CHAPTER 1

INTRODUCTION

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Malaysia is known for its richness in plants that have been around for millions of years. Strategically located on the equator where there exists a balance of hot and wet climate, therefore the environment has a wide range of plants to form a majestic plant kingdom. From ancient times, preparations of plants have been used as remedies against disease. Today, with the advent of modern technology scientists are able to identify the active principles of medicinal plants such as morphine in opium poppy which acts as a pain killer. By the end of nineteenth century organic chemists had begun the investigation on alkaloids and their usage in traditional or modern medicine. Therefore, the studies on various compounds such as alkaloids have been carried out widely due to the demand of the pharmaceutical industries.

Natural products have played an important role in drugs discovery. Natural products are those chemical compounds derived from living organisms such as plants, animals, insects, and the study of natural products is the investigation of their structures, formations, use, and purpose in the organism. Drug derived from natural products are usually secondary metabolites and their derivatives. Today those must be pure and highly characterized compounds. Until the late 1800's, organic chemistry was almost exclusively the study and use of natural products. The natural products that were studied and used tend to be the compounds that occurred in the largest amounts, mostly from plants, and were most easily isolated in a pure, or sometimes not very pure, from the technique such as simple distillation, steam distillation or extraction with acid or base. We now employ different solvents, for example hexane and CO_2 nowadays are used to extract the non-polar constituents, methanol and ethanol to extract the polar constituents. Modern isolation techniques include all types of

chromatography (HPLC, TLC, CC, GC), often guided by bioassays, to isolate the active compounds.

This thesis deals mainly with the isolation and structural elucidation of the alkaloids contained in bark and leaves of *Ochrosia oppositifolia* which belongs to the family of Apocynaceae.

The objectives of this research are:

i. to isolate the phytochemicals from the *Ochrosia oppositifolia*.

ii. to identify the chemical structures of the isolated compounds.

iii. to evaluate the antiplasmodial activity of the crudes and the isolated compounds.

1.1 Apocynaceae: Distribution and Habitat

The Apocynaceae or dogbane family is a family of flowering plants, including trees, shrubs, herbs, or lianas. Many species are tall trees, found in the tropical rainforest, and most are from the tropic and subtropics, but some grow in tropical environments. There are also some perennial herbs from temperate zones. Many of these plants have milky sap; ans some species are poisonous if ingested. Some genera of Apocynaceae, such as *Adenium* how ever, have both clear and milky latex sap, and others, such as *Pachypodium*, possess clear sap.[1]

The Apocynaceae family is one of the largest plant families; comprises of 400 genera and 1500 species. In Peninsular Malaysia, there are only 32 genera and 120 species. The alkaloids in the Apocynaceae are important in native medicine, such as bark of *Ochrosia oppositifolia* which had used as a medicine to treat the cancer in ancient Hawaii, while more recent tests of the crude alkaloid extract showed antiplasmodial properties [2]. These plants are distributed mainly in the tropical and sub tropical region.

1.2 General Appearance and Morphology [3, 4]

The leaves are simple, usually opposite and decussate, or whorled; stipules are usually absent. The flowers are bisexual and actinomorphic or sometimes weakly zygomorphic. The calyx is synsepalous and usually 5-lobed. The corolla is sympetalous and usually 5-lobed. The stamens are distinct, as many as corolla lobes and alternate with them, and adnate to the corolla tube (or erigynous zone). The anthers are introrse and commonly adherent to the surface of the stigma. The ynoecium consists of a single compound pistil of 2 carpels that may be distinct at the level of the superior or rarely partly inferior ovary but which are united by a single style. When distinct, each ovary typically has few to numerous ovules on marginal placentae; when connate, the placentation is axile or intruded parietal.

A nectary consisting of 5 glands or an annular ring is usually found at the base of the ovary. The fruit is commonly a follicle, capsule, or berry. The seeds usually are flat and winged or have a tuft of hairs at one end.

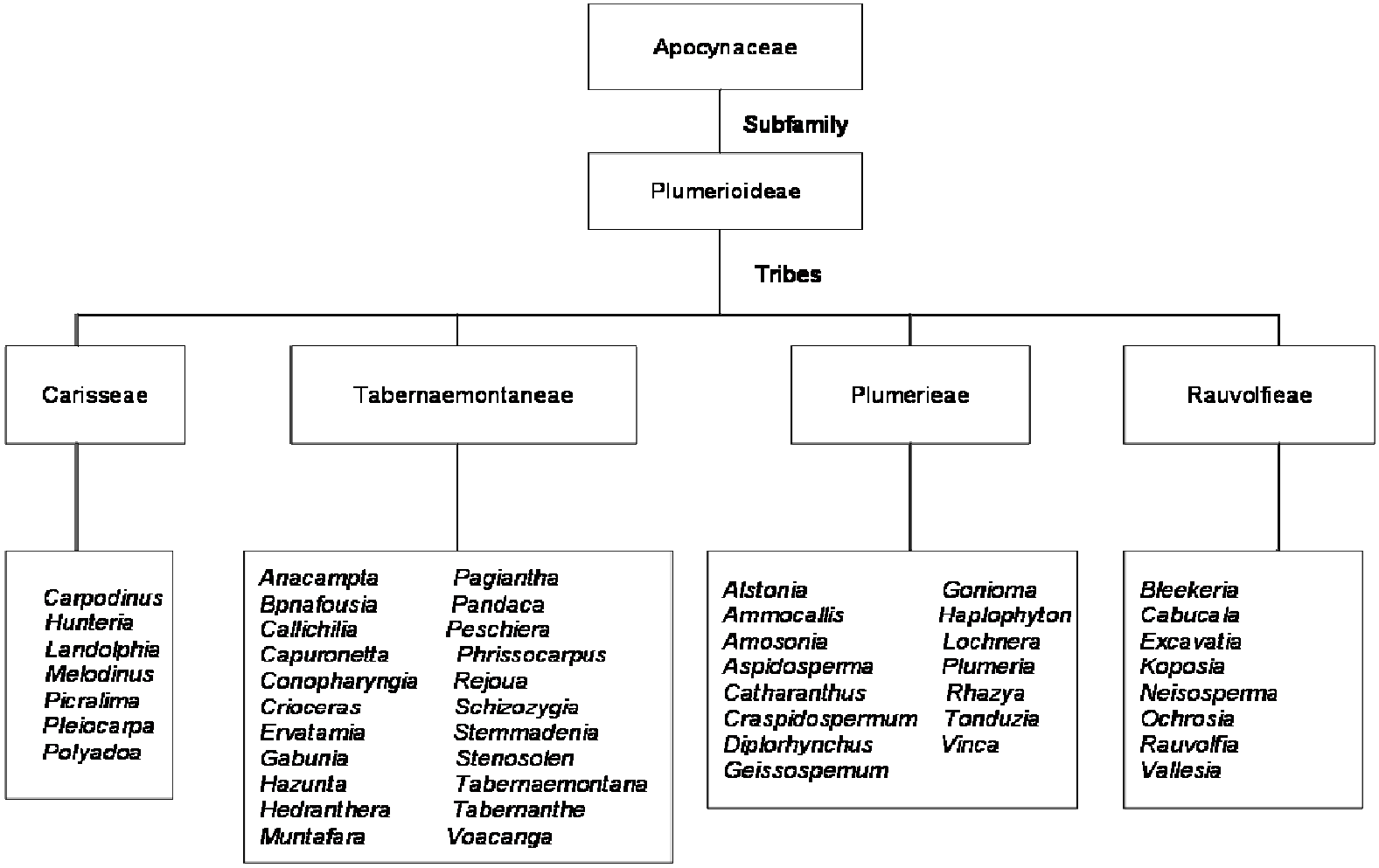
This is a large Family with about 1500 species found mainly in tropical regions. It includes many of the most well-known tropical ornamental plants (Oleander, Frangipani,Allamanda, Mandevilla). Many are large trees with buttress roots found in rainforests some are smaller, evergreen or deciduous trees, shrubs or climbers from other warm areas of the world, and one or two are found in temperate regions (Vinca). The sap of most plants is milky latex,which is often of economic importance for medicinal use, or further production of rubber. This sap is often toxic.

Fruit type is highly diversified in the family, and it is diagnostic of many genera. Genera 1-produce 1, 2-celled berries from a flower; genus 5 produces 2, 1-celled berries from a flower; 6 and 7 produce mostly fleshy follicles containing deeply indented seeds with ruminate endosperm; 8 has follicles and winged seeds; 9 produces

follicles and seeds with 2 comas; 10-12 have follicles with globose seeds; 13-18 have drupes mostly with fleshy mesocarp; 19 has samaroid fruit; 20 has spiny capsules with seeds winged all around; and 21-44 have free or fused follicles and comose seeds. Double flowers are known only from cultivated forms of *Nerium oleander*, *Tabernaemontana divaricata*, and *Wrightia religiosa*. Plants of the Apocynaceae are often poisonous and are rich in alkaloids or glycosides, especially in the seeds and latex. Some species are valuable sources of medicine, secticides, fibers, and rubber.

1.3 Classification of Apocynaceae

Apocynaceae can be divided into Plumerioideae subfamily, which is further divided into a few tribes (Scheme 1.1). Each tribe comprises of several genera. The genus *Ochrosia* is classified as member of the tribe Rauvolfieae. [5]



Scheme 1.1: Classification of Apocynaceae

1.3.1 Genus *Ochrosia*

Ochrosia is a genus of sea-shores, which was observed in Singapore a century ago. There are 32 *ochrosia* in Malaysia which mostly occurs in the Pangkor island.Trees with latex. Branches stout. leaves in whorls 3-5, rarely opposite, lateralveins numerous, sub parallel, almost at a right angle to midvein. Cymes sub terminal, peduncalate. Calyx deeply divided, usually without glands. Corolla salver form; tube slightly dilated above middle, to 1cm throat without scales; lobes overlapping to right. Stamens inserted in widening of corolla tube; anthers free from pistil head, narrowly oblong, rounded at base; disc absent. Ovaries 2, free or basally connate; ovules 2-6, biseriate on each side of a prominent placenta. Style filiform; pistil head shortly 2-cleff atapex. Drupes 1 or 2, smooth, endocapp thick, hard, seeds 2-4 per locale, flat, not compose; endosperm none; cotyledons large, flat.[6] The nearest related genus is probably *Cerbera.*

1.3.2 *Ochrosia oppositifolia*

Ochrosia oppositifolia is an evergreen tree that can vary greatly in size, growing to a maximum height of 15 m (50ft) or more. This species produces five-petalled white flowers with yellow centers. The flowers usually drop to the ground like confetti. The fallen flowers provide a clue to finding the tree. The fruit comes in pairs, is elliptical in shape, and is about 5-8 cm long and turns yellow when it is ripe. The seed is about 10-20 cm, apparently growing quite slowly.

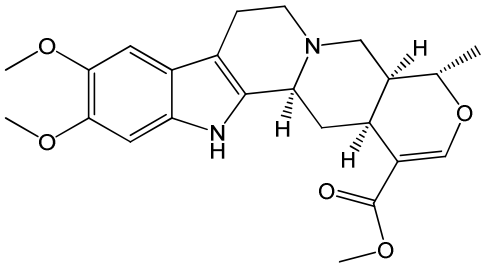
Ochrosia oppositifolia occurs in Indonesia and along the southern coastal region of Sri Lanka. This plant is commonly called Muda Kaduru in Sri Lanka. The timber is moderately hard and even grained. Its roots are reputed to nullify the effect of eating poisonous fish and crabs. It is also used as a bitter, stomachic and carminative. The seeds of *Ochrosia oppositifolia* are edible and also reputed in indigenous medicine. [7]

Ochrosia oppositifolia belonging to Apocynaceae family is an alkaloid-rich plant. Plants of this genus find wide use in the traditional system of medicine.[7] The presence of alkaloids was first observed by J.Polsson. A literature search on the alkaloids of *Ochrosia oppositifolia* revealed the presence of (isoreserpiline **1**, reserpiline, ochroposinine **7**, epirauvanine, bleekerine, 10-hydroxyapparicine, 10-methoxyapparacine, 10-methoxydihydrocorynantheol **10**, ochrolofuanine, reserpinine **3**, isoreserpinine **8** and 9-methoxyellipticine **4**).[8]

Table 1.2 shows the alkaloids isolated from *Ochrosia*.[9, 10]

Table 1.2 Alkaloids Isolated from *Ochrosia*

Compound	Mol.Wt.	Mol. Formula
Isoreserpiline **1**	412	$C_{23}H_{28}N_2O_5$
Neisosposinine **2**	428	$C_{23}H_{28}N_2O_6$
Reserpinine **3**	382	$C_{22}H_{26}N_2O_4$
9-methoxyellipticine **4**	276	$C_{18}H_{16}N_2O$
10-Hydroxyapparacine **5**	280	$C_{18}H_{20}N_2O$
10-methoxyapparacine **6**	294	$C_{19}H_{22}N_2O$
Ochropposinine **7**	411	$C_{23}H_{27}N_2O^{+}_5$
Isoreserpinine **8**	382	$C_{22}H_{26}N_2O_4$
Ochrolifuanine **9**	438	$C_{29}H_{34}N_4$
10-methoxydihydrocorynantheol **10**	382	$C_{22}H_{26}N_2O_4$
10- methoxycorynantheol **11**	326	$C_{20}H_{26}N_20_2$



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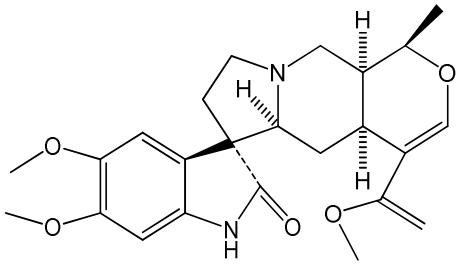

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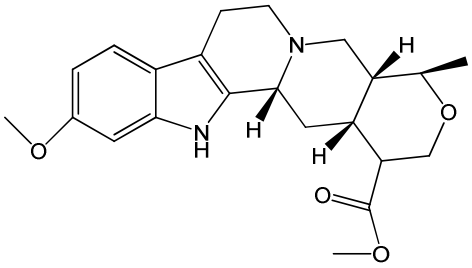

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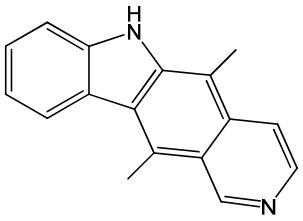

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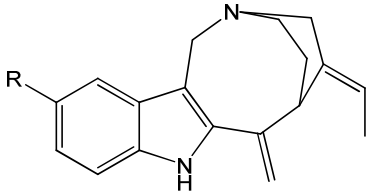
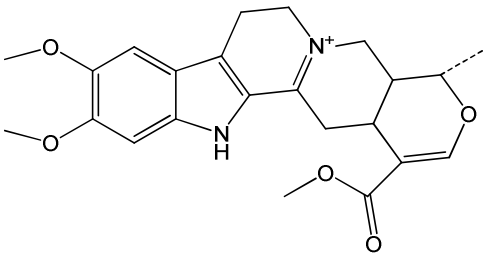


5 R= OH

6 R= CH_3



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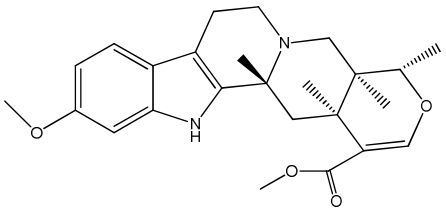

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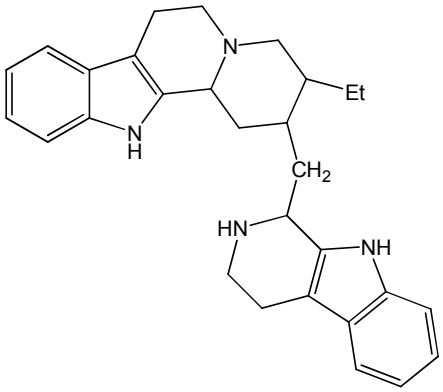

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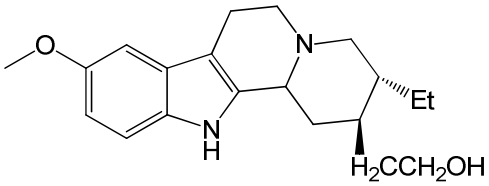

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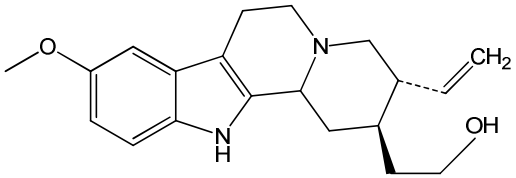

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Figure 1.1: Bark and Leaves of *Ochrosia oppositifolia*

CHAPTER 2
GENERAL CHEMICAL ASPECTS

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2.1 Alkaloids

Plants produced two types of metabolites, i.e. primary metabolite and secondary metabolite. The former includes polysaccharide, protein fatty acid and nucleic acid, and the latter includes chemicals with no apparent function in the primary metabolism of the organism and these substances tend to be of restricted taxonomic distribution and such metabolites have a history of use as bioactive agents like antiplasmodial activities.

2.2 Secondary Metabolites

Secondary metabolites are often unique to a particular species or group of organism and some act many act as antifeedants, sex attractants, repellents, antimalarial, antibacterial, antifungal, antiviral, and antibiotic agents. [11] While others may not have any apparent biological role.

Alkaloids are the most potent of all the secondary metabolites since the last century. Their potency extends to almost universal toxicity and it would be correct to start that the action of my alkaloid on the body is inherently stressful. [12] The following paragraph shall discuss briefly the alkaloids and their chemicals aspect.

2.3 Definition of Alkaloids [13-15]

The term alkaloids or alkali-like was first proposed by W. Meisner in 1819.Alkaloids are defined as nitrogen containing basic substances, having a complex structure, naturally origin and limited distribution on earth. Alkaloids always contain their nitrogen atom as part of the heterocyclic system, and they usually possess some pharmacological activity. Particular alkaloid is usually restricted to certain genera and families of plant kingdom, rarely being present in large groups of plants. They are biosynthetically exist as salt and related to acid amino such as ornithine and lysine. A precise definition of the term alkaloid (alkali-like) is somewhat difficult because there is no clear-cut boundary between alkaloids and naturally occurring complex amines[16]. Many of alkaloids are derived from plant sources. They are basic, contain one or more nitrogen atoms (usually in a heterocyclic ring) and usually have a marked physiological action on man or other animals (Table 2.1).

Alkaloids can be separately categorized into three groups based on their biogenesis; **true alkaloids**, **protoalkaloids** and **pseudoalkaloids.**

2.3.1 True Alkaloid

The true alkaloids normally contain nitrogen in heterocyclic rings, they derived from amino acid. They normally occur in the plants as the salt of an organic acid. Examples of this group are hygrine **12** and cryptostyline **13**.

2.3.2 Proto Alkaloid

The protoalkaloids are relative simple amine in which the amino acid nitrogen is not in a heterocyclic ring. Like the true alkaloids, they are derived from amino acid and are basic. Examples of this groups are mescalin **14**, ephedrine **15**, *N,N*-dimethyltripthamine **16**, serotonin **17** and cathinone **18**.

2.3.3 Pseudo Alkaloid

The pseudoalkaloids are not derived from amino acid precursor and usually basic. They are nitrogen containing in the molecule but they have a carbon skeletons derived from terpenoids (mono-, sesqui-, di-, and triterpenoids), steroidal, hemiterpenoids and other acetate derivatives. For example of this group are actinidine **19**, deoxynupharidine **20** and alchorneine **21**.

Alkaloids represent a fascinating group of natural product for a number of reasons. Many reveal important biological and pharmacological activities and for several decades have been therapeutically applied in the treatments of various diseases. Some of the alkaloids with impressive activities which have prompted the development of broadly applied drugs on the pharmaceutical market, include the well known cytotoxic bisindole alkaloids vincaleucoblastine **22** and vinblastine **23** from *Catharanthus*, the diterpenoid alkaloid taxol from *Taxus*, the highly important analgesic morphine **24**, the spasmolytics tubocurarine **25** and papaverine **26**, the vasodilating agents vincamine **27** and ajmalicine **28**, emetine **29** with its emetic activity, and the antiarrhythmic alkaloids quinidine **30** and ajmaline **31.** [17]

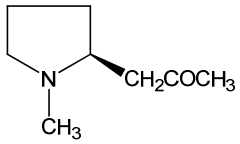
The diversity of alkaloid structures forced scientists to concentrate during recent decades on the elucidation of biosynthetic pathways at the enzymatic level. Now the first example exist for the detection of series of enzymes catalyzing multistep biosynthetic sequences, e.g. in the

field of isoquinoline alkaloids[18], indole bases[19] and pyrrolizadine alkaloids[20]. Successful study of the molecular genetics of alkaloid formation has been undertaken[21] including the first example of heterologous expression of appropriate enzymes catalyzing alkaloid

metabolism.[22-24]

Table 2.1 Physiological activities of some alkaloids [25, 45, 67]

Alkaloids	Physiological activities
Nicotine **41**	Toxic, stimulate respiratory processes, inhibition in all sympathetic and parasympathetic
Atropine **42**	Antagonism to muscarinic receptors (parasympathetic inhibition)
Cytochalasin D **43**	Cytotoxic and antitumor activity
Haplophyllidine **44**	Ataractic and sedative
Perforine **45**	Hypoglycemic activity
Bucharaine **46**	Suppress aggressive tendencies
Mescaline **47**	Inhibit the pressor effect epinephrine and produce hyperthemia and uterine contraction
Ephedrine **48**	Therapeutic agent
Gliotoxin **49**	Bacteriostatic agent
Papaverine **50**	Decrease the tonus of the smooth muscle.
Laudanosine **51**	Reduce intraocular pressure
(+)-Cepharanthine **52**	Effective against human tuberculosis and
(-)-mecambrine **53**	leprosy
Thebaine **54**	Increases motility of isolated rat or rabbit
Heroine **55**	Duodenum
Erysotrine **56**	Increased intestinal muscle tone in rabbit
Coccuvinine **57**	Exhibited significant cytotoxicity
Strychnine **33**	Act as a muscle contractor
Tylophorine **58**	Neuromuscular blocking agents
Canthin-6-one **59**	Antifungal and leishmanicidal activities
Tecomine **60**	Toxic to many bacteria and other unicellular organism
Toxiferine **70**	Causes muscle relaxation



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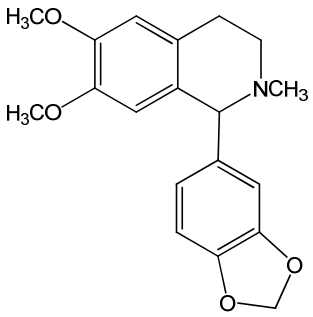

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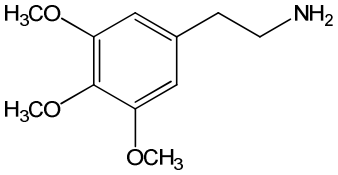

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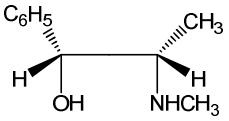

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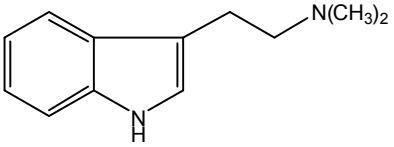

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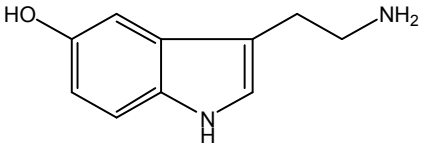

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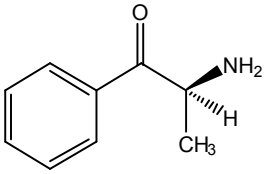

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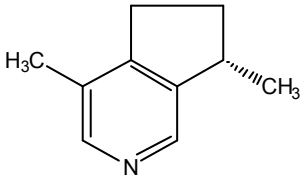

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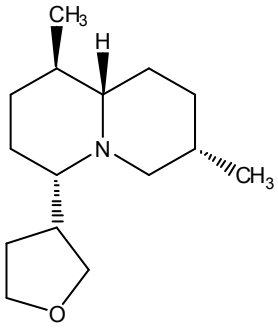

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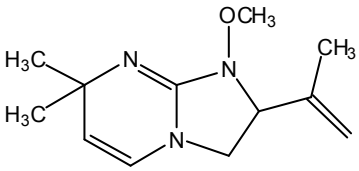

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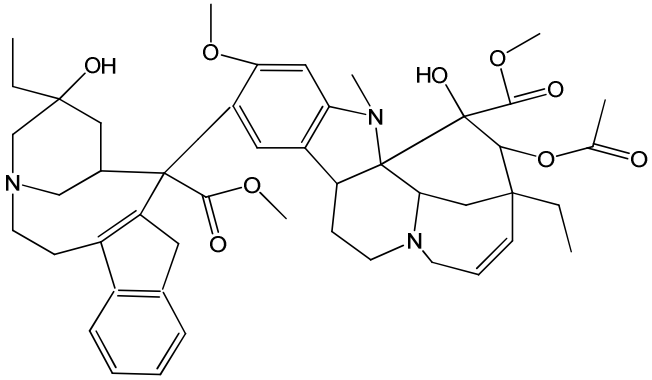

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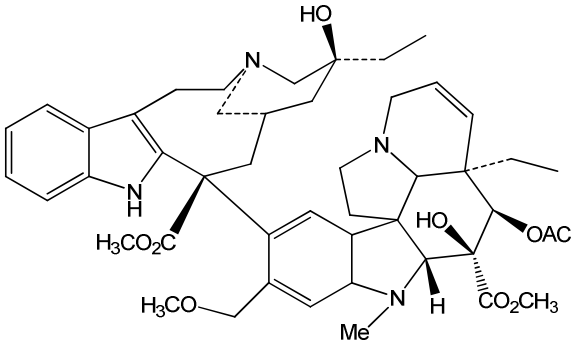

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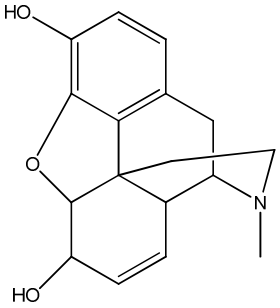

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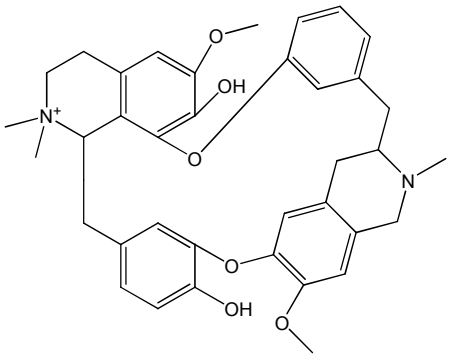

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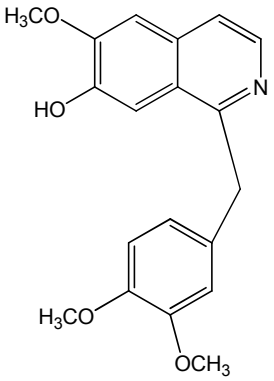

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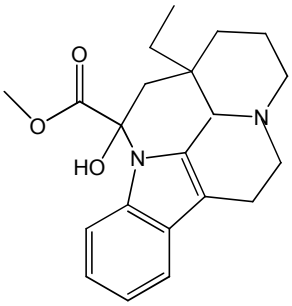

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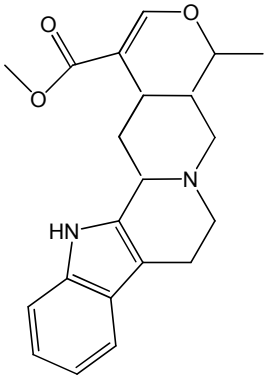

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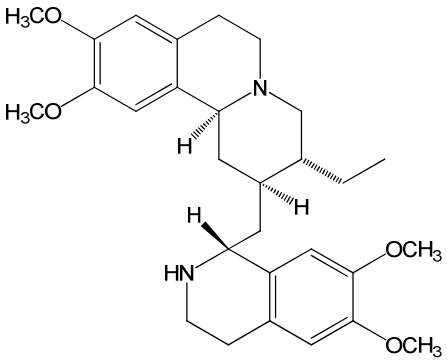

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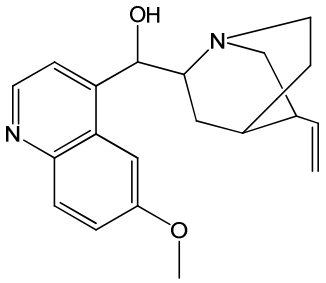

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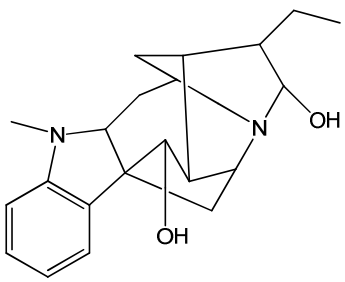


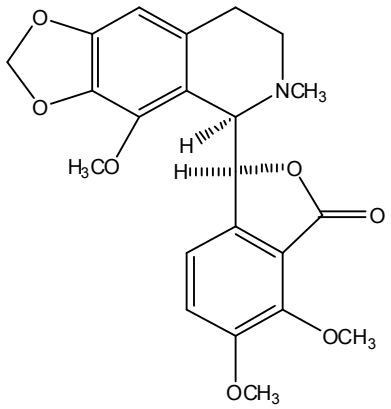
31

Usually all the alkaloids occur in multicomponent mixture and separation of alkaloids from other groups of natural product is the first requirement for detailed qualitative, quantitative and structural analysis of single alkaloids.

The first crude drug to be investigated chemically was opium, the dried latex of the poppy *Papaver somniferum*. In 1803, Derosne isolated a semipure alkaloid from opium and named it as narcotine **32**. Further examination of opium by Serturner in 1805 led to the isolation of morphine structure. In the years 1817-1820 in the laboratory of Pelletier and Caventou at the Faculty of Pharmacy in Paris, the researchers obtained many active alkaloids. Among the alkaloids obtained were strychnine **33**, emetine **29**, brucine **34**, piperine **35**, caffeine **36**, quinine **37**, cinchonine **38** and colchicine **39**. They also obtained coniine **40** in 1826.[26]

Certain families have a marked tendency to elaborate alkaloids: this is true for the monocotyledons (Annonaceae, Apocynaceae, Fumariceae, Lauraceae, Loganiaceae, Magnoliaceae, Menispermaceae, Papaveriaceae, Ranunculaceae, Rubiaceae, Rutaceae, and Solanaceae). Within these families, some genera produce alkaloids and others do not. The biological activity of the alkaloids, together with their impressive structural diversity, largely accounts for the enormous research effort that has been devoted to their characterization, pharmacologic evaluation and synthesis.

