CHEMICAL CONSTITUENTS OF *KOPSIA SINGAPURENSIS* RIDL. AND THEIR ANTIPLASMODIAL ACTIVITY

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FACULTY OF SCIENCE
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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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2012

UNIVERSITI MALAYA

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Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

CHEMICAL CONSTITUENTS OF *KOPSIA SINGAPURENSIS* RIDL. AND THEIR ANTIPLASMODIAL ACTIVITY

Field of Study: NATURAL PRODUCT CHEMISTRY

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ABSTRACT

The chemical constituents of the Malaysian Apocynaceae *Kopsia singapurensis* Ridl. (KL5334) has been carried out in this study. Seven substances ; methyl sinapate (IUPAC name : (*E*)-methyl-3-(4-hydroxy-3,5-dimethoxyphenyl) acrylate **82**, leuconolam **83**, *trans*-2,2`-dicarboxyazobenzene dioxide **84**, lonicerine **85**, 15-hydroxykopsinine **86**, singapurine **87** and N_4-hydroxymethyl kopsinic acid **88,** have been isolated using several chromatography techniques such as column chromatography and thin layer chromatography from the bark of *Kopsia singapurensis* Ridl.. Methyl sinapate **82** and *trans*-2,2`-dicarboxyazobenzene dioxide **84** are new natural compounds while singapurine **87** and N_4-hydroxymethyl kopsinic acid **88** are two new indole alkaloids. The structures of the isolated compounds were elucidated by using spectroscopic techniques namely NMR (^{1}H, ^{13}C and 2D), UV, IR and mass spectrometry.

ABSTRAK

Kandungan kimia yang terdapat dalam Malaysia Apocynaceae *Kopsia singapurensis* Ridl. (KL5334) telah dikaji dalam penyelidikan ini. Tujuh sebatian; metil sinapat (nama IUPAC ; (*E*)-metil-3-(4-hidroksi-3,5-dimetoksifenil) akrilat **82**, leukonolam **83**, *trans*-2,2`-dikarboksiazobenzen dioksida **84**, loniserina **85**, 15-hidroksikopsinina **86**, singapurina **87** dan N_4-hidroksimetil asid kopsinik **88** telah diasingkan menerusi beberapa kaedah kromatografi seperti kromatografi turus dan kromatografi lapisan nipis daripada kulit batang *Kopsia singapurensis* Ridl. Metil sinapat **82** dan *trans*-2,2`-dikarboksiazobenzen dioksida **84** adalah sebatian semulajadi yang baru manakala singapurina **87** dan N_4-hidroksimetil asid kopsinik **88** adalah dua alkaloid indola baru. Penentuan struktur kimia telah dibuat mengunakan teknik spektroskopi iaitu NMR (^{1}H, ^{13}C dan 2D), UV, IR dan spektrometri jisim.

ACKNOWLEDGEMENTS

Alhamdulillah thank you Allah for His grace that I can complete my thesis. First and foremost, I would like to express my greatest appreciation to my supervisor, Professor Dr. Khalijah Awang for her patience, advices, kindness and dedication throughout this course.

I also would like to thank Professor Datuk Dr. A. Hamid A. Hadi and late Associate Professor Dr. Mat Ropi Mukhtar for his valuable helps and cooperation.

My gratitude would also be due to the Herbarium staffs for their help in sample collection and the NMR and GCMS staffs for recording the spectra. Their kindness in guiding me, sharing experience and priceless consultation are really appreciated.

Last but not least, I wish to express my heart felt thanks to my beloved parents Othman@Ghazali bin Abdullah and Fatimah binti Ali and everybody in the Phytochemistry Lab for their concern, helps and friendships. Thank you so much for their assistance, support and helps.

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ABBREVIATIONS

CC	column chromatography
br s	broad singlet
dt	doublet of triplet
q	quartet
s	singlet
t	triplet
dd	doublet of doublets
ddd	doublet of doublet of doublets
δ	chemical shift
α	alpha
β	beta
λ	wavelength
g	gram
cm^{-1}	percentimeter
kg	kilogram
M	molar
mM	milimolar
ml	mililitre
m	meter
MHz	mega hertz
$CDCl_3$	deuterated chloroform
Hz	hertz
UV	ultraviolet

IR	infrared
ppm	part per million
eV	electron volt
MeOH	methanol
$CHCl_3$	chloroform
CH_2Cl_2	dichloromethane
DCM	dichloromethane
DMSO	dimethylsulphoxide
EA	ethyl acetate
OCH_2O	methylenedioxy
CH_3	methyl group
OCH_3	methoxyl group
OH	hydroxyl group
NH_3	ammonia
TLC	thin layer chromatography
PTLC	preparative thin layer chromatography
pH	power of hydrogen
HCl	hydrogen chloride

INTRODUCTION

1.1 General

For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases.[1] Just over 200 years ago, a 21 year old pharmacist`s apprentice named Friedrich Serturner isolated the first pharmacologically active pure compound from a plant: morphine from opium produced by a cut seed pods of the poppy, *Papaver somniferum*.[2] By 1990, about 80% of drugs were either natural products or analogs inspired by Second World War.[3]

Antibiotics (e.g., penicillin, tetracycline, erythromycin), antiparasitics (e.g., avermectin), antimalarials (e.g., quinine, artemisinin), lipid control agents (e.g., lovastatin and analogs), immunosuppressants for organ transplants (e.g., cyclosporine, rapamycins) and anticancer drugs (e.g., taxol, doxorubicin) revolutionized medicine.[3]

Today even with the advent of new technologies, the majority of new drugs is still generated from natural products (secondary metabolites) or from compounds derived from natural products.[3] An analysis of the origin of the drugs developed between 1981 and 2002 showed that natural products or natural product, derived drugs comprised 28% of all new chemical entities (NCEs) launched onto the market.[4] In addition, 24% of these NCEs were synthetic or natural mimic compounds related to natural products.[5] This combined percentage (52% of all NCEs) suggests that natural products are important sources for new

drugs and are also good lead compounds suitable for further modification during drug development.[1]

Besides, within 2000 to 2005, over 20 new drugs were launched on the market.[1] In fact, a recent analysis by Newman[6] et al. and Butler[7] et al., from 1981 to 2007 indicate that almost half of the drugs approved since 1994 are based on natural products. Thirteen natural product-derived drugs were approved in the United States between 2005 and 2007, with five of them being the first members of a new class.[7,8]

Most of the leads from natural products that are currently in development have come from either plant or microbial source.[8] Malaysia is known for its diverse variety of flora and fauna that have been around for millions of years. The flora is regarded as one of the richest and oldest in the world. This may be attributed to the climate in Malaysia which is constantly warm and nearly uniform, suitable for the growth of tropical rainforests. Hence it is an interesting site to search for medicinal plants and potential drugs or lead compounds. In addition, natural resources from tropical rain forest are a promising source of biologically active compounds.[9] The importance of the region`s diverse medicinal plants lies not only in their chemotherapeutic value in traditional health care but also in their potential as source of NCEs for drug discovery.[10]

In Malaysia, efforts in discovering new potential antiplasmodial drugs is still of great interest since, most malaria cases in this country are caused by *Plasmodium falciparum*.[9] Present drug have become ineffective because of the occurrence of resistance *P. falciparum*, particularly to chloroquine.[9] By 1963, chloroquine resistant has been reported

in Malaysia.[11] Currently, the only fully effective class of antimalarial drug is the artemisinin.[12] Artemisinin from *Artemesia annua* is a sesquiterpenoid that is effective against multi-drug-resistant *Plasmodium* species but is expensive for Third World patients.[3] In all malaria endemic countries, plants are used in traditional medicine for treatment of the diseases.[13]

In view of the fact that Malaysia has many plants of medicinal importance the author has embarked of *Kopsia singapurensis* Ridl an investigation or its chemical constituents and potential antiplasmodial activities. The objectives of this study are:

- To extract and isolate the compounds from *K. singapurensis* Ridl. using chromatographic methods.
- To elucidate the structure of the compounds using modern spectroscopic methods such as ^{1}H-NMR, ^{13}C-NMR, COSY, DEPT, HMBC, HMQC, IR, MS and UV.
- To determine antiplasmodial activity against the extract and the isolated compounds from *K. singapurensis*.

