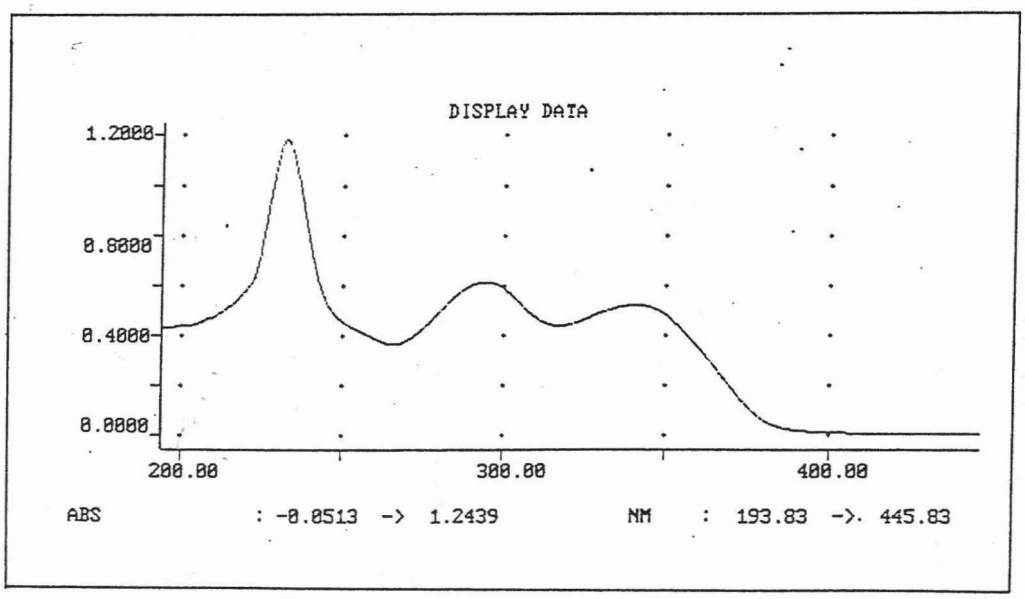
Spectrum 51: NOESY NMR spectrum of Compound VII



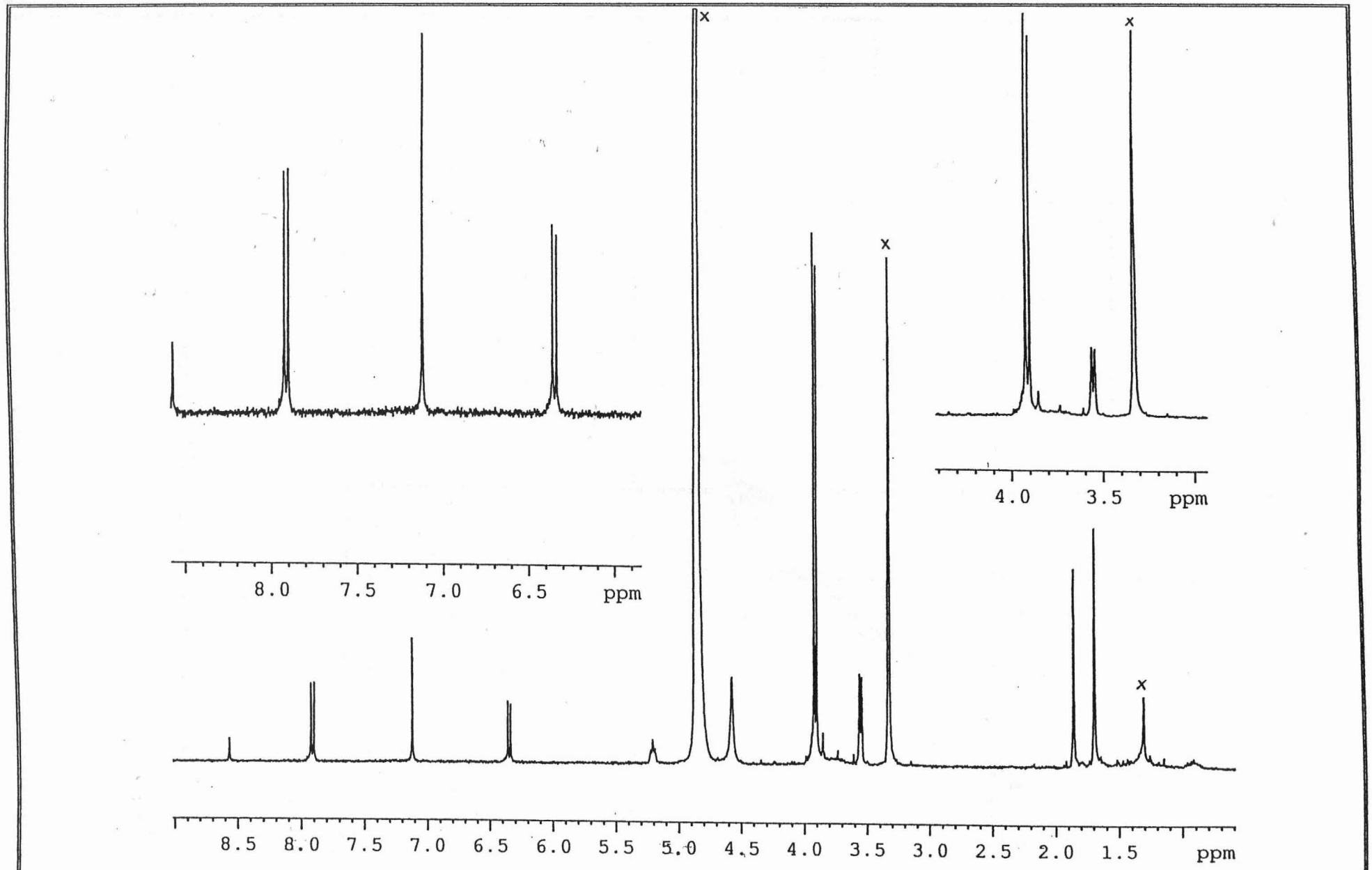
Spectrum 52: Mass spectrum of Compound VIII



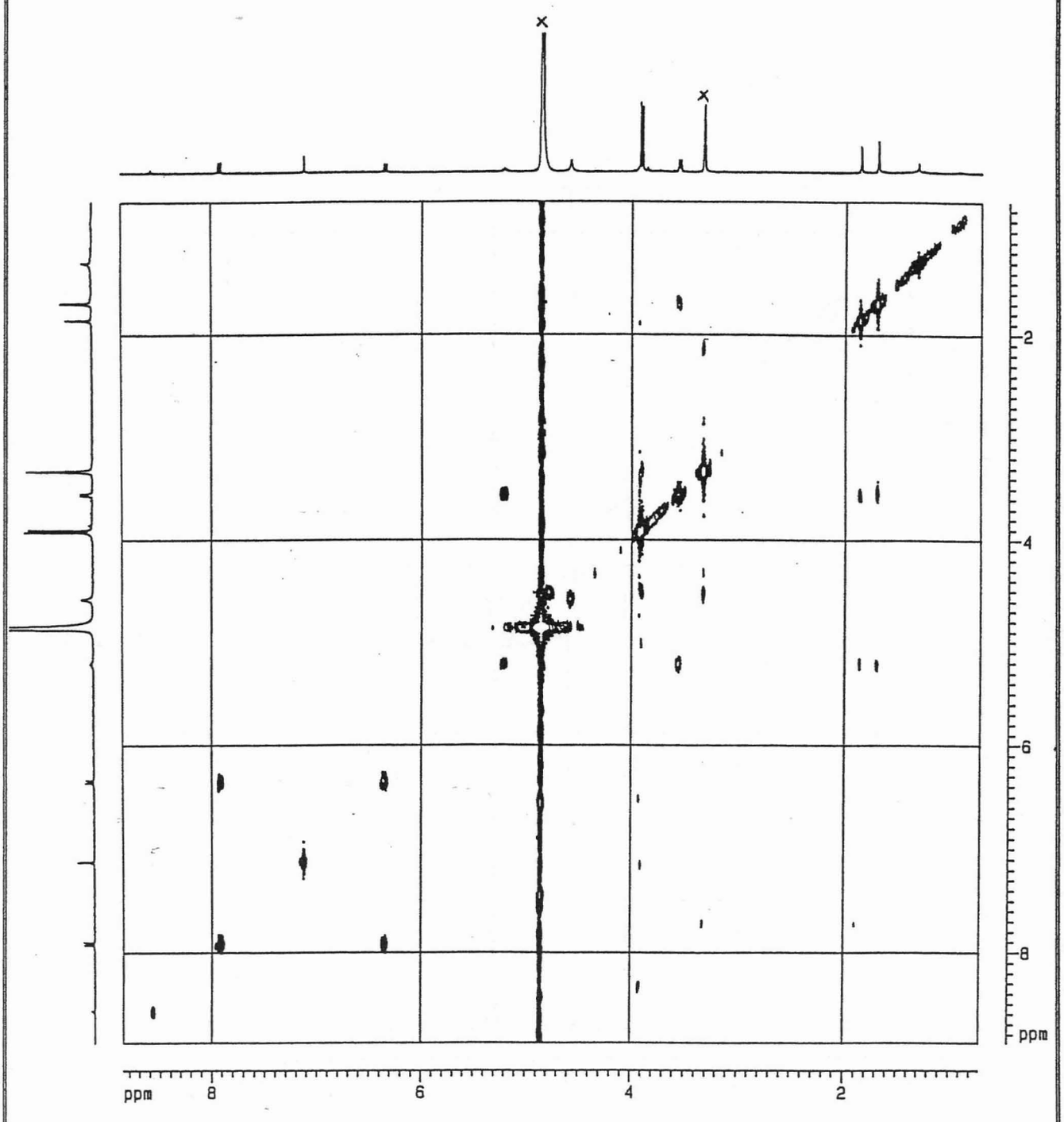
Spectrum 53: IR spectrum of Compound VIII



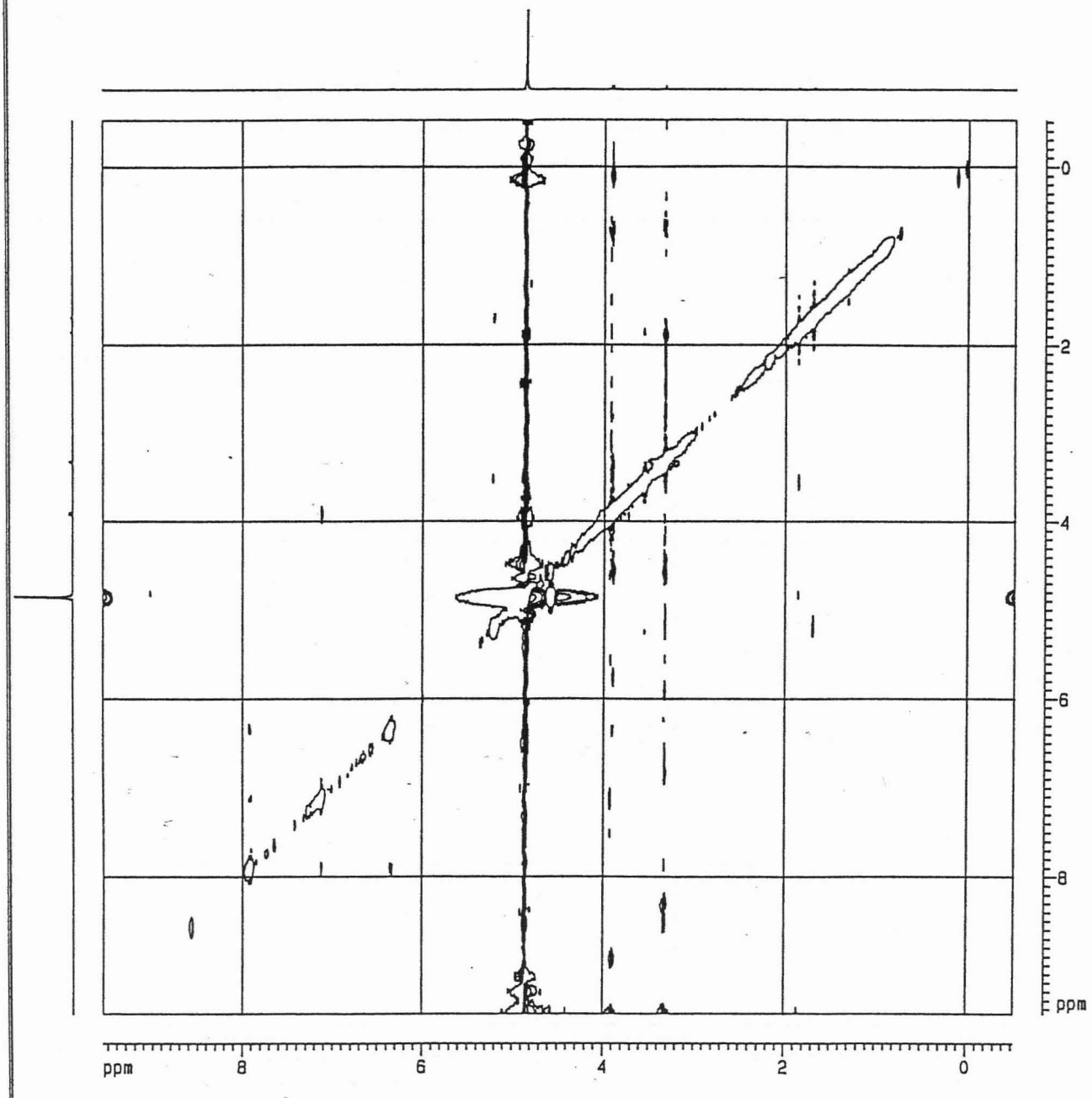
Spectrum 54: UV spectrum of Compound VIII