The probable function of alkaloids in plants seems to be to deter animals and herbivorous insects. A part from their toxicity, the action of the alkaloids is not easily summarized because each has its own individual character. Instead a list will give some idea of their range and categories. As the individual examples provided are the best known, and thus often the most notorious or dramatically active, a more toxic picture of the alkaloidal range may be conveyed than is actually justified.[27]



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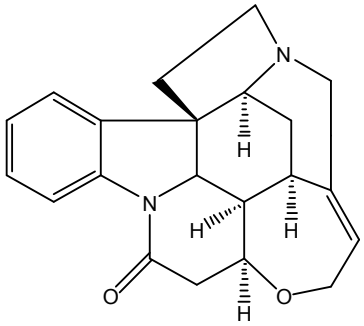

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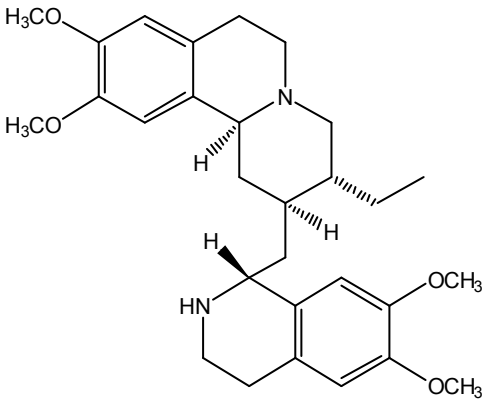

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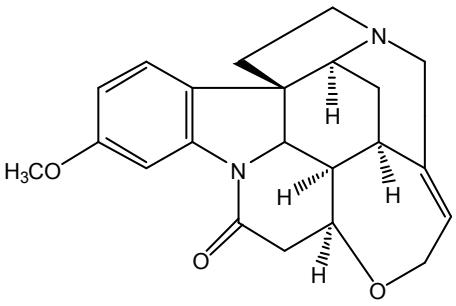

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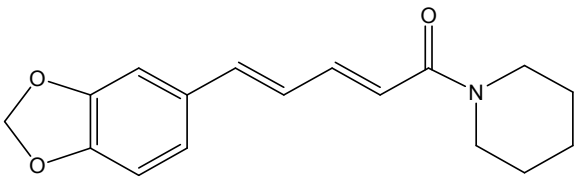

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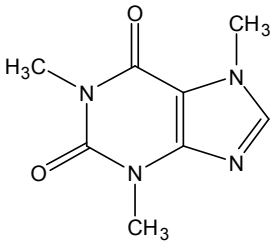

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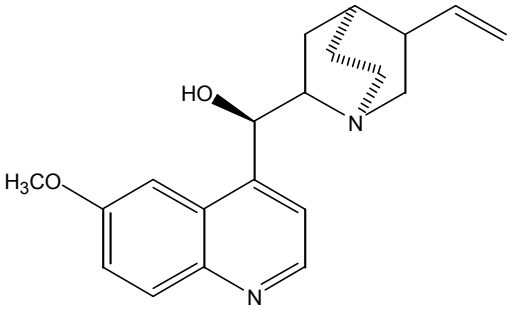

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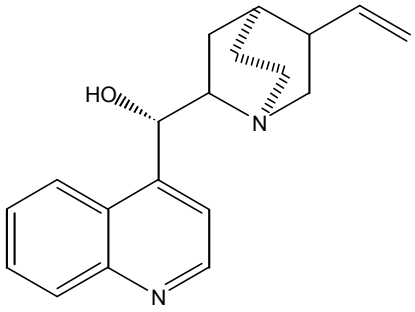

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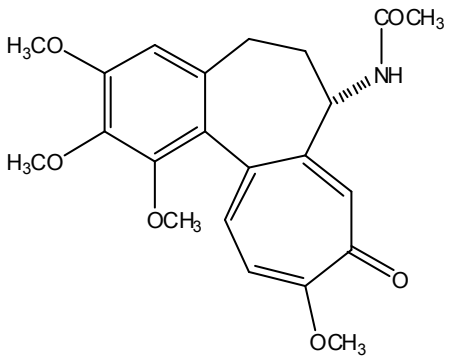

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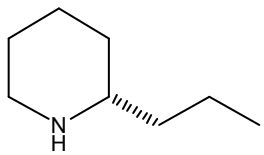

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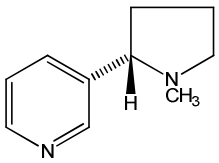

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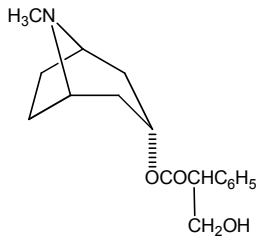

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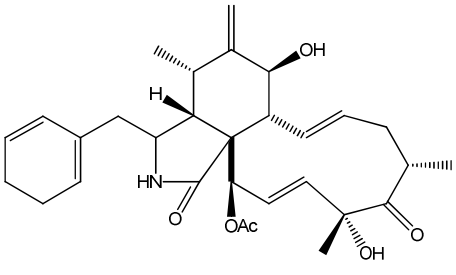

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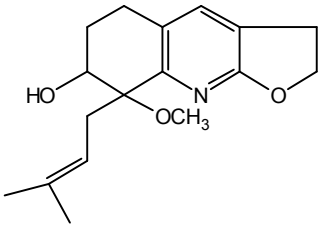

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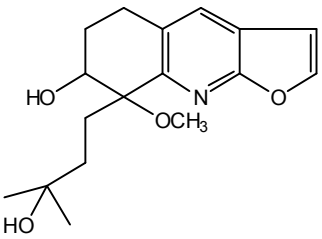

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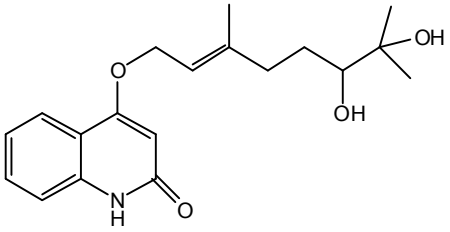

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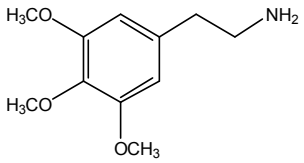

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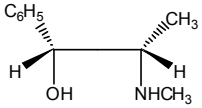

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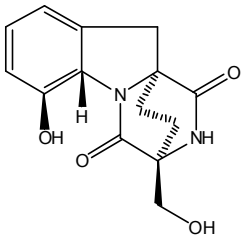

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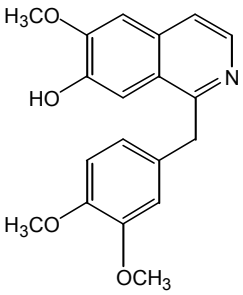

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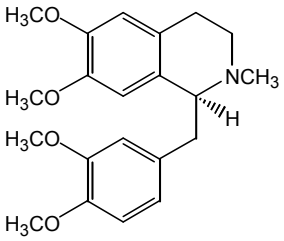

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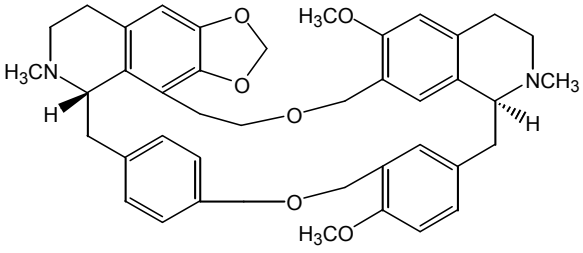

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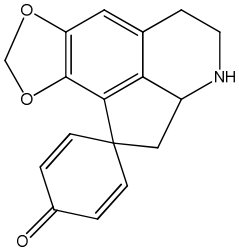

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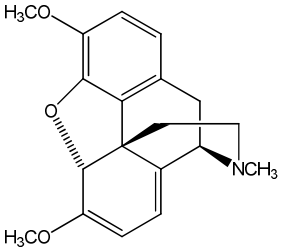

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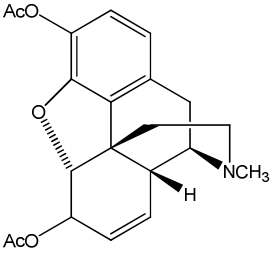

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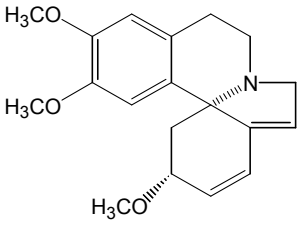

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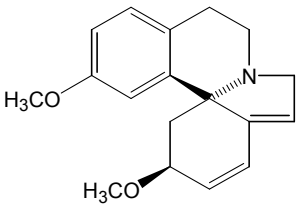

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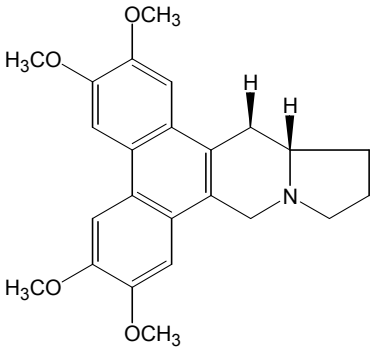

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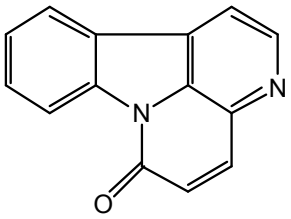

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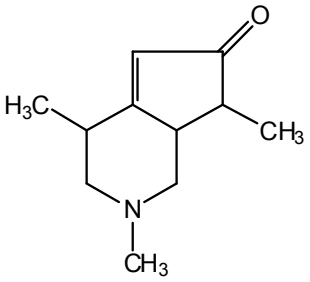

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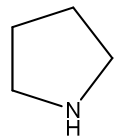
The role of alkaloids in the plants are still unknown, but the researchers suggested that the alkaloids are as the end product of metabolism or waste product, and it functions as a protection against predator attack, storage reservoir of nitrogen for protein synthesis, plants stimulants or regulator in many activities, such as growth, metabolism and reproduction and a model for the chemical synthesis of analogue with excellent properties. [28]

2.4 Classification of Alkaloids

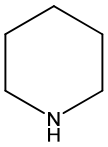
Alkaloids are usually classified according to their common structural motif, based on the metabolic pathway used to construct the molecule. When not much was known about the biosynthesis of alkaloids, they were grouped under the names of known compounds, even some non-nitrogenous ones, for example the phenanthrenes, since this moiety appeared in the finished product or by the plant or animals from which they were isolated. When more is learned about a certain alkaloid, the grouping is changed to reflect the new knowledge, usually talking the name of a biologically-important amine that stands out in the synthesis process. Alkaloids may be classified by various methods which are based on the nature of the classification such as: [29]

- Biogenesis.
- Structural relationship based on chromophore of fundamental skeleton.
- Nitrogen atom and its immediate environment.
- Botanical origin.
- Spectroscopic criteria.

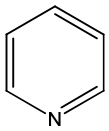
Several examples of common alkaloids ring skeleton are illustrated in Scheme 2.1.[30]



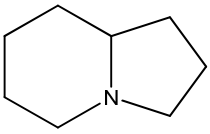
Pyrolidine



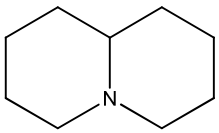
Piperidine



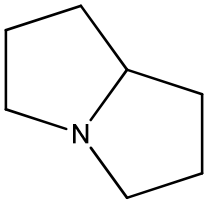
Pyridine



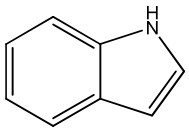
Indolizidine



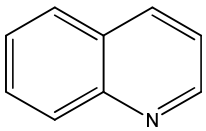
Quinolizidine



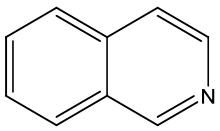
pyrolizidine



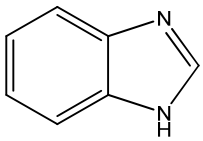
Indole



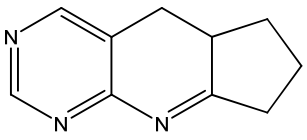
Quinoline



Isoquinoline



Purine



Quinazoline

Scheme 2.1: Example of alkaloid ring skeleton

2.4.1 Indole Alkaloids

Indole is an electron-rich aromatic compound with characteristic properties and is widely distributed in natural product and in proteins as the important constituent of essential amino acid tryptophan **71**. It is known to form a hydrophobic environment in proteins and to be involved in enzymatic reactions, in addition to the redox activities and various weak interactions.[31] It shows versatile metal binding abilities through the nitrogen and carbon atoms. They include the 'animal alkaloids'; adrenaline, noradrenaline, serotonin (5-hydroxytryptamine or 5-HT), the tranquillizing alkaloids of passion flower, the ophthalmic alkaloids related to physostigmine from the calabar bean, the uterine stimulants ergotamine and ergometrine from the fungus ergot of rye, and lysergic acid diethylamide (LSD). Also included are the alkaloids of the Indian snakeroot (*Rauwolfia serpentia*), including reserpine, having powerful hypotensive effects. In addition, there are some examples of central nervous stimulants: strychnine, johimbine and psilocybin. All these alkaloids have their effect on the neuromuscular system by interacting with adrenergic receptors. Finally, the infamous two antileukaemic medications vincristine and vinblastine isolated from the Madagascar periwinkle (*Catharanthus rosea*).[32]

Indole alkaloids exhibit numerous biological activities (anti-tumor, anti-microbial, anti-hypertensive and central nervous system stimulant 1). They can be found in plants of the Apocynaceae, Rubiaceae, and Loganiaceae families.

Among the Apocynaceae, the genus *Ochrosia* is rich in indole alkaloids. The previous chemical studies of genus *Ochrosia* have reported many occurrences of indole alkaloids especially of the corynanthean or C-type. [33] They are useful chemical markers of the genus, and also have a great value for the classification of the individual species within the genus which is very difficult if the classification was to be based only upon the morphological character of the plants.

The ^{1}H NMR and ^{13}C NMR proton and carbon values of simple indole, C_8H_7N, are given below:

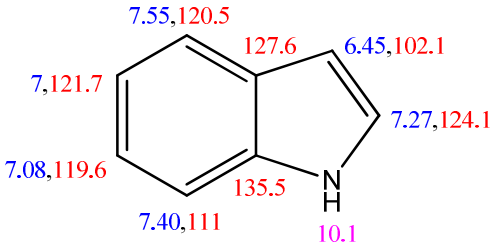
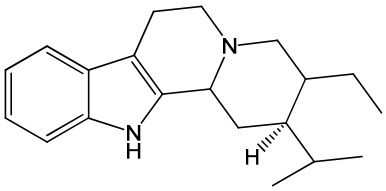
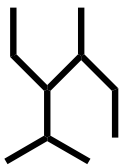
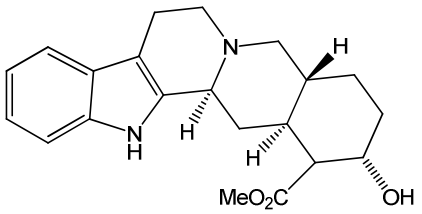
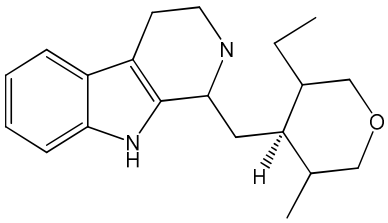
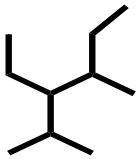
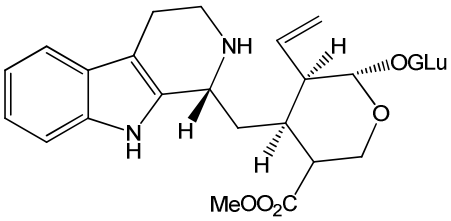
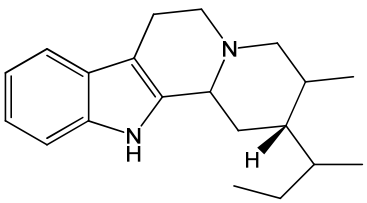
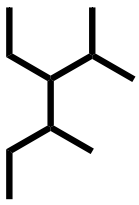
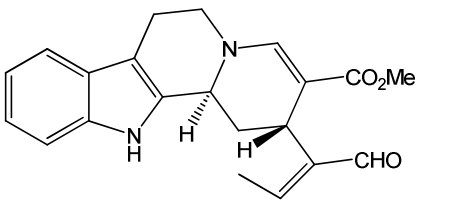


Figure 2.1: ^{1}H NMR and ^{13}C NMR of C_8H_7N

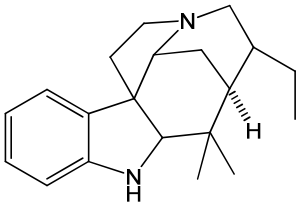
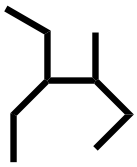
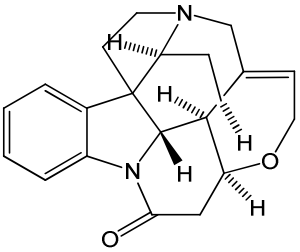
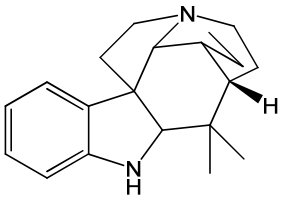
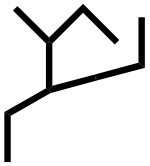
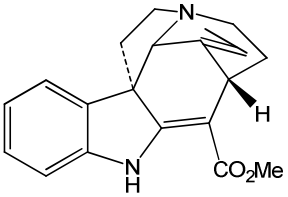
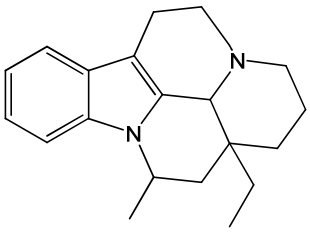
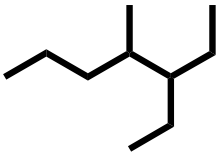
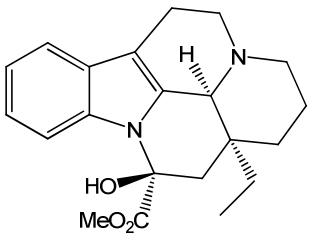
The majority of indole alkaloids from the plant of Apocynaceae can be classified into nine main types according to the structural characteristics of their skeleton (Table 2.2). [34-36]

Eight main types have been defined. There are vincosan, vallesiachotaman, corynanthean, strychnan, aspidospermatan (all belong to the class I skeleton with an intact secologanin), plumeran, eburnan (belong to the class II skeleton, rearranged secologanin) and ibogan (class III skeleton, rearranged monoterpene). The ninth type, tacaman (class III skeleton) has been added by Verpoorte and Beek to account for the isolation of a few tacamines (Table 2.2). [37, 38]

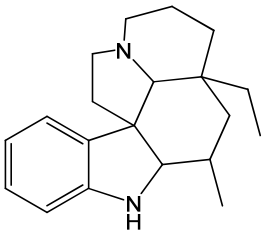
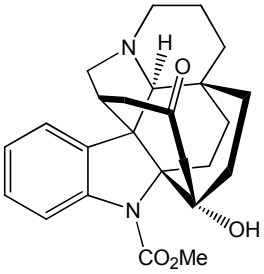
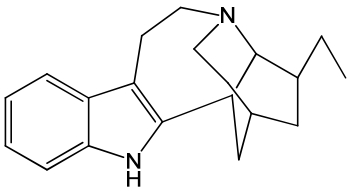
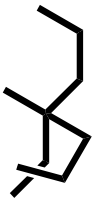
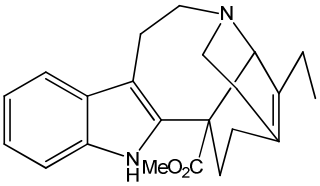
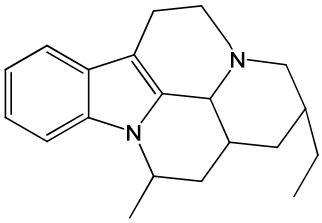
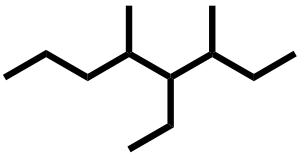
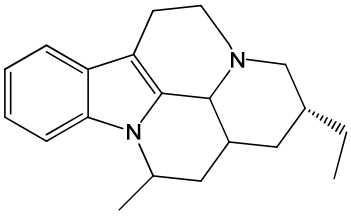
Table 2.2 : Types of Indole Skeleton, Classes and the Examples

Type of indole alkaloid	Skeleton	Example
		
Corynanthean or C-type	Class I	corynanthine
		
Vincosan or D-type	Class I	vincoside
		
Vallesiachtamon or V-type	Class I	vallesiachotamine

Types of Indole Skeleton, Classes and the Examples (continued)

Type of indole alkaloid	Skeleton	Examples
		
Strychnan or S-type	Class I	strychnine
		
Aspidospermatan or A-type	Class I	condylocarpine
		
Eburanan or E-type	Class II	vincamine

Types of Indole Skeleton, Classes and the Examples (continued)

Type of alkaloid	Skeleton	Examples
		
Plumeran or P-type	Class II	kopsine
		
Ibogan or J-type	Class III	catharantine
		
Tacaman type	Class III	tacamine

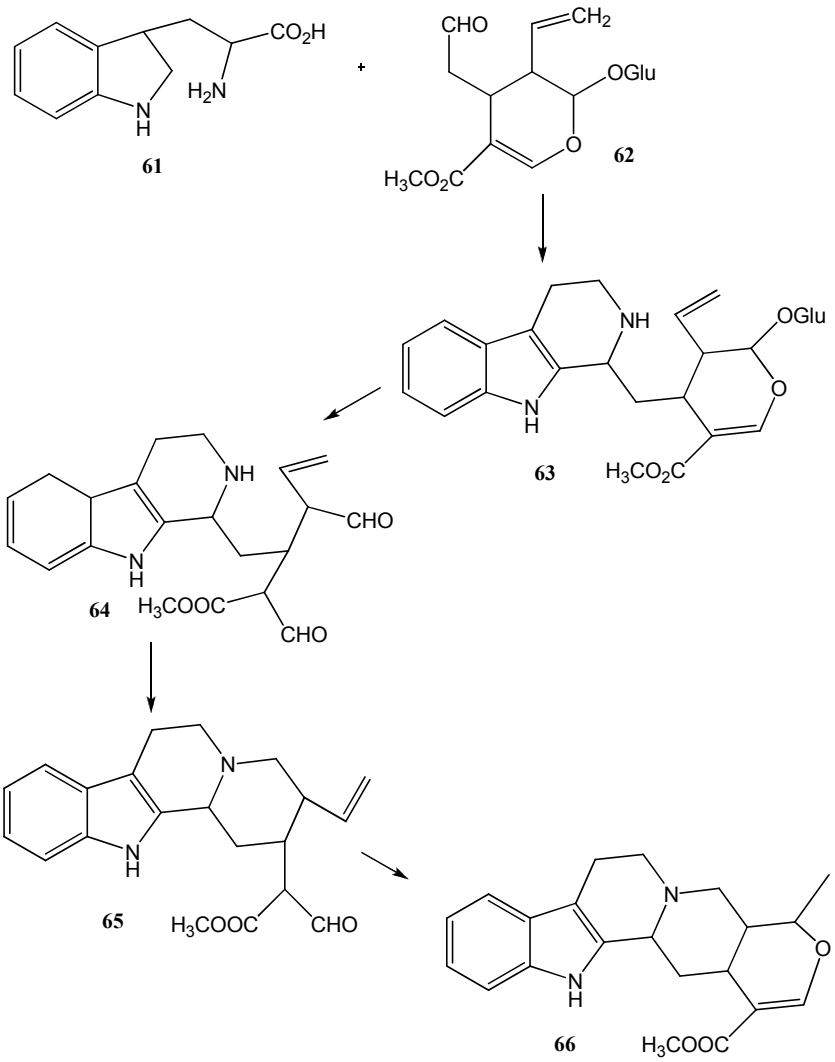
2.4.2 Biosynthesis of Indole Alkaloids: [39, 40, 41]

Biosynthesis refers to the manner in which organic substances are synthesized, altered or degraded by organism (plant or animal).

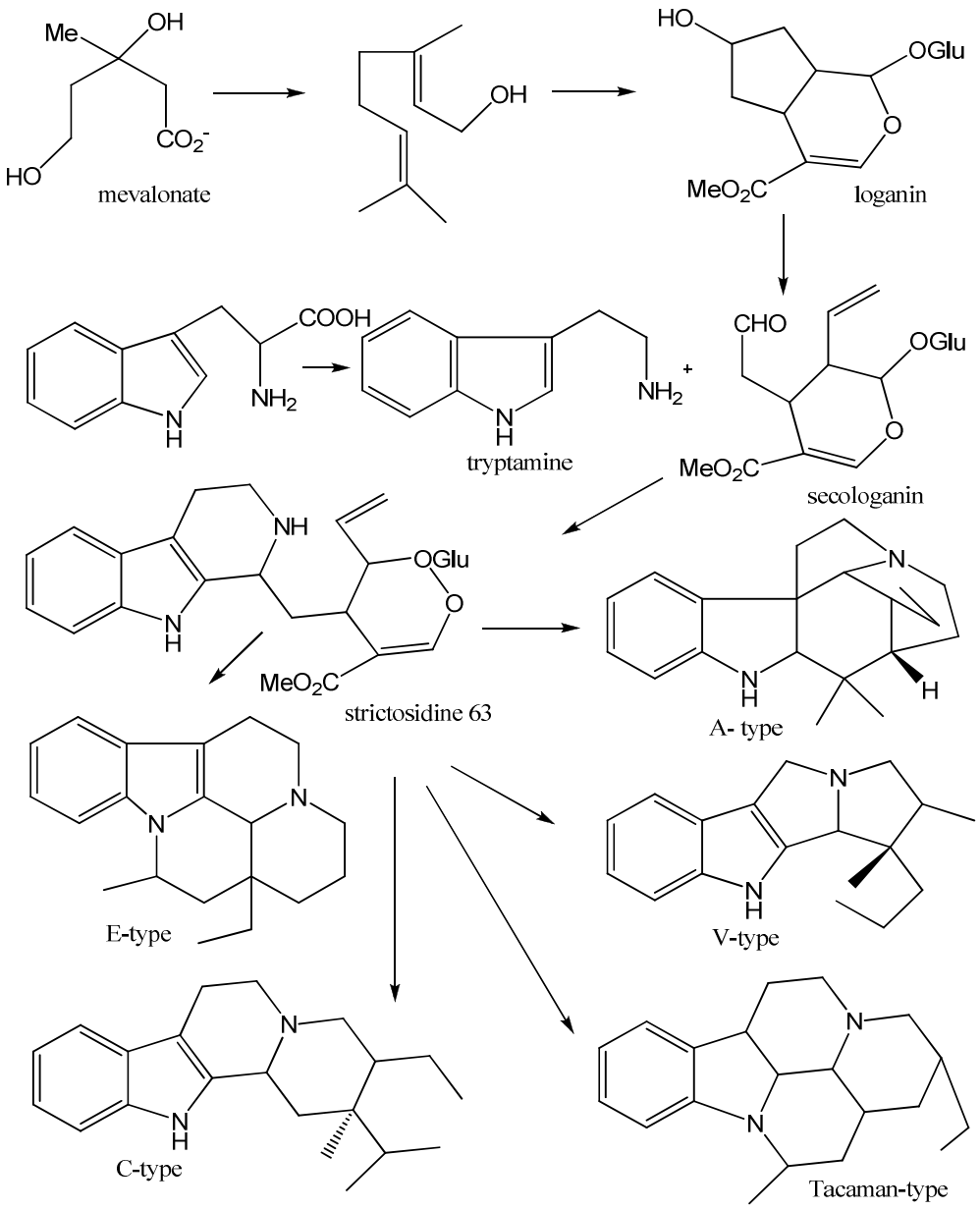
The complexity of indole alkaloid structure are formally derived from a Mannich condensation of tryptamine **61** as the indole nucleus and a C9 or C10 monoterpene moiety, derived from secologanin **62**. Secologanine is made up of two molecules of mevalonic acid.

Condensation of secologanine with tryptamine leads to strictosidine **63,** a vincosan skeleton alkaloid. Hydrolysis of the sugar residue and the opening of the cyclic acetal function give the dialdehyde **64**. Ring closure of **64** yields the tetracyclic system **65**. Minor rearrangements generate ajmalicine **66**, a corynanthian type of alkaloid (type C) which is the main alkaloid type in *Ochrosia* species. These transformations are described in (Scheme 2.2).

Strictosidine **63**[42] is also the precursor of many other type of indole alkaloids; (type A), (type C), (type V), (type E) and Tacaman type. (Scheme 2.3)



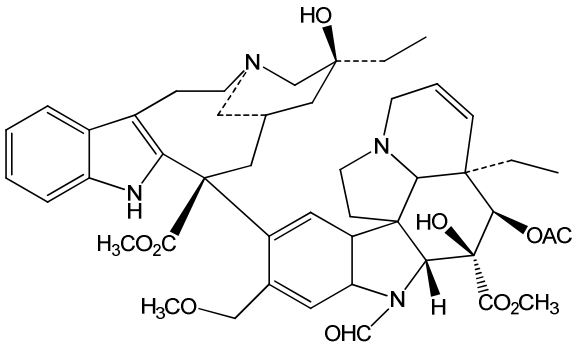
Scheme 2.2: Condensation of Secologanin with Tryptamine



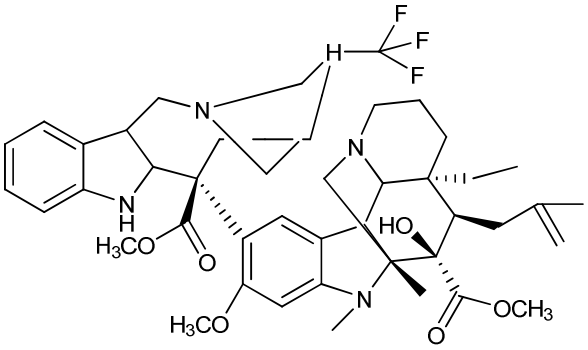
Scheme 2.3: Steps in the Biosynthesis of Indole Alkaloids

2.5 Pharmacological Activity

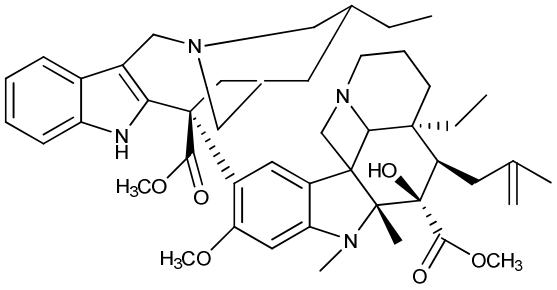
Before their recognition as useful therapeutic agents, alkaloids were renowned for their poisonous properties. Of the many biologically active indole alkaloids, only a few were used today as therapeutic agents of value in human medicine. For example vincristine **67** was used to treat acute leukemia.[43] The dimeric alkaloids from *Catharanthus roseus* form an important class of antitumour agents, widely used in combination chemotherapy regimens for treating leukamias and many solid tumours. Vinflunine **68** which is a novel *vinca* alkaloid synthesized from vinorelbine **69** using superacidic chemistry and characterised by superior in vivo activity to vinorelbine in preclinical tumour models. [44, 45]


HO
N
N
H
H_3CO_2C
HO
OAC
H_3CO
N
H
CO_2CH_3
OHC

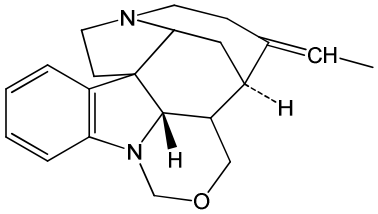
67


F
F
F
H
N
N
H
H_3CO
O
HO
H_3CO
N
OCH_3
O

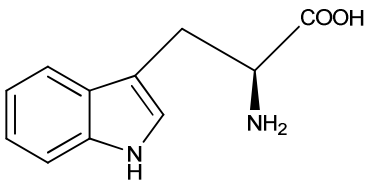
68


N
N
H
H_3CO
O
HO
H_3CO
N
O
OCH_3

69


N
CH
H
N
H
O

70


COOH
NH_2
N
H

71

CHAPTER 3

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

One Malaysian species from the family of Apocynaceae, *Ochrosia oppositifolia*, has been studied for its components content. The plant materials were collected from Pangkor islands in 2007. The sample was identified by L.E.Teo , University Malaya and deposited at the herbarium unit (specimen no; KL 5349).

The isolation process was carried out using the conventional methods and the structural elucidation was carried out using spectroscopic techniques, notably NMR, IR, MS and UV and also by comparison with the literature values.