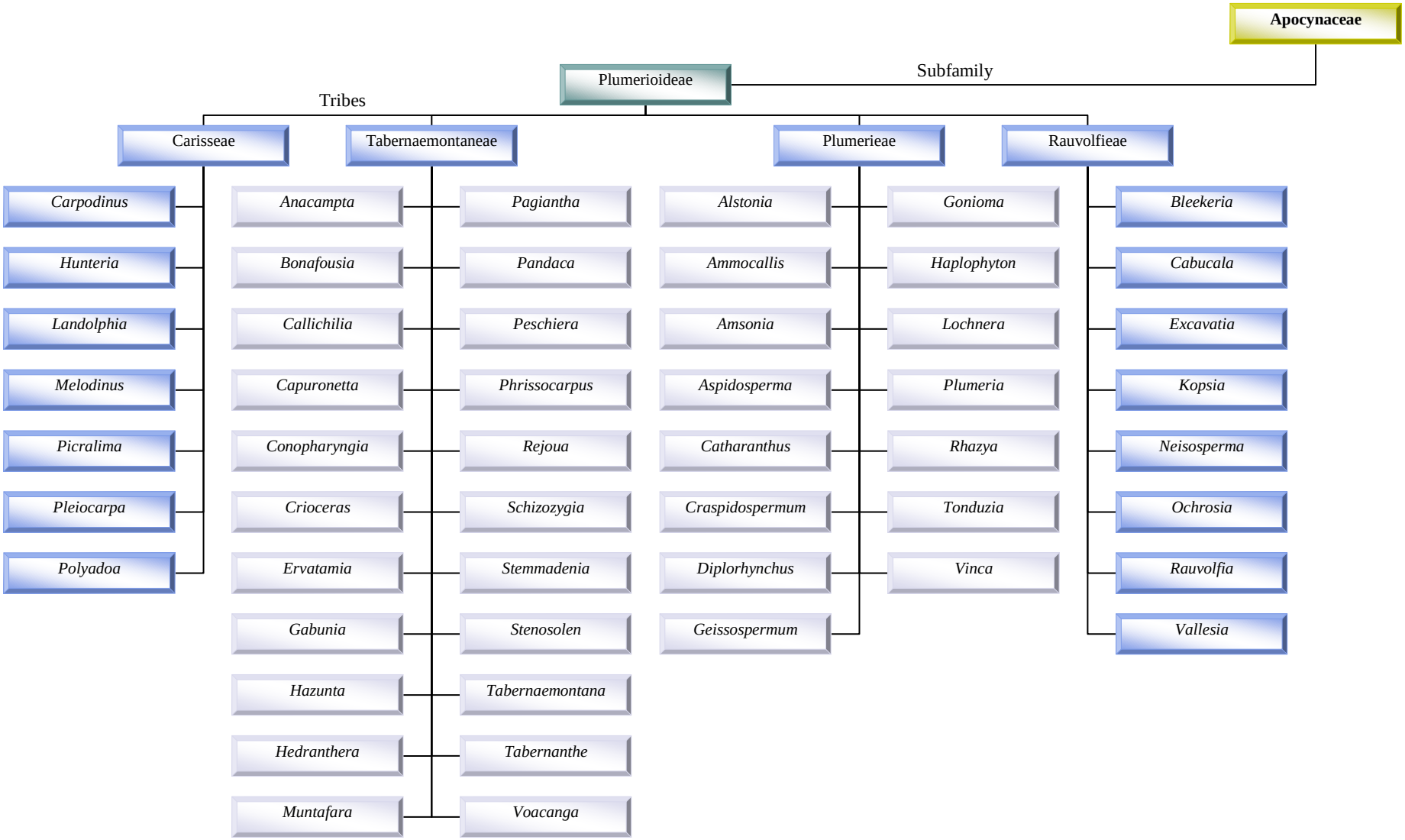
1.2 Apocynaceae

Apocynaceae is a family of flowering plants, including trees, shrubs and herbs. The family was currently recognized, comprising 1500 species classified in about 424 genera.[14] However in Malaysia nearly 120 species which are classified into 32 genera of this family are distributed over lowlands and mountains.[15]

1.3 Genus *Kopsia*

Genus *Kopsia* belongs to Rauvolfieae tribe, subfamily Plumerioidiae of the Apocynaceae family[16,17,18](Scheme 1.1). By contrast, Sevenet *et. al*[19] has reported that this genus belongs to the subtribe Vallesiinae, close to Ochrosiinae in Plumerioideae-Rauvolfioideae subfamily.

The genus *Kopsia* (Apocynaceae) comprises of 30 species which are native to China, India and southeast[20-25] and about 18 species are to be found in Malaysia. This genus was identified to generate a great number of biologically active indole alkaloids possessing interesting skeletons



Scheme 1.1: Classification of Apocynaceae Family

1.4 *Kopsia singapurensis* Ridl.

K. singapurensis Ridl. (White Kopsia)[26] or locally known as "selada" is a small evergreen tree with a conical crown, up to 25 feet high. In Malaya, this plant was distributed from Negeri Sembilan southward to Singapore and common in lowland marshy forest. The leaves are elliptic, thin with a distinct blunt tip up to 0.5 in long and 4.0 in wide, rather dark green. The bark is grayish buff to pale silvery brownish. The flowers are rather loose clusters on stalks 1.5 - 3.0 in long and 1.0 - 1.5 in or 2.0 - 3.0 in wide. The fruit is 1.5 in long and 0.75 in wide, flattened, triangular in outline, finely hairy but glabrous[26] (Figure 1.1).

The flower of *K. singapurensis*

The leaf of *K. singapurensis*

The bark of *K. singapurensis*

Figure 1.1: Various part of *K.singapurensis*

1.5 Alkaloids Isolated from *Kopsia singapurensis*

Previous investigation on *K. singapurensis* afforded many skeletal types of indoles such as aspidofractinine, mersinine, aspidosperma, vincorine and akuammiline.[25,27-35,18,36] Table 1.5.1 showed the list of different compounds isolated from *Kopsia singapurensis* Ridl..

Table 1.1: List the alkaloids isolated from *Kopsia singapurensis* Ridl..

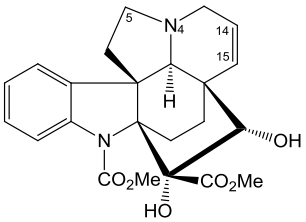
Skeletal type	Name	References	Class*
i) Aspidofractinine	Kopsiloscine A **1**	31	II
	Kopsiloscine B **2**	31	II
	Kopsiloscine C **3**	31	II
	Kopsiloscine D **4**	31	II
	Kopsiloscine E **5**	31	II
	Kopsiloscine F **6**	31	II
	Kopsiloscine G **7**	32, 57	II
	Kopsiloscine H **8**	32	II
	Kopsiloscine I **9**	32	II
	Kopsiloscine J **10**	32	II
	(17α)-17-hydroxy-Δ^{14}-kopsinine **11**	31, 32, 57	II
	Kopsidarine **12**	57	II
	Kopsinitarine A **13**	57	II
	Kopsinitarine B **14**	57	II
	Mersingine A **15**	57	II
	Singapurensine A **16**	34	II
	Singapurensine B **17**	34	II
	Singapurensine C **18**	34	II
	Singapurensine D **19**	34	II
	Kopsimaline A **20**	32	II
	Kopsimaline B **21**	32	II
	Kopsimaline C **22**	32	II
	Kopsimaline D **23**	32	II
	Kopsimaline E **24**	32	II
	Kopsimaline F **25**	57	II
	Kopsidine A **26**	57	II
	Kopsidine C **27**	57	II
	Kopsidine D **28**	31	II
	Kopsidine C *N*-oxide **29**	57	II
	Kopsinicine **30**	32	II
	Kopsofinone **31**	32	II

	Kopsinine **32**	31, 32, 57	II
	N-decarbomethoxykopsamine **33**	32	II
	Kopsilongine-*N*-oxide **34**	31	II
	16-epikopsinine **35**	31	II
	Kopsilongine **36**	31	II
	Kopsaporine **37**	31, 35	II
	Kopsingine **38**	31, 35, 57	II
	Kopsinganol **39**	31, 57	II
	11,12-methylenedioxykopsaporine **40**	34	II
	15-hydroxykopsinine-*N*-oxide **41**	18	II
	Singaporentine A **42**	56	II
	Kopsingarine **43**	35	II
	Kopsifoline A **44**	56	II
	Kopsinic acid **45**	56	II
ii) Mersinine	Mersirachine **46**	32, 33	
	Mersinaline **47**	32, 33	I
	Mersinine A **48**	25	I
	Mersinine B **49**	25	I
	Mersinine C **50**	25	I
	Mersifoline A **51**	25	I
	Mersifoline B **52**	25	I
	Mersifoline C **53**	25	I
	Mersiloscine **54**	25	I
	Mersiloscine A **55**	25	I
	Mersiloscine B **56**	25	I
	Mersidasine A **57**	25	I
	Mersidasine B **58**	25	I
	Mersidasine C **59**	25	I
	Mersidasine D **60**	25	I
	Mersidasine E **61**	25	I
	Mersidasine F **62**	25	I
	Mersidasine G **63**	25	I
iii) Aspidosperma	Rhazinilam **64**	31, 32, 57	II
	Rhazinal **65**	31	II
	Rhazinicine **66**	31	II
	5,21-Dihydrorhazinilam **67**	32, 57	II
	Leuconolam **68**	31, 32	II
	Leuconoxine **69**	32	II
iv) Condylocarpine	14α-hydroxycondylocarpine **70**	32	I

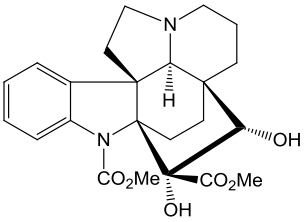
v) Vincorine	Vincophylline **71**	31	I
vi) Aspidodasycarpine	Aspidodasycarpine **72** Lonicerine **73** Aspidophylline A **74** Aspidophylline B **75**	31, 32, 57 31, 32, 57 31 57	I I I I
vii) Akuammiline	Akuammidine **76** Deacetylakuammiline **77** 16-epiakuammiline **78** 16-epideacetylakuammiline **79**	31, 32, 57 32 31, 32, 57 31, 32, 57	I I I
viii) Yohimbine	Tetrahydroalstonine **80**	31, 32	I
ix) Pleoicarpamine	16-hydroxymethylpleiocarpamine **81**	32	I

* Please refer to Scheme 2.1 and Section 2.2

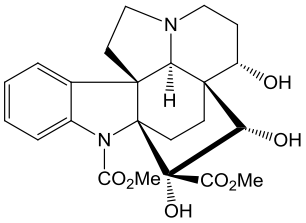
i) Aspidofractinine type



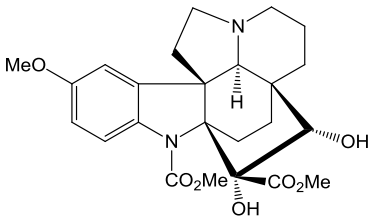
Kopsiloscine A **1**



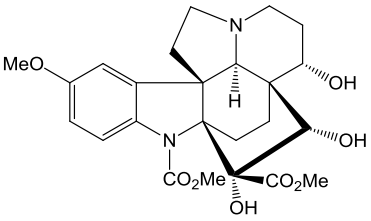
Kopsiloscine B **2**



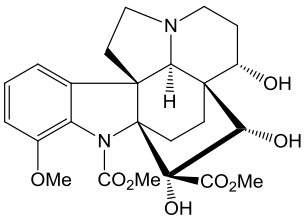
Kopsiloscine C **3**



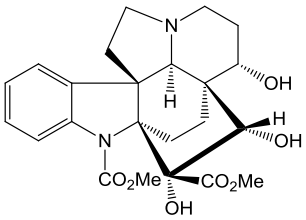
Kopsiloscine D **4**



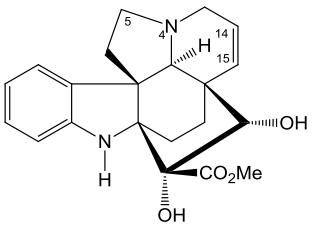
Kopsiloscine E **5**



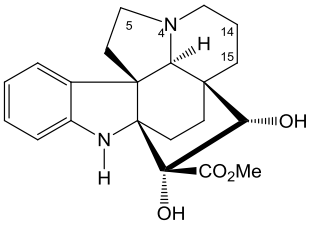
Kopsiloscine F **6**



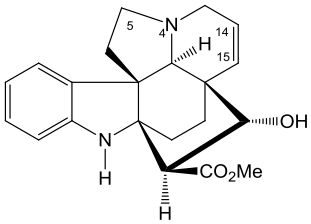
Kopsiloscine G **7**



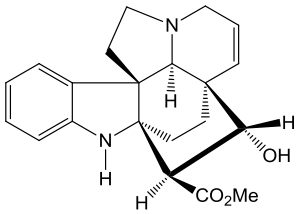
Kopsiloscine H **8**



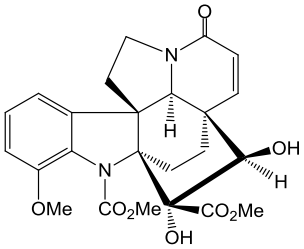
Kopsiloscine I **9**



Kopsiloscine J **10**

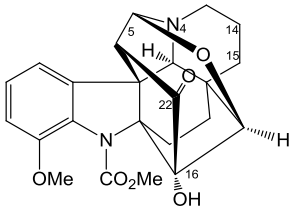


(17α)-17-hydroxy-Δ^{14}-kopsinine **11**

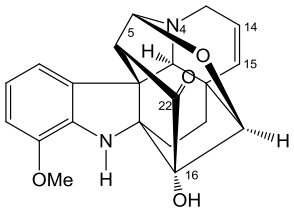


Kopsidarine **12**

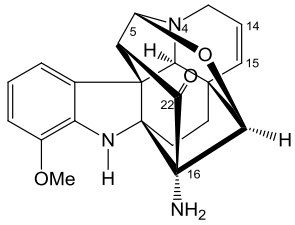
Continued….