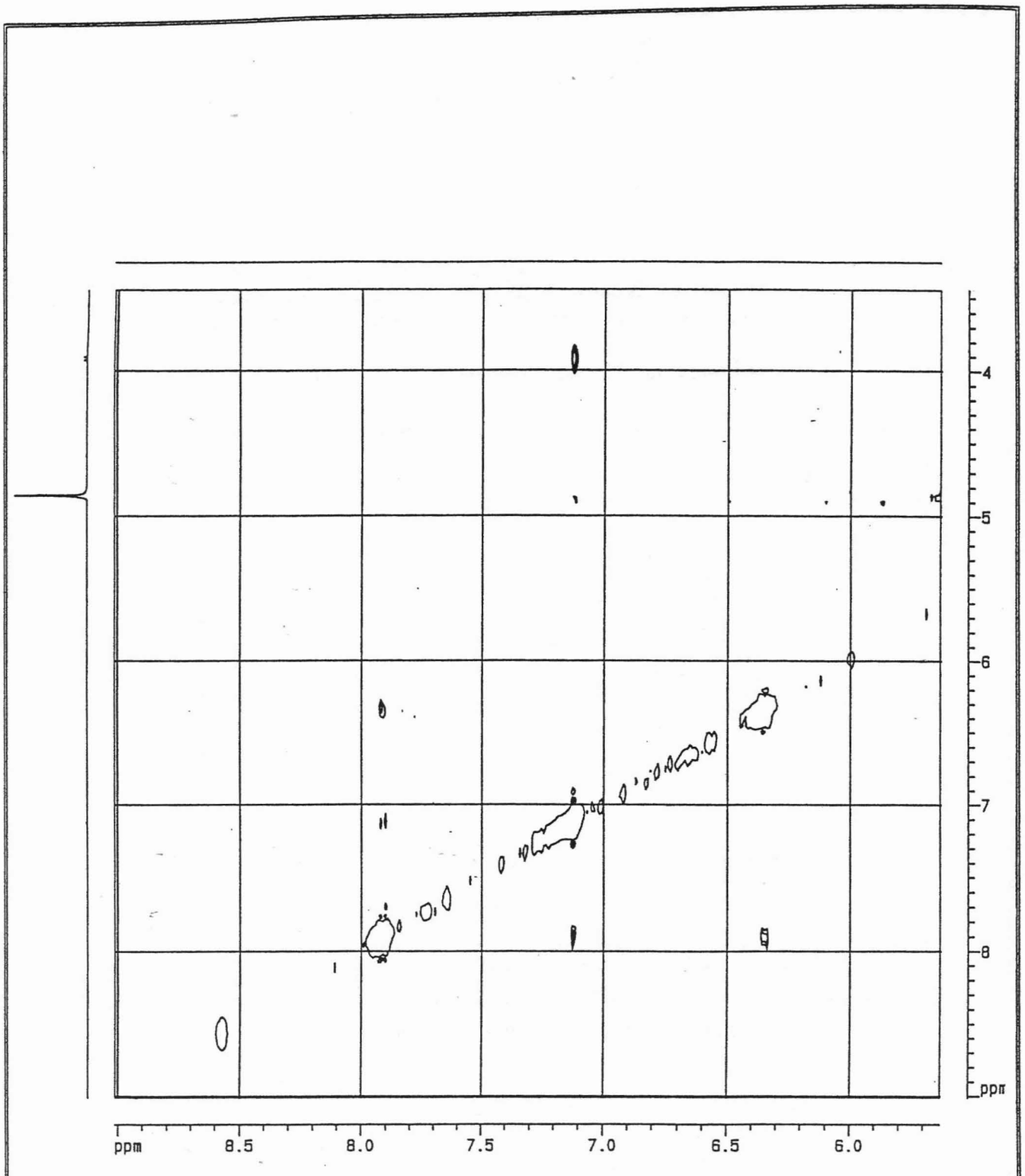
Spectrum 55: ^{1}H NMR spectrum of Compound VIII



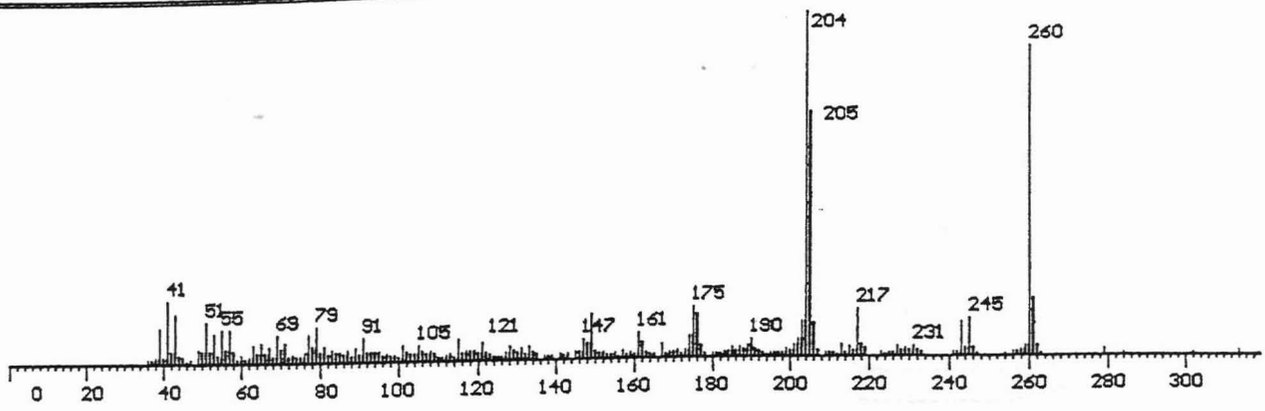
Spectrum 56: COSY NMR spectrum of Compound VIII



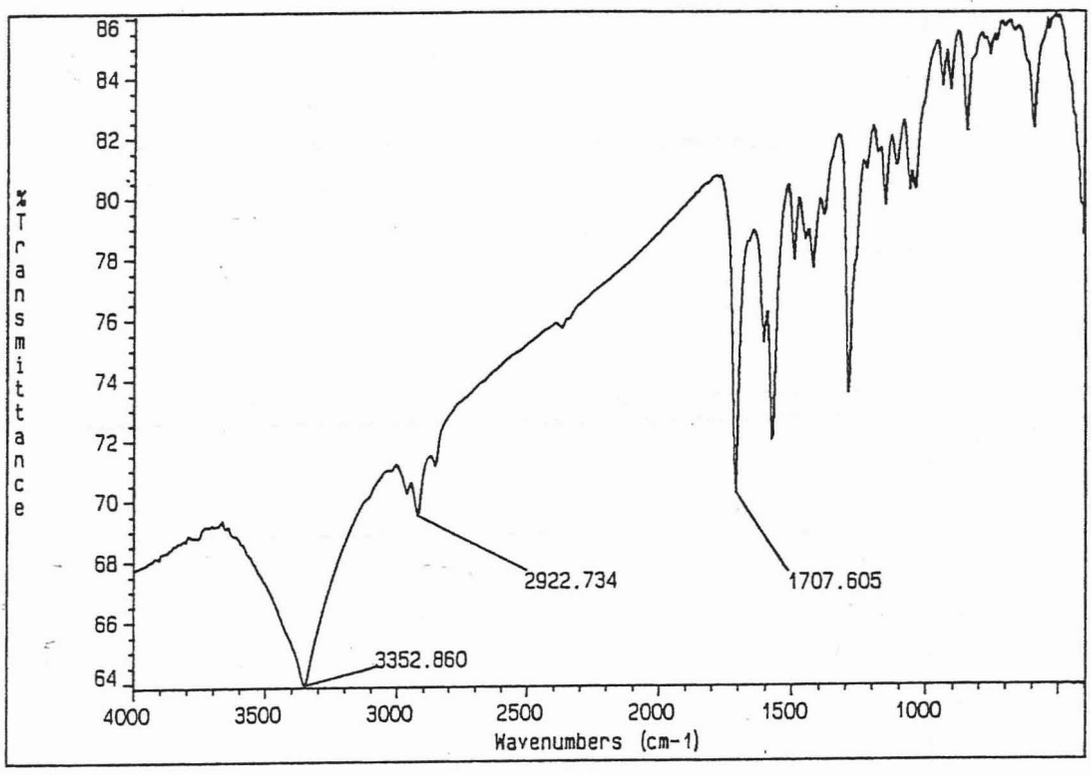
Spectrum 57a: NOESY NMR spectrum of Compound VIII



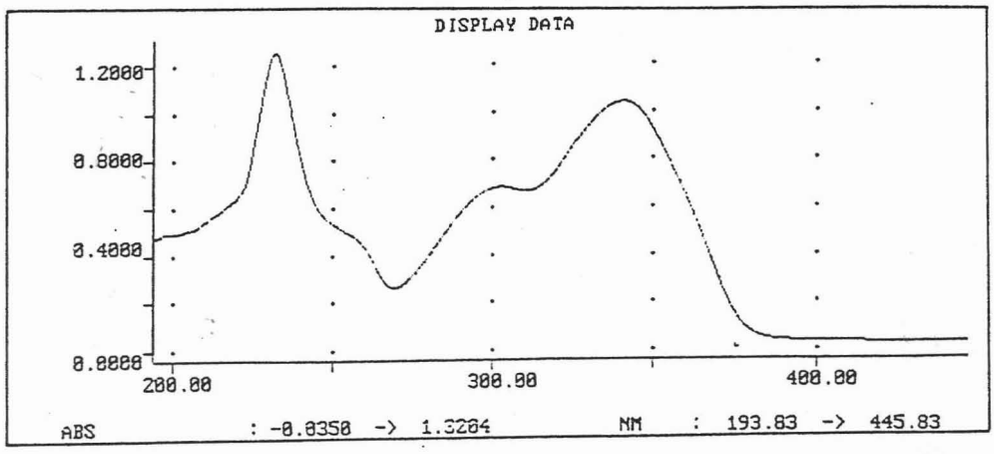
Spectrum 57b: NOESY NMR spectrum of Compound VIII