3.1 Isolation and Structural Elucidation of Compounds from *Ochrosia oppositifolia*

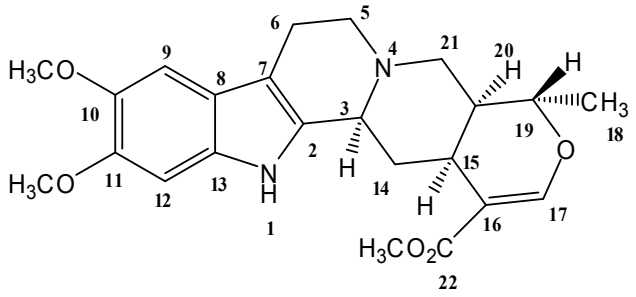
Six compounds were isolated. The investigation on the crude extract from the bark of *Ochrosia oppositifolia* have resulted in the isolation of three compounds, one alkaloid namely, isoreserpiline **1**, and two ferulic acid ester derivatives; 2-propenoic acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)-,methylester **73** and 17-methoxy-carbonyl-14-heptadecaenyl- 4-hydroxy-3-methoxy cinnamate **74.**

Isolation and structural elucidation of the crude alkaloids from the leaves of *Ochrosia oppositifolia* yielded three alkaloids; neisosposinine **2**, reserpinine **3** and alkaloid D **72.**

The following sub chapters will discuss briefly the structural elucidation of all compounds.

3.2 Alkaloid from the Bark of *Ochrosia oppositifolia*

3.2.1 Alkaloid A: Isoreserpiline. 1[46]



1

Isoreserpiline **1** was isolated as a brownish amorphous solid. The mass spectrum showed

pseudo-molecular ion peak at m/z 413.2, $[M+H]^+$, which was consistent with the molecular formula of $C_{23}H_{28}N_2O_5$. Another significant fragmentation was observed at m/z 353 $(M\text{-}COOCH_3)^+$ as depicted in Scheme 3.2.

The UV spectrum revealed maxima at 226, 299 nm which were characteristic for an indole system [47]. In addition, the IR spectrum showed a peak at 1692 cm^{-1} which indicate the presence of the carbonyl group, [46] and a band at 3370 cm^{-1} presence of the NH/OH groups. (Figure 3.3)

The ^1H NMR spectrum (Figure 3.1) indicated the presence of two aromatic protons at δ 6.88 and 6.79 attached to C-9 and C-12 respectively, and a deshielded singlet at δ 7.54 which belongs to H-17, indicative of a corynanthean skeleton. [47]

Another three singlets appeared at δ 3.90, 3.87 and 3.46 can be attributed to the protons of three methoxyls attached to C-10, C-11 and C- 22 respectively.

The presence of the N-H protons was confirmed by the existence of a broad downfield signal at δ 7.73 in the ^{1}H NMR spectrum.

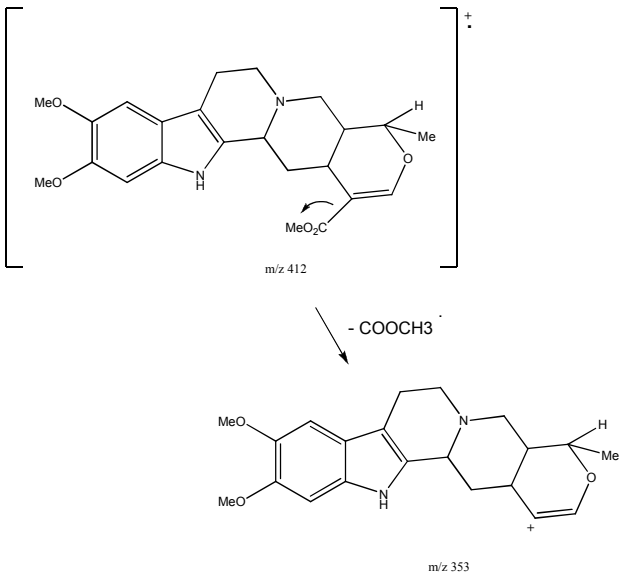
There were a total of twelve aliphatic proton signals observed in the ^{1}H NMR spectrum. Eight methylene proton signals appeared between δ 1.47- 3.05 which belong to the protons attached to C-5, C-6, C-14 and C-21. The signal for another four aliphatic protons δ 3.30, 2.8, 4.4 and 1.5 were assigned to H-3, H-15, H-19 and H-20 respectively (Figure 3.2). Finally a doublet in the high field region at δ 1.4 (d, 6.8 Hz) may be attributed to the methyl group attached to C-19.

The ^{1}H, ^{13}C data (Table 3.2.1) and DEPT indicated the presence of 23 carbons; 8 quaternary carbons; δ 168.2 (C-22), 146.5 (C-11), 144.9 (C-10), 133.3 (C-2), 130.2 (C-13), 120.0 (C-8), 109.6 (C-16) and 107.9 (C-7), 7 methines; δ 155.8 (C-17), 100.4 (C-9), 94.9 (C-12), 72.6 (C-19), 60.0 (C-3), 38.5 (C-20) and 31.4 (C-15), 4 methylenes; δ 56.5 (C-21) , 53.7 (C-5), 34.4 (C-14) and 21.9 (C-6), 1 methyl; δ 18.6 (C-18), and 3 methoxyls at δ 56.4 (C_{10}-OMe), 56.5 (C_{11}-OMe) and 50.8 (C_{22}- OMe) respectively.

The carbonyl (C-22) appeared at δ 168.1. Two aromatic methine signals were observed at 100.4 and 94.9 could be assigned to C-9 and C-12 respectively. C-17 appeared at δ 155.8 and the carbonyl is proven by the correlation between carbonyl and H-17 in HMBC (Figure 3.5). The HMBC spectrum also showed correlation between C-20 and the methyl protons of C-18, while H-14 (δ 1.5) revealed correlation with C-3. Other correlations were shown in Figure 3.5.

The complete assignments of carbons and protons were confirmed with DEPT, HMQC and HMBC spectra. Analysis of all spectral data obtained and comparison with literature

confirmed the identify of alkaloid A as isoreserpiline **1** which was previously isolated from *Rauvolfia grandiflora* Mart. [46]



Scheme 3.2: Possible Mass Fragmentation of Isoreserpiline A **1**

Table 3.2.1: ^{1}H NMR [400 MHz, 1_H (J,Hz)] and ^{13}C NMR [100 MHz, $б_C$] of **1** in $CDCl_3$

Position	^{1}H (J,Hz)	^{13}C	^{13}C [45]
2		133.3	133.14
3	3.30 (br d, 11.48 Hz)	60.0	59.91
4			
5	2.5 (m) 2.9 (m)	53.7	53.67
6	2.7 (m) 2.81 (m)	21.9	21.82
7		107.90	107.82
8		120.0	119.94
9	6.88 (s)	100.4	100.35
10		144.9	144.78
11		146.50	146.41
12	6.79 (s)	94.9	94.82
13		130.2	130.13
14	2.6 (m) 1.5 (q , 9.76 Hz)	34.4	34.26
15	2.8 (m)	31.4	31.29
16		109.6	109.49
17	7.54 (s)	155.8	155.75
18	1.4 (d , 6.8 Hz)	18.6	18.49
19	4.4 (m)	72.6	72.47
20	1.5 (m)	38.5	38.41
21	3.05 (m) 2.7 (dd , 1.6, 12.2 Hz)	56.5	56.25
22		168.2	168.03
OMe	3.90 (s)	56.4	56.25
OMe	3.87 (s)	56.5	56.41
OMe	3.46 (s)	50.8	51.10
NH	7.73 (s)		

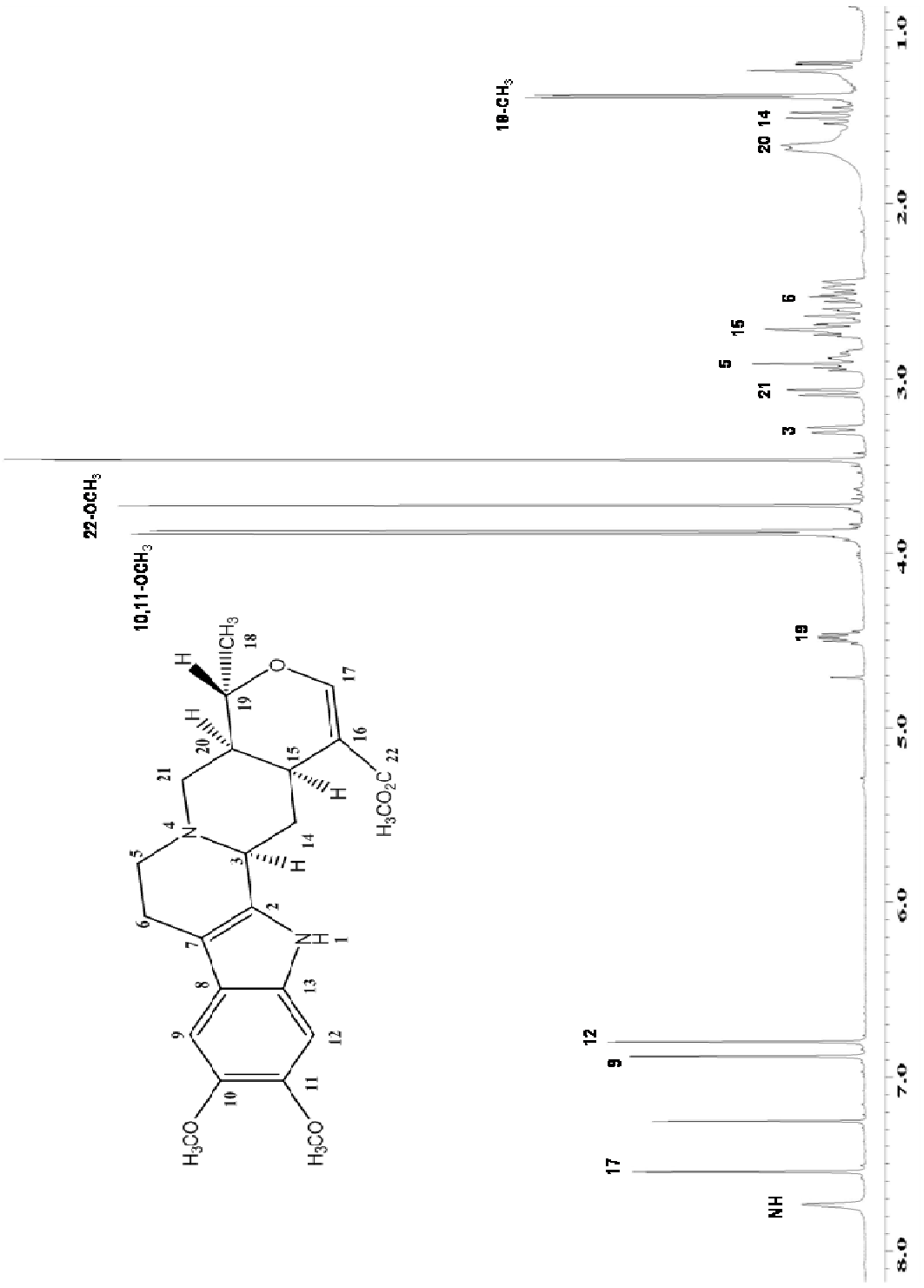


Figure 3.1: ^{1}HNMR Spectrum of Isoreserpiline

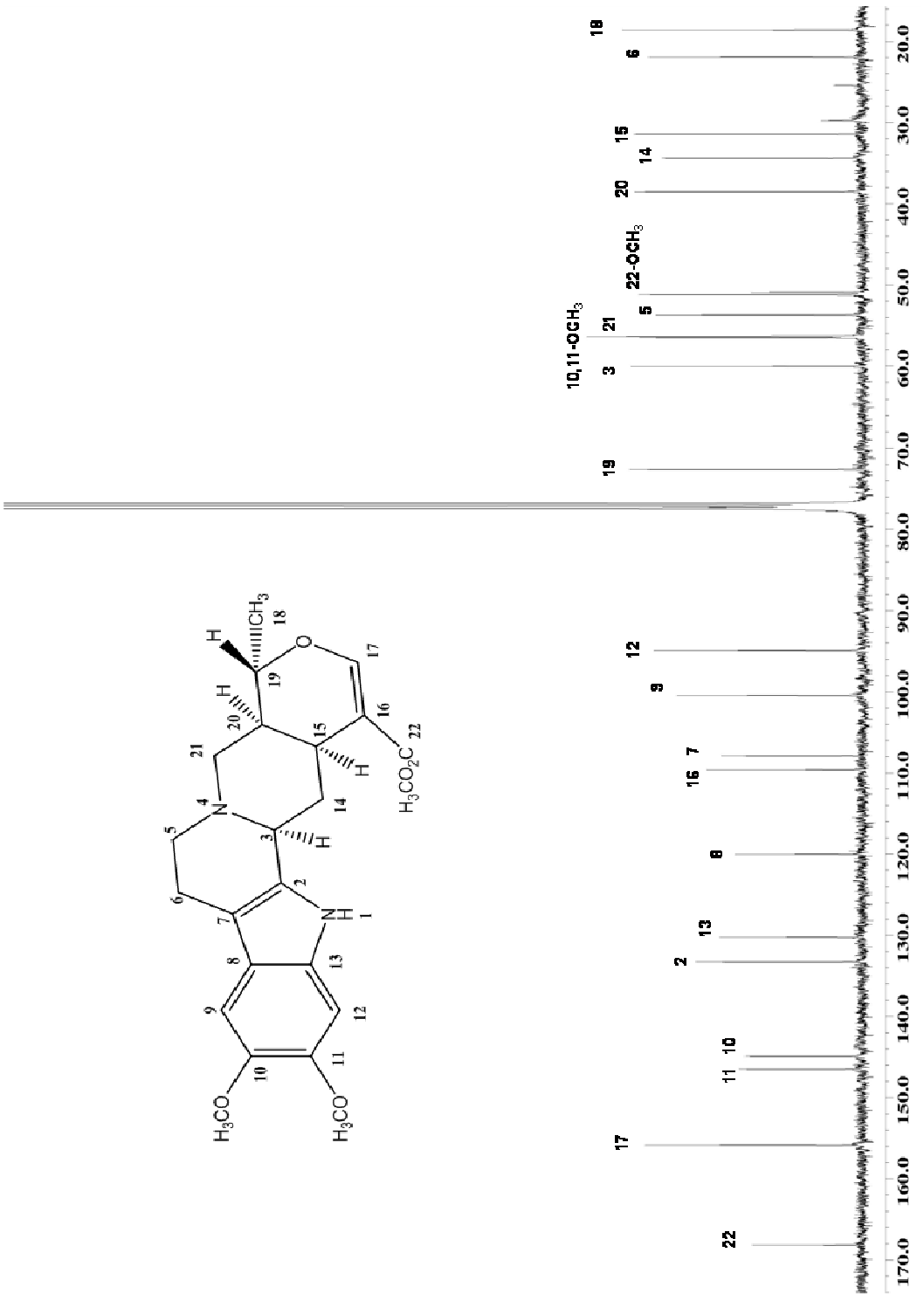


Figure 3.2: ^{13}C NMR Spectrum of Isoreserpiline

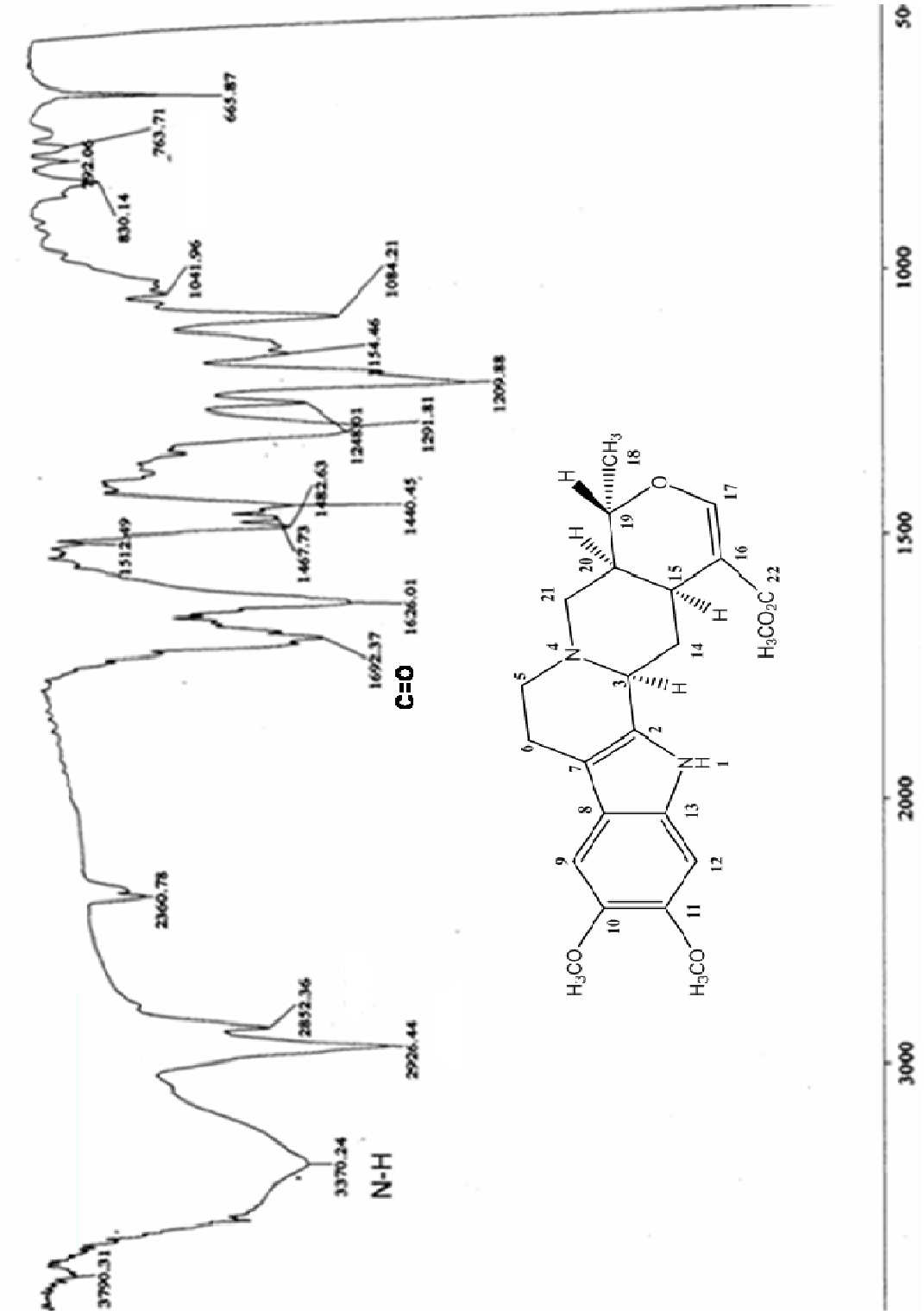


Figure 3.3: IR Spectrum of Isoreserpiline

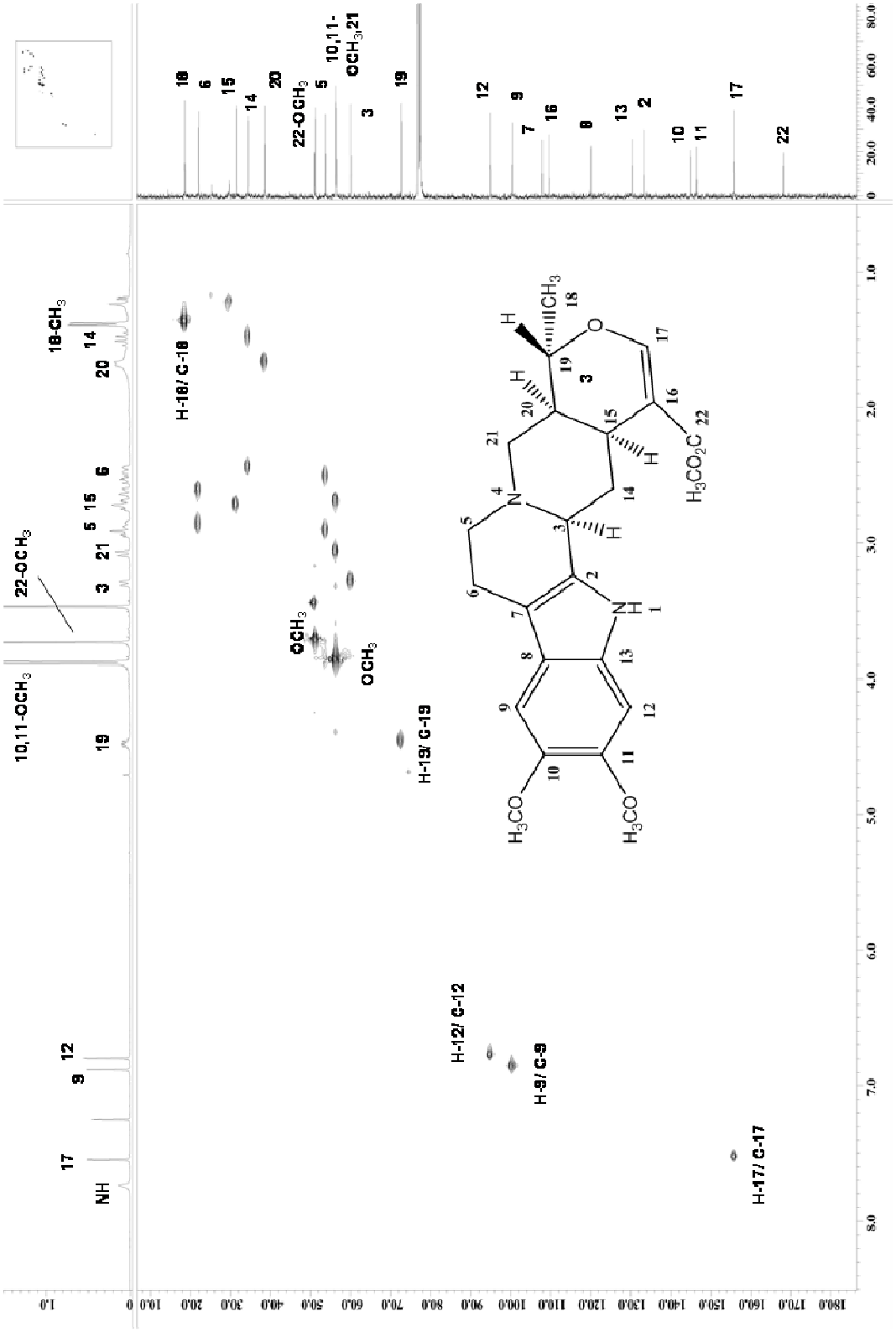


Figure 3.4: HSQC Spectrum of Isoreserpiline

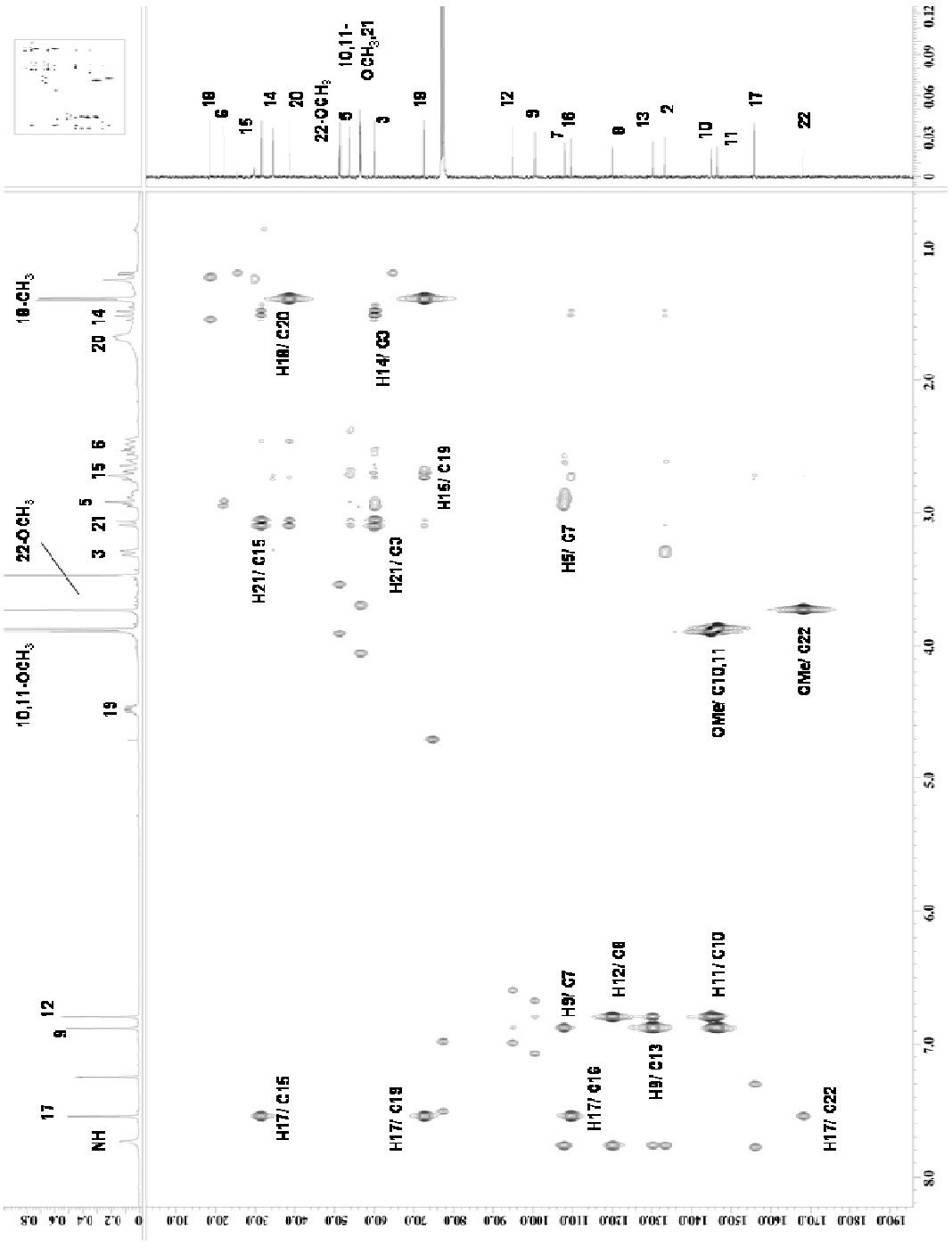
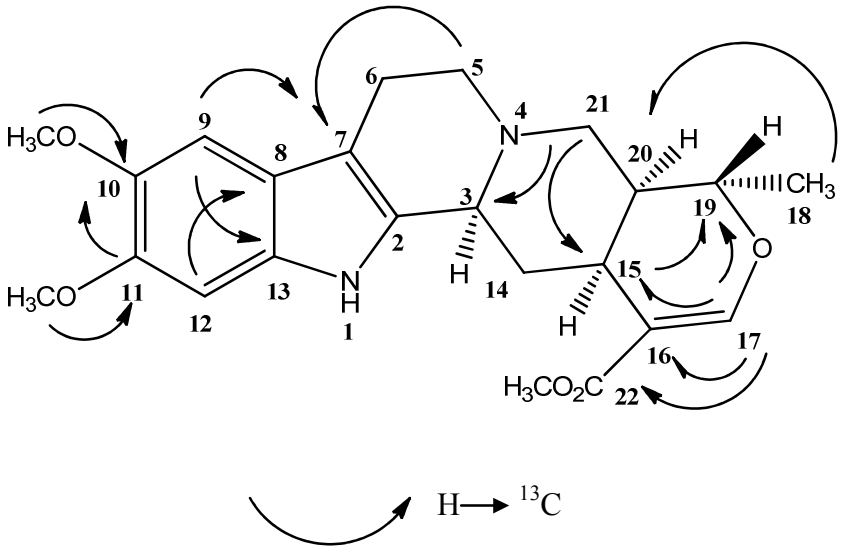


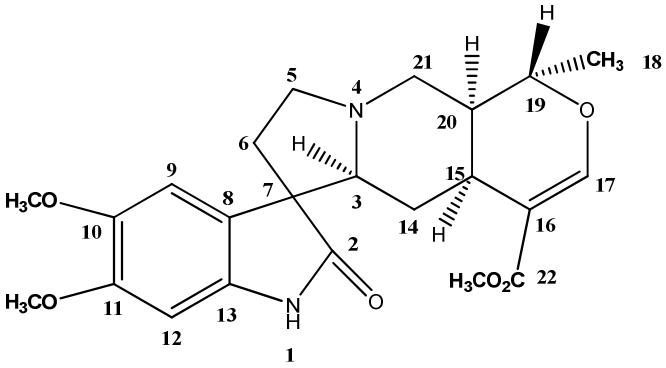
Figure 3.5: HMBC Spectrum of Isoreserpiline



Scheme 3.6: The HMBC Correlations of Isoreserpiline **1**

3.3 Alkaloids from the Leaves of *Ochrosia oppositifolia*

3.3.1 Alkaloid B: Neisosposinine. 2 [48]



2

Neisosposinine **2** was isolated as a brownish amorphous solid. The mass spectrum revealed a pseudo-molecular ion peak at m/z 429.1 $[M+H]^+$ corresponding to the molecular formula of $C_{23}H_{28}N_2O_6$. The other prominent fragmentation peaks were observed at m/z 413, 397 and 369 due to the loss of the methyl group $[M\text{-}CH_3]^+$, methoxyl group $[M\text{-}OCH_3]^+$ and carbomethoxyl group $[M\text{-}CO_2CH_3]^+$ respectively. The fragmentation patterns are shown in Scheme 3.3.

The UV spectrum revealed maxima at 215, 246 and 302 nm which were characteristic for an oxindole system.[49] The IR spectrum (Figure 3.9) displayed a band at 3583 cm^{-1} presence of the NH/OH groups. In addition, a peak was observed at 1704 cm^{-1} (C=O) and 1190 cm^{-1} (C-O band). [48]

The ^1H NMR spectrum (Figure 3.7) showed three singlets at δ 7.40, 6.81 and 6.49 which were assigned to the isolated an olefinic proton H-17 and two aromatic protons

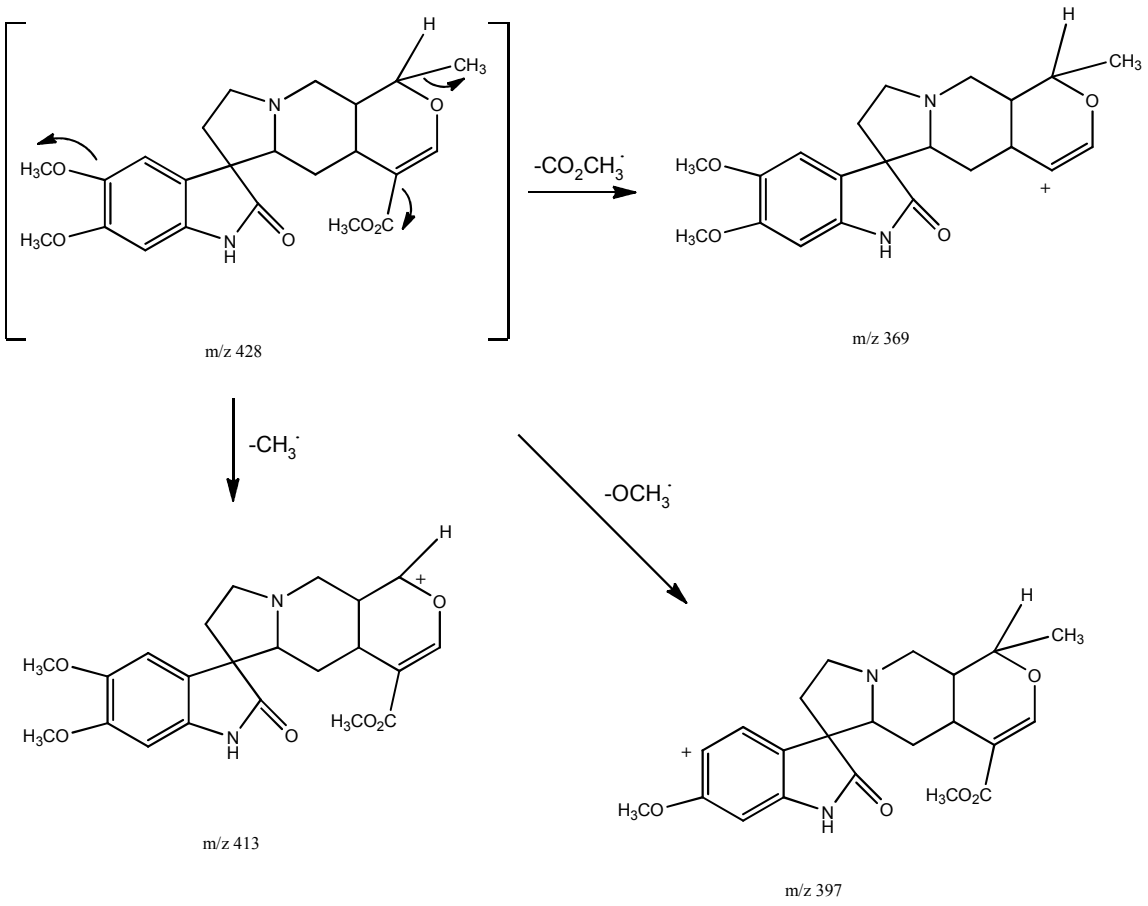
H-9 and H-12 respectively. The spectrum also showed a broad singlet in down-field region at δ 7.79 due to the presence of NH suggested the possibility of an oxindole skeleton.[47] This was further confirmed by the existence of a carbon signal at δ 182.2 (C=O).