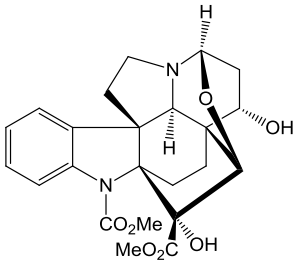
Kopsinitarine A **13**



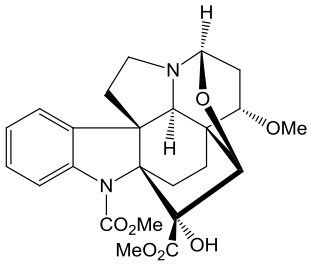
Kopsinitarine B **14**



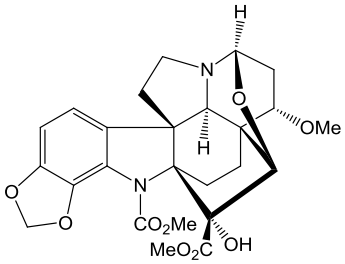
Mersingine A **15**



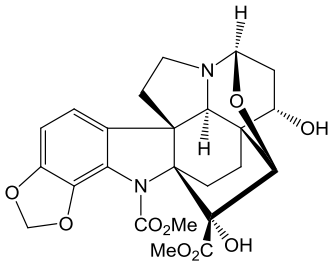
Singapurensine A **16**



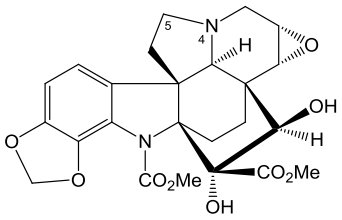
Singapurensine B **17**



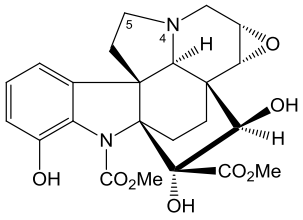
Singapurensine C **18**



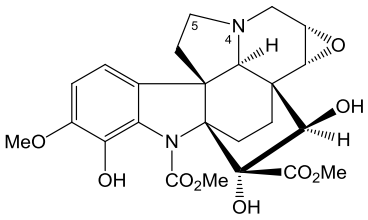
Singapurensine D **19**



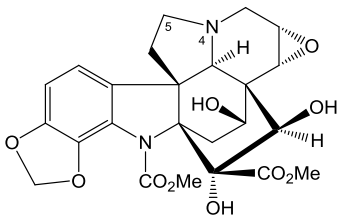
Kopsimaline A **20**



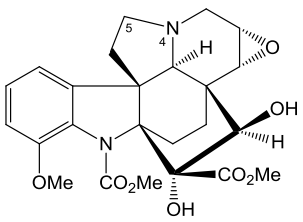
Kopsimaline B **21**



Kopsimaline C **22**



Kopsimaline D **23**



Kopsimaline E **24**

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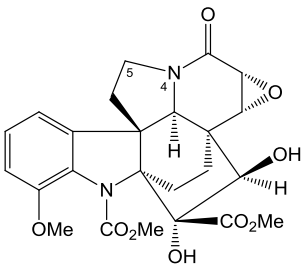
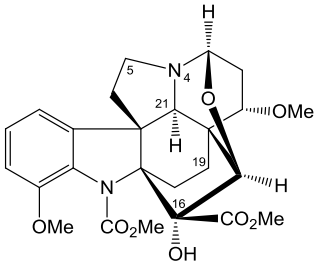
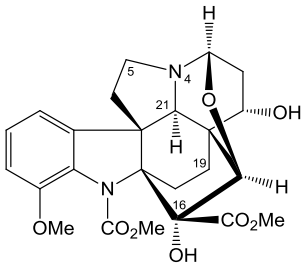


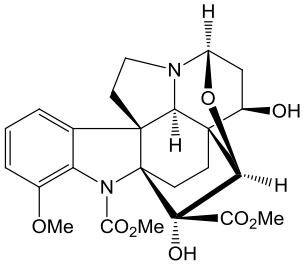
Kopsimaline F **25**



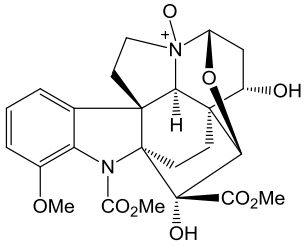
Kopsidine A **26**



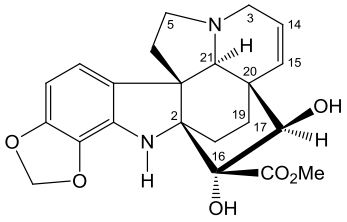
Kopsidine C **27**



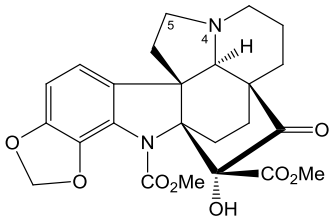
Kopsidine D **28**



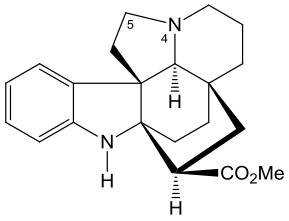
Kopsidine C *N*-oxide **29**



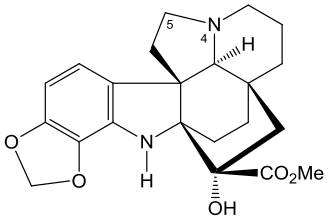
Kopsinicine **30**



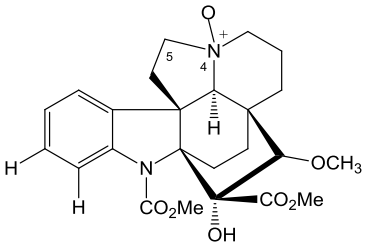
Kopsofinone **31**



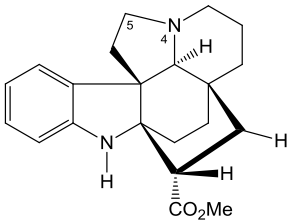
Kopsinine **32**



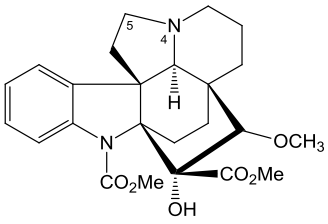
N-decarbomethoxykopsamine **33**



Kopsilongine-*N*-Oxide **34**



16-Epikopsinine **35**



Kopsilongine **36**

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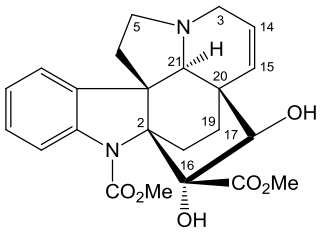
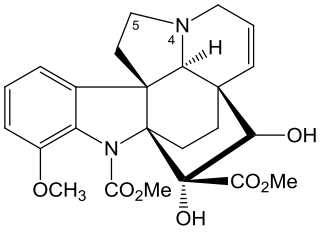
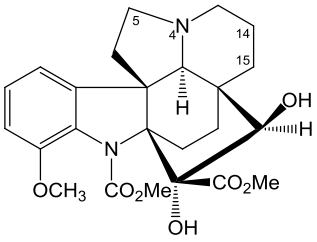