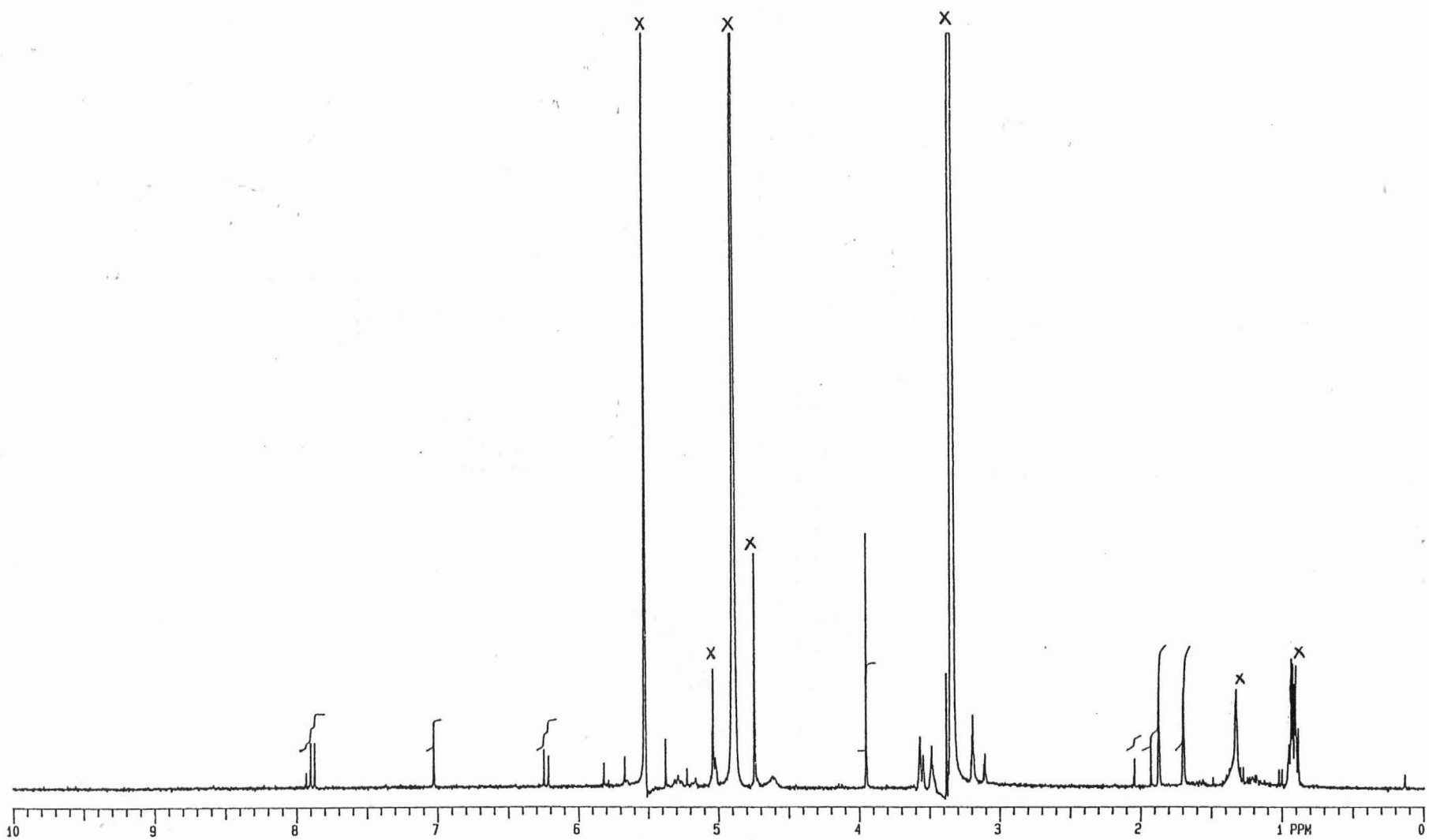
Spectrum 58: Mass spectrum of Compound IX



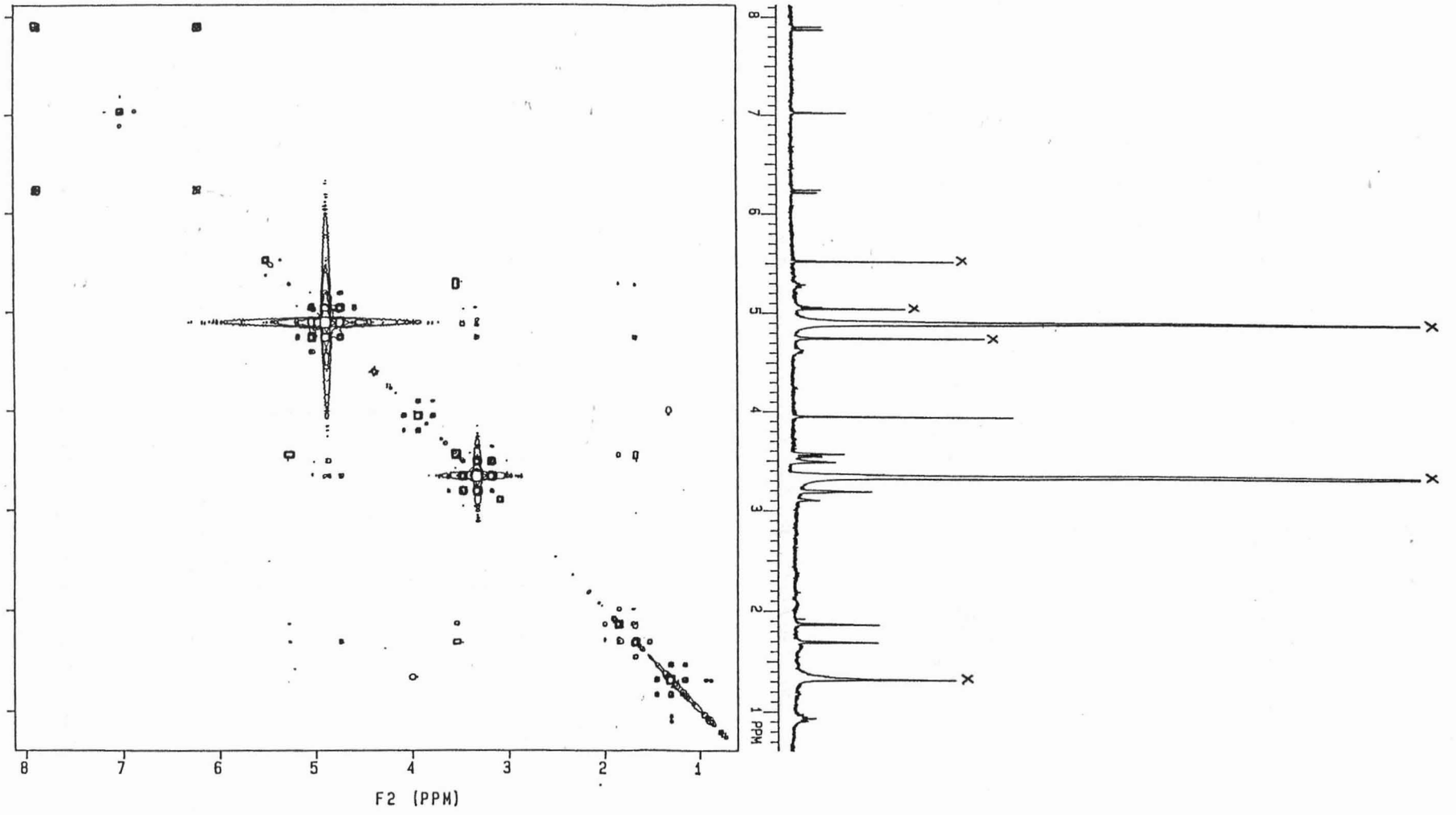
Spectrum 59: IR spectrum of Compound IX



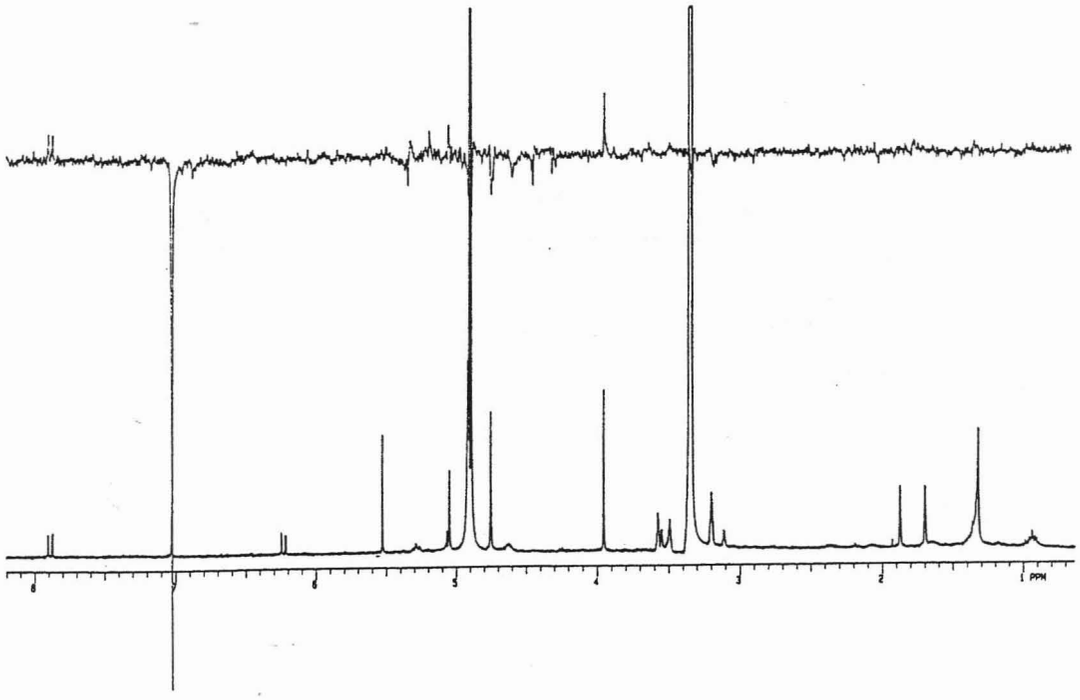
Spectrum 60: UV spectrum of Compound IX



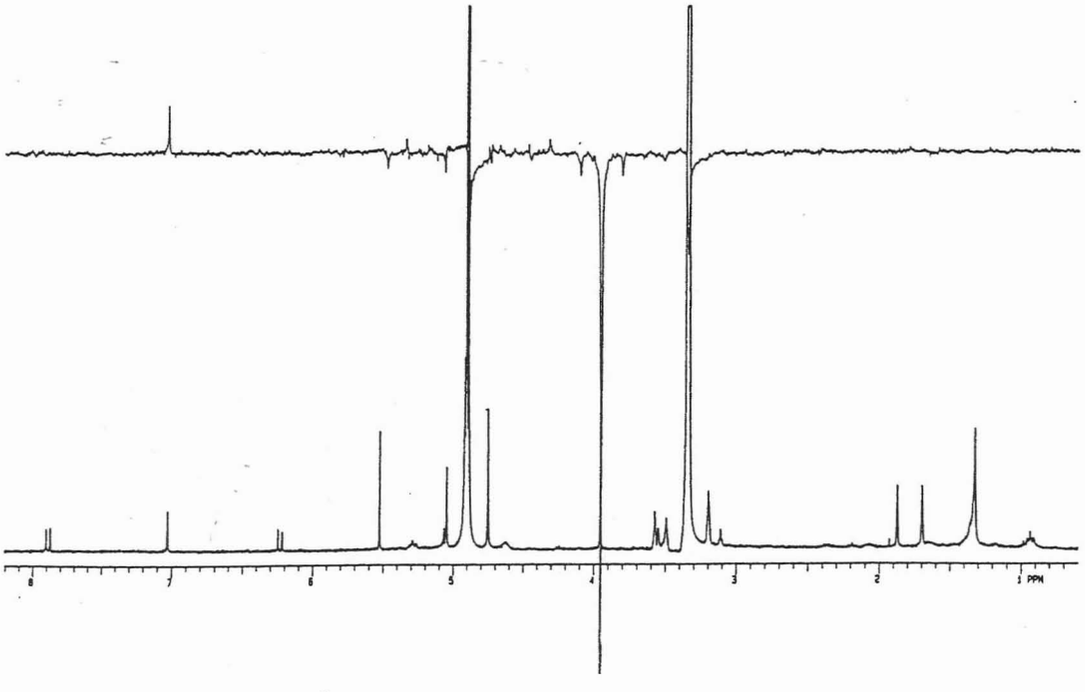
Spectrum 61: ^{1}H NMR spectrum of Compound IX



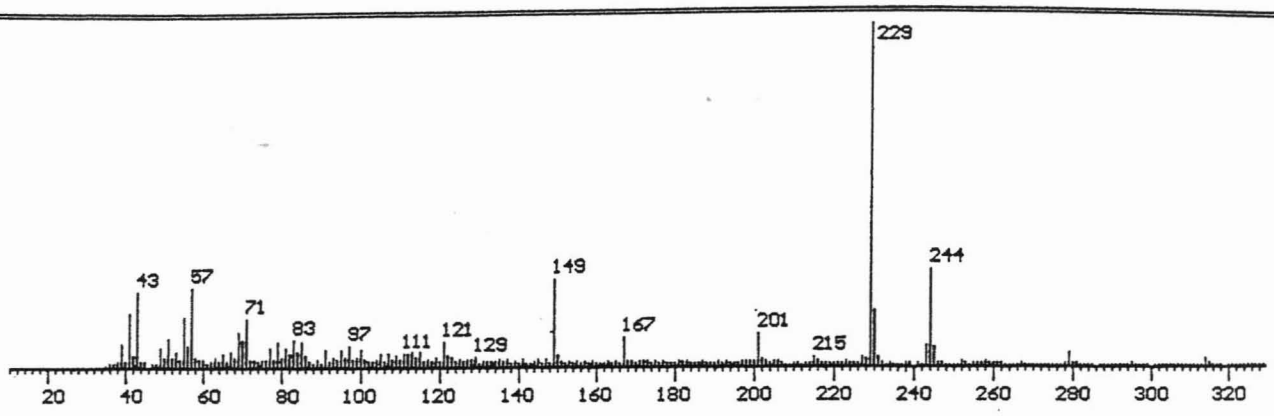
Spectrum 62: COSY NMR spectrum of Compound IX