In high field region, three singlet signals were appeared at δ 3.87, 3.85 and 3.60 attributed to three methoxyl groups attached to C-10, C-11 and C- 22 respectively.

The typical signal at δ 1.25 confirmed the existence of one methyl group C-18 attached to C-19. This signal appeared as a doublet with *J* value 5.96 Hz. The hypothesis was also supported further by the 1H-1H COSY experiment that showed the following fragments; C-3-C-15, C-5-C-6 and C-18-C-21 which were similar to the previous alkaloid (Isoreserpiline **1**).

The ^{13}C NMR (Figure 3.8), DEPT and HMQC showed the presence of 23 carbon atoms; 8 quaternary carbons; δ 181.2 (C-2), 168.0 (C-22), 146.2 (C-11), 144.9 (C-10), 133.8 (C-13), 124.2 (C-8), 109.9 (C-16) and 56.3 (C-7), 7 methines; δ 155.0 (C-17), 109.3 (C-9), 95.8 (C-12), 72.0 (C-19), 71.3 (C-3), 37.9 (C-20) and 30.4(C-15), and 4 methylene; δ 54.08 (C-21), 54.06 (C-5), 34.5 (C-14) and 30.1 (C-6), 1 methyl; δ 18.5 (C-18), and 3 methoxyls at δ 56. 7 (C_{10}-OMe), 56.6 (C_{11}-OMe) and 51.1 (C_{22}-OMe) respectively.

Detailed analysis of the spectral data and also data comparison with the literature value (Table 3.3.1) confirmed that Alkaloid B **2** is assigned as neisosposinine which was isolated previously from *Neisosperma oppositifolia*. [48]



Scheme 3.3: Possible Mass Fragmentation of Neisosposinine B **2**

Table 3.3.1: ^{1}H NMR [400 MHz, 1_H (J,Hz)] and ^{13}C NMR [100 MHz, $б_C$] of **2** in $CDCl_3$

Position	^{1}H (J,Hz)	^{13}C	^{13}C [48]
2		181.2	181.3
3	2.48 (m)	71.3	72.2
4			
5	3.20 (t , 6.84 Hz) 3.09 (d , 11.88 Hz)	54.06	53.67
6	1.59 (m) 0.86 (q , 11.8 Hz)	30.1	30.1
7		56.3	57. 2
8		124.2	124.6
9	6.81 (s)	109.3	109. 5
10		144.9	144.2
11		146.2	145. 8
12	6.49 (s)	95.8	95.9
13		133.8	133.6
14	1.96 (m) 2.48 (m)	34.5	34.4
15	2.40 (m)	30.4	30.4
16		109.9	110.0
17	7.40 (s)	155.0	155.1
18	1.25 (d , 5.96 Hz)	18.5	18.4
19	4.32(m)	72.0	72.4
20	1.48 (m)	37.9	38.0
21	2.38 (m) 2.40 (m)	54.08	56.3
22		168.0	167.7
OMe	3.87 (s)	56.7	56.8
OMe	3.85 (s)	56.6	56.3
OMe	3.60 (s)	51.1	51.0
NH	7.79 (s)		

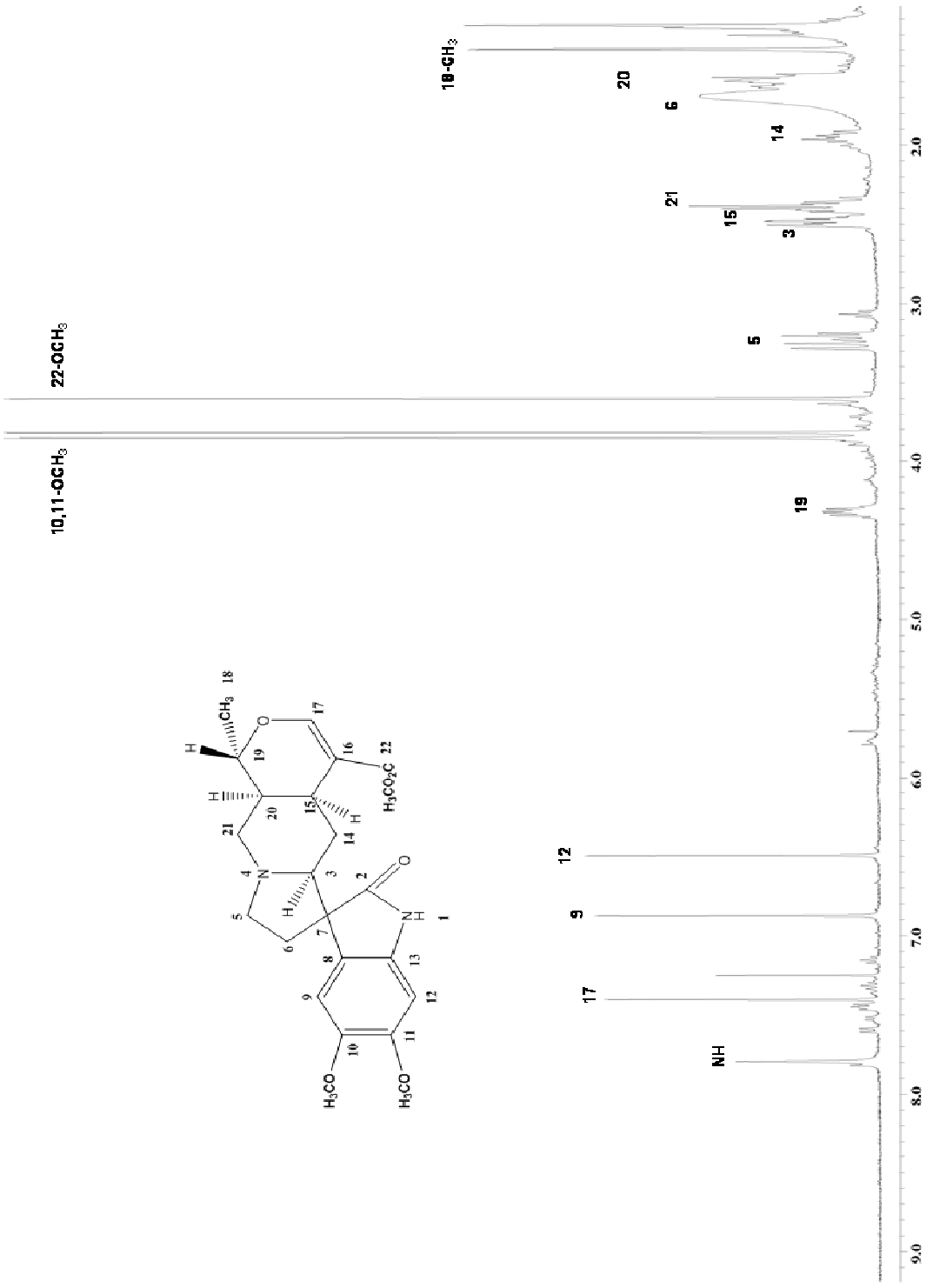


Figure 3.7: ^{1}HNMR Spectrum of Neisuposinine

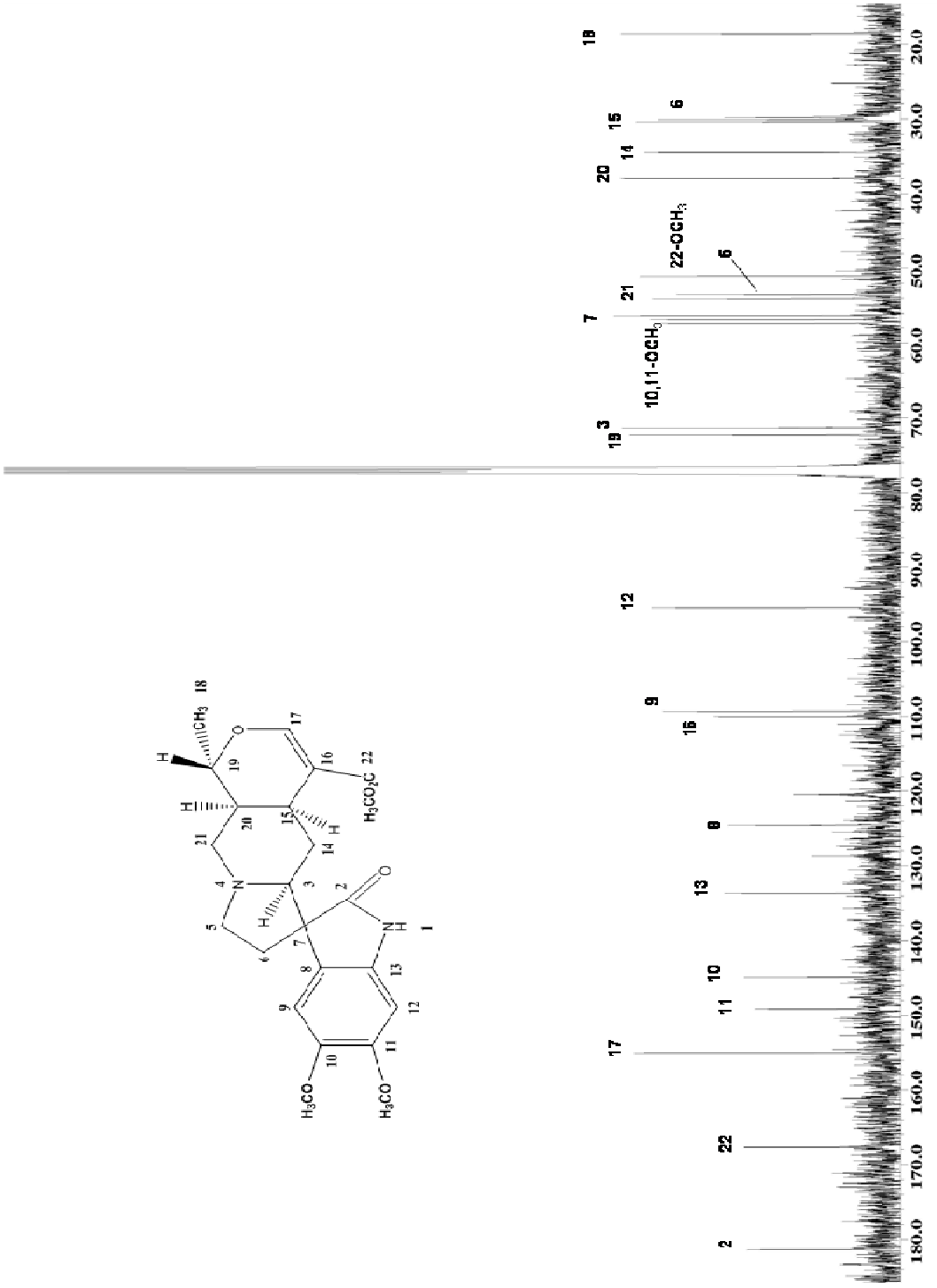


Figure 3.8: ^{13}C NMR Spectrum of Neisuspeimine

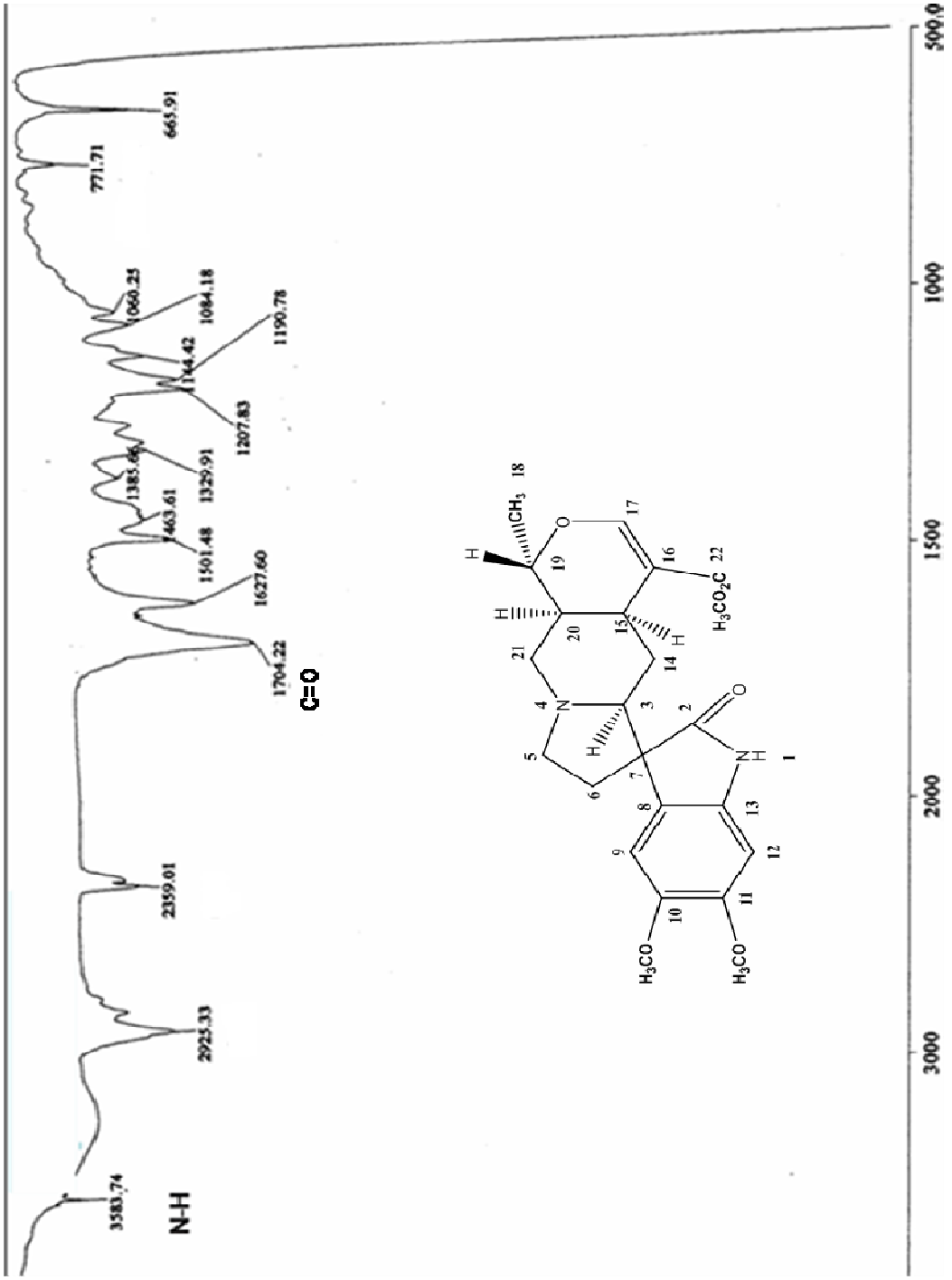


Figure 3.9: IR Spectrum of Nelumpotinine

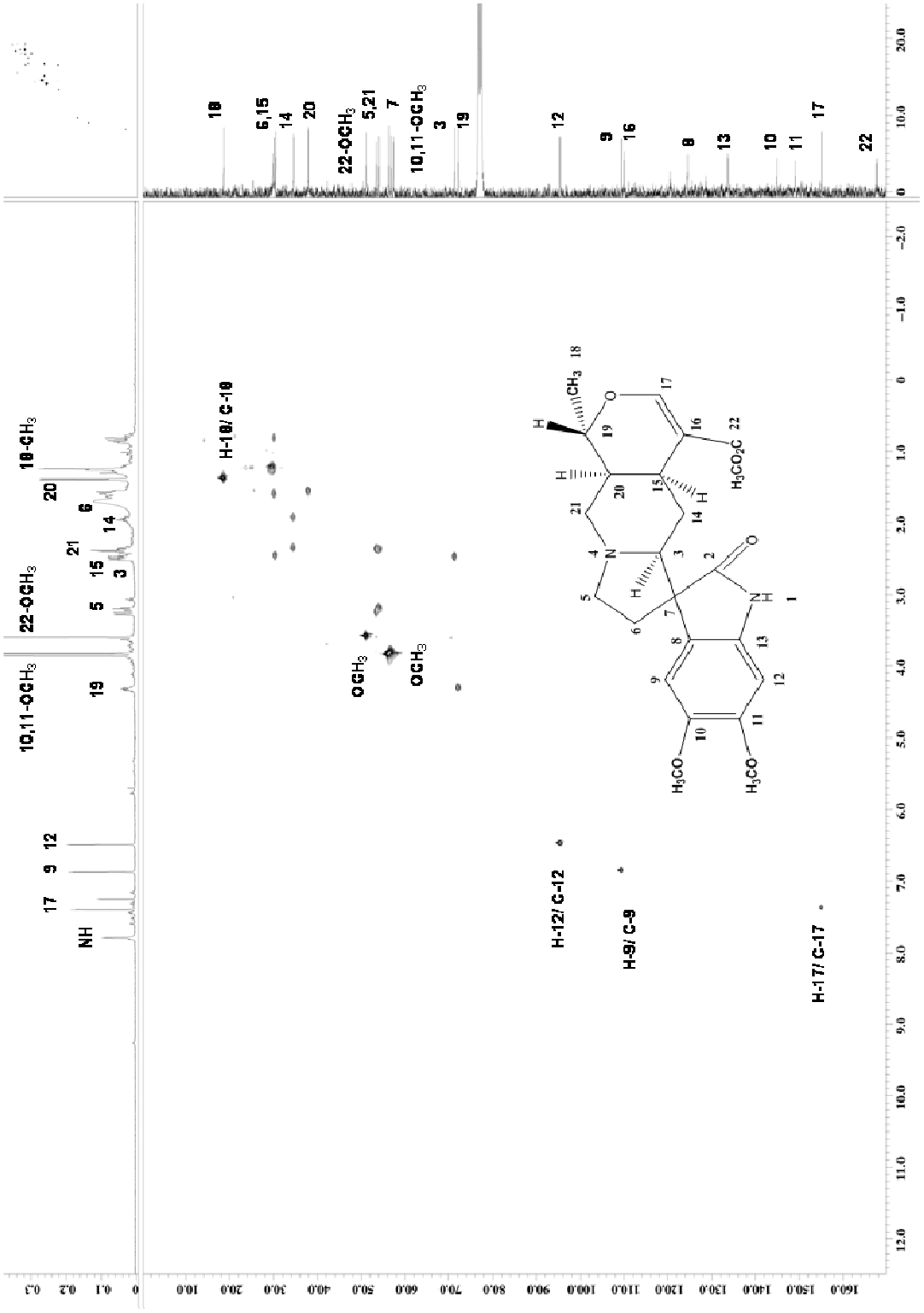


Figure 3.10: HSQC Spectrum of Neisposinine

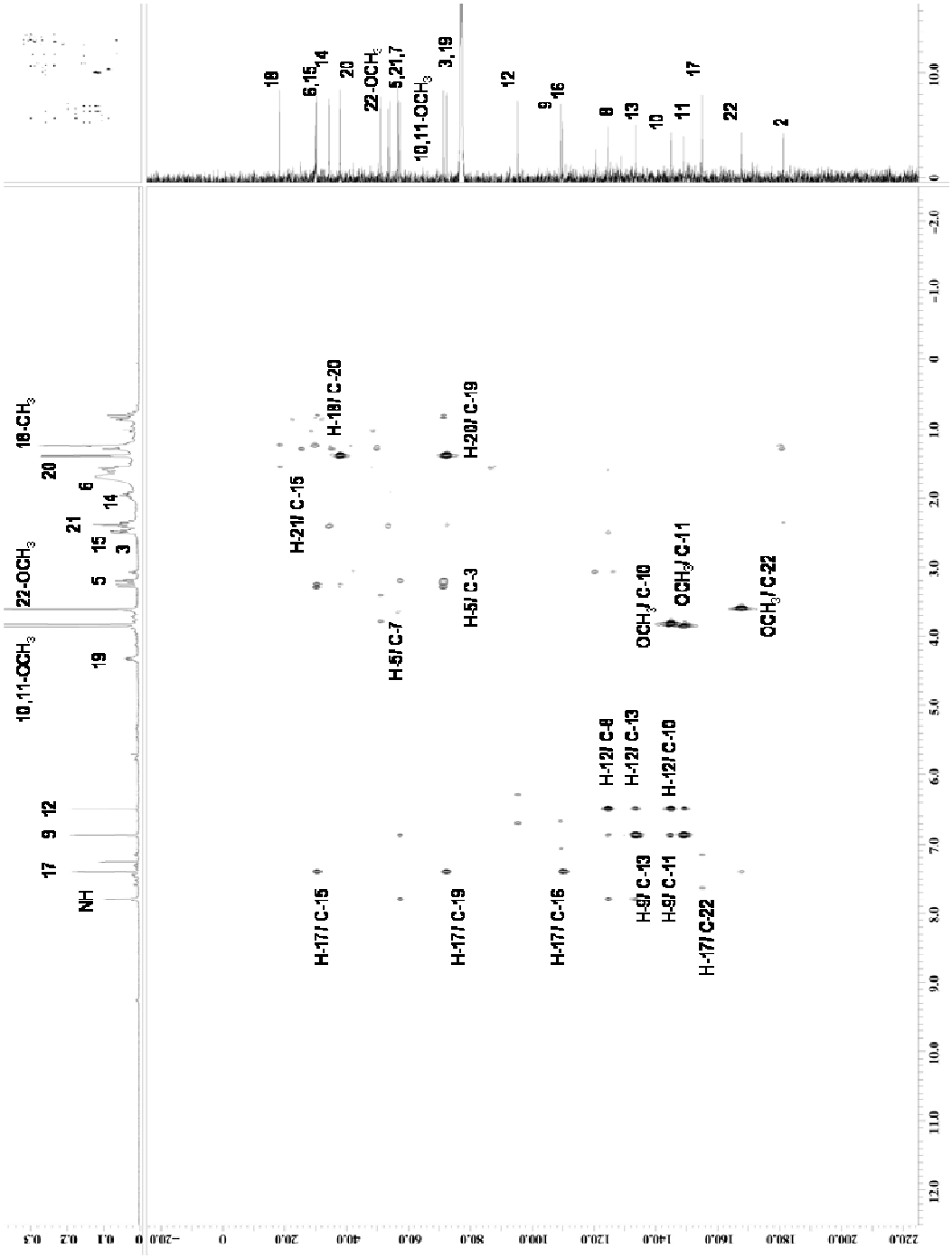
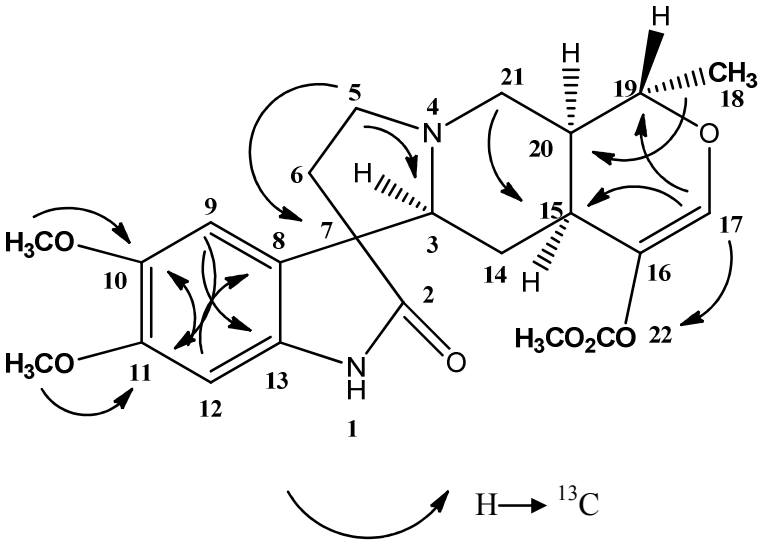
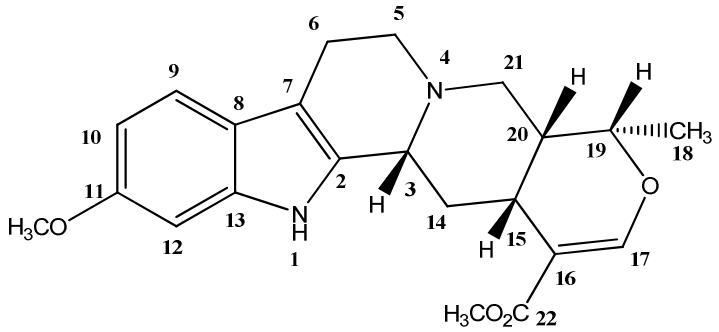


Figure 3.11: HMBC Spectrum of Neisposinine



Scheme 3.12: The HMBC Correlations of Neisosposinine **2**

3.3.2 Alkaloid C: Reserpinine. 3 [50]



3

Reserpinine **3** was isolated as a brownish amorphous solid. The mass spectrum showed a $[M+H]^+$ ion at m/z 383.2 corresponding to the molecular formula of $C_{22}H_{26}N_2O_4$. The UV spectrum revealed maxima at 225 and 300 nm which were attributed typical of an indole system.[51] The IR spectrum (Figure 3.15) displayed a stretching of NH/OH at 3402 cm^{-1}. In addition, a peak was observed at 1706 cm^{-1} which indicated the presence of the carbonyl group. [50]

The ^1H NMR spectrum (Figure 3.13) of alkaloid C **3** is reminiscent of alkaloid A **1** (isoreserpiline). The different between isoreserpiline **1** and resrpinine **3** is existing of one methoxyl group which attached to the C-11 in the isoreserpiline. The spectrum indicated the presence of three aromatic protons at δ 7.28; 6.76 and 6.71 attached to C-9, C-12 and C-10 respectively. The deshielded signal of H-17 appeared at δ 7.53 for confirming the corynanthean skeleton type of reserpinine **3**. In the aliphatic region, two singlets observed at δ 3.80 and 3.70 may be attributed to two methoxyl groups attached to C-11 and C- 22 respectively. The signal for another four aliphatic protons

δ 3.27, 2.8, 4.6 and 1.7 were assigned to H-3, H-15, H-19 and H-20 respectively. Finally a doublet in the high field region at 1.4 (d, 6.8 Hz) may be attributed to the methyl group attached to C-19.

The ^{13}C NMR (Figure 3.14) and DEPT showed the presence of 22 carbon atoms; 7 quaternary carbons; δ 168.2 (C-22), 156.0 (C-11), 136.8 (C-2), 133.4 (C-13), 121.7 (C-8), 109.6 (C-16) and 107.8 (C-7), 8 methines; δ 155.9 (C-17), 118.6 (C-9), 109.5 (C-10), 95.07 (C-12), 72.3 (C-19), 59.9 (C-3), 38.5 (C-20) and 31.4 (C-15), 4 methylens; δ 56.3 (C-21), 53.6 (C-5), 34.2 (C-14) and 21.8 (C-6), 1 methyl; δ 18.6 (C-18), and 2 methoxyls at δ 55.8 (C_{11}-OMe) and 51.2 (C_{22}-OMe) respectively.