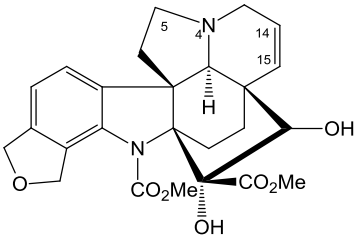
Kopsaporine **37**



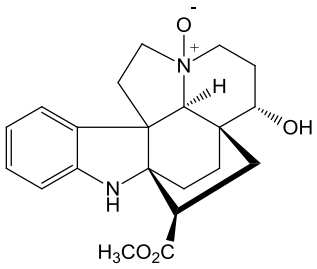
Kopsingine **38**



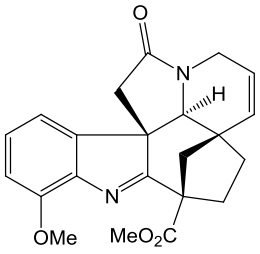
Kopsinganol **39**



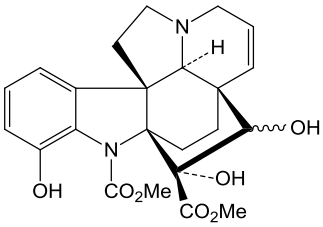
11,12-Methylenedioxykopsaporine **40**



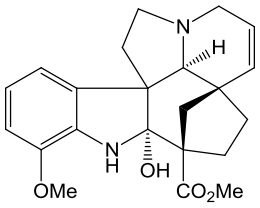
15-Hydroxykopsinine-*N*-oxide **41**



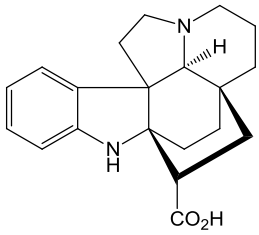
Singaporentine A **42**



Kopsingarine **43**

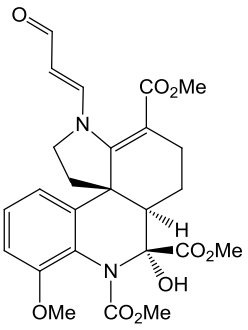


Kopsifoline A **44**

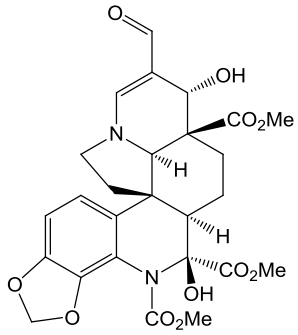


Kopsinic acid **45**

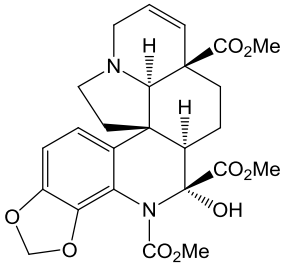
ii) Mersinine type



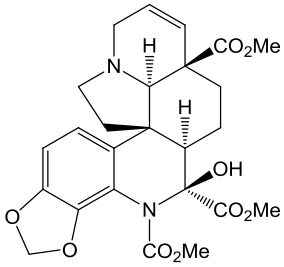
Mersirachine **46**



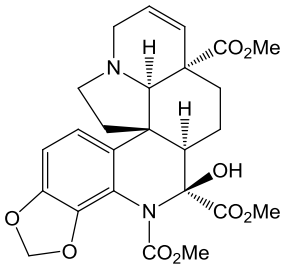
Mersinaline **47**



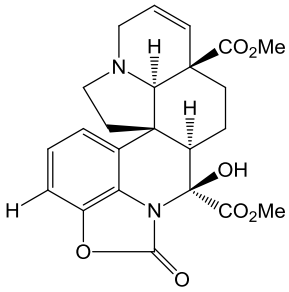
Mersinine A **48**



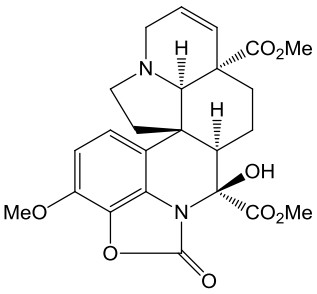
Mersinine B **49**



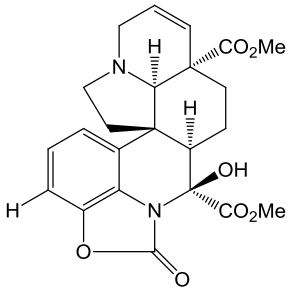
Mersinine C **50**



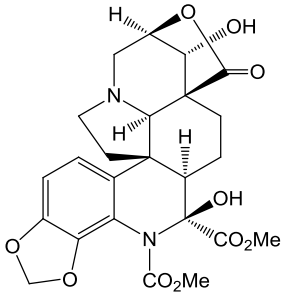
Mersifoline A **51**



Mersifoline B **52**



Mersifoline C **53**



Mersiloscine **54**

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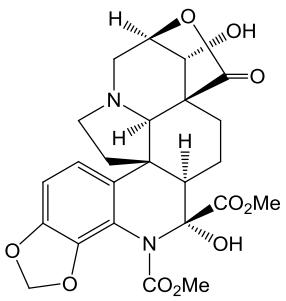
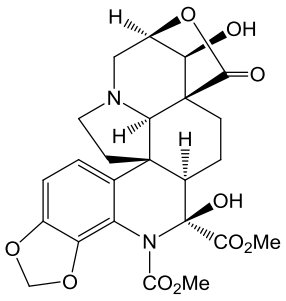
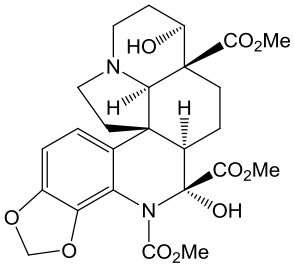


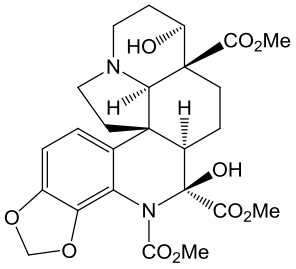
Mersiloscine A **55**



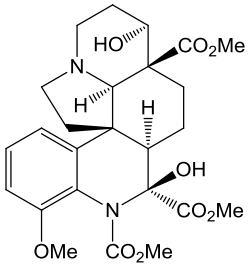
Mersiloscine B **56**



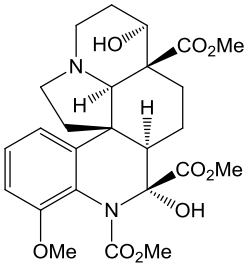
Mersidasine A **57**



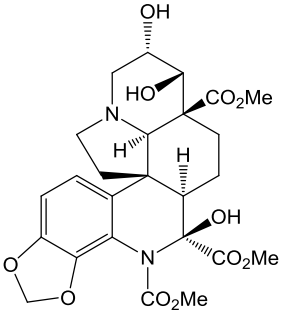
Mersidasine B **58**



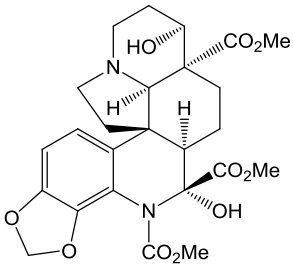
Mersidasine C **59**



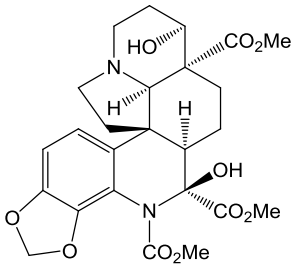
Mersidasine D **60**



Mersidasine E **61**

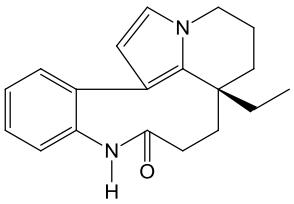


Mersidasine F **62**

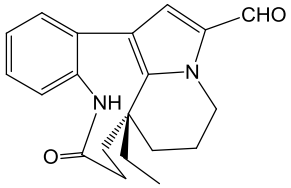


Mersidasine G **63**

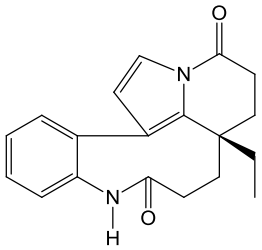
iii) Aspidosperma type



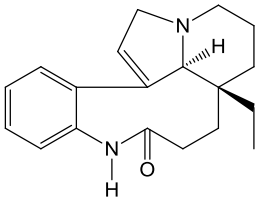
Rhazinilam **64**



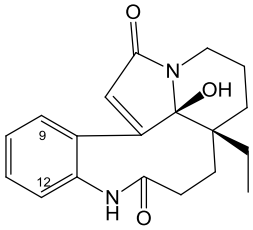
Rhazinal **65**



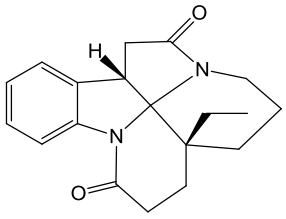
Rhazinicine **66**



5,21-Dihydrorhazinilam **67**

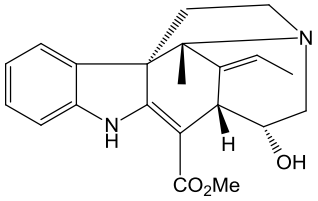


Leuconolam **68**



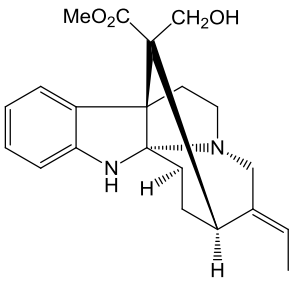
Leuconoxine **69**

iv) Condylocarpine type



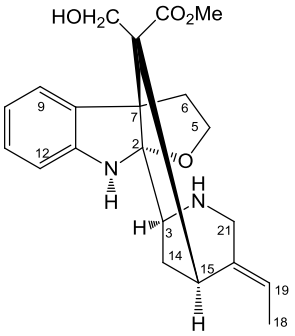
14α- Hydroxycondylocarpine **70**

v) Vincorine type

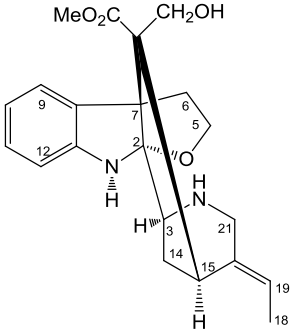


Vincophylline **71**

vi) Aspidodasycarpine type



Aspidodasycarpine **72**



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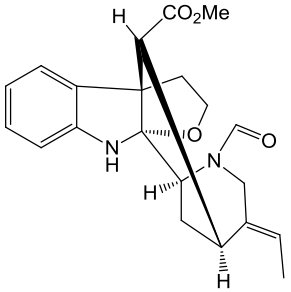
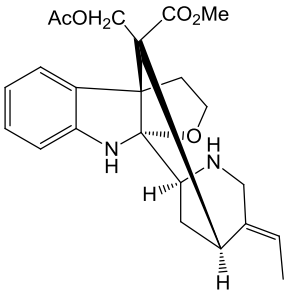
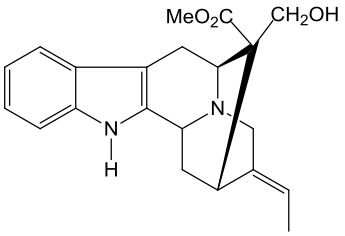


Aspidophylline A **74**



Aspidophylline B **75**

vii) Akuammiline type



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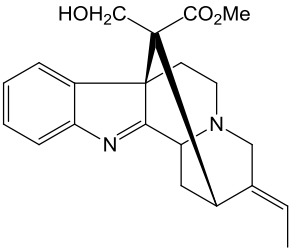
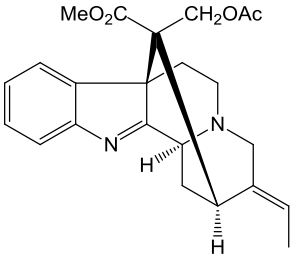
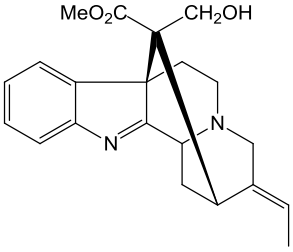