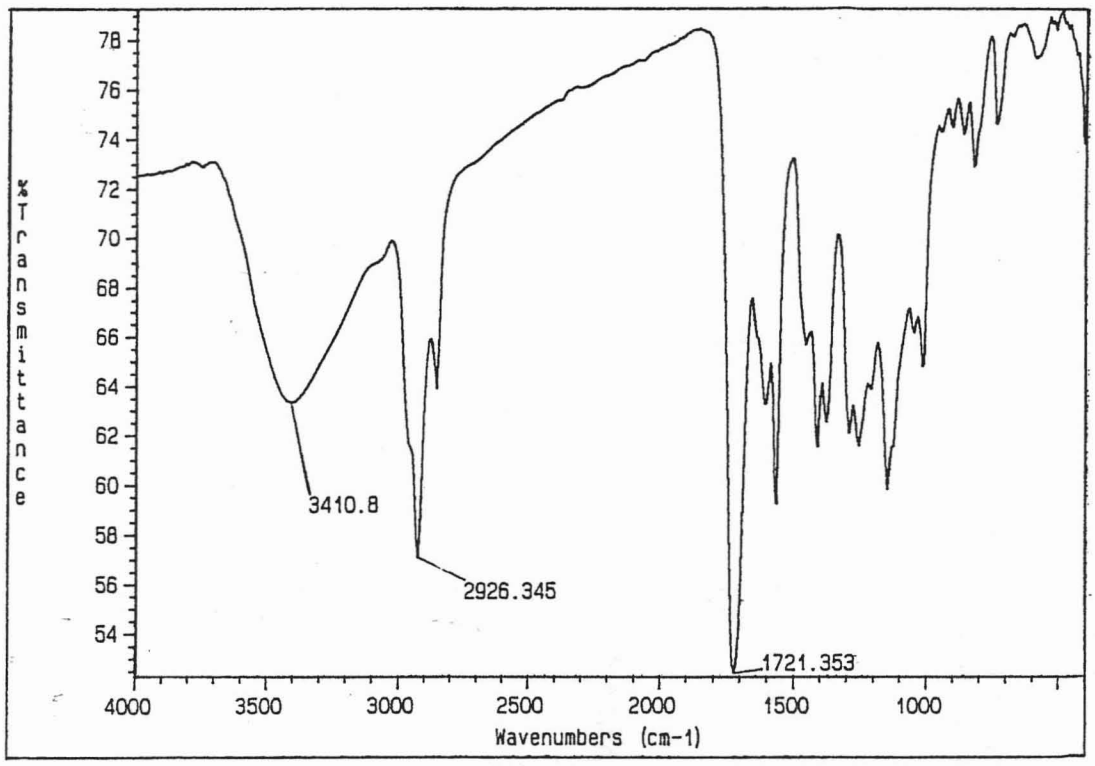
Spectrum 63a: NOESY NMR spectrum of Compound IX



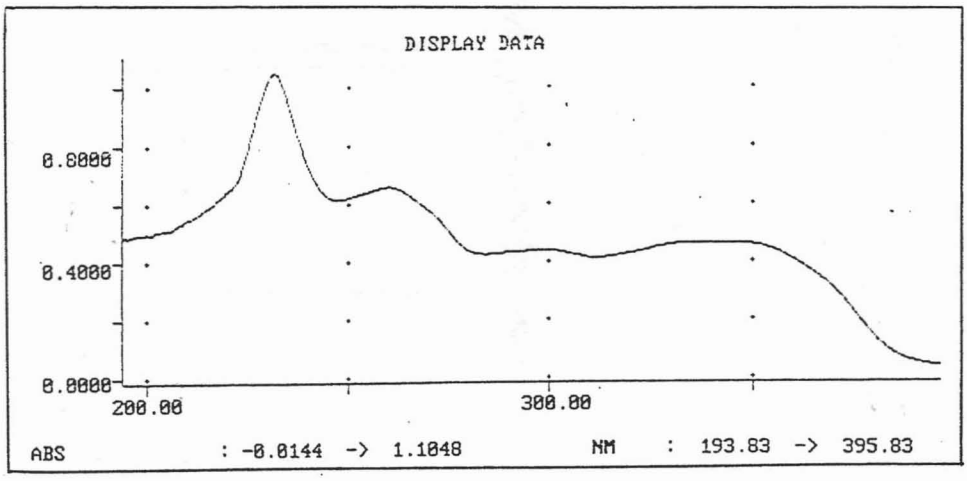
Spectrum 63b: NOESY NMR spectrum of Compound IX



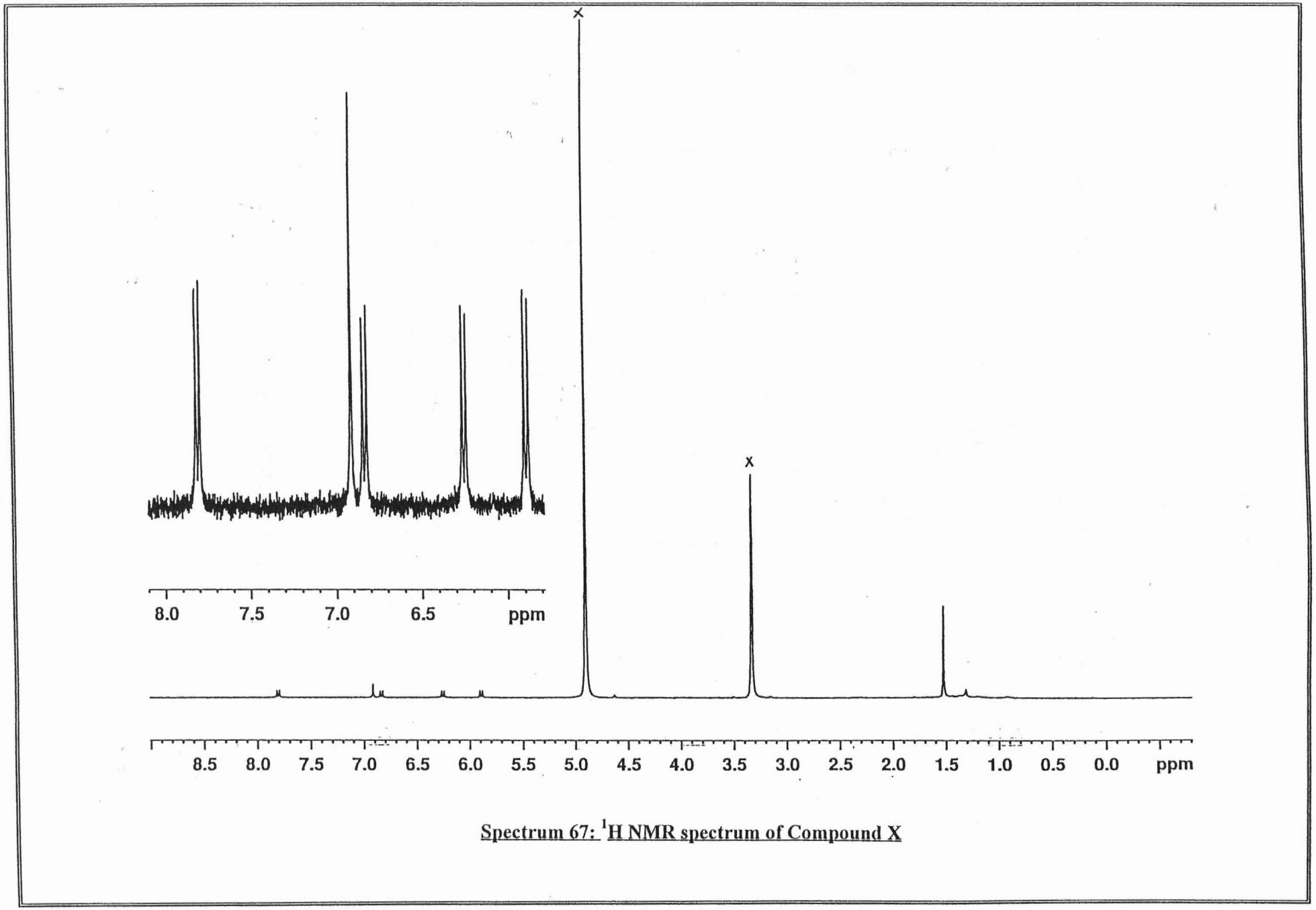
Spectrum 64: Mass spectrum of Compound X