Detailed Analysis of the spectral data obtained and assessment with literature value confirmed that alkaloid C **3** is reserpinine which was previously isolated from *Rauvolfia bahiensis*.[50] Complete assignments of compound C **3** were listed in table 3.3.2

Table 3.3.2: ^{1}H NMR [400 MHz, 1_H (J,Hz)] and ^{13}C NMR [100 MHz, $б_C$] of **3** in $CDCl_3$

Position	^{1}H (J,Hz)	^{13}C	^{13}C [49]
2		136.8	133.14
3	3.27 (br d, 11.28 Hz)	59.9	60.0
4			
5	2.5 (m) 2.9 (m)	53.6	53.67
6	2.8 (m) 2.7 (m)	21.8	21.82
7		107.8	107.7
8		121.7	121.9
9	7.28 (d, 8.56 Hz)	118.6	118.2
10	6.71 (dd, 2.4, 8.7 Hz)	109.5	108.7
11		156.0	155.8
12	6.76 (d, 2.2 Hz)	95.07	95.0
13		133.4	130.13
14	2.5 (m) 1.6 (q , 9.86 Hz)	34.2	34.2
15	2.8 (m)	31.4	31.29
16		109.6	109.49
17	7.53 (s)	155.9	155.7
18	1.4 (d , 6.8 Hz)	18.6	18.49
19	4.6 (m)	72.3	72.47
20	1.7 (m)	38.5	38.41
21	3.2 (m) 2.7 (dd , 1.6, 12.2 Hz)	56.3	56.25
22		168.2	168.03
OMe	3.8 (s)	55.8	56.25
OMe	3.7 (s)	51.2	51.10
NH	7.8 (s)		

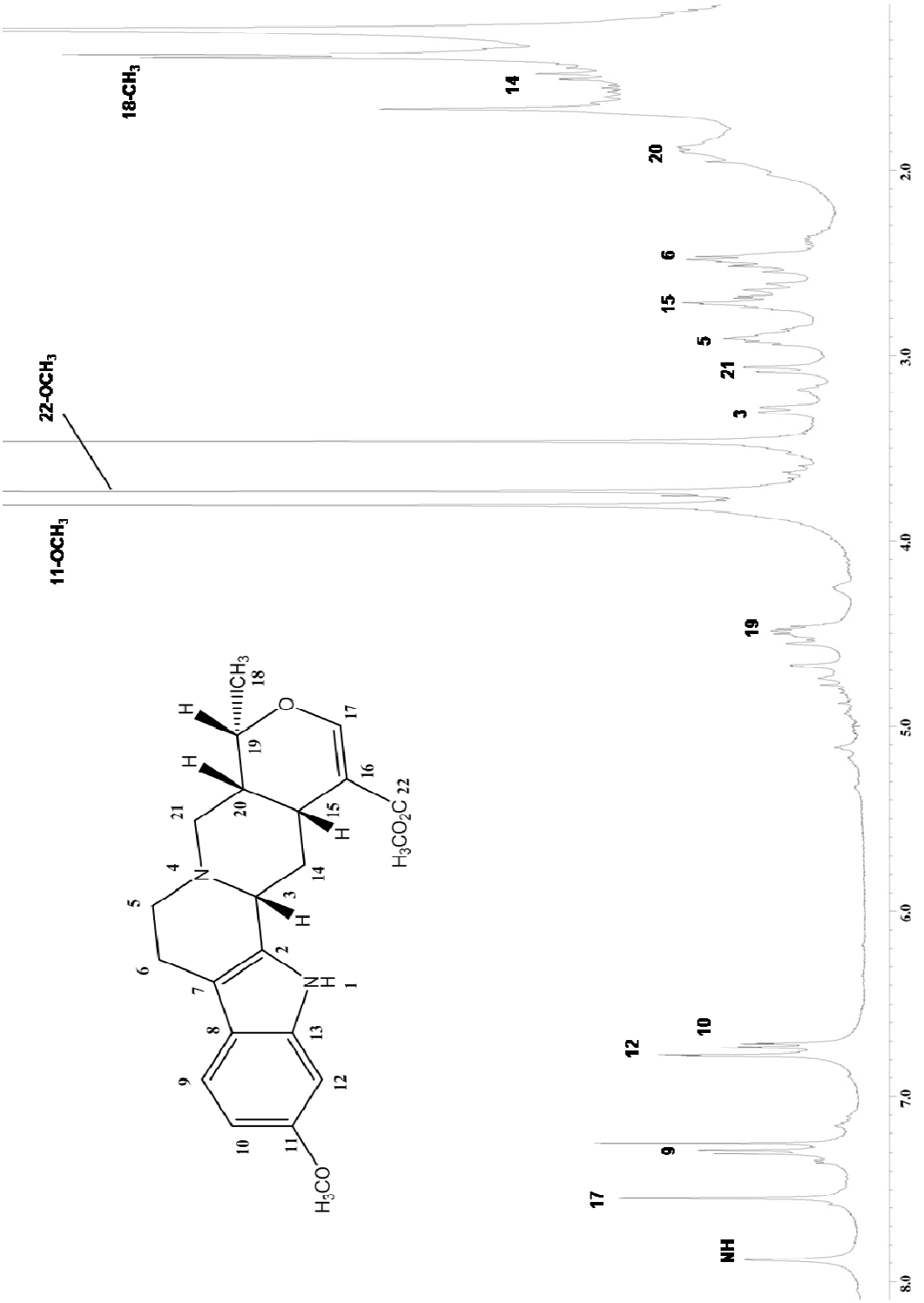


Figure 3.13: ^{1}HNMR Spectrum of Reserpine

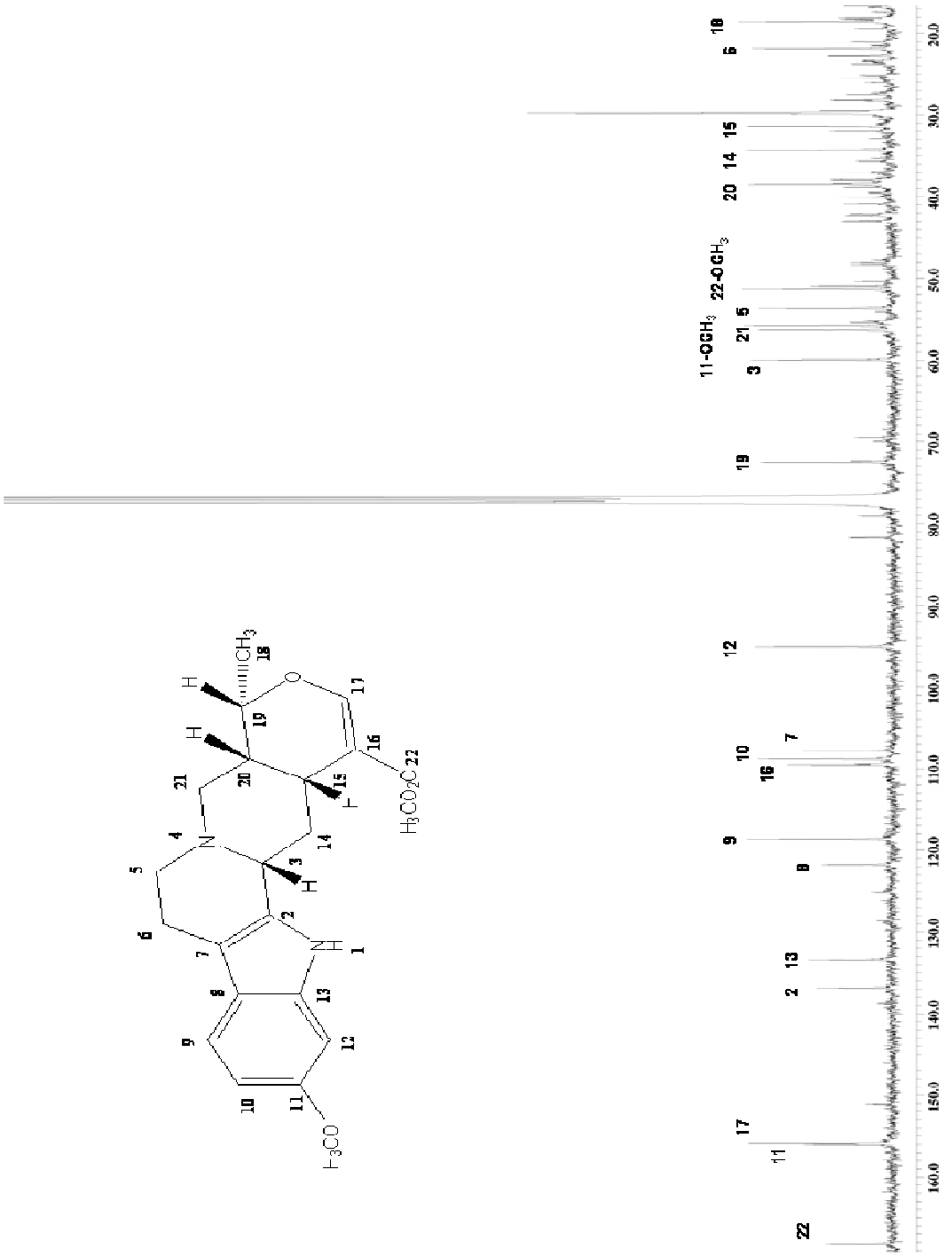


Figure 3.14: ^{13}C NMR Spectrum of Reserpinine

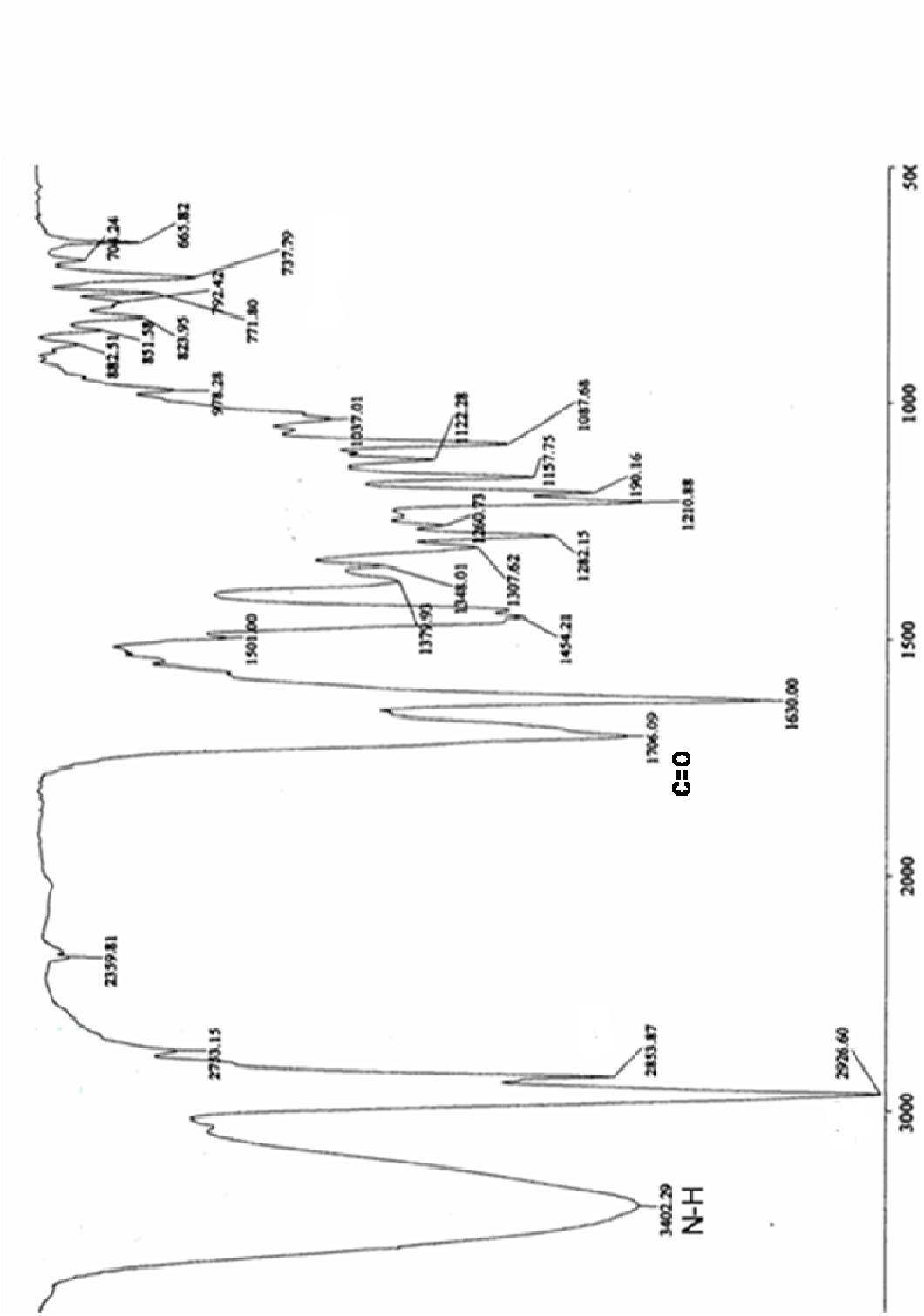


Figure 3.15: IR Spectrum of Reserpine

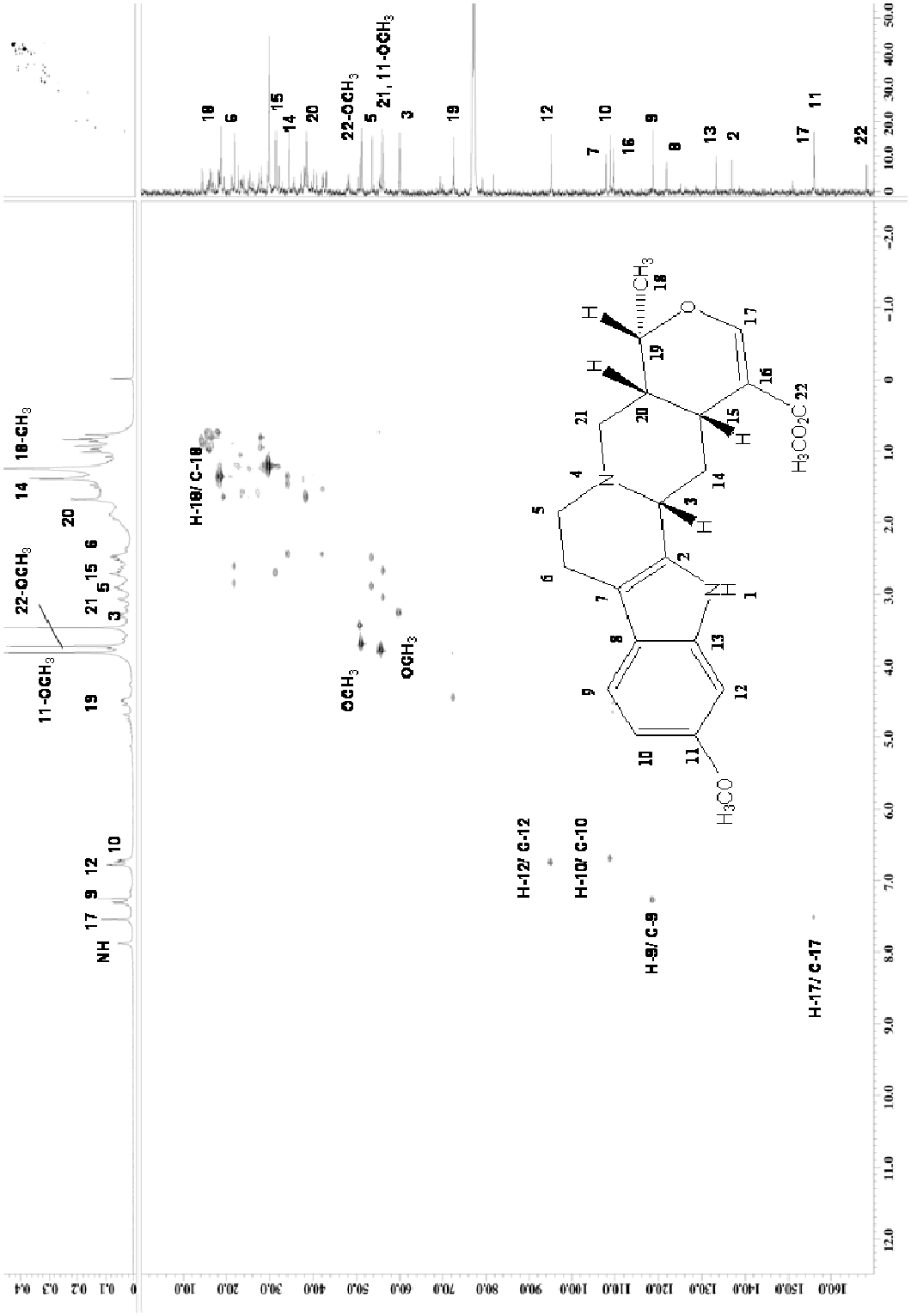


Figure 3.16: HSQC Spectrum of Reserpine

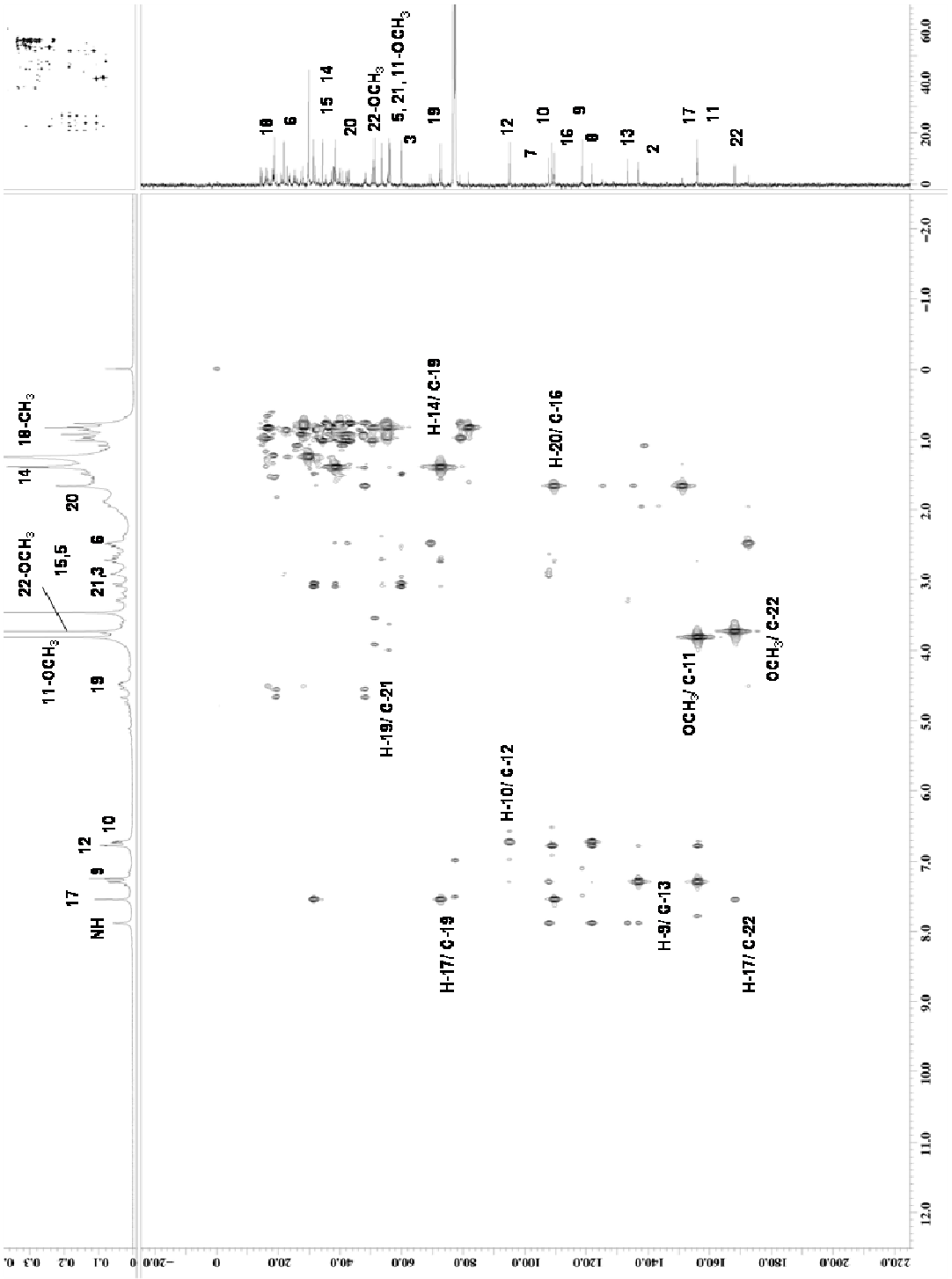
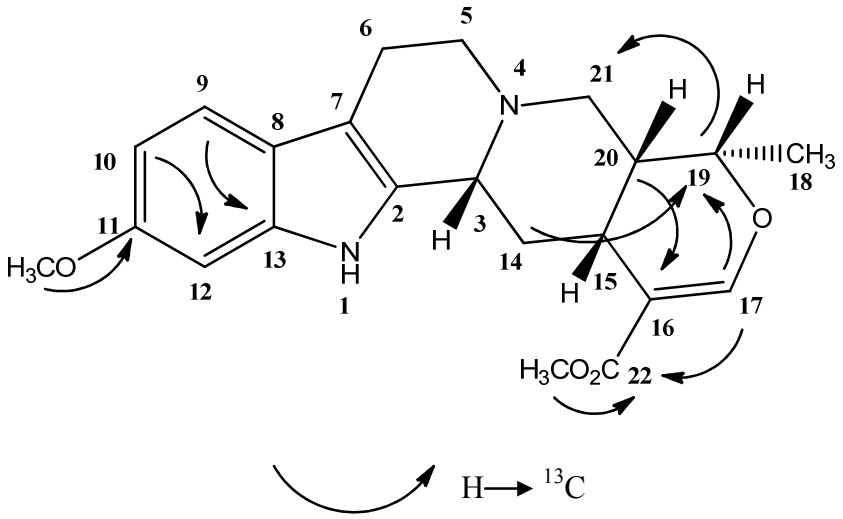
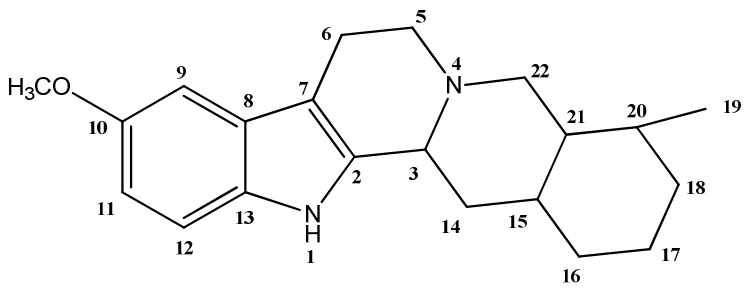


Figure 3.17: HMBC Spectrum of Reserpinine



Scheme 3.18: The HMBC Correlations of Reserpinine **3**

3.3.3 Alkaloid D: 72



72

Alkaloid D **72** was isolated as a brownish amorphous solid. The mass spectrum revealed a pseudo-molecular ion peak at m/z 325.1 $[M+H]^+$ corresponding to the molecular formula of $C_{21}H_{28}N_2O$. The UV spectrum revealed maximum at 250, 285 nm. The IR spectrum (Figure 3.21) showed a band of NH/OH at 3378 cm^{-1}.

The ^1H NMR spectrum (Figure 3.19) indicated the presence of three aromatic protons at δ 7.1, 6.8 and 6.7 attached to C-12, C-9 and C-11, respectively.

In up field region, one singlet peak was appeared at δ 3.8 attributed to one methoxyl group.

There were a total of twelve aliphatic proton signals observed in the ^1H NMR spectrum. Fourteen methylene proton signals appeared between δ 1.2- 3.3 which may be attributed to the protons attached to C-5, C-6, C-14, C-16, C-17, C-18 and C-22. The signal for another four aliphatic protons δ 3.2, 1.5, 1.3 and 3.6 were assigned to H-3, H-15, H-20 and H-21 respectively (Figure 3.19). Finally a doublet in the high field region at δ 0.9 may be attributed to the methyl group attached to C-20.

The ^{13}C NMR (Figure 3.20) and DEPT showed the presence of 21 carbon atoms; 5 quaternary carbons; δ 154.0 (C-10), 135.8 (C-2), 131.2 (C-13), 127.4 (C-8) and 107.9 (C-7), 7 methines; δ 111.5 (C-12), 111.05 (C-11), 100.4 (C-9), 60.03(C-3), 55.8 (C-21), 41.7 (C-15) and 37.1 (C-20), 7 methylens; δ 60.5 (C-22), 53.2 (C-5), 35.5 (C-14), 35.3 (C-18), 30.0 (C-16), 23.5 (C-17) and 21.7 (C-6), 1 methyl; δ 11.1 (C-19) and 1 methoxyl; δ 55.9 (C_{10}-OMe).

The assignments of the position of each carbon were established with DEPT, HMQC and HMBC spectra. Complete assignments of compound D **72** were listed in table 3.3.3 that led to the proposed structure as depicted above (alkaloid D **72**).

Experiments such as NOESY and HRMS need to be done to confirm the structure of alkaloid D **72**, however, these are not achievable at the moment due to lack of sample.

Table 3.3.3: ^{1}H NMR [400 MHz, 1_{H} (J,Hz)] and ^{13}C NMR [100 MHz, $б_{C}$] of **72** in $CDCl_3$

Position	^{1}H (J,Hz)	^{13}C
2		135.8
3	3.2 (d, 11.0 Hz)	60.03
4		
5	2.9 (m) 2.4 (m)	53.2
6	2.8 (m) 2.3 (m)	21.7
7		107.9
8		127.4
9	6.8 (s)	100.4
10		154.0
11	6.7 (d, 6.4 Hz)	111.05
12	7.1 (d, 10.2 Hz)	111.5
13		131.2
14	1.2 (m) 1.4 (m)	35.5
15	1.5(m)	41.7
16	1.1 (m) 1.3 (m)	30.0
17	1.0 (m) 1.7(m)	23.5
18	1.9 (m) 2.1(m)	35.3
19	0.9 (d, 15.1)	11.1
20	1.3 (m)	37.1
21	3.6 (m)	55.8
22	1.9 (m) 3.5 (m)	60.5
OMe	3.8 (s)	55.9
NH	8.0 (s)	