Deacetylakuammiline **77**

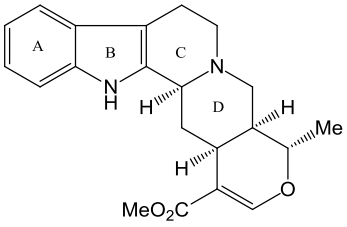


16-Epiakuammiline **78**



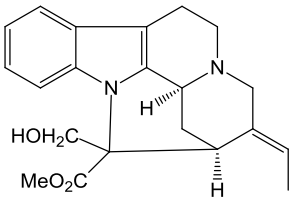
16-Epideacetylakuammiline **79**

viii) Yohimbine type



Tetrahydroalstonine **80**

ix) Pleoicarpamine type



16-Hydroxymethylpleiocarpamine **81**

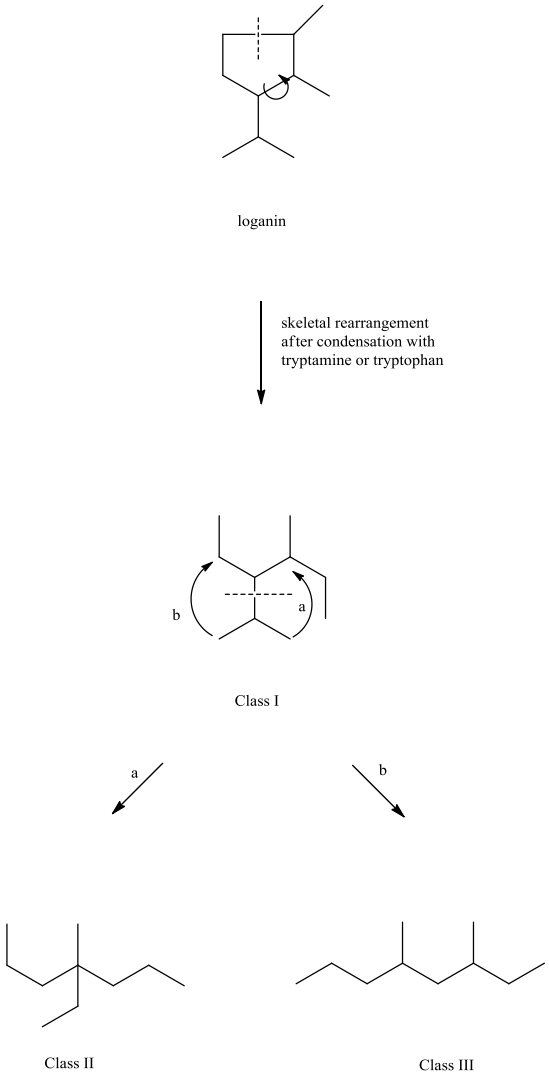
GENERAL ASPECTS OF INDOLE ALKALOIDS

2.1 General

Indole alkaloids have been the subject of many chemical and biosynthetic studies due to their complex and diversed chemical structures and also their interesting biological activities. In the following sections, the author shall discuss the classification and biosynthesis of indole alkaloids.

2.2 Classification of Indole Alkaloids

All indole alkaloids can be classified into five main classes from the structure of the carbon skeleton of the non-tryptamine unit.[37] Class I to Class III is derived from secologanin while Class IV is not derived from secologanin and Class V consists of binary indole alkaloids. (See Scheme 2.1: The three major skeletal classes from loganin). After condensation with tryptophan **94**, these skeletal systems can be determined in an unrearranged form as α- or β-condensation products. (Refer to 2.3: Biosynthesis of Indole Alkaloids).



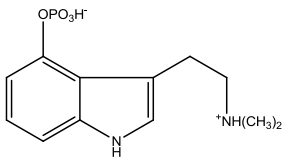
Scheme 2.1: The three major skeletal classes from loganin

2.3 Biosynthesis of Indole Alkaloids

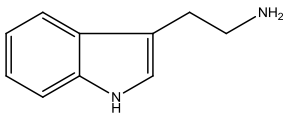
The indole group is highly structurally varied. Simple tryptamines such as psilocybin **89** the hallucinogenic principle of the Mexican ceremonial mushroom, are found in nature.[38] The indole ring is mechanistically available for alkylation's or oxidation both at α- and β-

positions; β-methylation (and spontaneous cyclization of the amine) of a tryptamine **90** can thus lead to physostigmine **91**, an African ordeal poison; β - oxidative coupling yields the *Calycanthaceae* group; folicanthine **92**. The Mannich condensation (with an equivalent of CH_3CHO) proceeds at α-position to furnish β-carboline group; harmine **93**.[38]

More complex indole alkaloids are generally derived from tryptamine **90** by Mannich condensation either at the indolic α- and β- positions.[38] According to Battersby[39,40] *et al*. and Blackstock[41,40] *et al*., condensation of tryptamine **90** (or tryptophan **94**) with secologanin **95**, a monoterpene glucoside, to give rise to a nitrogeneous glucoside, vincoside **96**. Many of these indole alkaloids contain the skeletal fragment biogenetically symbolized in **97**.[38] They are shown in heavy lines in some of the examples but in some molecules, particularly those from *Tabernanthe* species; voacangine **98** and *Hunteria* species; eburnamonine **99** and the aspidospermine **100** are rearranged. The circled carbon in **97**, as in yohimbine **101** may have its origin in an oxidized N-methyl.[38]



89



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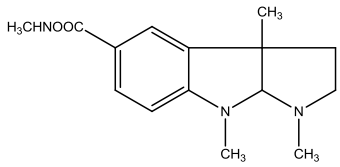

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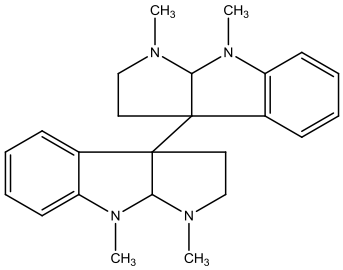

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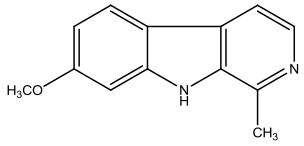

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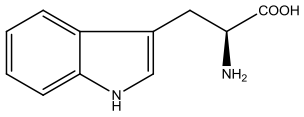

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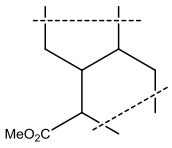

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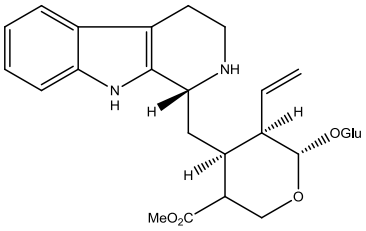

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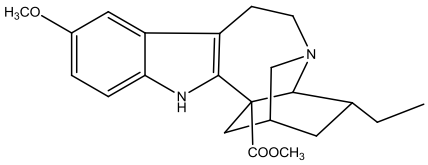

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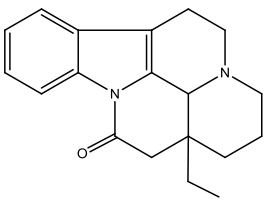

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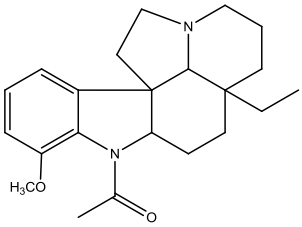

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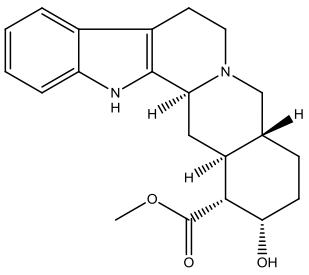
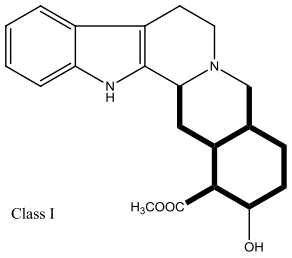


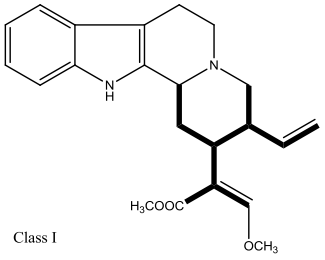
101

The Mannich condensation of the aldehydic secologanin complex with the indole **97** resulted in two groups. Cyclization involving the α-position usually leads to true indoles, as in yohimbine **101**, corynantheine **102**, cinchonamine **103** and ajmaline **104**; whereas β-condensation must block indolic unsaturation with a quartenary center as in mitraphylline **105** and caracurine VII **106**. A number of other alkaloids are biogenetically derived from these by a few further transformations, as in the conversion of cinchonamine (or a close relative) to the quinine **107** family skeleton via opening of the indole ring and enclosure of the aniline nitrogen on the side chain. Indeed Leete finds radioactive tryptophan **94** a precursor of this group. Interesting if somewhat more involved schemes may be constructed to afford stemmadenine **108**, ellipticine **109** and gelsemine **110** from a starting point of the caracurine type **106**.[38]



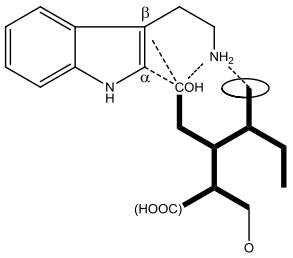
Class I

101



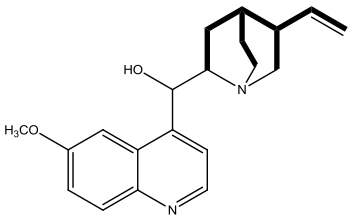
Class I

102



97

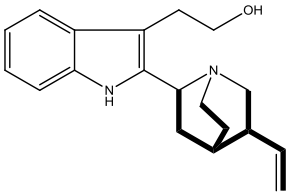
(tryptamine + monoterpene secologninn)