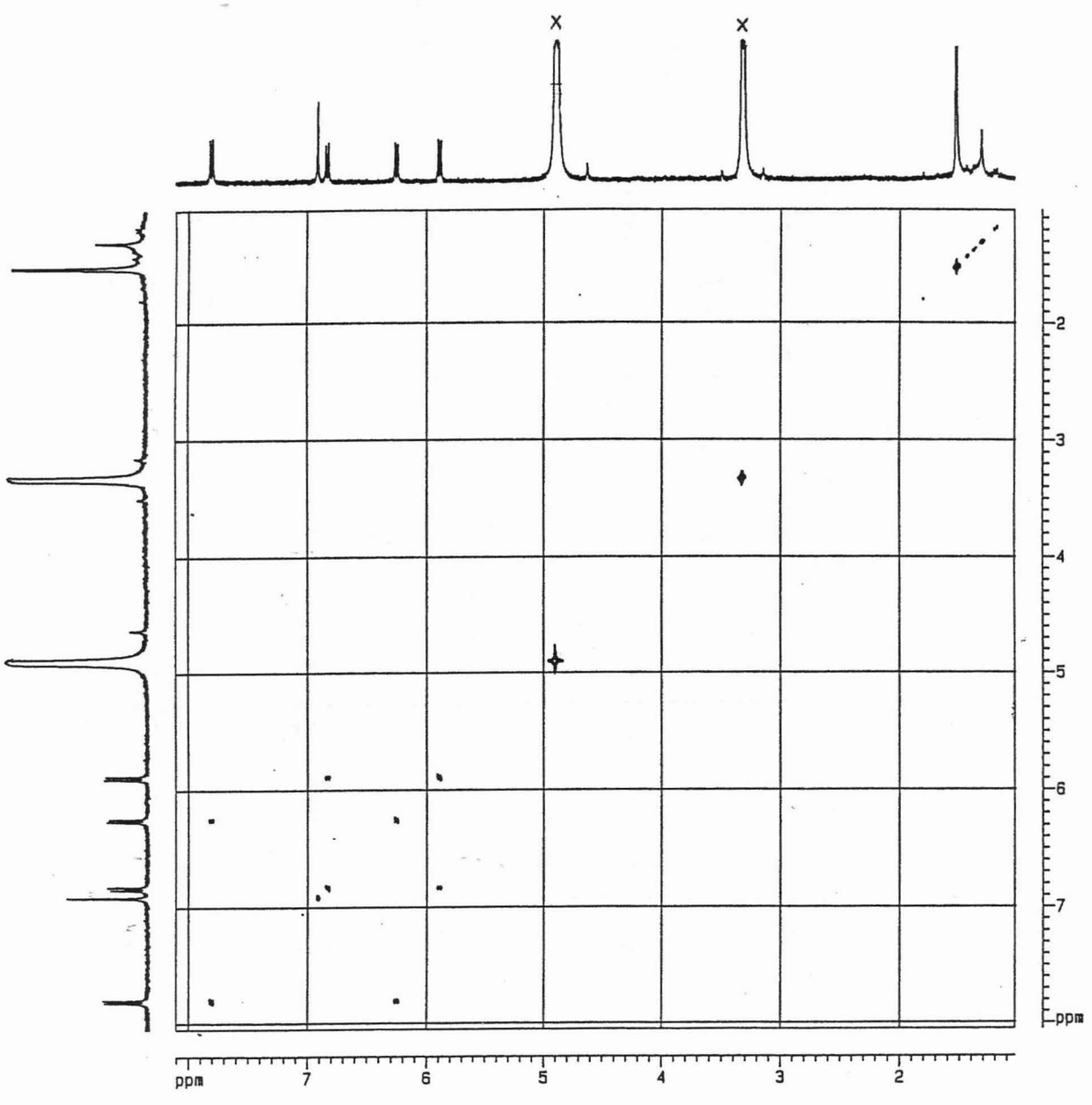
Spectrum 65: IR spectrum of Compound X



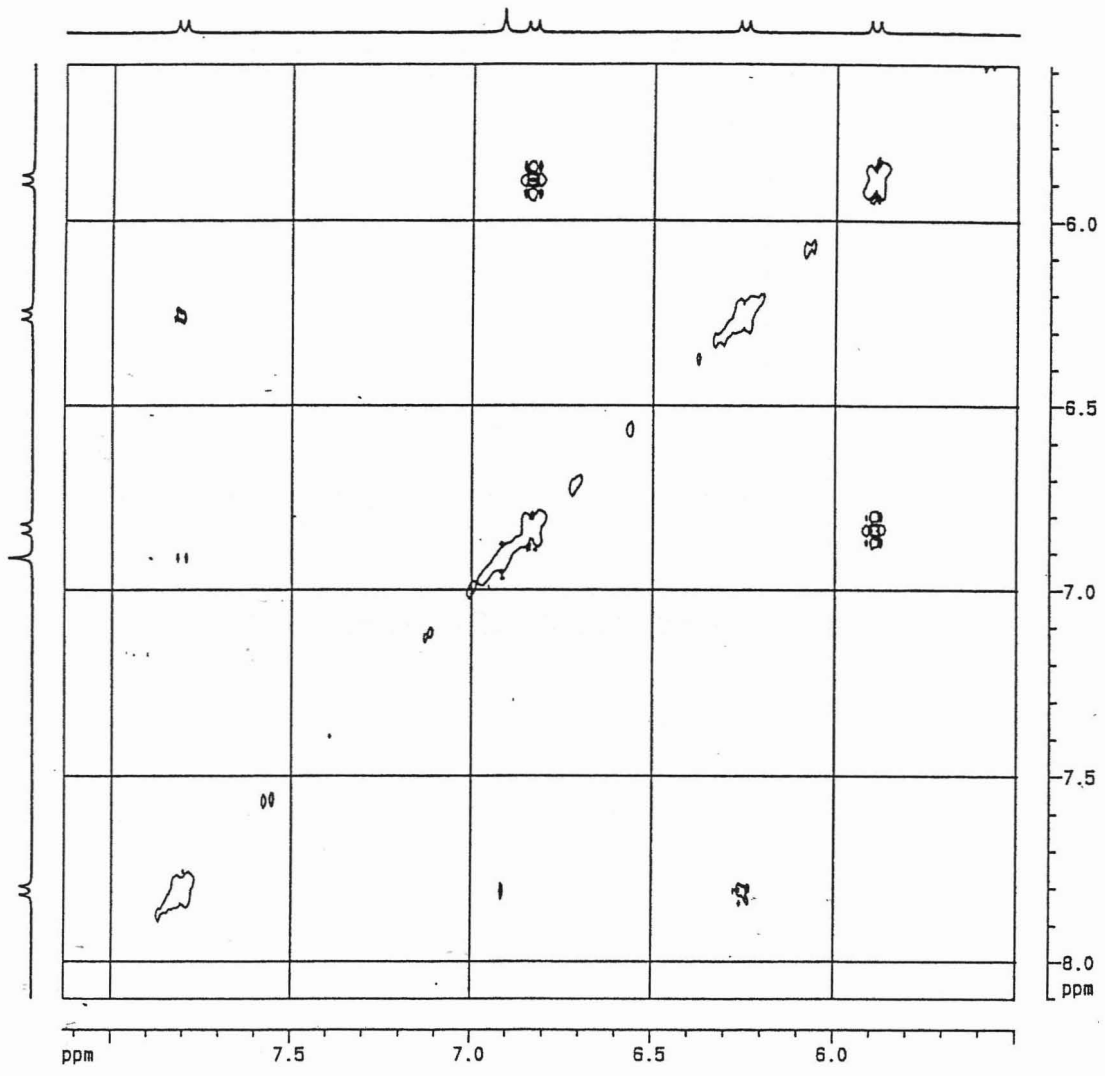
Spectrum 66: UV spectrum of Compound X



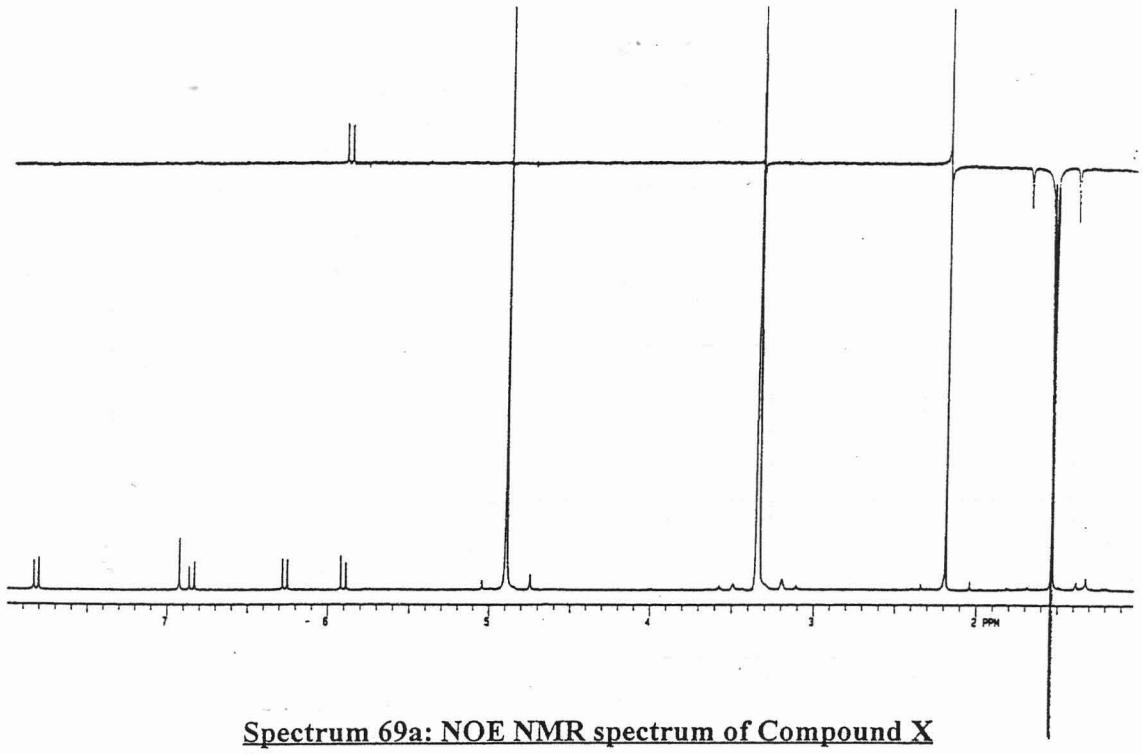
Spectrum 67: ^{1}H NMR spectrum of Compound X



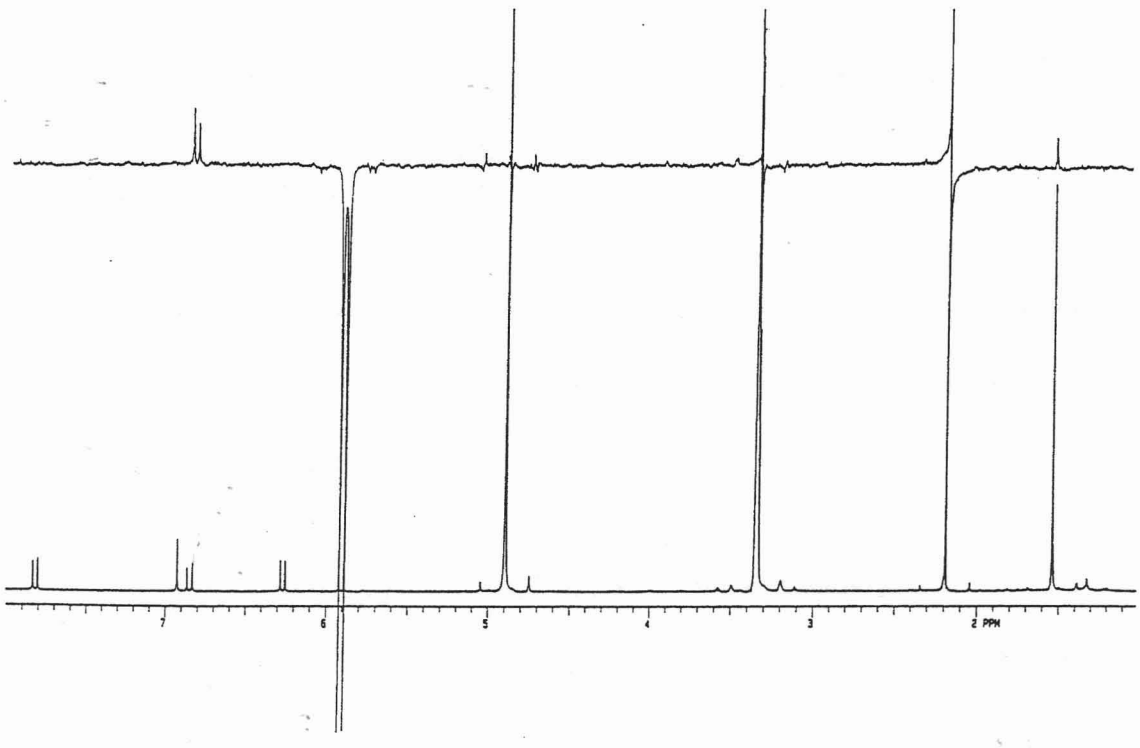
Spectrum 68: COSY NMR spectrum of Compound X



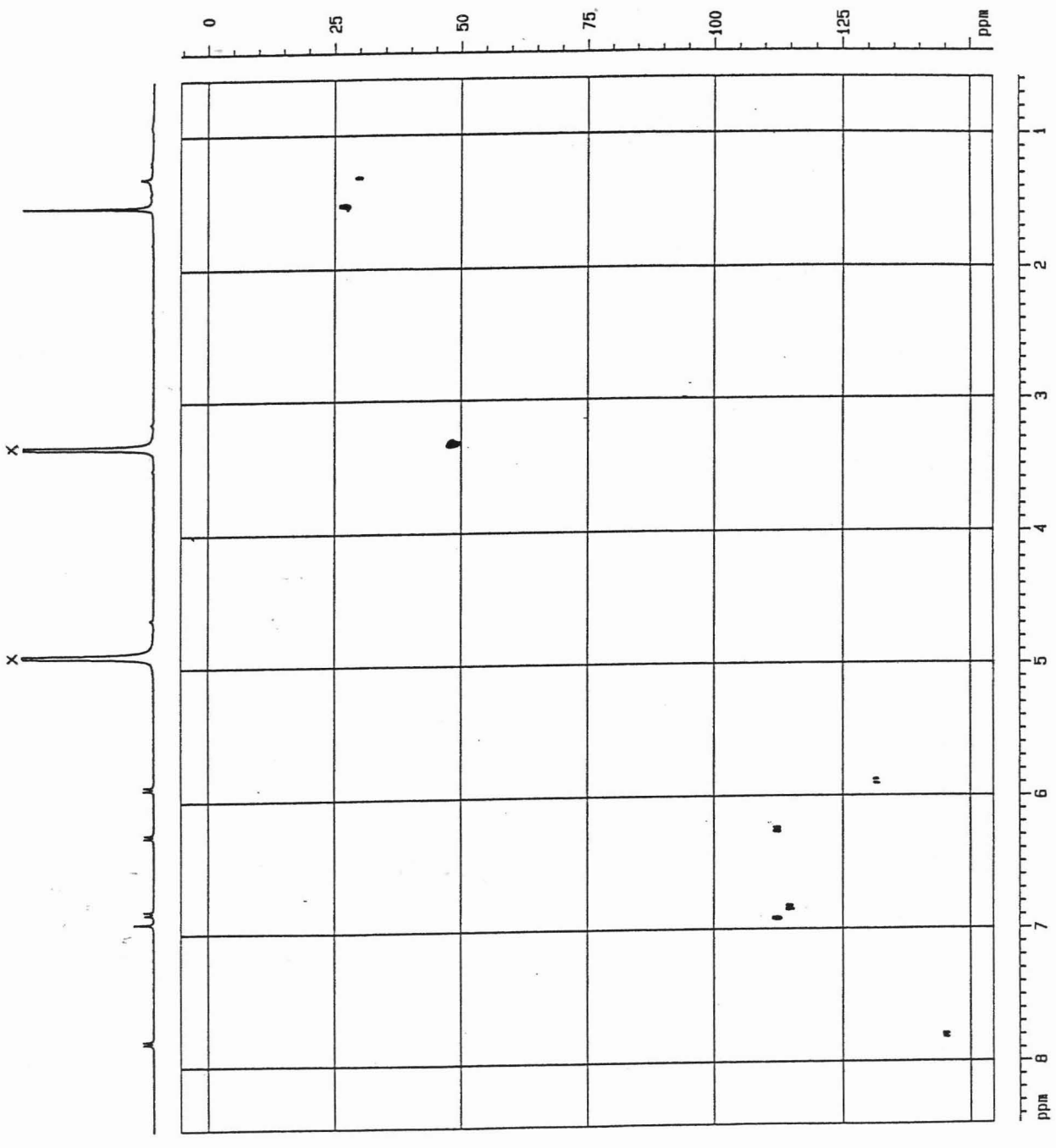
Spectrum 69: NOESY NMR spectrum of Compound X