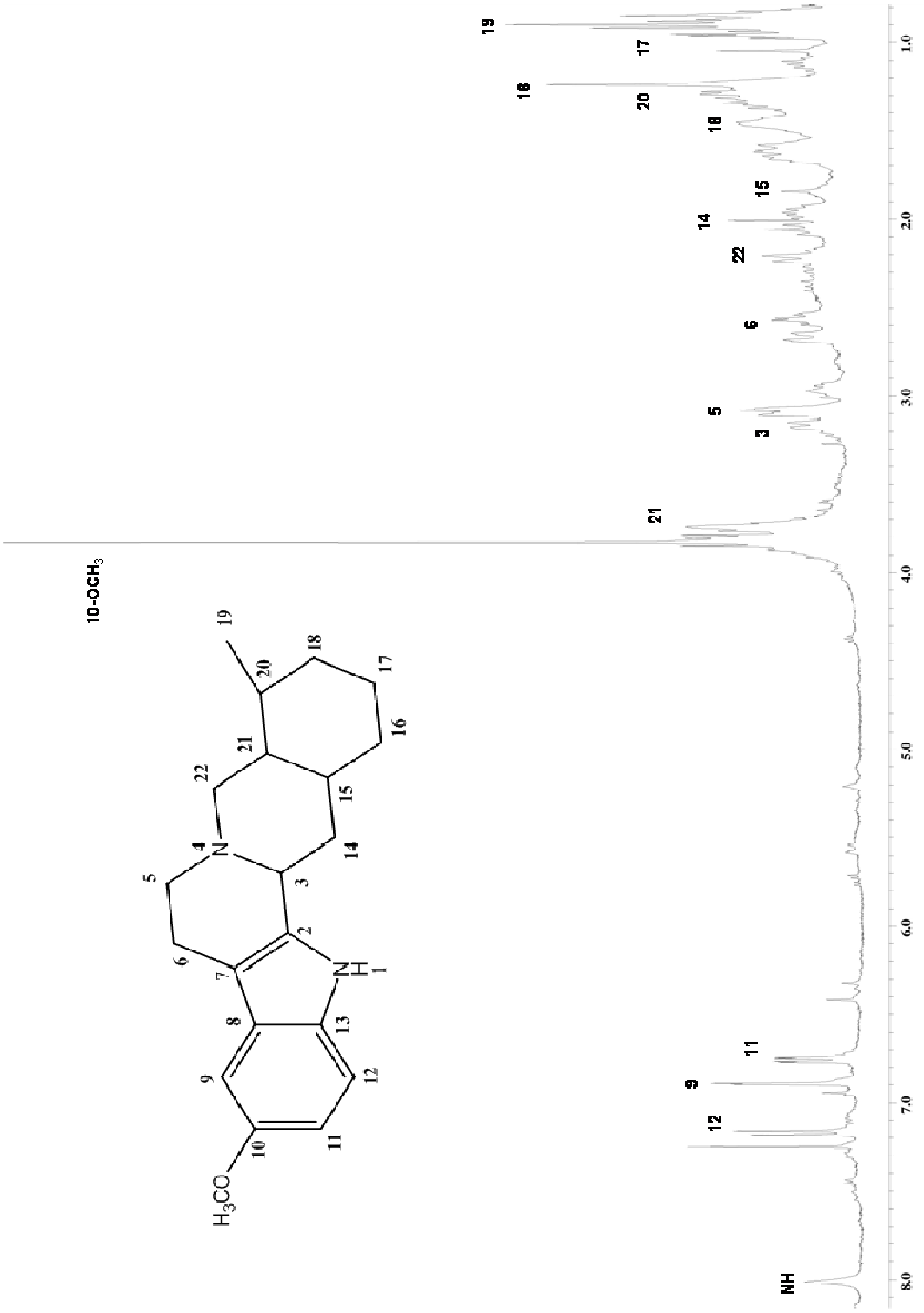


Figure 3.19: ^{1}HNMR Spectrum of Alkaloid D 72

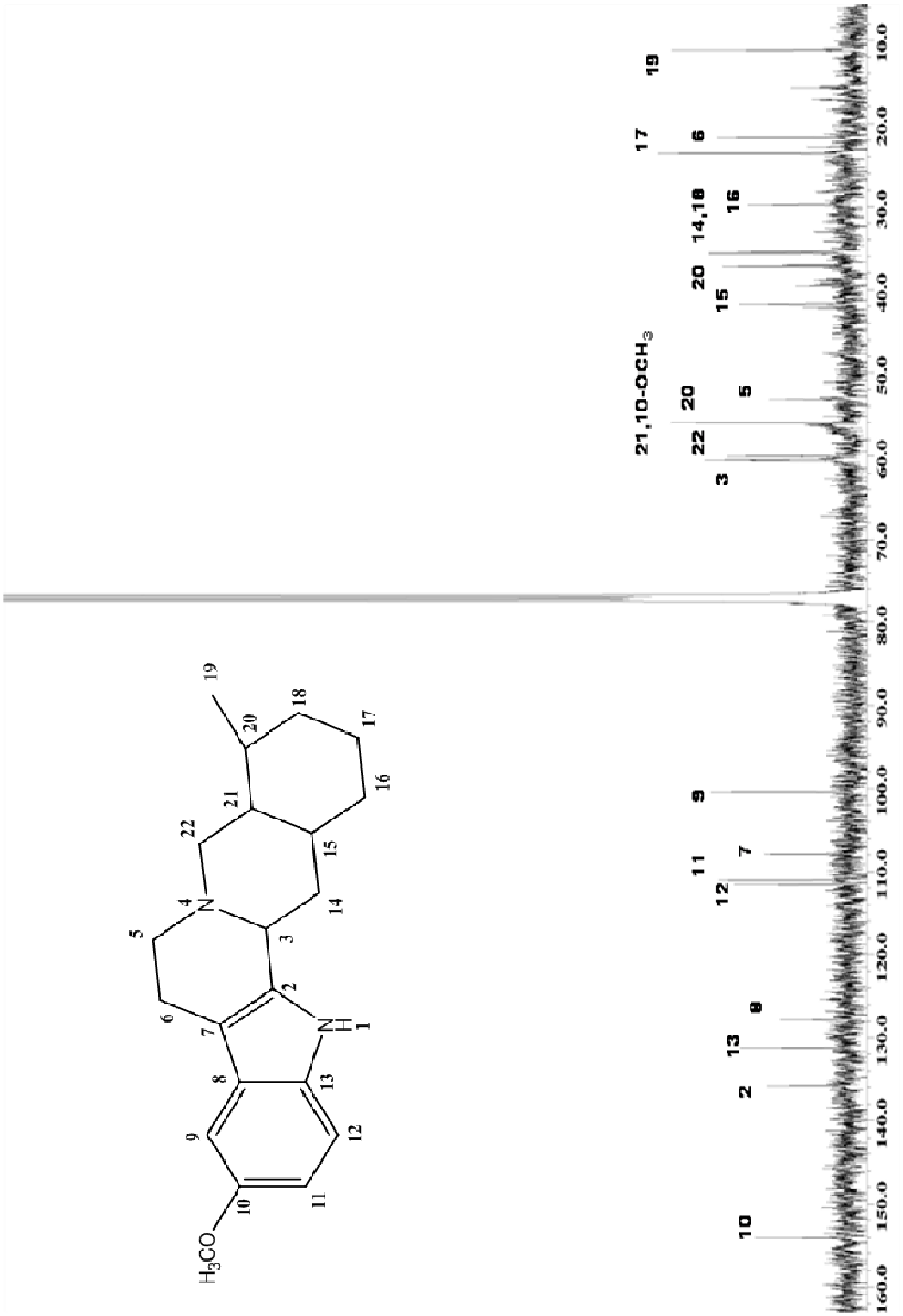


Figure 3.20: ^{13}C NMR Spectrum of Alkaloid D 72

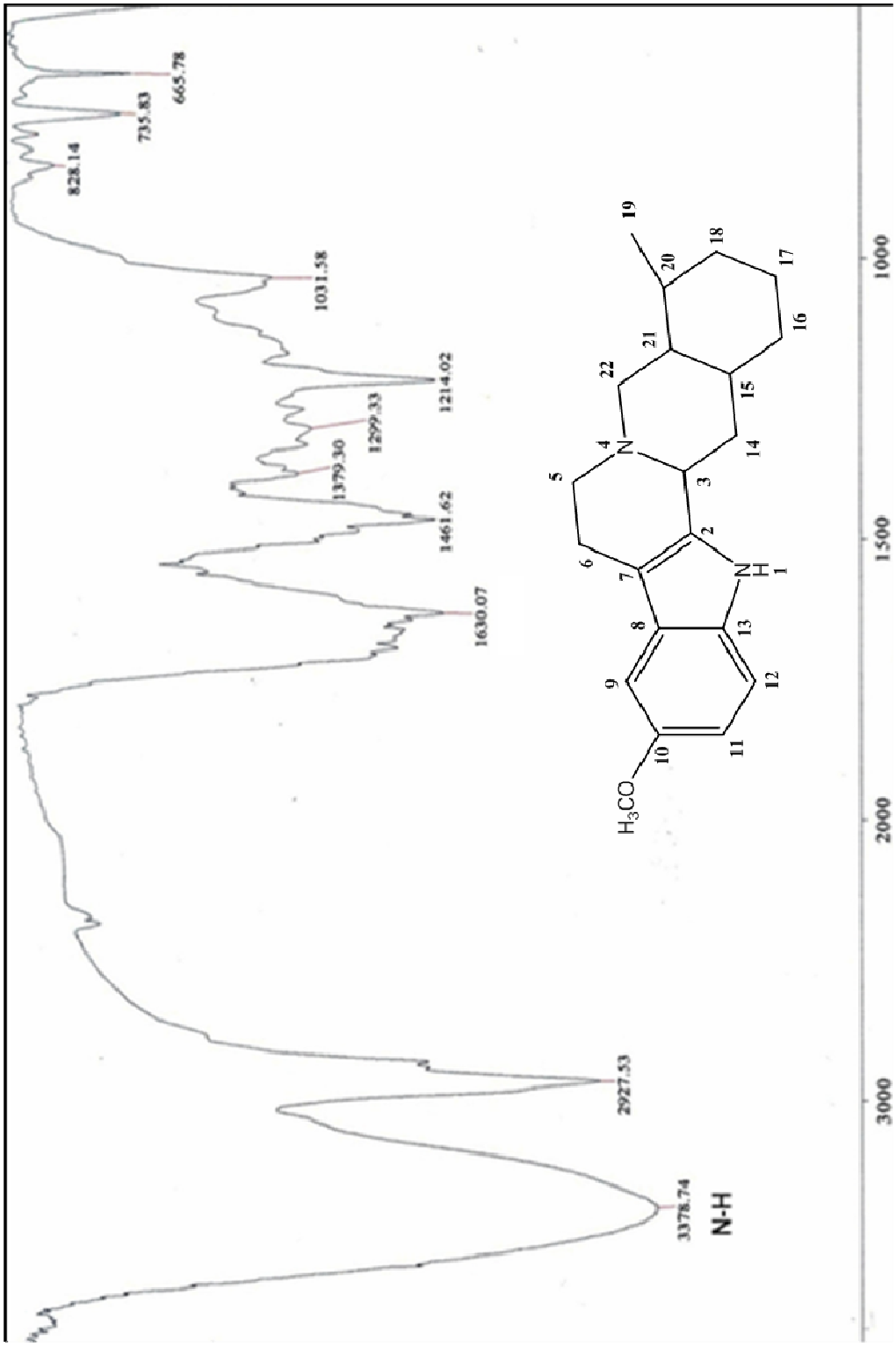


Figure 3.21: IR Spectrum of Alkaloid D 72

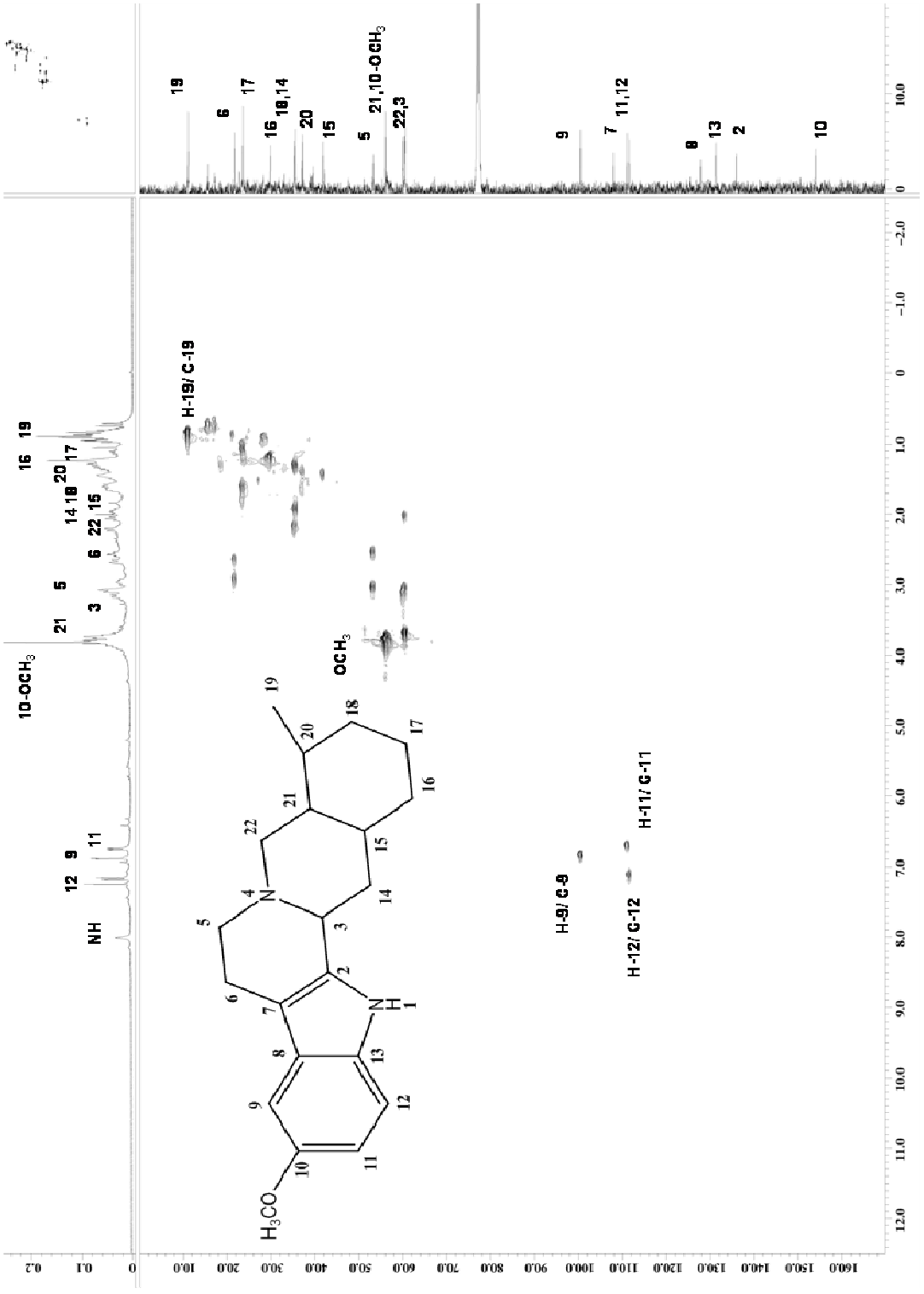


Figure 3.22: HSQC Spectrum of Alkaloid D 72

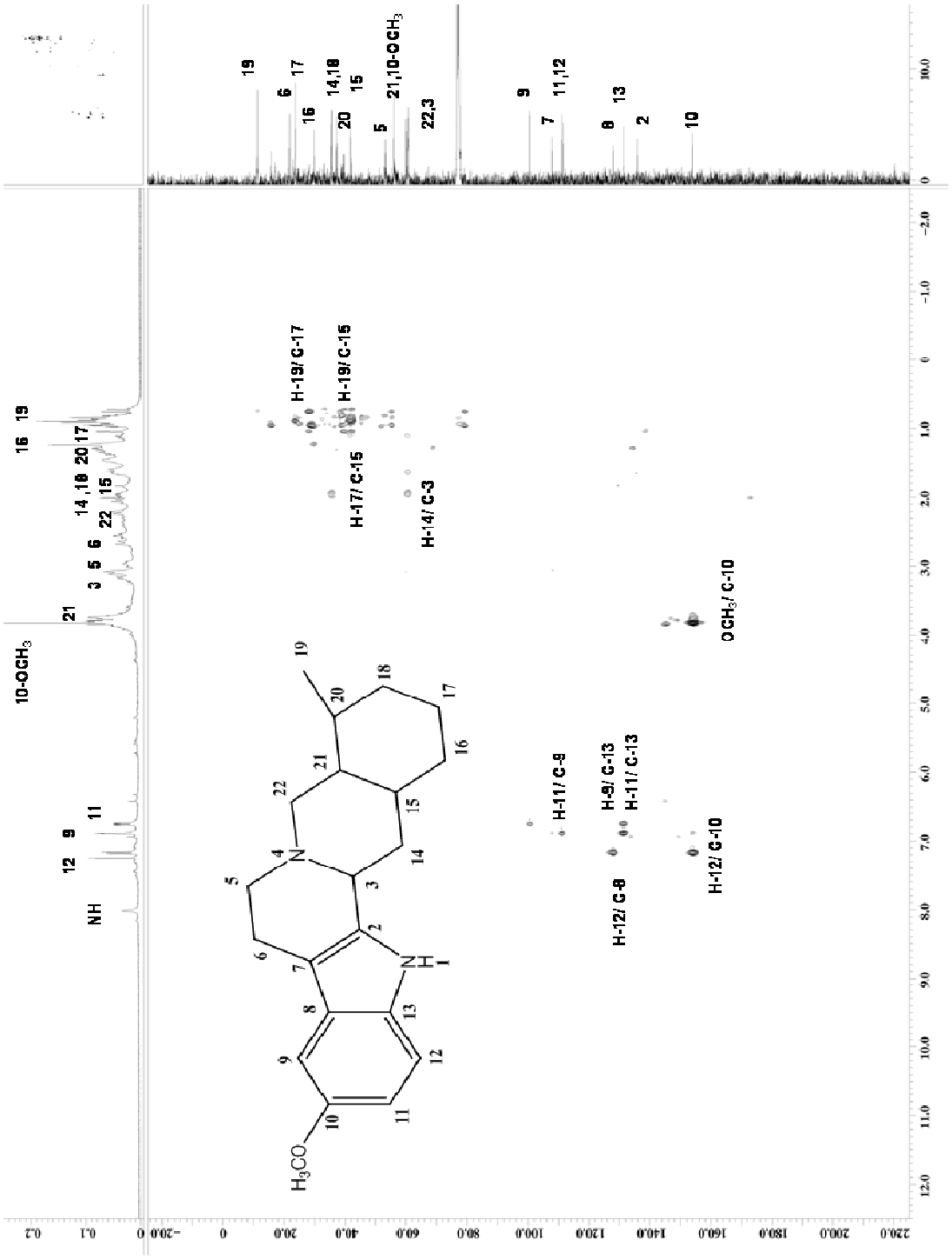
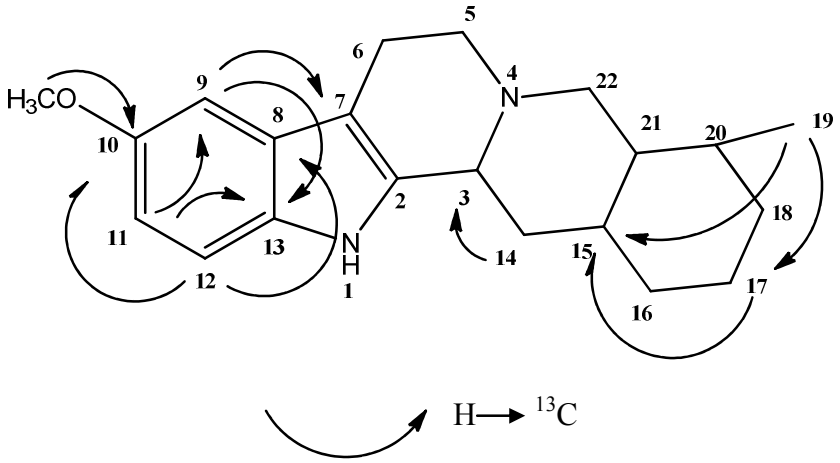


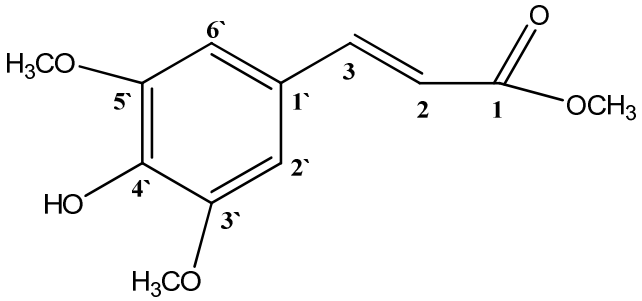
Figure 3.23: HMBC Spectrum of Alkaloid D 72



Scheme 3.24: The HMBC Correlations of Alkaloid D **72**

3.4 Ferulic Acid Esters from the Bark of *Ochrosia oppositifolia*

3.4.1 Ferulic Acid Ester E: 2-propenoic acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)-, methyl ester. 73



73

Ferulic acid ester E **73** was isolated as a whitish amorphous solid. The mass spectrum revealed a pseudo-molecular ion peak at m/z 238.09 $[M+H]^+$ corresponding to the molecular formula of $C_{12}H_{14}O_5$. The UV spectrum revealed maximum at 196, 273 and 328 nm. The IR spectrum (Figure 3.27) showed a band of OH at 3401 cm^{-1}. In addition, a peak was observed at 1704 cm^{-1} which indicated the presence of the conjugated carbonyl of an ester.

The ^1H NMR spectrum (Figure 3.25) showed two overlapping aromatic protons at δ 6.77 attributed to H-6` and H-2` respectively. In the up field region, three singlets appeared at δ 3.92 and 3.91 and 3.79 attributed to three methoxyls attached to C- 3`, C- 5` and C- 1 respectively.

The hypothesis was also supported further by the cross peaks of COSY experiment that showed existence of two sets of doublet (J = 16 Hz) in the ^{1}H NMR at δ 6.29 and 7.59 which corresponded to the resonances of H-2 and H-3.

The ^{13}C NMR (Figure 3.26) and DEPT showed the presence of 12 carbon atoms; four quaternary δ 147.2 (C-3` and C-5`), 137.1 (C-4`), and 125.9 (C-1`); four methines δ 145.2 (C-3), 115.6 (C-2), and 105.09 (C-2` and C-6`); three methoxyl δ 56.4 (OMe 3` and OMe 5`) and 51.7 (OMe 1). One peak appeared in δ 167.6 which is carbonyl of (C-1). The cross peak between H-3 and C-1 as observed from the HMBC spectrum of compound E **73** (Figure 3.29) indicated that C-1 is vicinal to the olefinic carbon, C-3.

The comprising of data; DEPT, HMQC and HMBC spectra, established the identify of compound E **73**.[52] Complete assignments of compound E **73** were listed in table 3.4.1.

Table 3.4.1: ^{1}H NMR [400 MHz, 1_H (J,Hz)] and ^{13}C NMR [100 MHz, $б_C$] of **73** in $CDCl_3$

Position	^{1}H (J,Hz)	^{13}C
1		167.6
2	6.29 (d, 16 Hz)	115.6
3	7.59 (d, 16 Hz)	145.2
1`		125.9
2`	6.77(s)	105.1
3`		147.2
4`		137.1
5`		147.2
6`	6.77 (s)	105.1
OMe 3`	3.92 (s)	56.4
OMe 5`	3.91 (s)	56.4
OMe 1	3.79 (s)	51.7
OH	5.80 (s)	

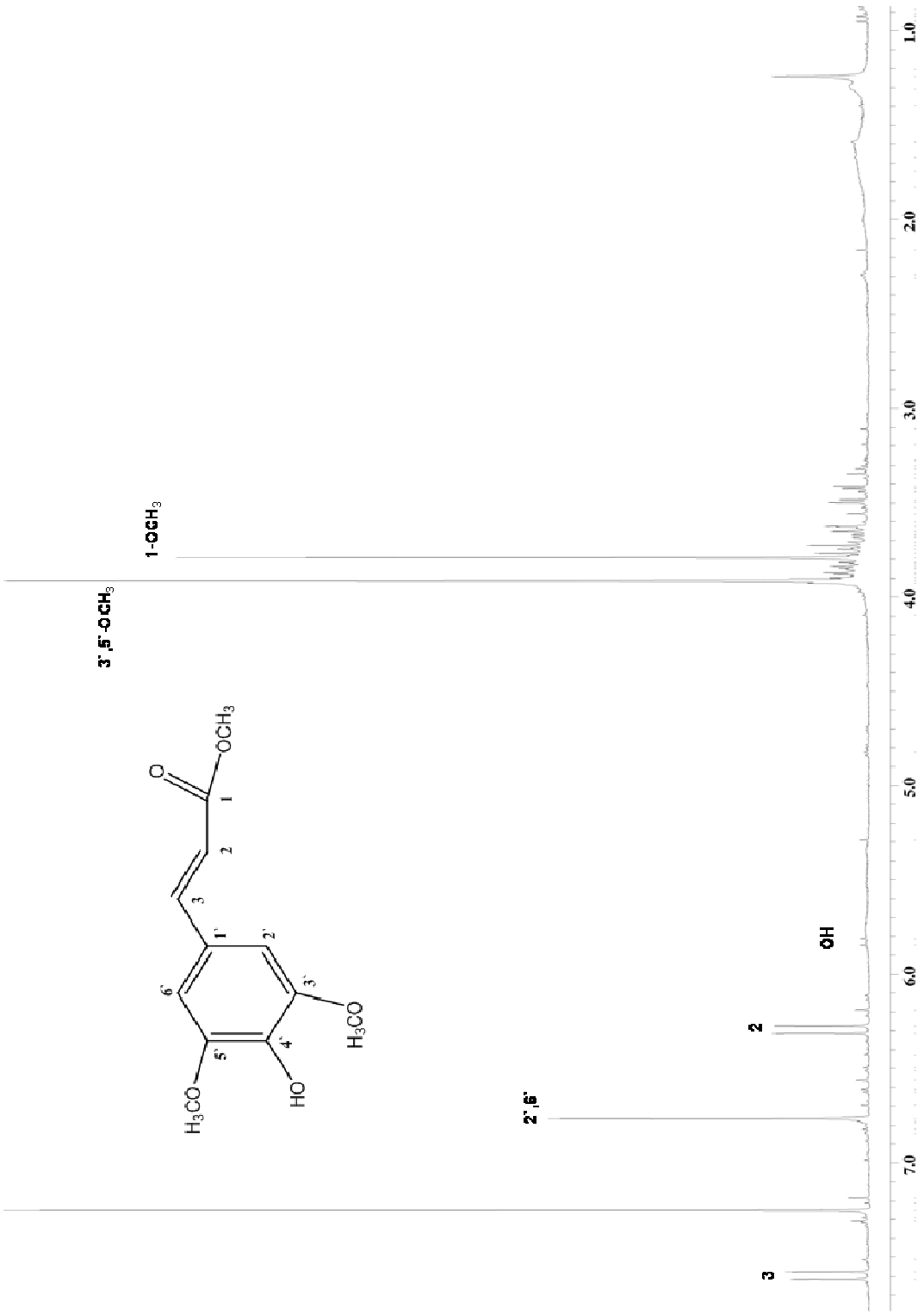


Figure 3.25: ^{1}HNMR Spectrum of 2-propenoic acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)-, methyl ester

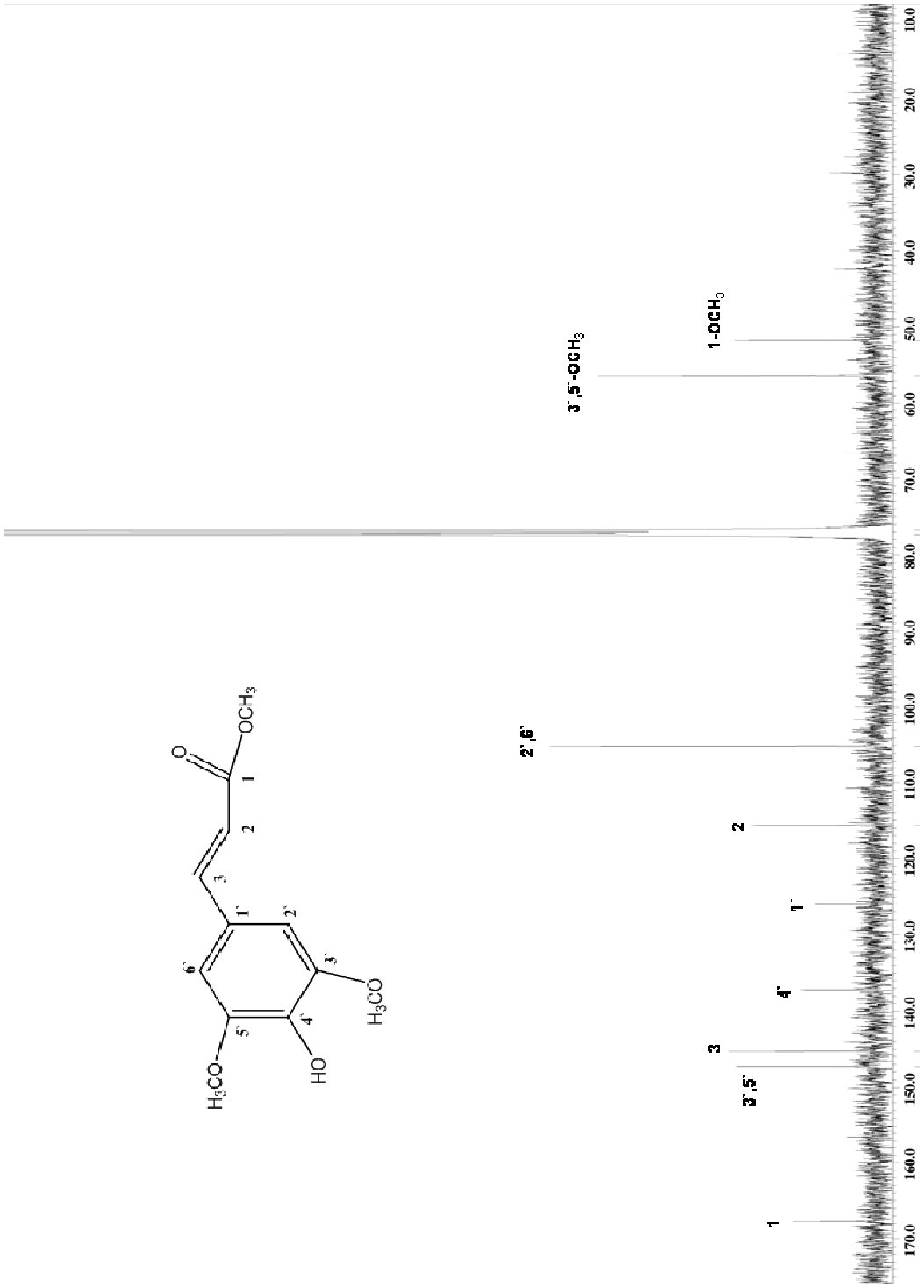


Figure 3.26: ^{13}C NMR Spectrum of 2-propenoic acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)-, methyl ester

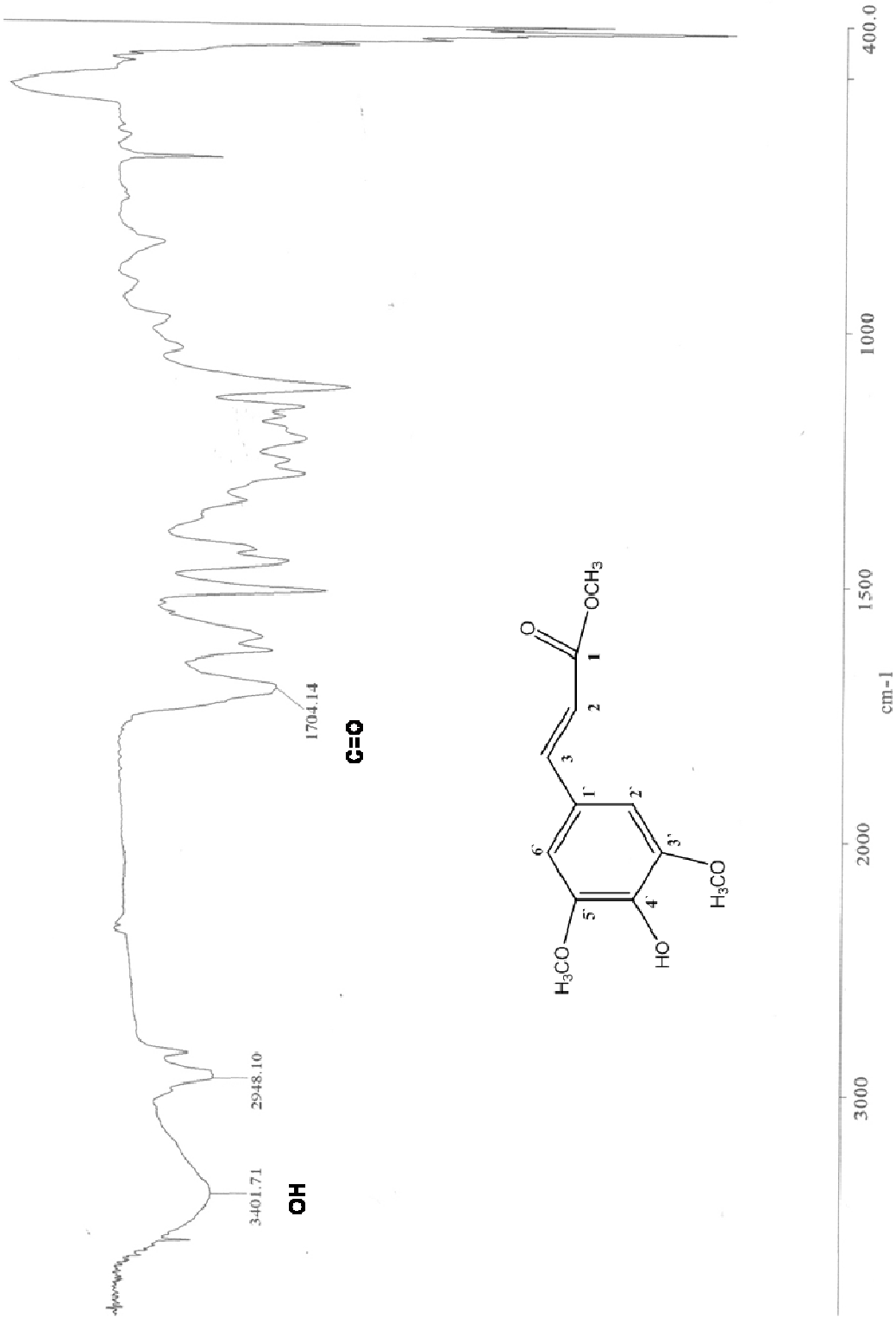


Figure 3.27: IR Spectrum of 2-propenoic acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)-, methyl ester

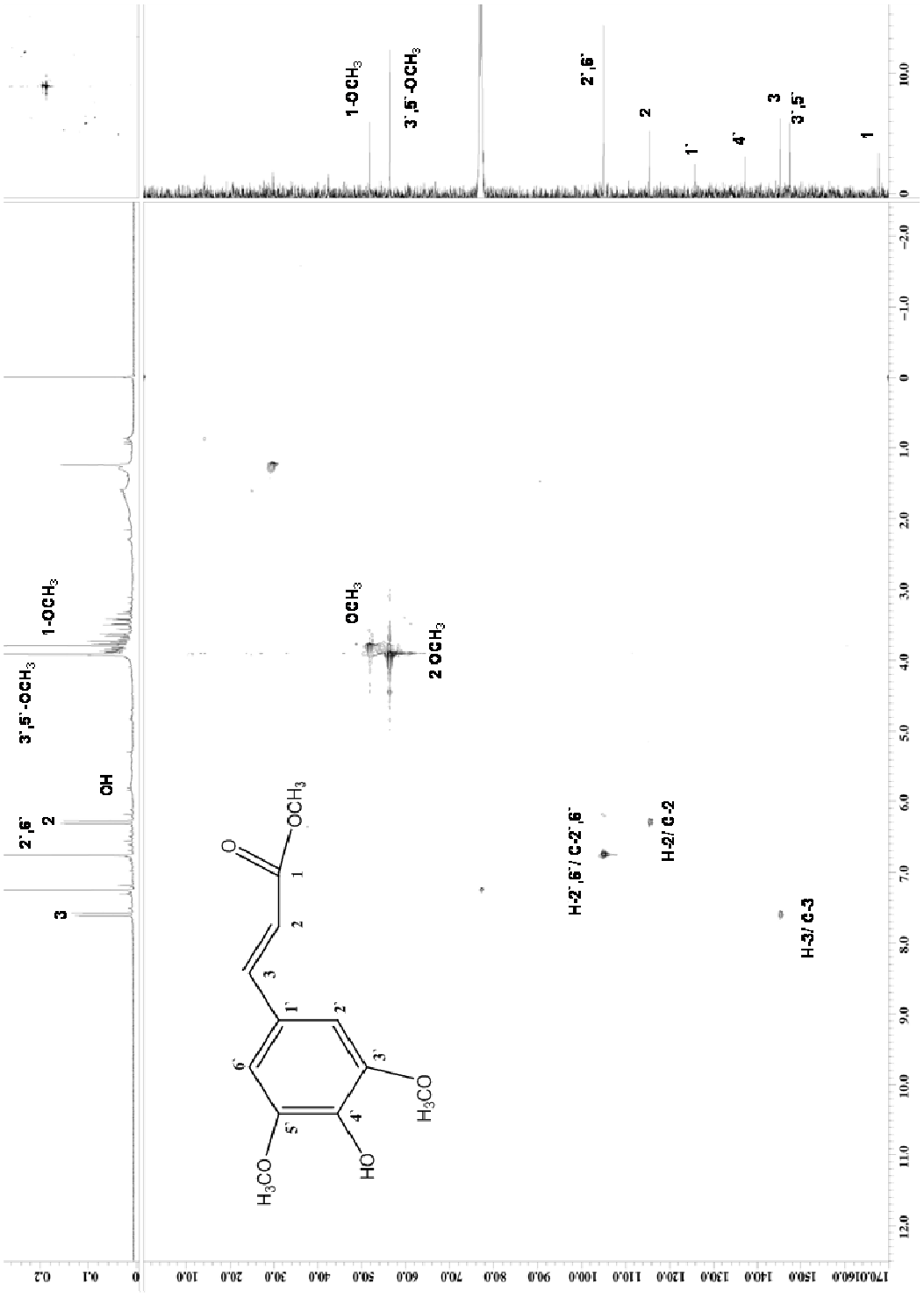


Figure 328: HSQC Spectrum of 2-propenoic acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)-, methyl ester