Class I

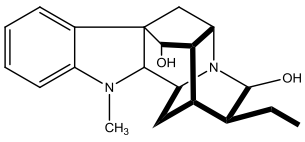
107

Class I



103

Class I



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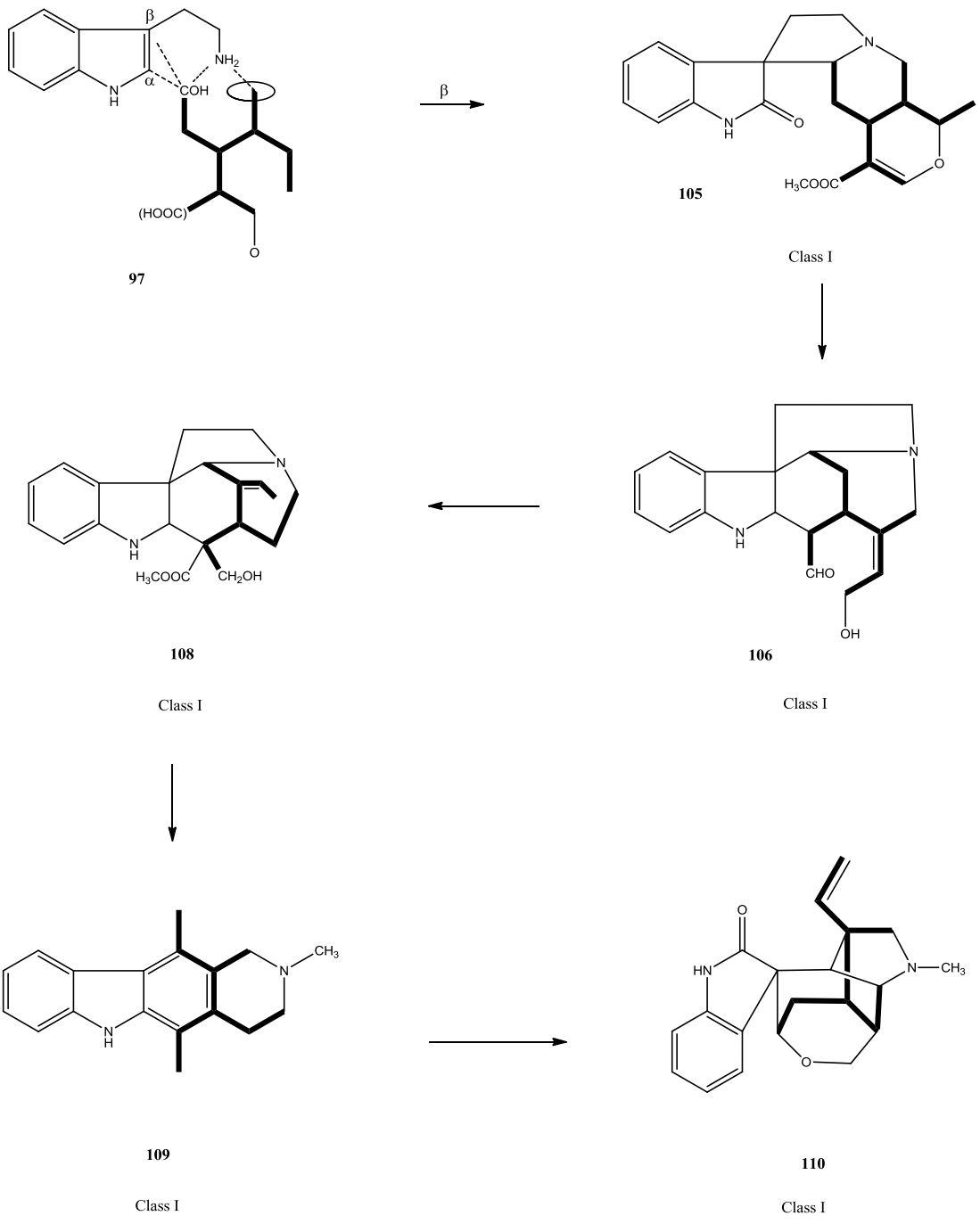
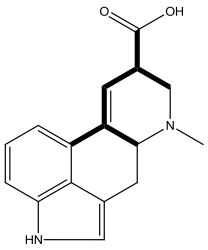


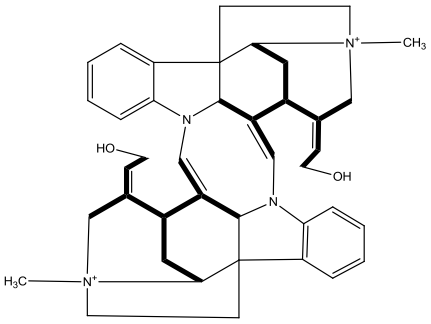
The hallucinogens from the rye fungus (ergot), thought to have been the cause of St. Vitus`s Dance in medieval times, are peptide amides of the interesting lysergic acid **111**, to be a biosynthetic product of tryptamine **90** and mevalonic acid **112**. The most complex bases are the dimeric indole alkaloids such as the many constituents of calabash curare,

derived from caracurine by a simple dehydrative dimerization and exemplified in the common toxiferine **113** and voacamine **114** and the very similar vinblastine **115**, an anticancer agent isolated from the humble periwinkle.



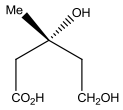
lysergic acid **111**

Class III

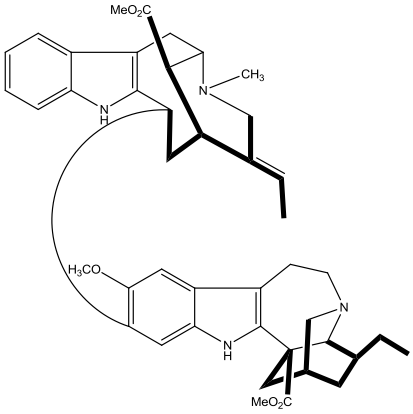


toxiferine **113**

Class V - (I-I class)

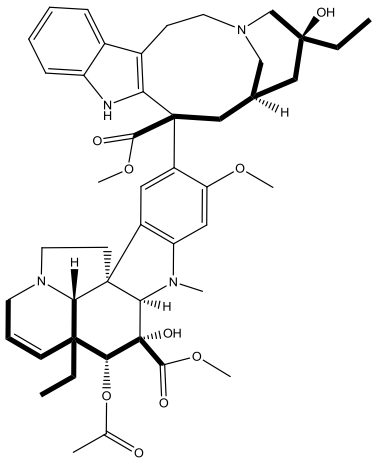


mevalonic acid **112**



voacamine **114**

Class V - (I-III class)

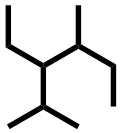


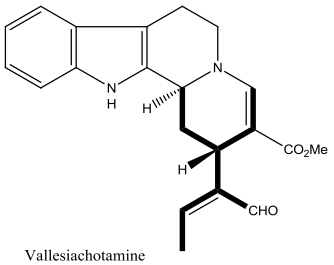
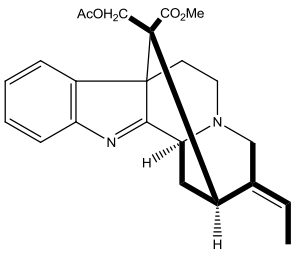
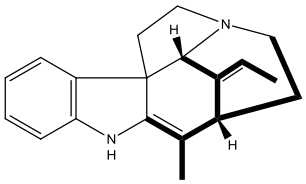
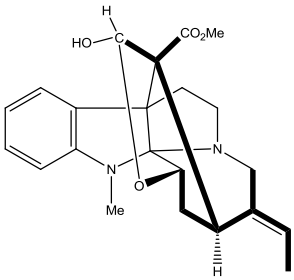
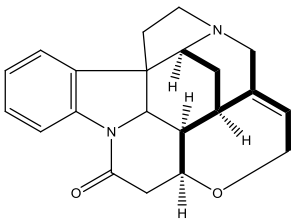
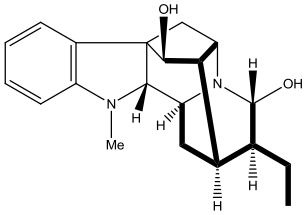
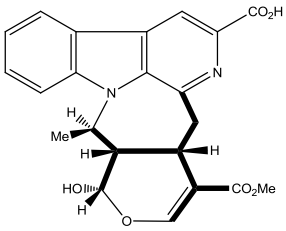
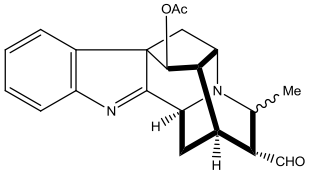
vinblastine **115**

Class V - (II-III class)

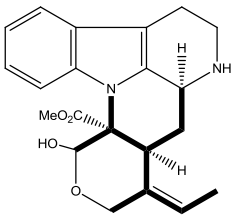
Table 2.1: Biogenetic Classification of Indole Alkaloids[42,43,36]

Class I



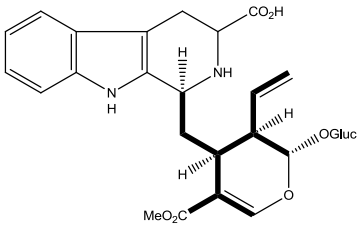
Vallesiachotamine group	Picraline group
 Vallesiachotamine	 Akuammiline
Condylocarpine group	**Corynine group**
 Condylocarpine	 Corynine
Strychnine group	**Ajmaline group**
 Strychine	 Ajmaline
Adifoline group	**Perakine group**
 Adifoline	 Perakine