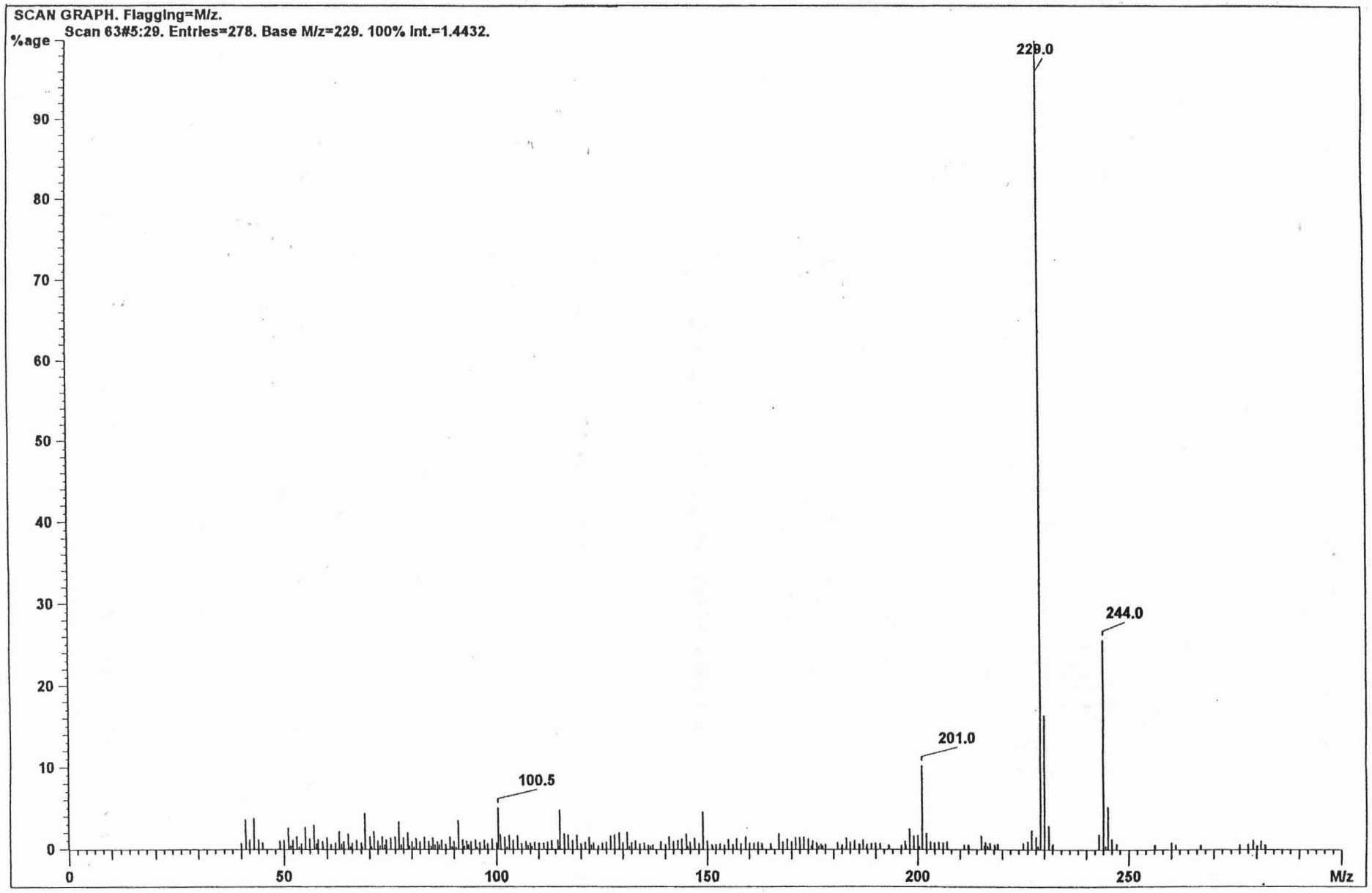
Spectrum 69a: NOE NMR spectrum of Compound X



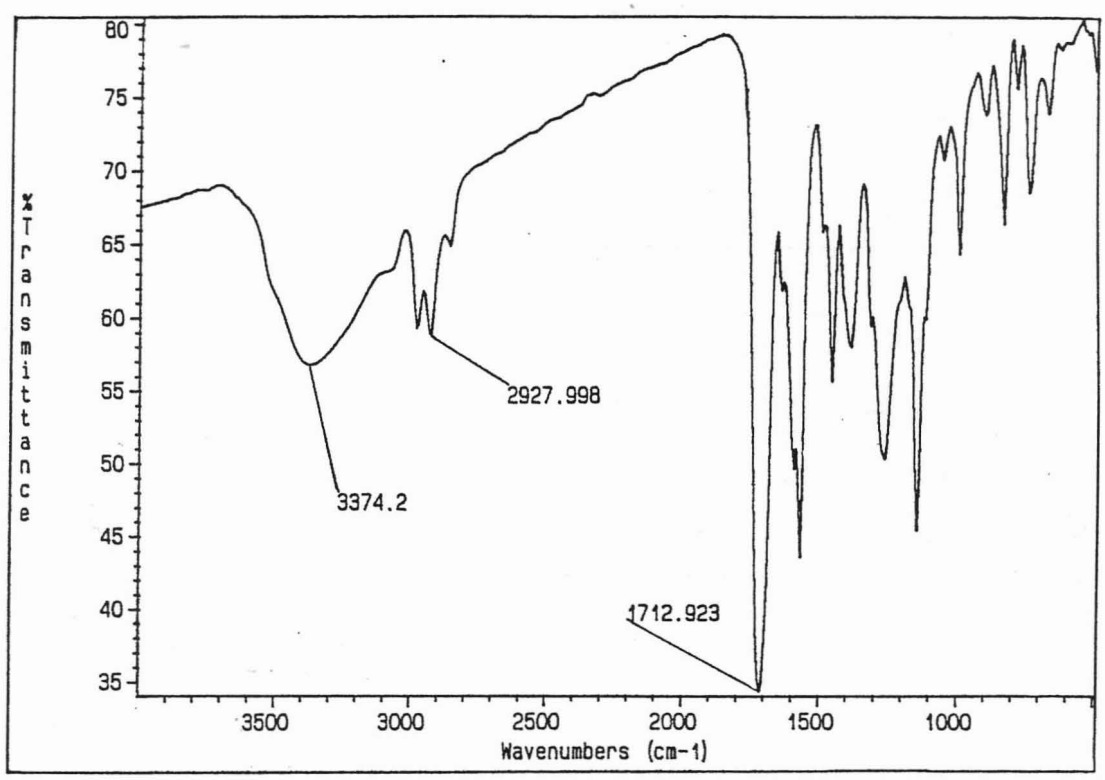
Spectrum 69b: NOE NMR spectrum of Compound X



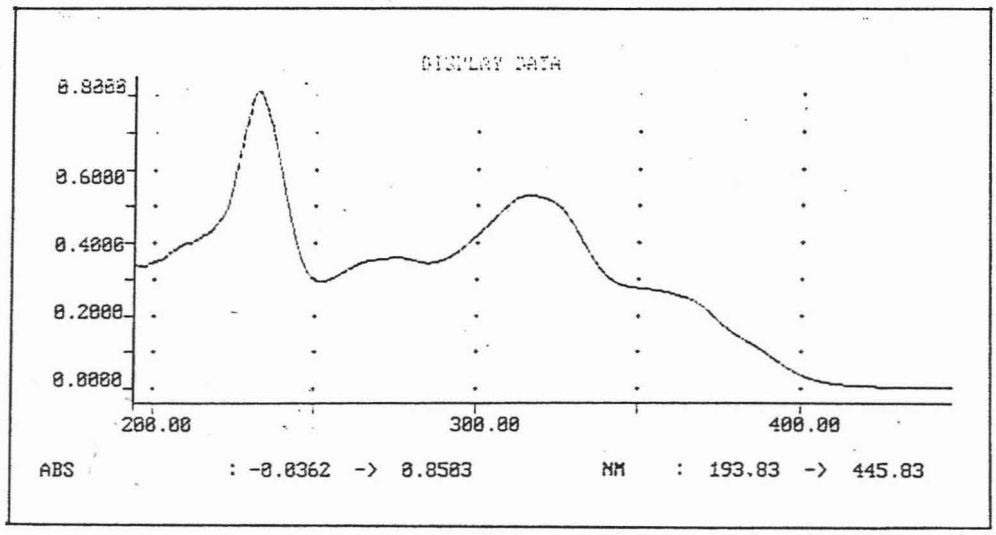
Spectrum 70: HMQC NMR spectrum of Compound X



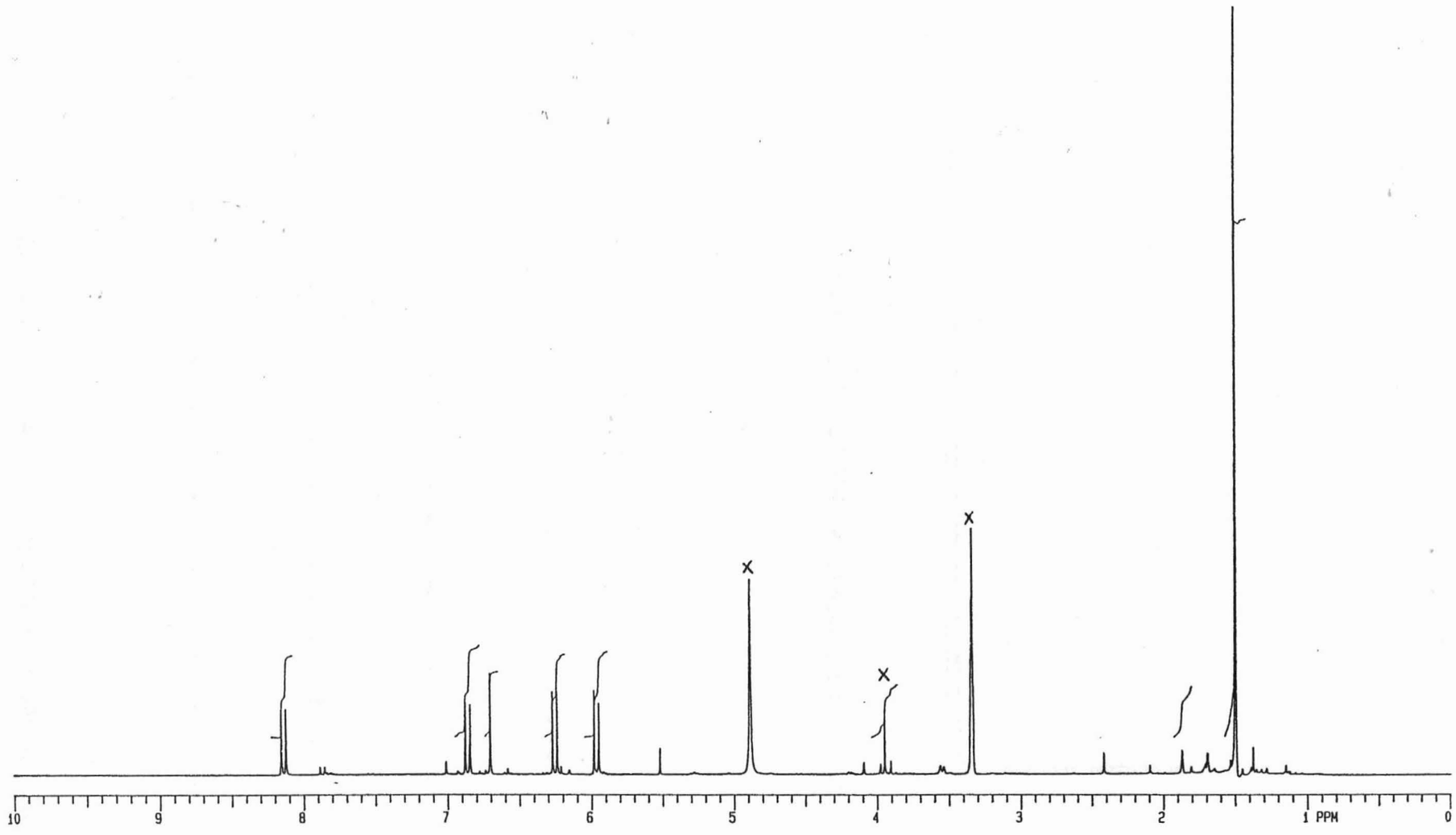
Spectrum 71: Mass spectrum of Compound XI