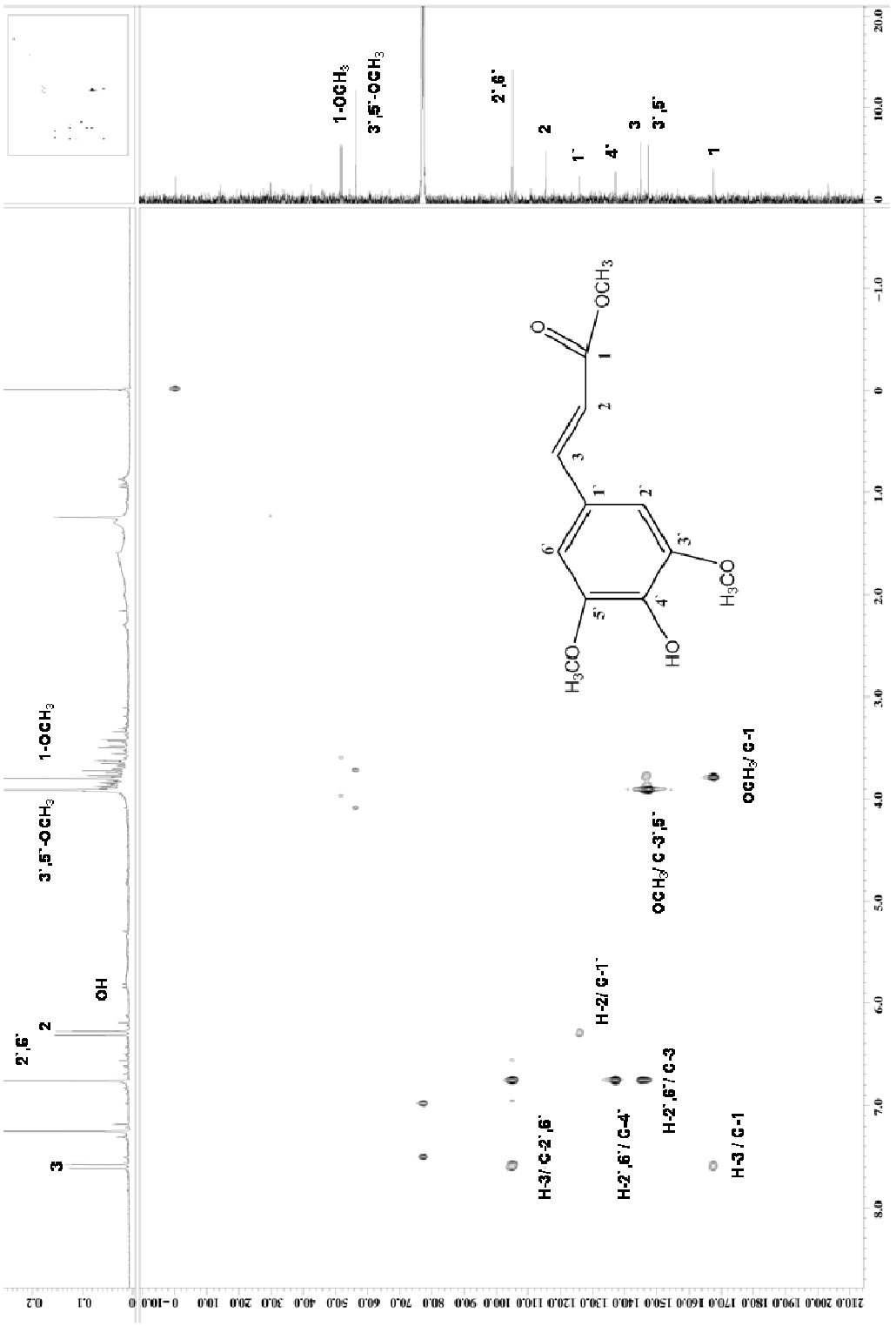
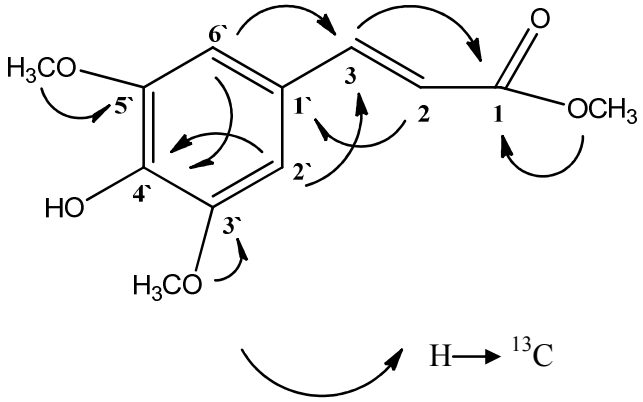
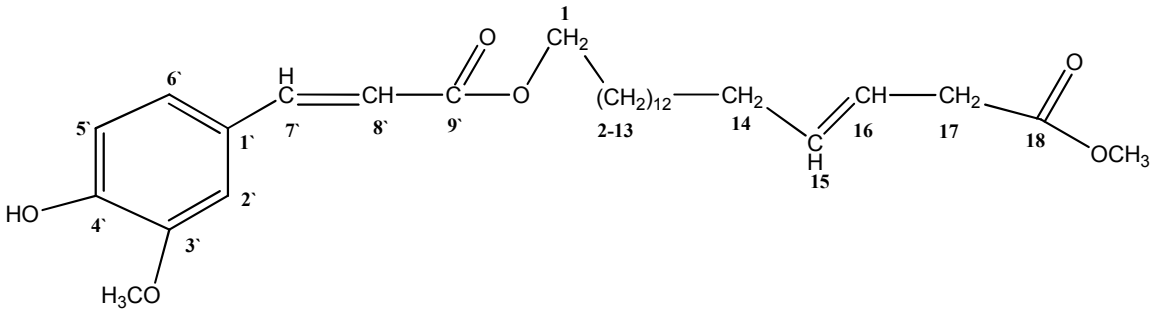


Figure 3.29: HMBC Spectrum of 2-propenoic acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)-, methyl ester



Scheme 3.30: The HMBC Correlations of: 2-propenoic acid, 3-(4-hydroxy-3,5-dimethoxyphenyl)-, methyl ester **73**

3.4.2 Ferulic Acid Ester F: 17-methoxy-carbonyl-14- heptadecaenyl- 4-hydroxy-3-methoxy cinnamate. 74



74

Ferulic acid ester F **74** was isolated as a whitish amorphous solid. The mass spectrum revealed a pseudo-molecular ion peak at m/z 488.3 $[M+H]^+$ corresponding to the molecular formula of $C_{29}H_{44}O_6$. The UV spectrum revealed maximum at 196, 273 and 328 nm. The IR spectrum (Figure 3.33) showed a band of OH at 3431 cm^{-1}. In addition, two peaks were observed at 1711 and 1738 cm^{-1} which implied the presence of two carbonyl group.

The ^1H NMR spectrum (Figure 3.31) indicated the presence of three aromatic protons at δ 7.10, 7.01 and 6.89 attached to C-6`, C-2` and C-5`, respectively.

In up field region, two singlets appeared at δ 3.91 and 3.64 attributed to two methoxyl groups attached to C-3` and C-18 respectively.

The cross peak of COSY showed one coupling set (H-7`- H-8`). This was confirmed by the existence of two sets of doublet (J = 16 Hz) in the ^1H NMR at δ 7.50 and 6.27 which corresponded to the resonances of H-7` and H 8`, respectively.

The correlation between H-14 and C-15, 16 in the HMBC established the position of double bond in the long chain and also correlation between H-1 and C=O 9` supported the chain connections. (Figure 3.35).

The ^{13}C NMR (Figure 3.32) and DEPT showed the presence of 29 carbon atoms; three quaternary carbons; δ 147.9 (C-4`), 146.8 (C-3`), and 127.1 (C-1`), seven methines; δ 144.7 (C-7`), 129.9 (C-15,16), 123.1 (C-6`), 115.7 (C-8`), 114.7 (C-5`) and 109.3 (C-2`), fifteen methylenes; δ 64.6 (C-1), 34.02 (C-17) and the rest thirteen methylens overlapped in the region between δ 29.2 until 29.8.Two methoxyls; δ 56.01 ($C_{3`}$-OMe) and 51.5 ($C_{18}$-OMe). There are two carbonyls which appeared at δ 174.4 (C=O 18) and 167.4 (C=O 9`).

The assignments of the position of each carbon were confirmed with DEPT, HMQC and HMBC spectra. Complete assignments of compound F **74** were listed in table 3.4.2.

Table 3.4.2: ^{1}H NMR [400 MHz, 1_H (J,Hz)] and ^{13}C NMR [100 MHz, $б_C$] of **74** in $CDCl_3$

Position	^{1}H (J,Hz)	^{13}C
1	4.1 (t, 6.8, Hz)	64.6
2-13	1.28-1.58	29.2-29.7
14	2.1 (d, 5.4 Hz)	29.8
15	5.3 (m)	129.9
16	5.3(m)	129.9
17	2.3 (t, 7.7 Hz)	34.02
18		174.4
1`		127.1
2`	7.01(s)	109.3
3`		146.8
4`		147.9
5`	6.90 (d, 8.2 Hz)	114.7
6`	7.10 (d, 8.2 Hz)	123.1
7`	7.50 (d, 16 Hz)	144.7
8`	6.27(d, 16 Hz)	115.7
9`		167.4
OMe 18	3.64 (s)	51.5
OMe 3`	3.91 (s)	56.01
OH	5.9 (s)	

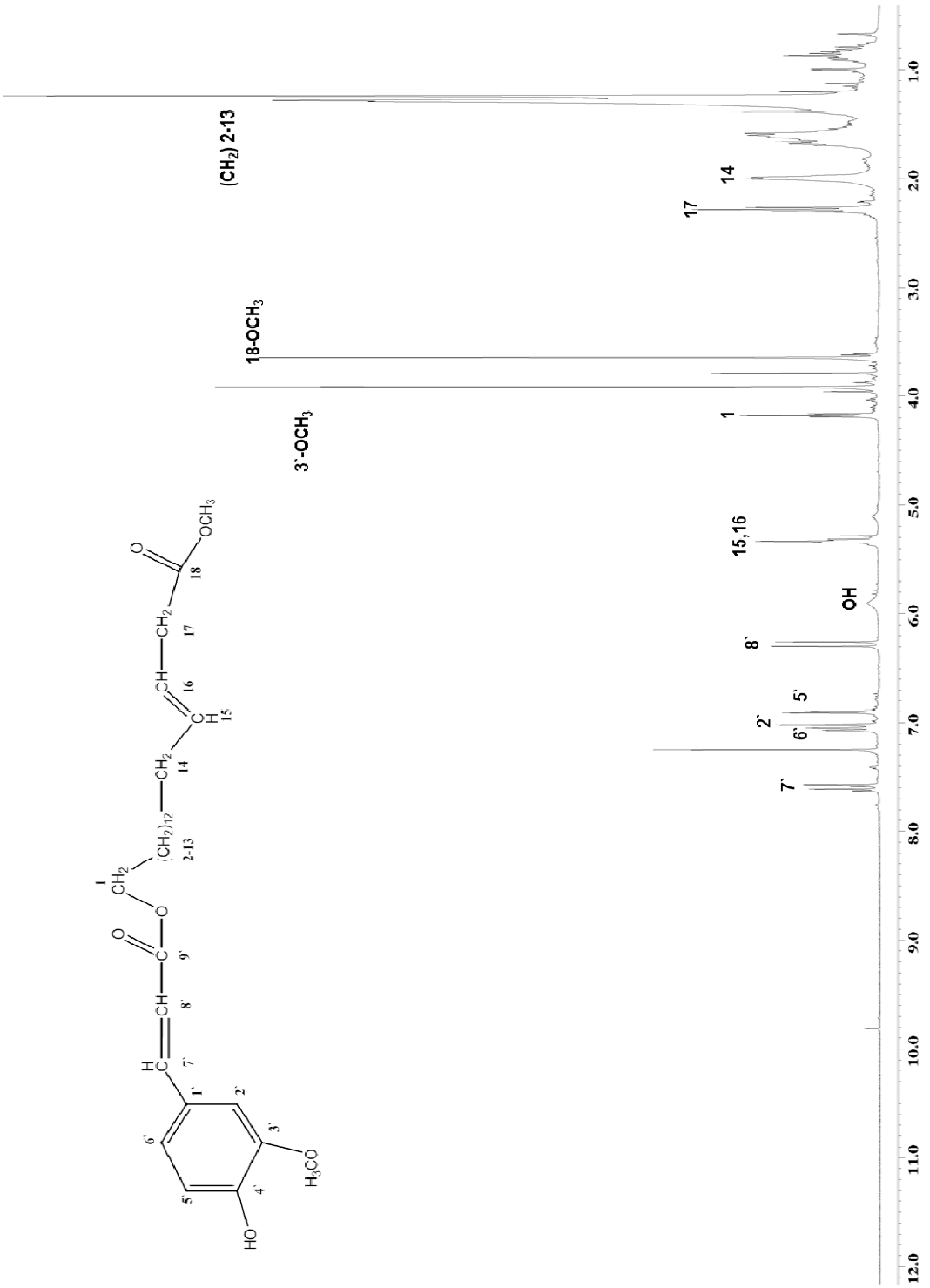


Figure 3.31: ^{1}HNMR Spectrum of 17-methoxy-carbonyl-14- heptadecaenyl- 4-hydroxy-3-methoxy cinnamate

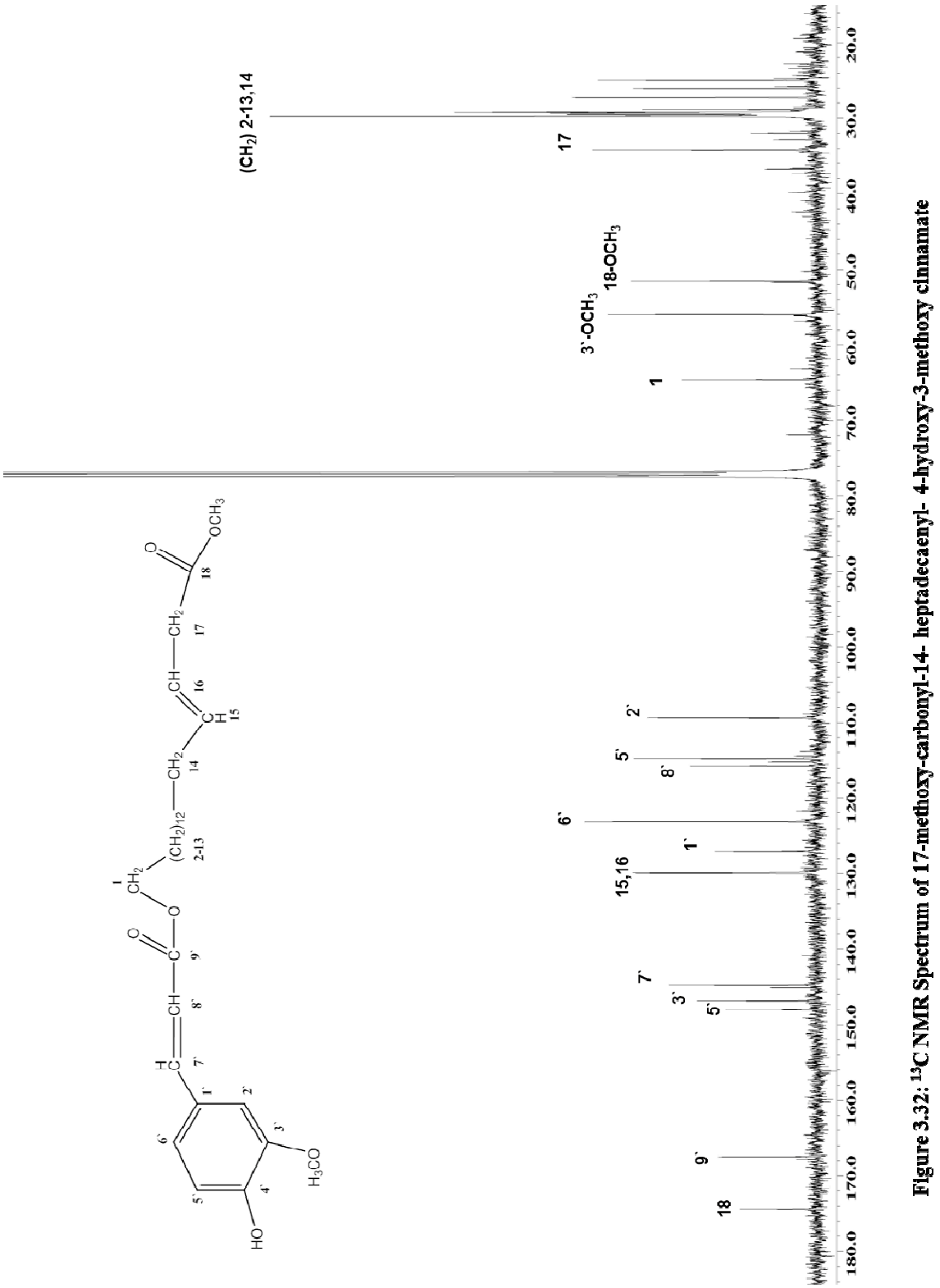


Figure 3.32: ^{13}C NMR Spectrum of 17-methoxy-carbonyl-14- heptadecaenyl- 4-hydroxy-3-methoxy cinnamate

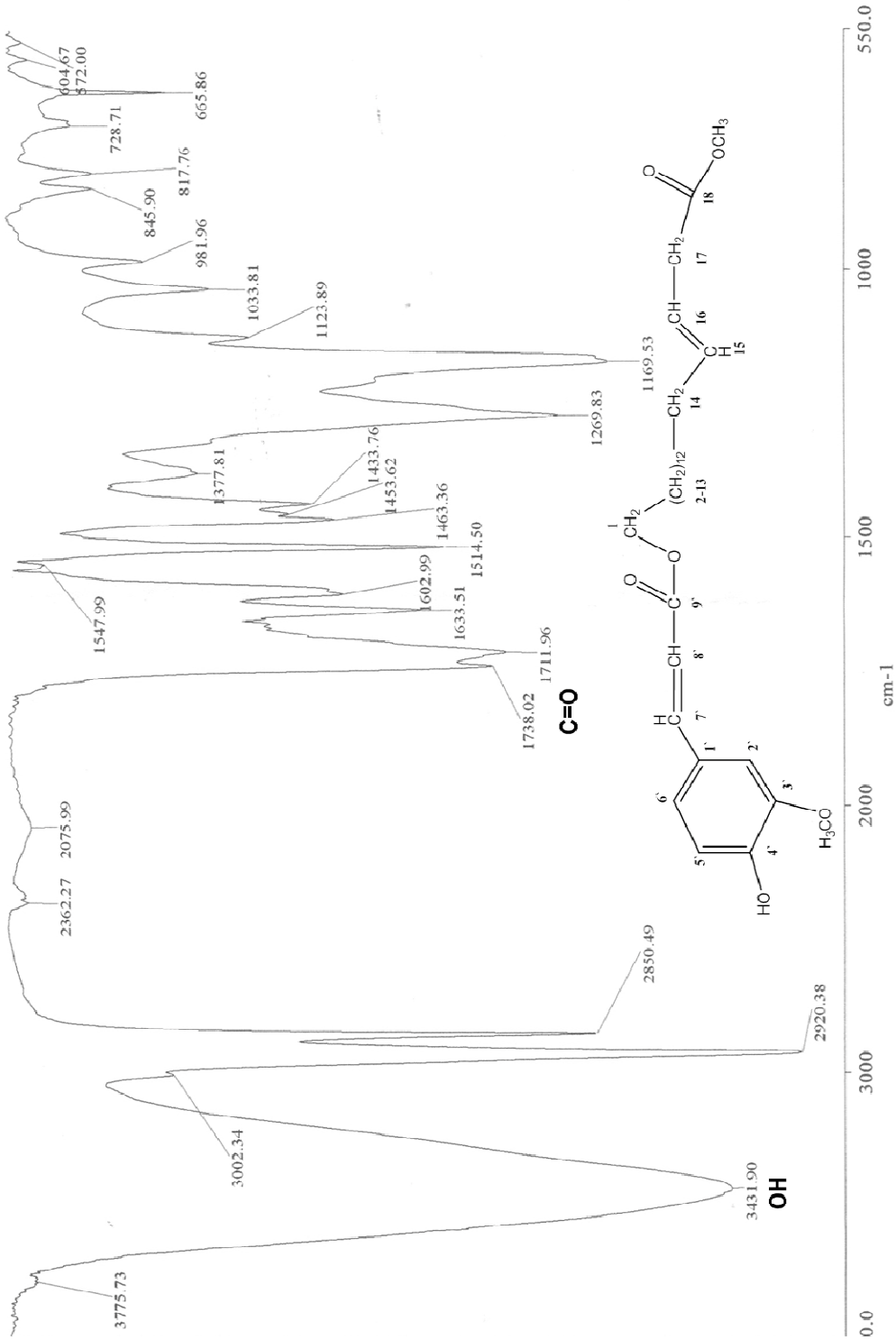


Figure 3.33: IR Spectrum of 17-methoxy-carbonyl-14- heptadecaenyl- 4-hydroxy-3-methoxy cinnamate

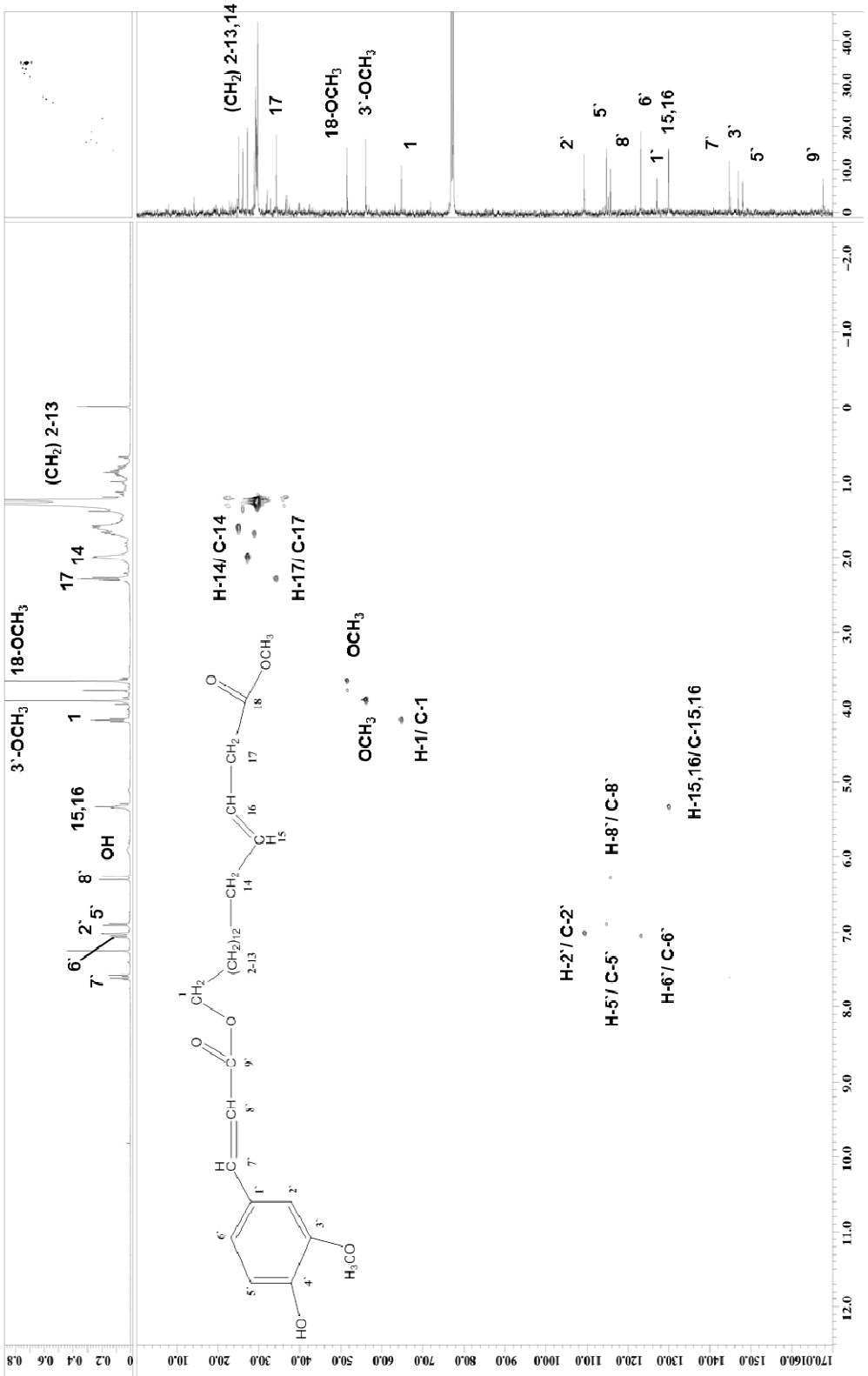


Figure 3.34: HSQC Spectrum of 17-methoxy-carbonyl-14- heptadecaenyl- 4-hydroxy-3-methoxy cinnamate

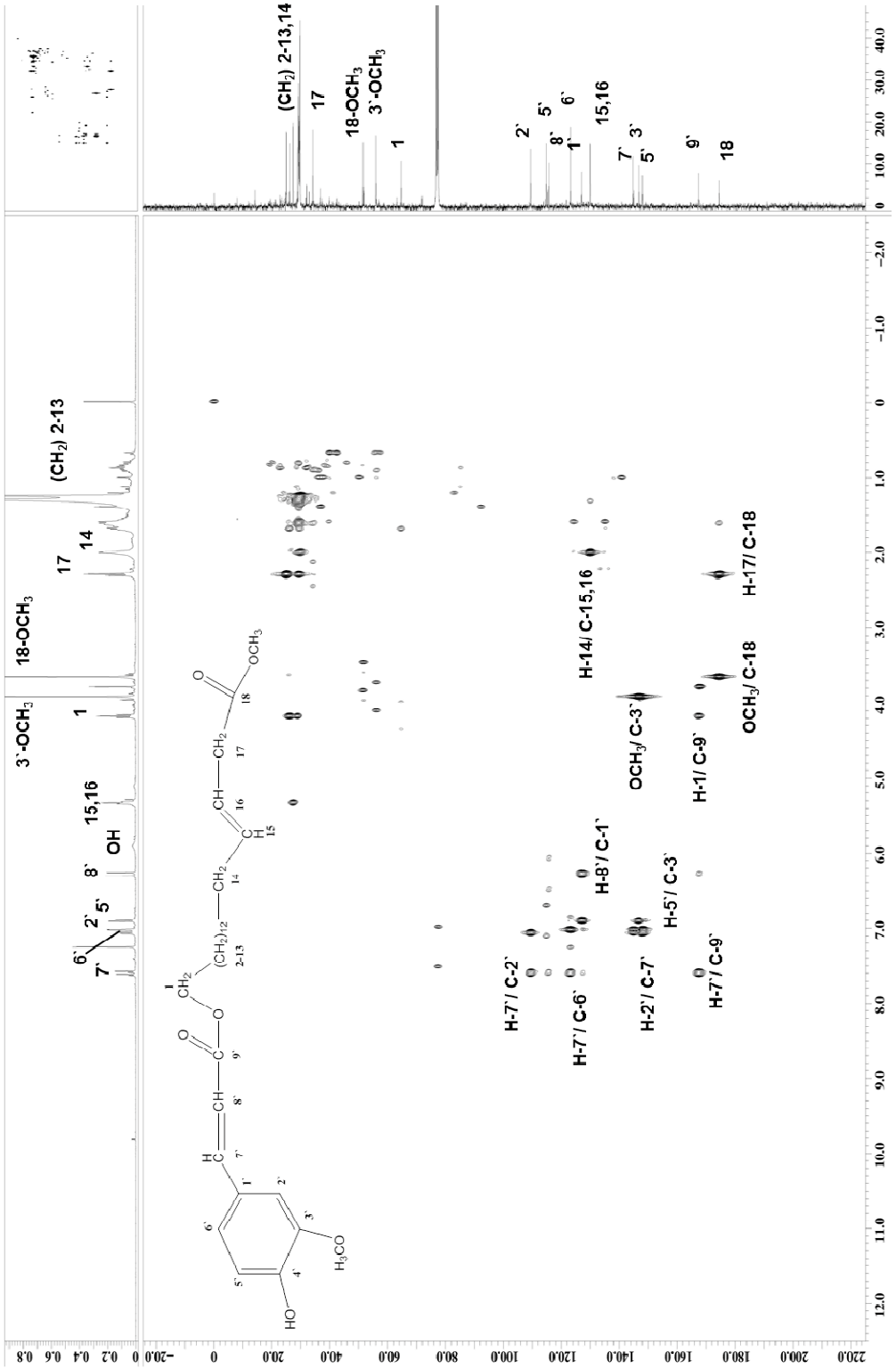
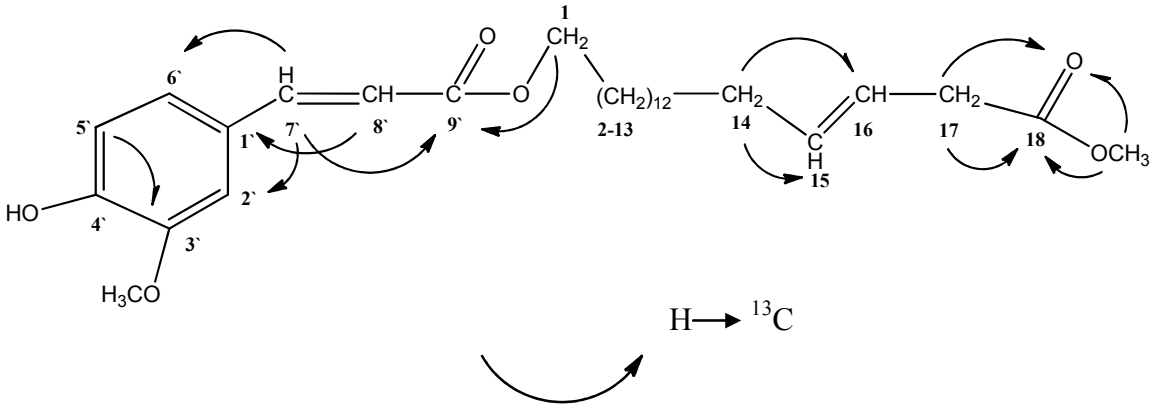


Figure 3.35: HMBC Spectrum of 17-methoxy-carbonyl-14- heptadecaenyl- 4-hydroxy-3-methoxy cinnamate



Scheme 3.36: The HMBC Correlations of: 17-methoxy-carbonyl-14- heptadecaenyl- 4-hydroxy-3-methoxy cinnamate **74**

CHAPTER 4
ANTIPLASMODIAL ACTIVITY

ANTIPLASMODIAL ACTIVITY

4.1 Introduction

In this studies the antiplasmodial activity of the crude extracts and pure compounds of *Ochrosia oppositifolia* were investigated.

Among the natural products, indole alkaloids represent an interesting class of compounds. Screening carried out to date has revealed several substances active in vitro under the micro molar range and with a good selectivity index. Nevertheless, in vivo activity has been confirmed only in a small number of cases, and there is a need to undertake research focused on the mode of action of these compounds.

Antiplasmodial indole alkaloids can be separated into three main categories.

The first category contains the alkaloids with a molecular weight higher than 400 and an important steric crowding: (1) indole analogues of emetine (usambarensine, ochrolifuanine, strychnopentamine); (2) and other bisindole alkaloids such as voacamine and ergoline derivatives, matopensines and isosungucines. Several of these alkaloids have been shown to be much more active against chloroquine-resistant strains. This phenomenon deserves to be elucidated. Considering the complexity of this group of compounds, very few attempts have been made to modify them chemically, as has been the case (in cancer therapy) for vinca alkaloids. This approach could nevertheless be very interesting.

The second category of antiplasmodial indole alkaloids contains unsaturated monomeric heterocycle. These are chemically much more attainable. There are two main models that have been developed: (1) derivatives of cryptolepine (the

most interesting compound being 2, 7- dibromocryptolepine); (2) derivatives of tryptanthrin. Considering this last group, there is still a lot of investigation needed concering their in vivo potentiality.

Finally, the last group of interesting indolic compounds includes monoindole alkaloids able to reverse the resistance to chloroquine. Among these, the most interesting compound seems to be malagashanine, found in strychnos myrtoides from Madagascar. This compound is active *in vivo*, but further clinical assays will be necessary to confirm the interest of this unconventional approach[53].

Malaysia is known with its green tropical vegetation and forest. Its diverse nature and uses are claimed to possess medicinal value. The Malaysians also practice traditional and herbal remedies as an alternative choice of treatment of malaria.