Talbotine group



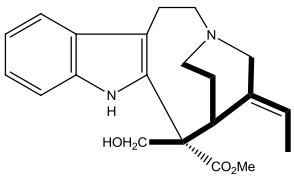
Talbotine

Vincoside group



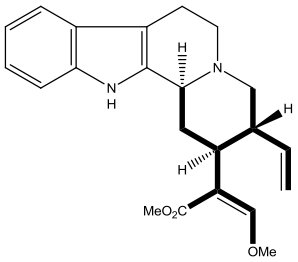
5α-Carboxystrictosidine

Stemmadenine group



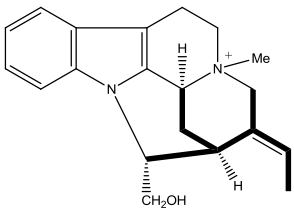
Stemmadenine

Corynantheine group



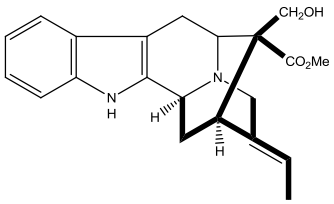
Corynantheine

Mavacurine group



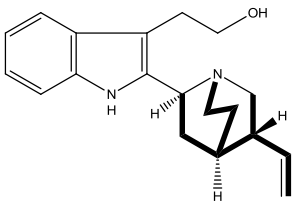
Mavacurine

Sarpagine group



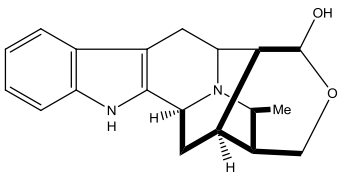
Polyneuridine

Cinchonamine group



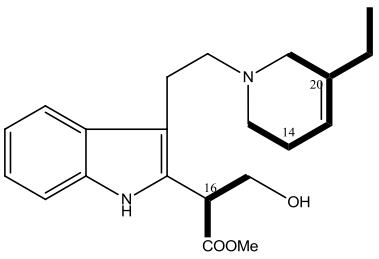
Cinchonamine

Peraksine group



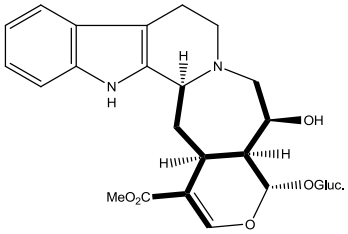
Peraksine

Secodine group



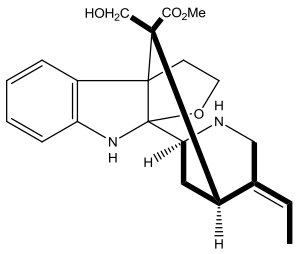
16, 17-dihydrosecodine-17-ol

Cadambine group



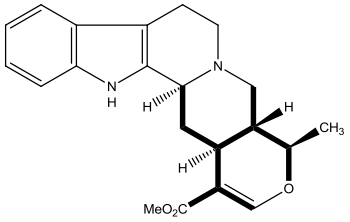
3α-Dihydrocadambine

Aspidodasycarpine group



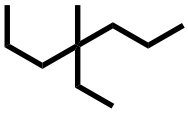
Aspidodasycarpine

Ajmalicine group

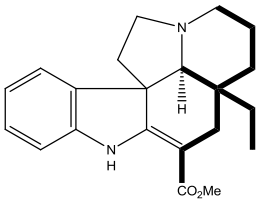


Ajmalicine

Class II

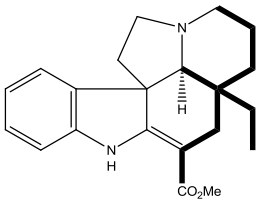


Aspidospermine group



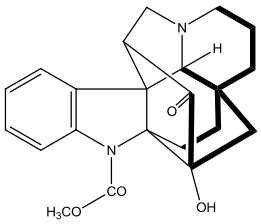
Vincadifformine

Pleiocarpine group



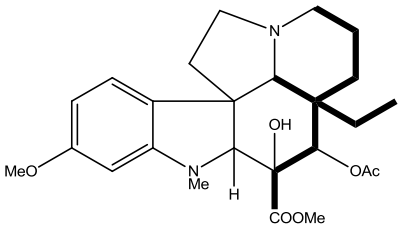
Aspidofractinine

Kopsine group



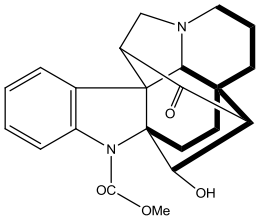
Kopsine

Vindoline group



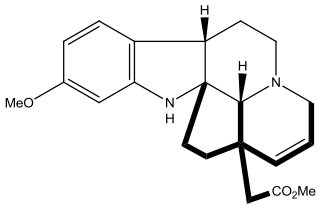
vindoline

Fruticosine group



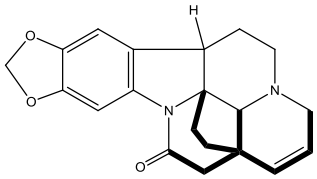
Fruticosine

Schizophylline group



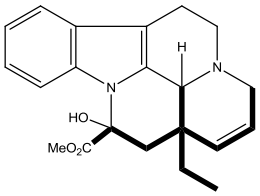
Schizophylline

Schizozygine group



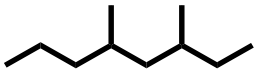
Schizozygine

Vincamine group

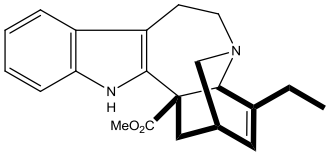


Vincamine

Class III

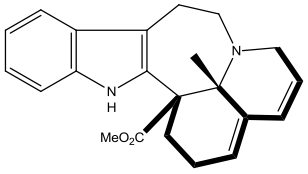


Catharanthine group



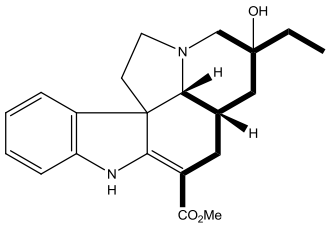
Catharanthine

Andranginine group



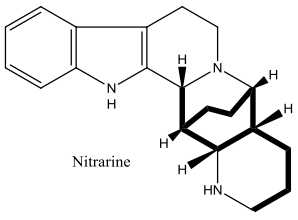
Andranginine

Pandoline group



Pandoline

Nitramidine group

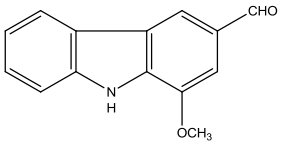


Nitrarine

Class IV (not derived from secologanin)

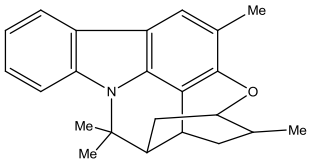
i) Non-tryptophan indole alkaloids (carbazole alkaloids)

Murrayanine group



Murrayanine

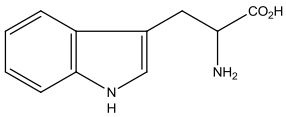
Currayangine group



Currayangine

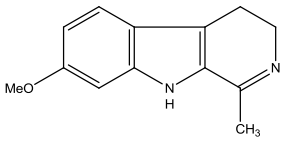
ii) Non-isoprenoid tryptophan alkaloids

Tryptophan group



Tryptophan

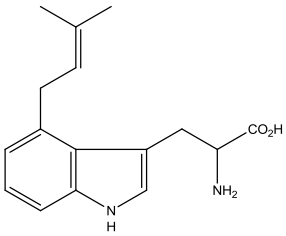
Harmaline group



Harmaline

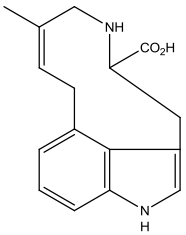
iii) Isoprenoid tryptophan alkaloids (fungal indole alkaloid)

Isopentenyl tryptophan group



4-isopentenyl tryptophan

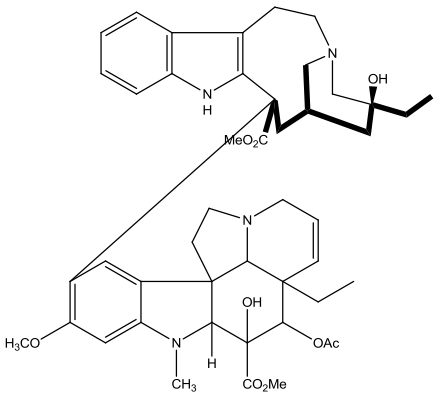
Clavicipitic acid group



Clavicipitic acid

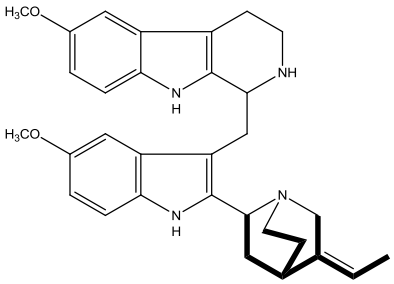
Class V (binary indole alkaloids)

II-III group



Vinblastine

I-IV group



Cinochophyllamine

RESULTS AND DISCUSSION

3.0 Introduction

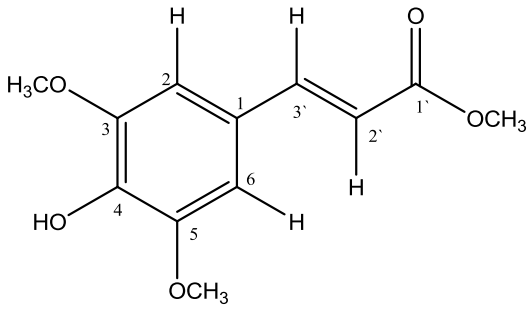
The present work deals with the isolation and structural elucidation of alkaloids from *Kopsia singapurensis* (bark). Seven compounds were isolated and characterized; methyl sinapate (IUPAC name ; (*E*)-methyl-3-(4-hydroxy-3, 5-dimethoxyphenyl) acrylate **82**, leuconolam **83**, *trans*-2,2`-dicarboxyazobenzene dioxide **84**, lonicerine **85**, 15-hydroxykopsinine **86**, singapurine **87** and N_4-hydroxymethyl kopsinic acid **88** (Table 3.0).

Various chromatographic techniques such as column chromatography and TLC have been used to isolate the compounds. The structures were determined by a combination of spectroscopic methods; 1D-NMR (^{1}H, ^{13}C, DEPT), 2D-NMR (COSY, NOESY, HMQC/HSQC and HMBC), UV, IR, MS (GCMS, LCMS and HRMS). The structures of the compounds were also elucidated by comparison with the previous work. The following subchapters/paragraphs will discuss on structural elucidation of all compounds isolated.

Table 3.0: Compounds isolated from *Kopsia singapurensis*

COMPOUND	Yield (g)
COMPOUND **A ;** methyl sinapate (IUPAC name : (*E*)-methyl-3-(4-hydroxy-3,5-dimethoxyphenyl) acrylate **82**	0.0022
COMPOUND **B ;** leuconolam **83**	0.0020
COMPOUND **C ;** *trans*-2,2`-dicarboxyazobenzene dioxide **84**	0.0040
COMPOUND **D ;** lonicerine **85**	0.0065
COMPOUND **E ;** 15-hydroxykopsinine **86**	0.0183
COMPOUND **F ;** singapurine **87**	0.0044
COMPOUND **G ;** N_4-hydroxymethyl kopsinic acid **88**	0.0073

3.1 **COMPOUND A;** Methyl sinapate **82**



Compound **A** was obtained as yellowish amorphous. The HREIMS exhibited a pseudo molecular ion peak at m/z 239.0732 $[M+H]^+$, suggesting a molecular formula of $C_{12}H_{14}O_5$. Absorptions at 3398, 1706 and 1113 cm^{-1} in IR spectrum indicated the presence of hydroxyl (OH), carbonyl (C=O) and (C-O) groups respectively[44].