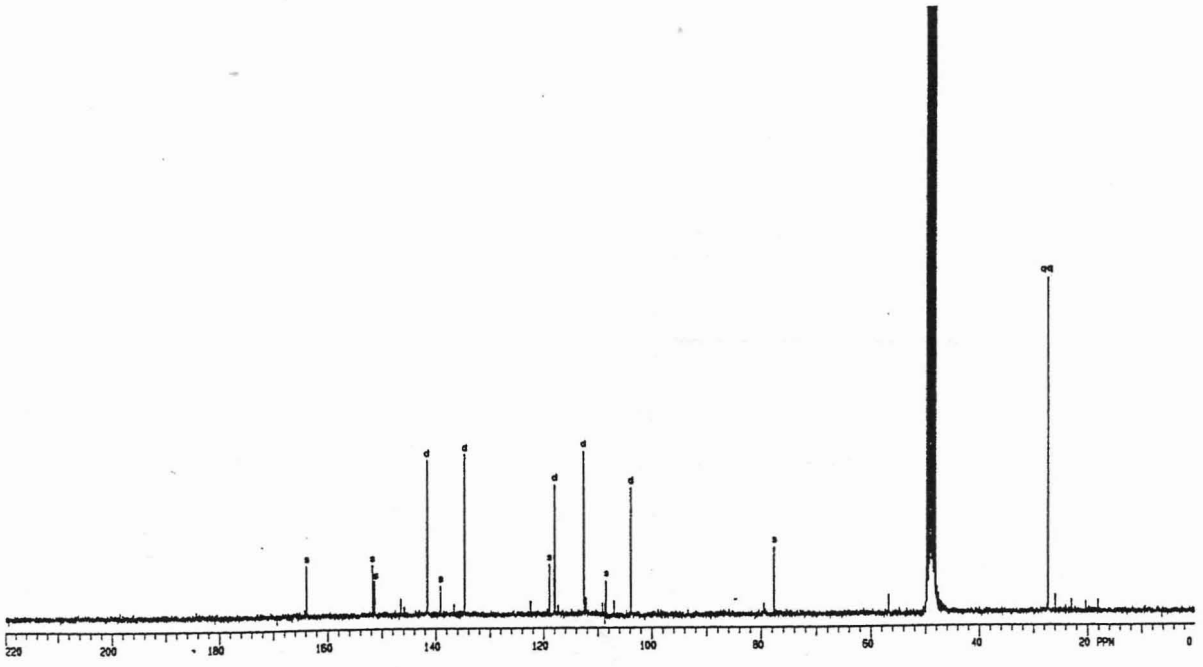
Spectrum 72: IR spectrum of Compound XI



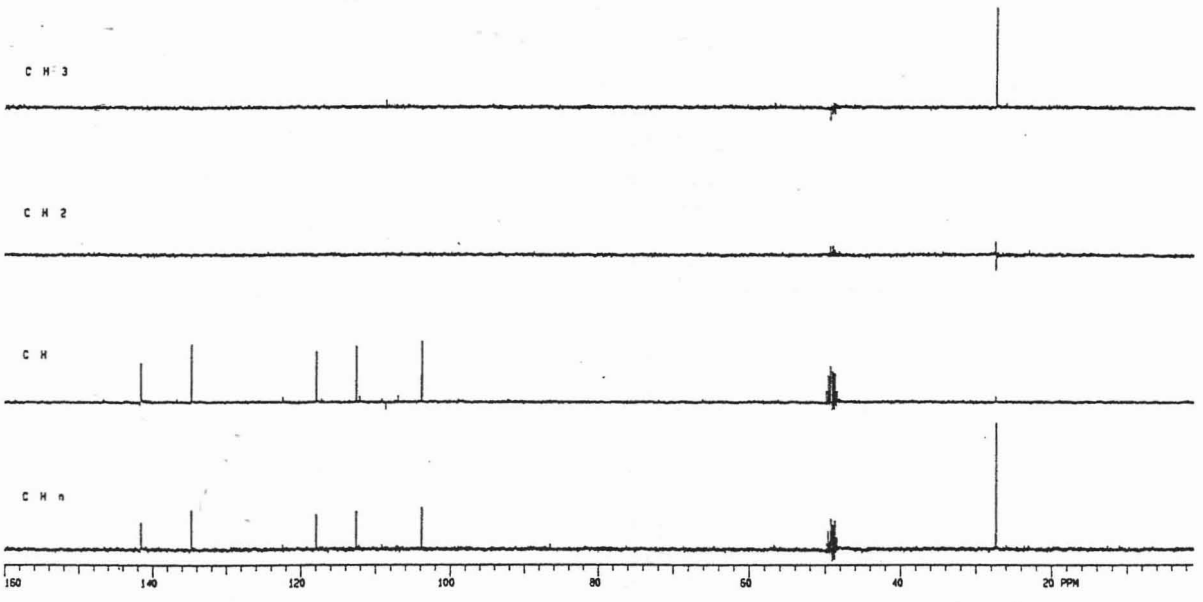
Spectrum 73: UV spectrum of Compound XI



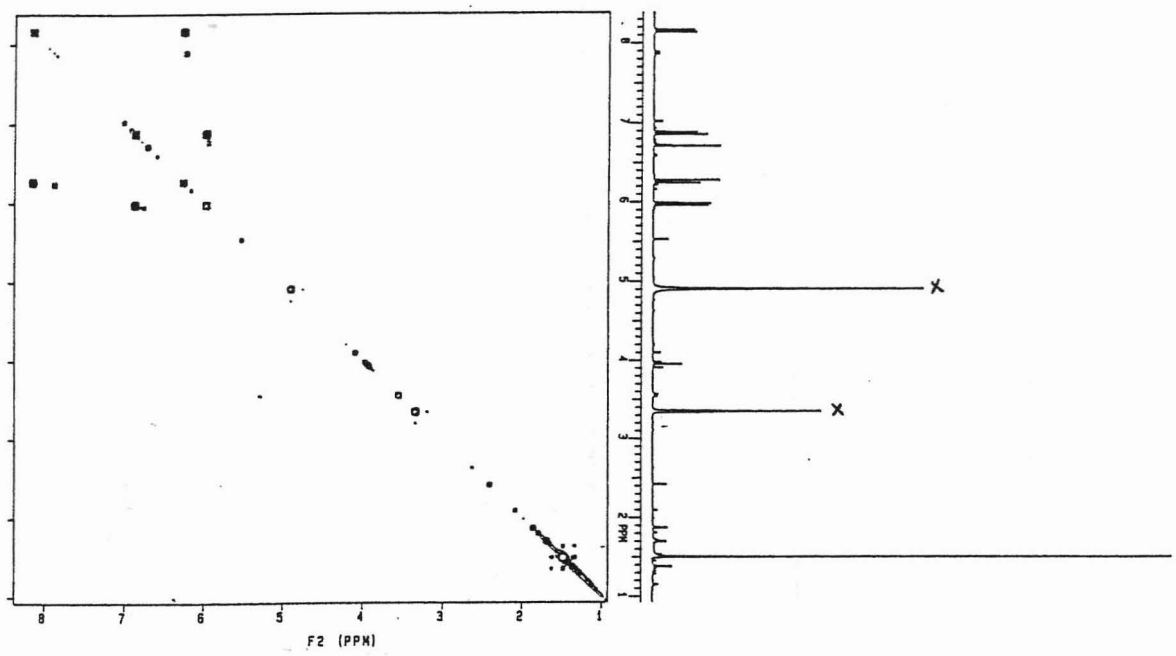
Spectrum 74: ^{1}H NMR spectrum of Compound XI



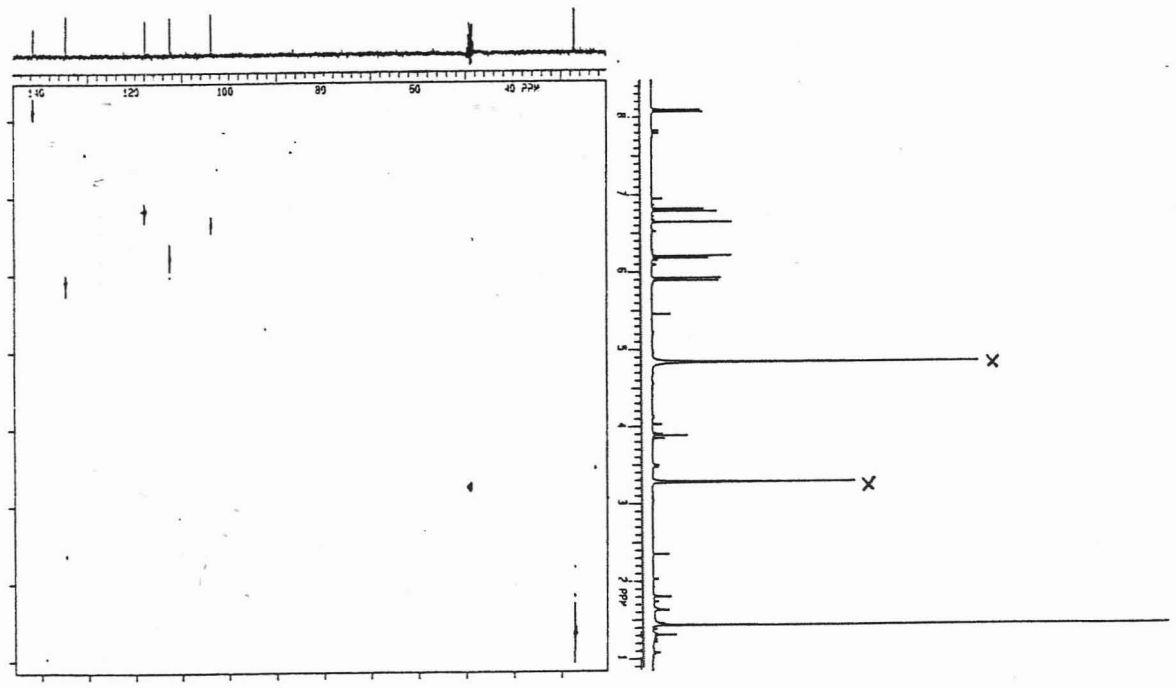
Spectrum 75: ^{13}C NMR spectrum of Compound XI



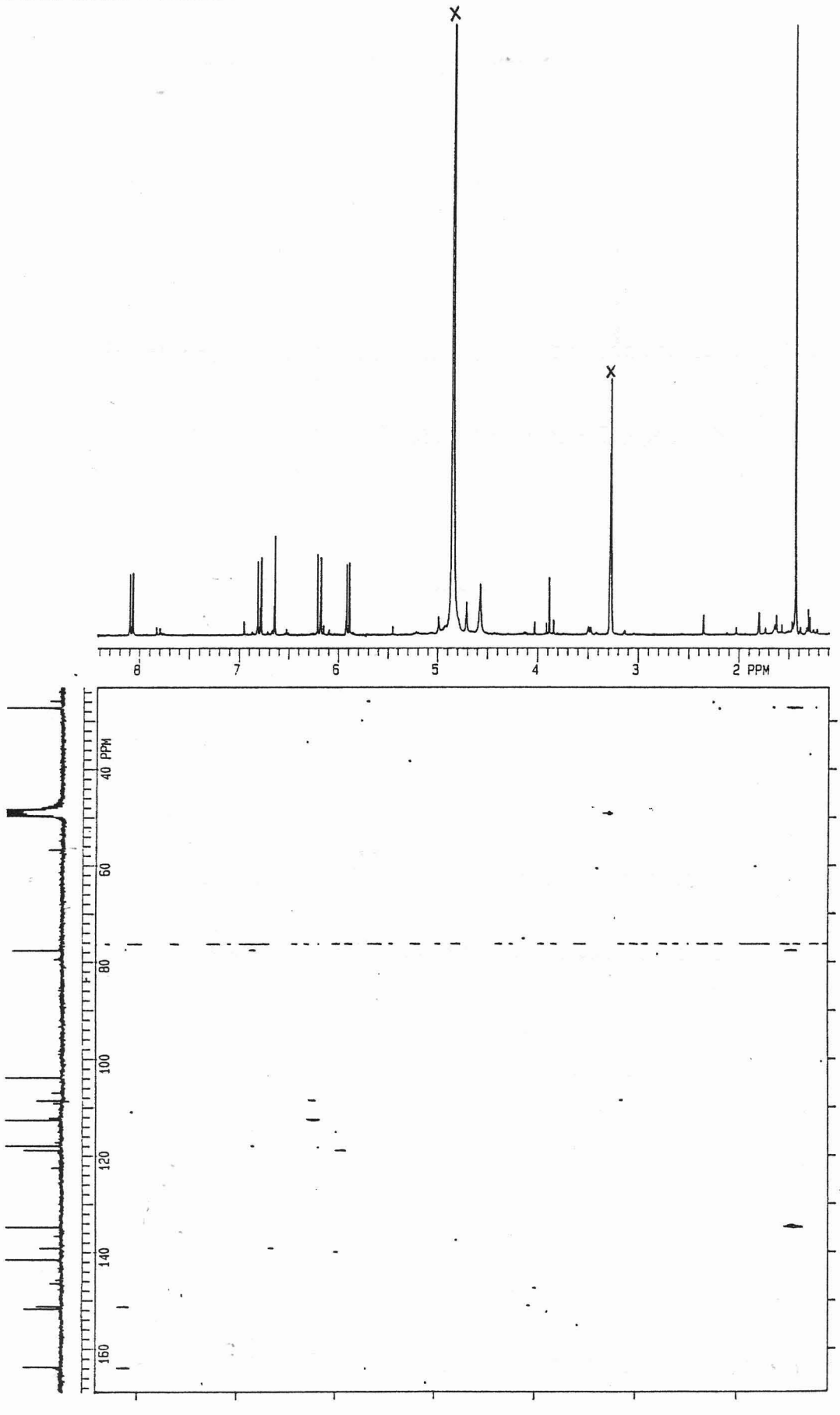
Spectrum 76: ADEPT NMR spectrum of Compound XI