It is therefore of interest to evaluate the effect of several local Malaysian plants on *Plasmodium falciparum in vitro* and *in vivo* (Table 4.1).

Table 4.1[54]**:** Species Used for Antiplasmodial Evaluation in Malaysia

Species	Family	Part used	Local name
Piper sarmentosum	Piperaceaea	Leaves	Kaduk/ Kudak
Andrographis paniculata	Acanthaceae	Whole plant	Hempedu Bumi
Tinospora crispa	Menispermaceae	Stem	Patawali

4.2 Result and Discussion

Seven samples were tested including three crude extracts and four isolated compounds for in-vitro inhibitory activity against *P. falciparum*. Among the crude extracts, hexane crude extract of bark showed the most potent inhibitory activity, with the IC_{50} value of 0.05051 μg/mL (Table 4.2), the other crude extracts and compounds especially hexane crude of leaves showed very weak or no inhibitory activity against *P. falsiparum*.

Four indole alkaloids showed inhibitory activity; inhibitory concentration ranged from 0.0123 to 1.1251 μmol L^{-1} (Tables 4.3). The result for the antiplasmodial activity of the pure alkaloids indicated the alkaloid D possess the most potent activity with the IC_{50} value of 0.0123 μmol L^{-1}.

Table 4.2: Inhibition Growth Percentage of Plasmodium falciparum and Probit Analysis with SPSS 11.5 (crude extracts)

Crude extracts	% Inhibition at Concentration (μg/mL)					IC_{50} (μg/mL)
	100	10	1	0.1	0.01	
DCM crude of Leaves	100	68.24	39.54	30.12	16.20	1.61338
DCM crude of Bark	100	71.26	54.45	39.07	34.61	0.46951
Hexane crude of Bark	100	91.78	69.73	63.65	22.04	0.05051

Table 4.3: Inhibition Growth Percentage of *Plasmodium falciparum* and Probit Analysis with SPSS 11.5 (alkaloids)

Alkaloids	% Inhibition at Concentration (μg/mL)					IC_{50} (μmol L^{-1})
	10	1	0.1	0.01	0.001	
Reserpinine **3**	58.84	53.83	49.15	38.14	22.92	1.1251
Neisosposinine **2**	55.13	54.43	51.55	41.88	31.37	0.7473
Isoreserpiline **1**	76.31	64.4	40.59	33.33	29.25	0.2911
Alkaloid D **72**	100	99.64	61.3	56.1	46.59	0.0123

CHAPTER 5

CONCLUSION

CONCLUSION

The first part of the study was dedicated to the investigation of the alkaloid components of *Ochrosia oppositifolia* (KL 5349) from Apocynaceae family, collected from Pangkor Island (Perak). This plant produced monoterpene indole alkaloids and have been the subject of several studies.[7,8] Four indole alkaloids were isolated from the bark and leaves of *Ochrosia oppositifolia* namely; isoreserpiline **1**, neisosposinine **2**, reserpinine **3** and alkaloid D **72** which showed high antiplasmodial activity. In addition two ferulic acid esters; ferulic acid esters **E 73** and F **74,** were isolated from the methanol crude extract of the bark.

Isoreserpiline and reserpinine belong to the corynanthian type of alkaloid while neisosposinine is an oxindole alkaloid.

The second part discussed the antiplasmodial activity of the crudes and isolated compounds. In antiplasmodial assay, hexane crude of bark showed most potent inhibitory activity, with IC_{50} 0.05051 µg/mL against *P.falsiparum* while hexane crude of leaves displayed very weak inhibitory activity against *P.falsiparum.*

Four pure indole alklaloids namely reserpinine **3**, neisosposinine **2**, isoreserpiline **1** and alkaloid D **72** showed inhibitory activity ranging from 0.0123 to 1.1251 µmol L^{-1} (Table 4.3).among the pure alkaloids alkaloid D showed the most potent activity with an IC 50 value of 0.0123 µmol L^{-1}. Further studies of this plant can be done such as mechanism studies which can serve to provide lead compounds for the treatment of malaria.

CHAPTER 6

EXPERIMENTAL

EXPERIMENTAL

6.1 General Methods

Spectra were recorded on the following instruments as follows:

Ultra Violet Spectra (UV)

Ultra Violet spectra were obtained in methanol on a Shimadzu UV-250 uv-visible spectrophotometer and the wavelength which the spectrum was recorded is 190-500nm.

Infrared Spectra (IR)

The infrared spectra were recorded on a Perkin Elmer FTIR (model 1600) spectrophotometer. Solvent used for dilution the sample is $CHCl_3$.

Optical Rotation (OR)

The optical rotation was obtained on Jasco DIP-1000 Digital polarimeter with tungsten lamp at 25^oC.

Mass Spectra (MS)

The mass spectra were measured on Waters Micromass ZQ. The Automass Thermofinnigan was used for HR ESI^+ and ESI^- analysis.

NMR Spectra

NMR spectra were recorded in deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OH) on a JEOL JNM-FX 400MHz, JEOL ECA 400MHz, Bruker Top Spin 400 MHz and Varian 600MHz. Chemical shifts (δ) were expressed in ppm and the coupling constants are given in Hz.

Column Chromatography (CC) and Thin Layer Chromatography (TLC)

Column chromatography were prepared by using Silica Gel 60F, 70-230 mesh ASTM (Merck 7734); Silica Gel 60F, 230-400 mesh ASTM (Merck 9385); Silica gel 40-63µm (Silicycle R12030B); Silica gel 60-200µm (Silicycle R10040B); Silica Gel $60GF_{254}$, (Merck 1.07730.1000). Analytical Thin Layer Chromatography (TLC) was performed on commercially Aluminium supported silica gel $60F_{254}$ TLC sheets (Merck 1.05554.0001); Glass supported silica gel $60F_{254}$ TLC plates (Merck 1.05715.0001); Glass backed TLC Amino plates (Silicycle TLG-R52030B-203).

6.2 Reagents

Mayer's Reagent (Potassium mercuric iodide) was used for screening the alkaloid compounds. A positive result indicated when white precipitate was formed under acidic condition. The Mayer's Reagent was prepared as follows:

1.4 g mercuric iodide was dissolved in 60 ml distilled water were mixed with a solution of 5.0 g of potassium iodide in 10 ml of distilled water.

Dragendorff's Reagent (Potassium bismuth iodide) was also used to identify the presence of alkaloids spotting on TLC. A positive result is indicated by the formation of orange spots on the developed TLC. The Dragendorff's Reagent was made as follows:

Solution A: Bismuth (III) nitrate (1.7 g) was dissolved in a mixture of 20 ml glacial acetic acid and 80 ml of distilled water.

Solution B: Potassium iodide (16 g) was dissolved in 40 ml of distilled water.

Solution A and B were mixed together to give stock solution. Finally, the spray reagent was prepared by diluting stock solution (40 ml) with 40 ml glacial acetic acid in 120 ml distilled water.

6.3 Plant Material

The plant materials were collected from Pangkor Islands in 2007. The sample was identified by L.E.Teo , University Malaya and deposited at the herbarium unit (specimen no. KL 5349).

The location of the plant species was shown in Table 6.1.

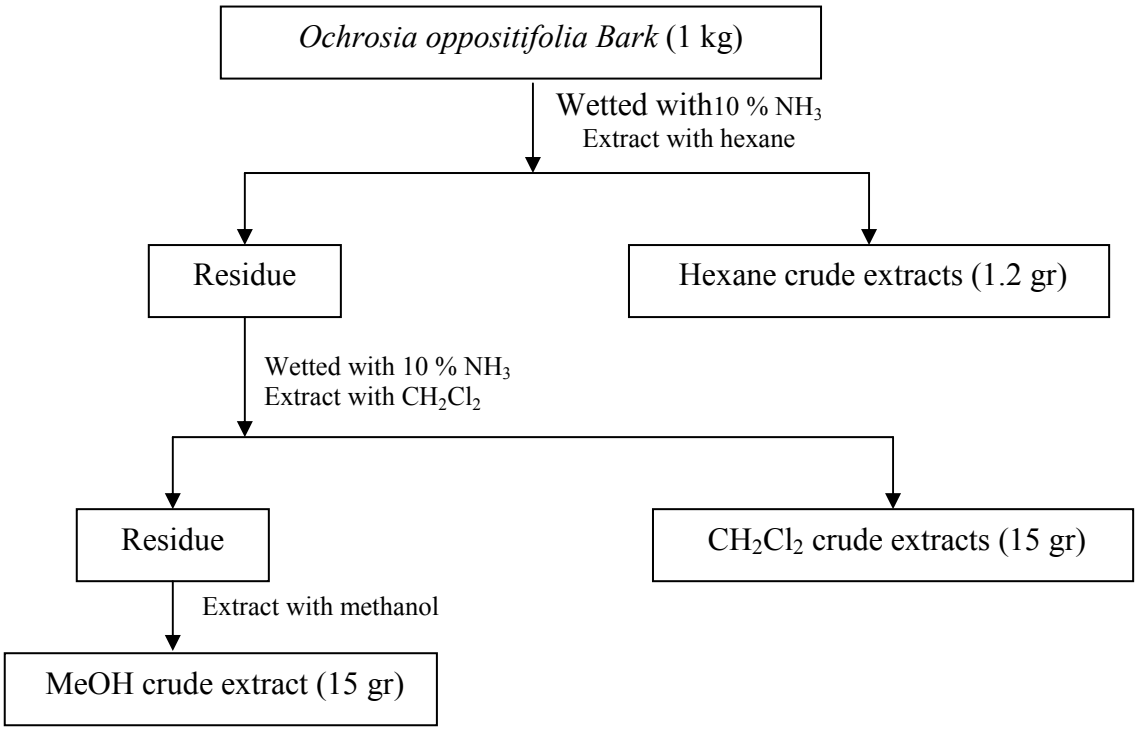
Table 6.1: Plant Species and Location

Species	Specimen Number	Location
Ochrosia oppositifolia	KL 5349	Pangkor Island, Perak

6.4 Extraction

6.4.1 Extraction of *Ochrosia oppositifolia* (Bark)

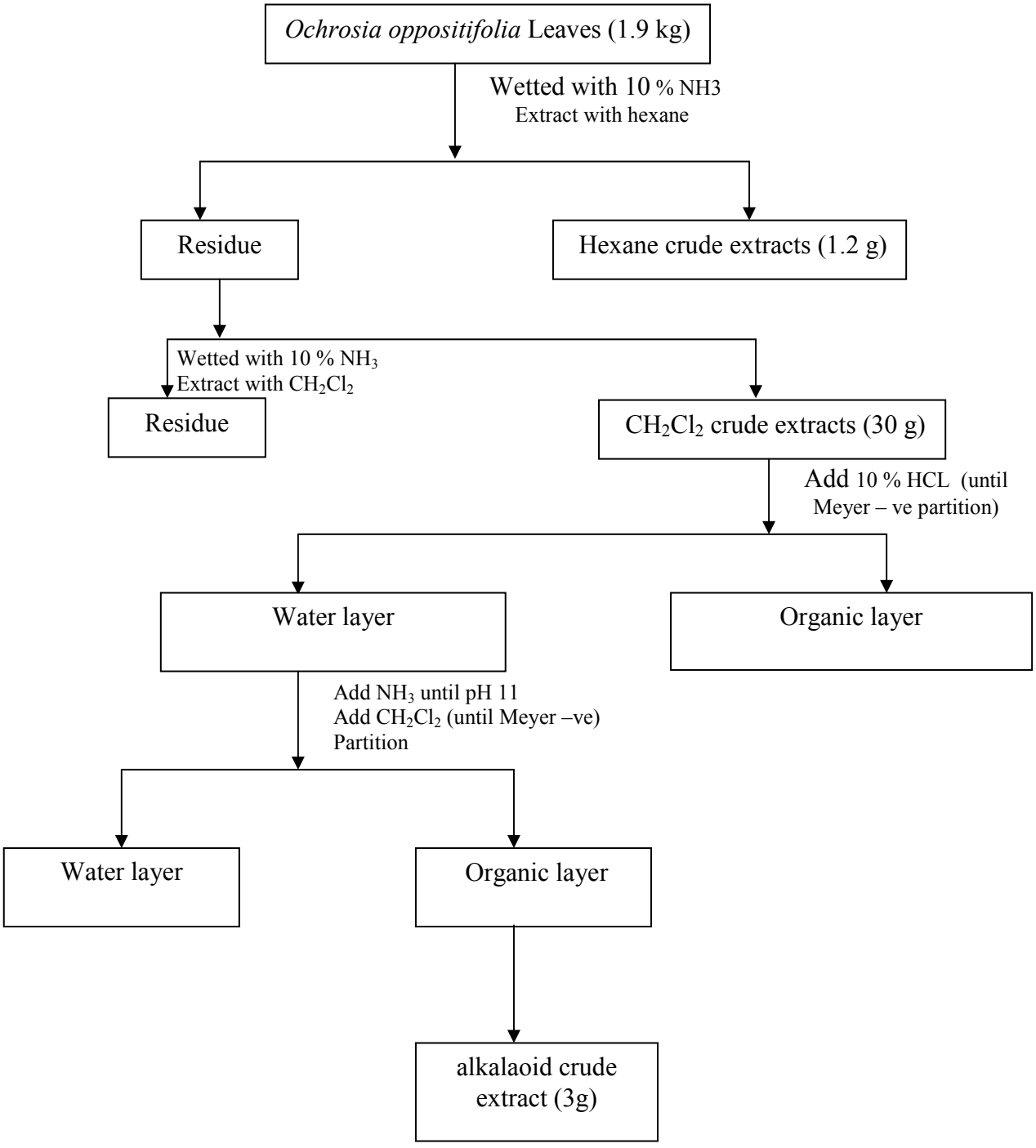
The extraction of the bark (1kg) was carried out by extracted exhaustively with hexane after wetting with 10% ammonia solution for 48 hours to removed non-polar organic compound, waxes and fats. Then the extract was dried on the rotary evaporator. The plant material was dried and again wetted with 10 % ammonia solution and left for overnight. They were then re-extracted successively with dichloromethane (CH_2Cl_2) and methanol (MeOH). After removal of the solvents, the hexane crude extract (1.2 g), dichloromethane (15 g) and methanol (15 g) were obtained. The extraction procedure was shown in Scheme 6.1.



Scheme 6.1: Extraction of *Ochrosia oppositifolia* (Bark)

6.4.2 Extraction of *Ochrosia oppositifolia (*leaves*)*

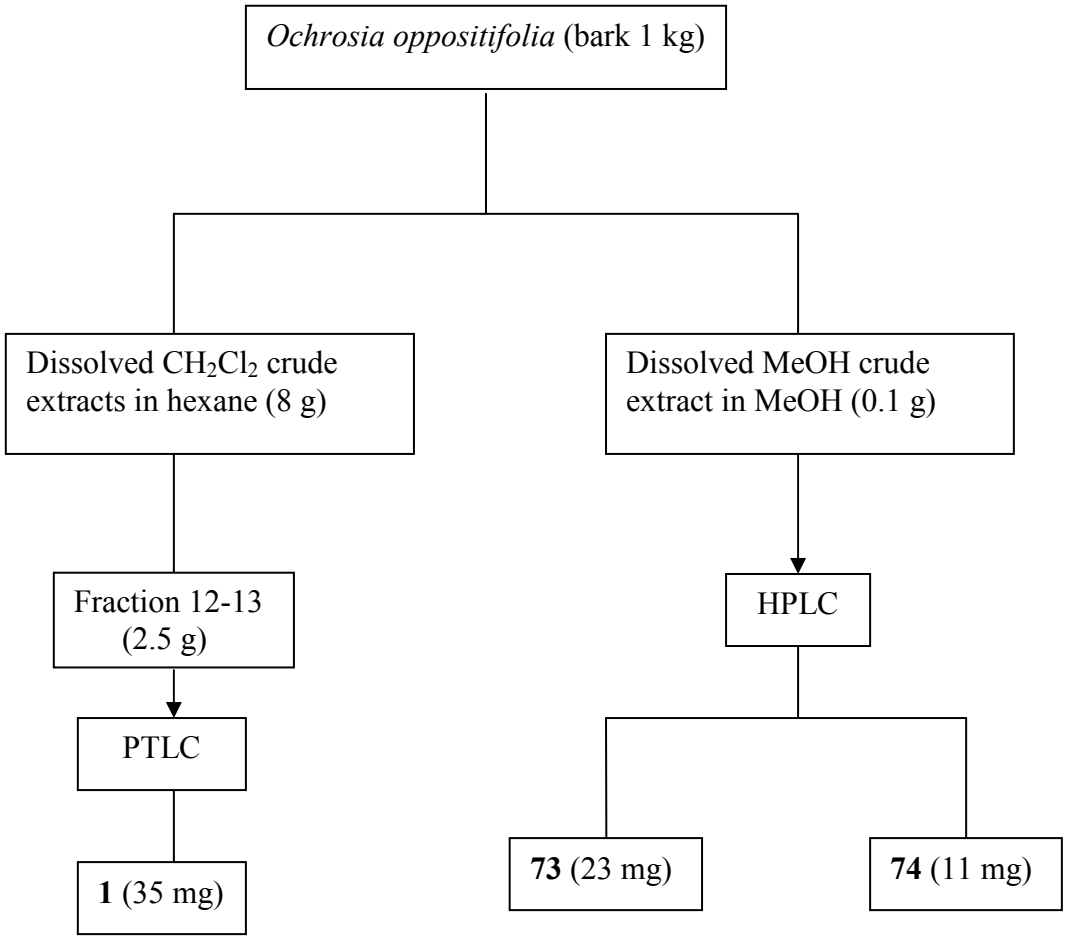
The extraction of the leaves (1.9 kg) were first wetted with 10 % ammonia solution and left for 2 hours then defatted in hexane for 48 hours. Then the extract was dried on the rotary evaporator. The plant material was dried and again wetted with 10 % ammonia solution and left for 2 hours. They were then re-extracted successively with dichloromethane (CH_2Cl_2). After removal of the solvents, the hexane crude extract (1.2 g) were collected. Dichloromethane crude extract was dissolved in CH_2Cl_2 and re-extracted with 5% hydrochloric acid (HCl) until a negative result was formed with mayers reagent. The combined extracts were then basified with 25% NH_3 solution (pH 11) and re-extracted with CH_2Cl_2 until a negative mayers test was obtained and later washed with distilled water and sodium chloride solution and dried with sodium sulfate anhydrous. Finally, the extract was evaporated to dryness to give an alkaloid crude extract (3 g). The extraction procedure was shown in Scheme 6.2.



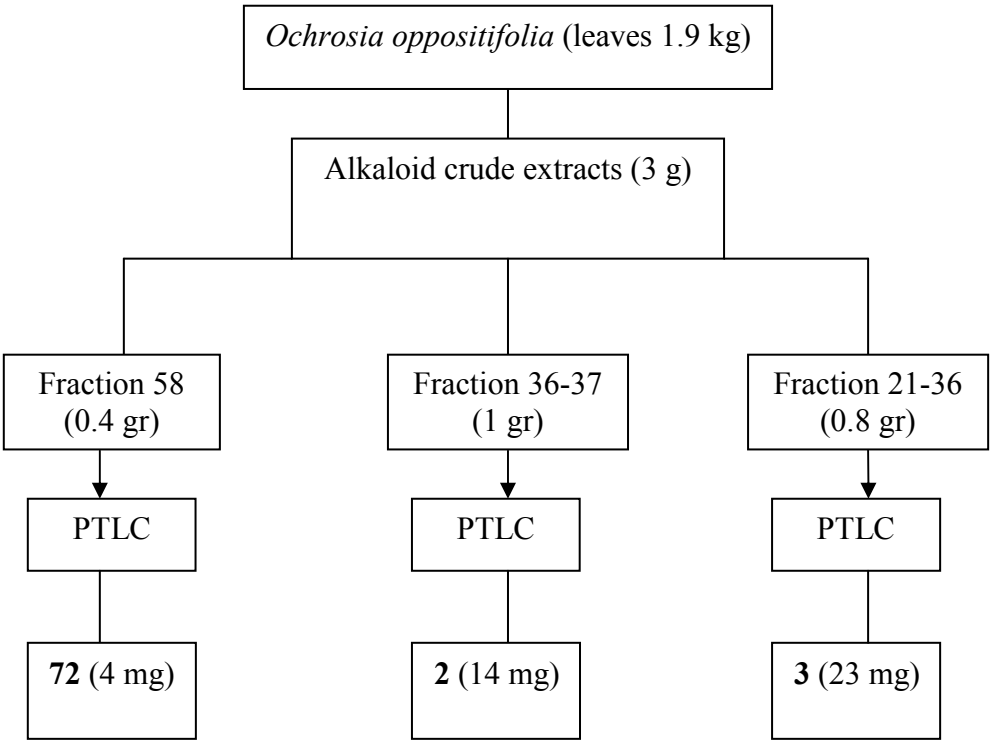
Scheme 6.2: Extraction of *Ochrosia oppositifolia* (Leaves)

6.5 Isolation and Purification

The crude extract from the plant (bark and leaves) were subjected to column chromatography over silica gel. The column was eluted with solvent mixtures of increasing polarity (CH_2Cl_2, CH_2Cl_2 / MeOH and MeOH) and fractions having spots with the same R_f value were grouped into a series of fractions (monitored by TLC). Each series of fractions were then treated separately by extensive column chromatography and preparative TLC to purify the alkaloids. The isolation and purification procedure were summarized in the flow diagram shown in Scheme 6.3 and 6.4.



Scheme 6.3: Isolation and Purification of Compounds from *Ochrosia oppositifolia* (Bark)



Scheme 6.4: Isolation and Purification of Alkaloids from *Ochrosia oppositifolia* (Leaves)

6.6 Physical and Spectral Data of Isolated Alkaloids

Ochrosia oppositifolia Compounds**:**

Isoreserpiline 1 : pale brownish amorphous solid

: $C_{23}H_{28}N_2O_5$

UV λ_{max} (MeOH), nm : 226,299

IR ν_{max} ($CHCl_3$), cm^{-1} : 3361, 2928, 1702, 665

Mass spectrum, m/z : 413 (100), 204, 435

^1H NMR ($CDCl_3$), ppm : See figure 3.1

^{13}C NMR ($CDCl_3$), ppm : See figure 3.2

Neisosposinine 2 : pale brownish amorphous solid

: $C_{23}H_{28}N_2O_6$

UV λ_{max} (MeOH), nm : 246,302

IR ν_{max} ($CHCl_3$), cm^{-1} : 3583, 2359, 1704, 665

Mass spectrum, m/z : 429 (100), 187

^1H NMR ($CDCl_3$), ppm : See figure 3.7

^{13}C NMR ($CDCl_3$), ppm : See figure 3.8

Reserpinine 3 : pale brownish amorphous solid

: $C_{22}H_{26}N_2O_4$

UV λ_{max} (MeOH), nm : 225,302

IR ν_{max} ($CHCl_3$), cm^{-1} : 3402, 2926, 1706, 665

Mass spectrum, m/z : 383 (100), 853

^1H NMR ($CDCl_3$), ppm : See figure 3.13

^{13}C NMR ($CDCl_3$), ppm : See figure 3.14

Alkaloid D 72 : pale brownish amorphous solid

: $C_{21}H_{28}N_2O$

UV λ_{max} (MeOH), nm : 250, 285

IR ν_{max} ($CHCl_3$), cm^{-1} : 3378, 1630, 735

Mass spectrum, m/z : 325 (100), 475, 701

^1H NMR ($CDCl_3$), ppm : See Figure 3.19

^{13}C NMR ($CDCl_3$), ppm : See Figure 3.20

Ferulic Ester E 73 : whitish amorphous solid

: $C_{12}H_{14}O_5$

UV λ_{max} (MeOH), nm : 196, 273,328

IR ν_{max} ($CHCl_3$), cm^{-1} : 3401, 1704

Mass spectrum, m/z : 238 (100), 412, 528

^1H NMR ($CDCl_3$), ppm : See Figure 3.25

^{13}C NMR ($CDCl_3$), ppm : See Figure 3.26

Ferulic Ester F 74 : whitish amorphous solid

: $C_{29}H_{44}O_6$

UV λ_{max} (MeOH), nm : 196, 273,328

IR ν_{max} ($CHCl_3$), cm^{-1} : 3431, 1711

Mass spectrum, m/z : 488 (100), 462, 588

^1H NMR ($CDCl_3$), ppm : See Figure 3.31

^{13}C NMR ($CDCl_3$), ppm : See Figure 3.32

6.7 Antiplasmodial Test Against *Plasmodium Falciparum Strains*

This protocol for assessing compound efficacy against *Plasmodium Falciparum* in vitro uses as a marker for inhibition of parasite growth.[55,56] Many alternative protocols exist, including ones based on microscopic detection of Giemsa-stained, assays based on production of parasite lactate dehydrogenase, and the use of flow cytometry. [57]

6.7.1 Preparation of the Antiplasmodial Test [58-66]

6.7.1.1 Parasite Strain

Several well- characterized strains (refer Table 6.2) are available, either from academic laboratories or from www.malaria.mr4.org (reagents available to registered users). One recommendation would be to test activity against a drug-sensitive line such a 3D7 (west Africa), D6 (sierra leone) or D10 (Papua New Guinea), as well as a drug-resistant line such as W2 or Dd2 (both from Indochina), FCB (SE Asia), 7G8 (Brazil) or K1 Thailand).

Table 6.2: Standard *Plasmodium Falciparum* Strains

Name	Clone	Origin	Resistant to	Multiplication
Dd2	yes (from WR82)	Indochina	CQ,QN,PYR,SDX	5-6
W2	yes (from Indochina-3)	Indochina	CQ,QN,PYR,SDX	5-6
HB3	yes	Honduras	PYR	4
3D7	yes (from NF54)	Apparently West Africa	-	4
D6	yes (from Sierra Leon-1)	Sierra Leone	-	4
D10	yes	Papua New Guinea		4
CAMP	no	Malaysia	PYR	4-5
FCB	no	Apparently SE Asia	CQ,QN,CYC	7-9
7G8	yes	Brazil	CQ,PYR,CYC	4-5
K1	no	Thailand	CQ,PYR	4-5

CQ, chloroquine; QN, quinine; PYR, pyrimethamine; SDX, sulfadoxine; CYC, cycloguanil.
Multiplication rate refers to increase in total numbers of viable parasites per 48-hr generation.
These rates and the drug phenotypes refer to data from the Fidock Laboratory (Albert Einstein College of Medicine, NY) and may not be the same elsewhere.

6.7.1.2 Malaria Culture Media

RPMI 1640 medium containing L-glutamine (Catalog number 31800, Invitrogen), 50 mg/liter hypoxanthine, 25 mM Hepes, 10 µg/ ml gentamicine, 0.225% NaHCO3 and either 10% human serum or 0.5% Albumax I or II (purified lipid-rich bovin serum albumin, Invitrogen). Medium is typically adjusted to a pH of 7.3 to 7.4.

6.7.1.3 Low Hypoxanthine Media

Same as above except that the hypoxanthine concentration is reduced to 2.5 mg/liter. Serum (as opposed to Albumax) is important for culturing fresh isolates, and for maintaining properties of cytoadherence and gametocyte production (the latter is required for transmission back to mosquitoes). Some strains also prefer to propagate in serum. Batch -to-batch variation is nonetheless a problem, with occasional batches not supporting robust parasite growth. Accordingly, many laboratory lines have been adapted to propagate in the presence of Albumax, which typically gives more consistent growth between batches (variation was a problem in the past, but now appears to have been resolved). Albumax appears to reduce both the rate at which erythrocytes deteriorate in vitro and pH drift when cultures are exposed to ambient air, i.e. during tissue culture hood manipulations.

6.7.1.4 Preparation of Host Erythrocytes

Human erythrocytes for parasite culture are prepared by drawing blood into heparin-treated tubes and washing several times in RPMI 1640 medium to separate the erythrocytes from the plasma and buffy coat. Separation can be achieved by centrifuging the blood at 500x g for 5 minutes in a swing-out rotor. Leukocyte-free erythrocytes are typically stored at 50% hematocrit. i.e. 1.

Volume of malaria culture media for 1 volume of packed erythrocytes, corresponding to approximately 5 x 10^9 cells/ml.

6.7.1.5 Parasite Culture Conditions

P. *falciparum* asexual blood stage parasites are propagated at 370 °C in malaria culture media at 3-5% hematocrit in a reduced axygen environment (e.g. a custom mixture of 5% CO_2, 5% O_2 and 90% N_2). Lines can be conveniently cultured in 6-24 well tissue culture plates in a modular chamber (billups- Rothenberg, Del Mar, CA, www.brincubator.com), with plates containing sterile water on the bottom to increase humidity and minimize desiccation. These chambers can be immersed with the low O_2 gas and mainained at 37 °C in an incubator designed to minimize temperature fluctuations.

Parasites can also be cultured in flasks that are individually gassed, or alternatively placed in flasks that permit gas exchange through the cap (in which case the incubator needs to be continuously infused with a low O_2 gas mixture). Depending on the line, parasites typically propagate 3-8 fold every 48 hours, thus care must be taken to avoid parasite cultures attaining too high a parasitemia, i.e. percentage of erythrocytes that are parasitized for healthy growth. Most lines grow optimally at 0.5-4% parasitemia. Parasites are most suitable for drug assays when they are 2-5% parasitemia, and mostly ring stages with few or no gametocytes.

6.7.1.6 Compounds

Compounds can be often be dissolved in 100% dimethyl sulfoxide (DMSO) and stored at -20 °C. Particles size of insoluble compounds can be reduced by ball-milling or sonication. For the drug assays, serial drug dilutions are made in low hypoxanthine medium and added to 96-well culture plates at 100 ml per well. Drugs are added to columns 2012 (test samples), with columns 1 and 2 reserved for wells with low hypoxanthine medium without compound. All drugs are typically tested in duplicate for each parasite line. Once completed, plates are placed into their own modular chamber, gassed and placed at 37 °C. These plates should be set up no more than a few hours to addition of the parasites.

6.7.1.7 Drug Assay Conditions

Parasites are diluted to a 2 times stock consisting of 0.6% to 0.9% parasitemia (depending on the growth rate of the line) and 3.2% hematocrit in low hypoxanthine medium, and 100 ml are added per well already containing 100 ml of low hypoxanthine medium with or without compound (present at different concentration). Plates are then incubated in a gassed modular chamber at 37 °C for 48 hours. After this time, 100 ml of culture supernatant from each well is removed and replaced with 100 ml of low hypoxanthine medium containing a final concentration of 7.5 μCi/ml of chloroquine. After a further 24 hours, the plates are placed at -20 °C for at least 1 hour to freeze the cells.

Plates are then thawed and the cells are harvested onto glass fiber filters (Wallac, Turku, Finland). Filters are dried for 30 minutes at 80 °C, placed in sample bags (Wallac), and immersed in scintillation fluid (Ecoscint A; National Diagnostic, Atlanta, GA). Radioactive emissions are counted in a 1205 Betaplate reader (Wallac). Mean counts per minute (cpm) are generally in the range 20,000-60,000, with an acceptable minimum of 10,000. percentage reduction in chloroquine uptake is equal to 100 x ((geometric mean cpm of no drug samples)- (mean cpm of test samples)) / (geometric mean cpm of no drug samples). Percentage reductions are used to plot percentage inhibition of growth as a function of drug concentrations.

IC_{50} values are determined by linear regression analayses on the linear segments of the curves (IC_{90} values can also be determined by curve-fitting and can provide an useful measure of variation between experiments). Assays are typically repeated on two or three separate occasions. Within each experiment, standard deviations are typically less than 10% of the mean. Different in parasite stages of development can lead to up to two-fold shifts in the IC_{50} values between experiments; however, these differences rarely affect the overall relationships between the parasite lines in terms of their differences in drug response.