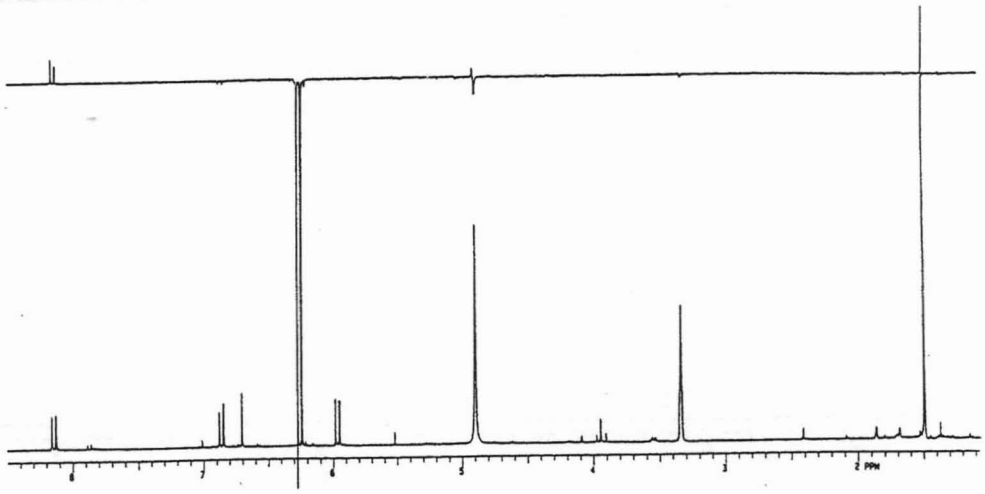
Spectrum 77: COSY NMR spectrum of Compound XI



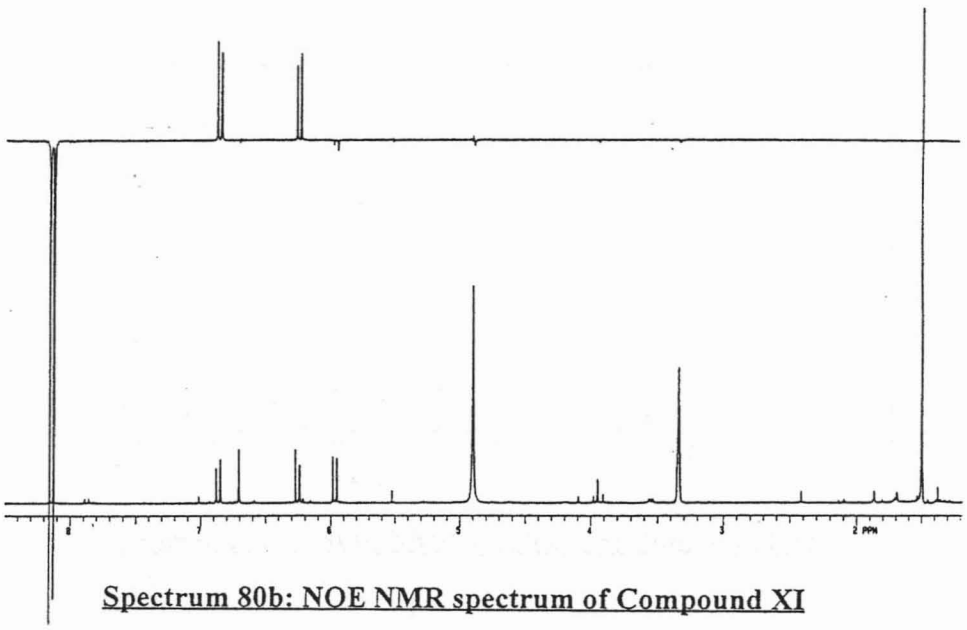
Spectrum 78: HETCOR NMR spectrum of Compound XI



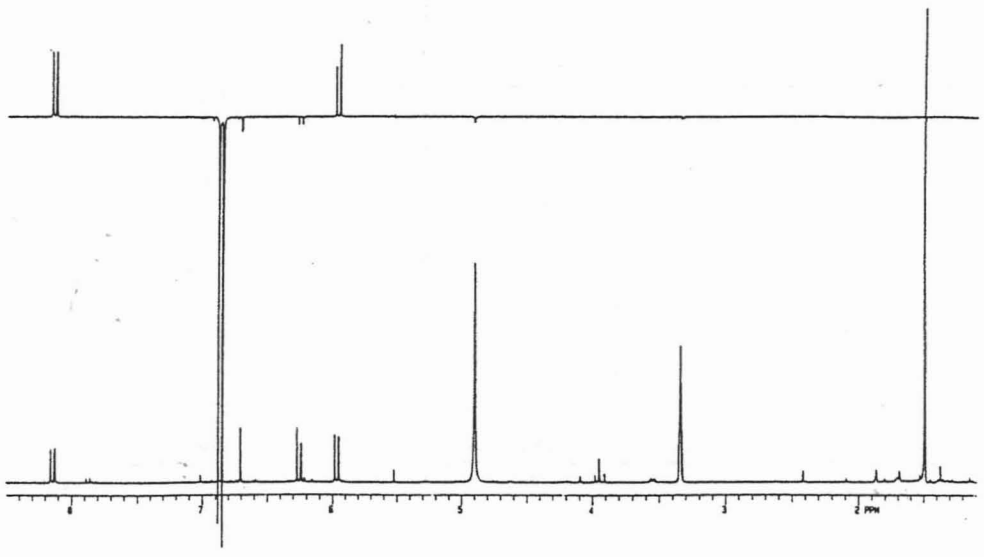
Spectrum 79: DELAYED HETCOR NMR spectrum of Compound XI



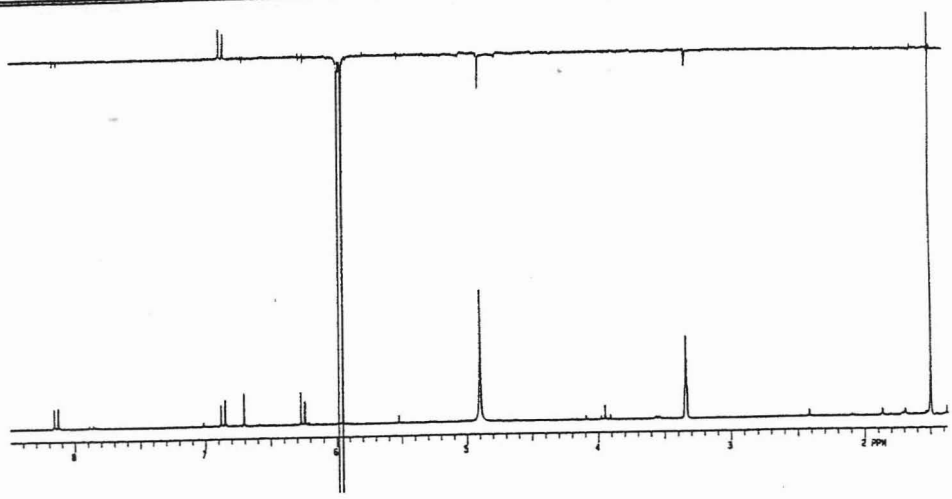
Spectrum 80a: NOE NMR spectrum of Compound XI



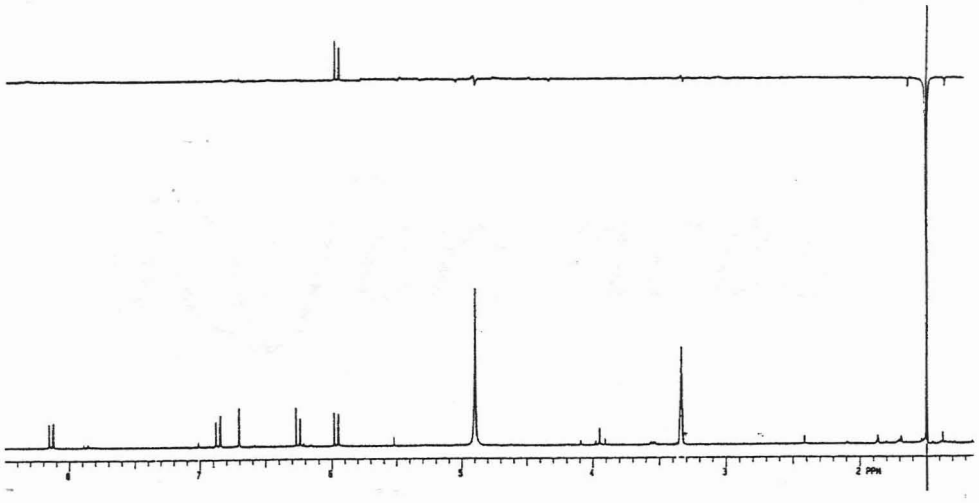
Spectrum 80b: NOE NMR spectrum of Compound XI



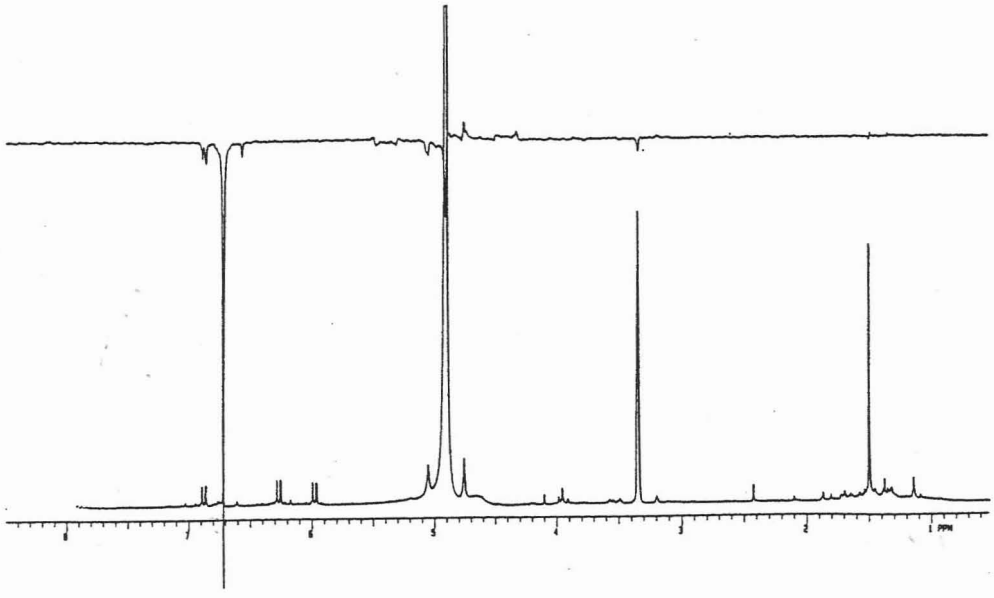
Spectrum 80c: NOE NMR spectrum of Compound XI



Spectrum 80d: NOE NMR spectrum of Compound XI



Spectrum 80e: NOE NMR spectrum of Compound XI



Spectrum 80f: NOE NMR spectrum of Compound XI

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