The ^{1}H-NMR spectrums showed a singlet corresponding to two aromatic protons at δ 6.77 which may be attributable to H-2 and H-6 respectively. Methoxyls attached to C-3 and C-5 resonated as a singlet at δ 3.92. Another singlet corresponding to the methoxyl attached to C-1` was also observed at δ 3.80. H-2` and H-3` resonated as a pair of doublet (J = 16 Hz) at δ 6.30 and δ 7.60 respectively. The presence of these two protons was further confirmed by using ^{1}H-^{1}H COSY spectrum.

The ^{13}C-NMR (Figure 3.2), DEPT (Figure 3.3) spectrum of compound **A** further confirmed the presence of twelve carbons. The DEPT spectrum showed four methines; δ 105.4 (C-2 and C-6), 115.9 (C-2`) and 145.5 (C-3`) and four quartenary carbon signals at δ 126.5 (C-1), 147.6 (C-3 and C-5) and 167.8 (C-1`) respectively (Table 3.1). The HMBC spectrum (Figure 3.5) showed the correlations between H-2 with C-6, C-1, C-4 and C-3`, whereas H-6 showed crosspeaks with C-2, C-1, C-4 and C-3`. The NOE-diff experiment

showed enhancement of H-2 and H-6 upon irradiation of the methoxyl protons of C-3 and C-5 indicating that C-4 is substituted by an OH group.

Based on the spectral data observed from DEPT, HSQC, HMBC, NOE-diff and comparison with literature compound **A** is indeed the known methyl sinapate[45,46] [IUPAC name; (*E*)-methyl-3-(4-hydroxy-3,5-dimethoxyphenyl) acrylate]. This is the first report on the occurrence of methyl sinapate **A** in nature.

Table 3.1: ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of compound **A** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
1		126.5
2	6.77 (*s*)	105.4
3		147.6
4-OH	5.76 (*s*)	137.5
5		147.6
6	6.77 (*s*)	105.4
1`		167.8
2`	6.30 (*d*, 16.0 Hz)	115.9
3`	7.60 (*d*, 16.0 Hz)	145.5
1`-OCH_3	3.80 (*s*)	52.1
3-OCH_3	3.92 (*s*)	56.7
5-OCH_3	3.92 (*s*)	56.7

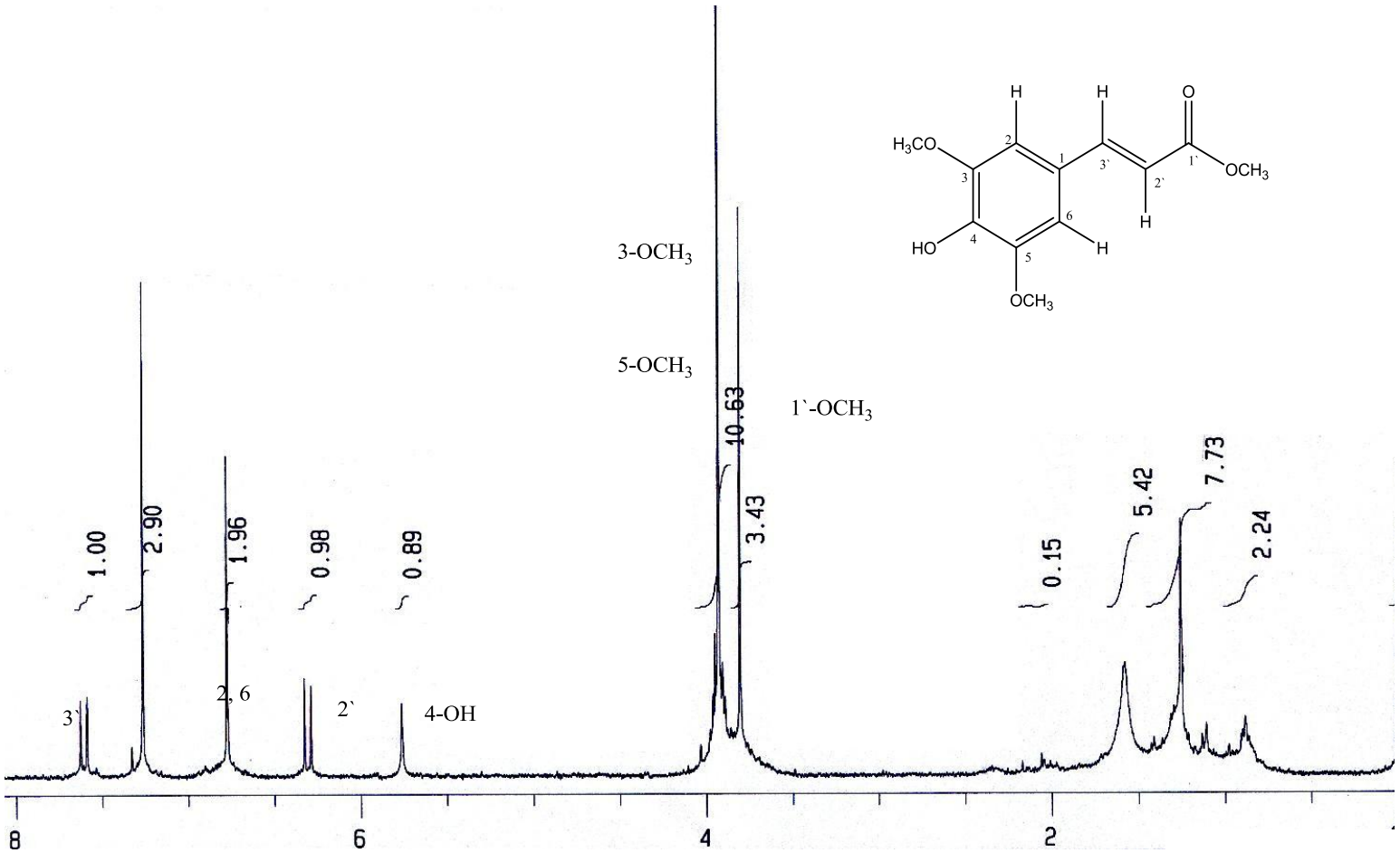


Figure 3.1: ^{1}H-NMR Spectrum of Compound **A**

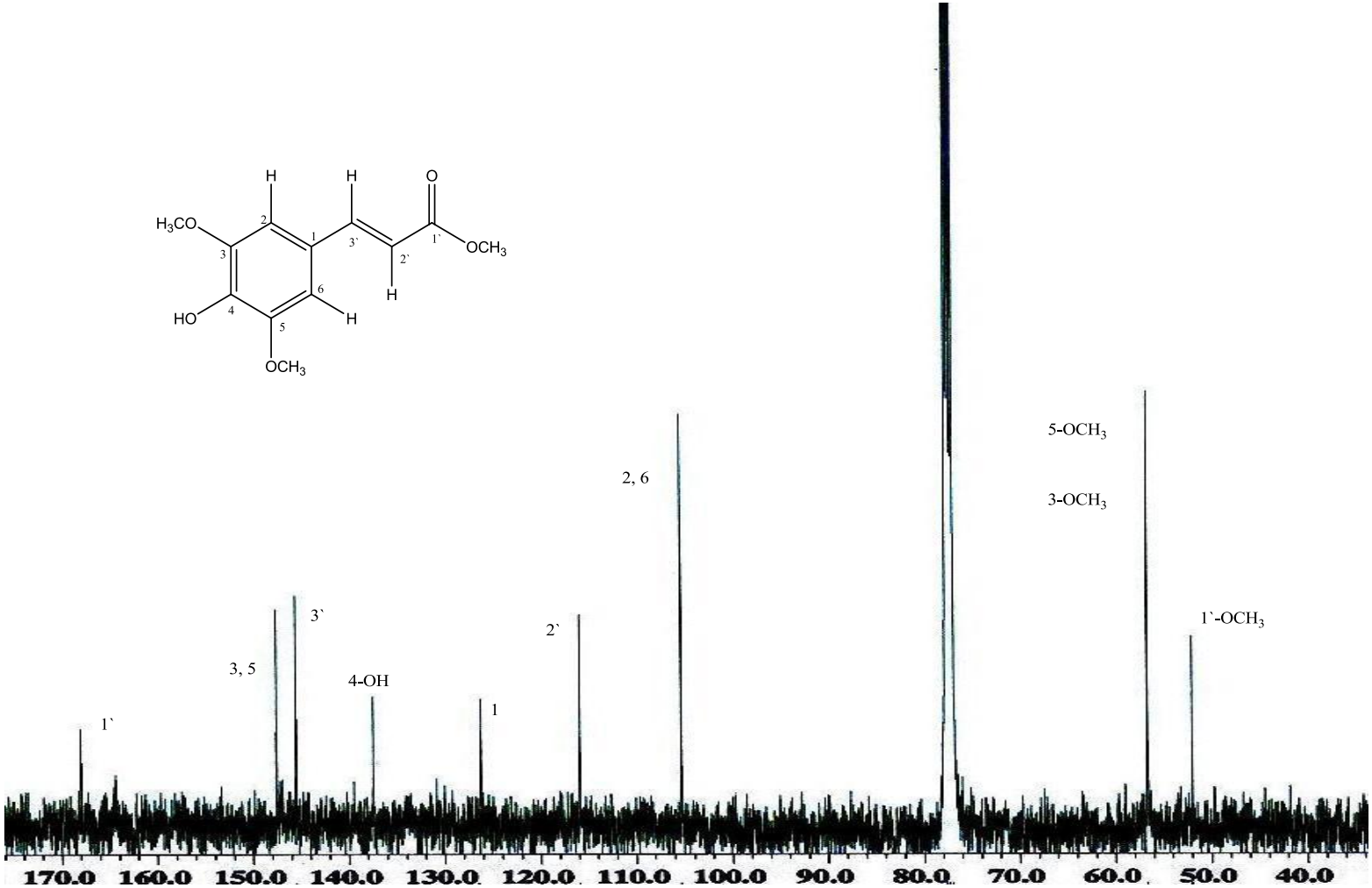


Figure 3.2: ^{13}C-NMR Spectrum of Compound **A**

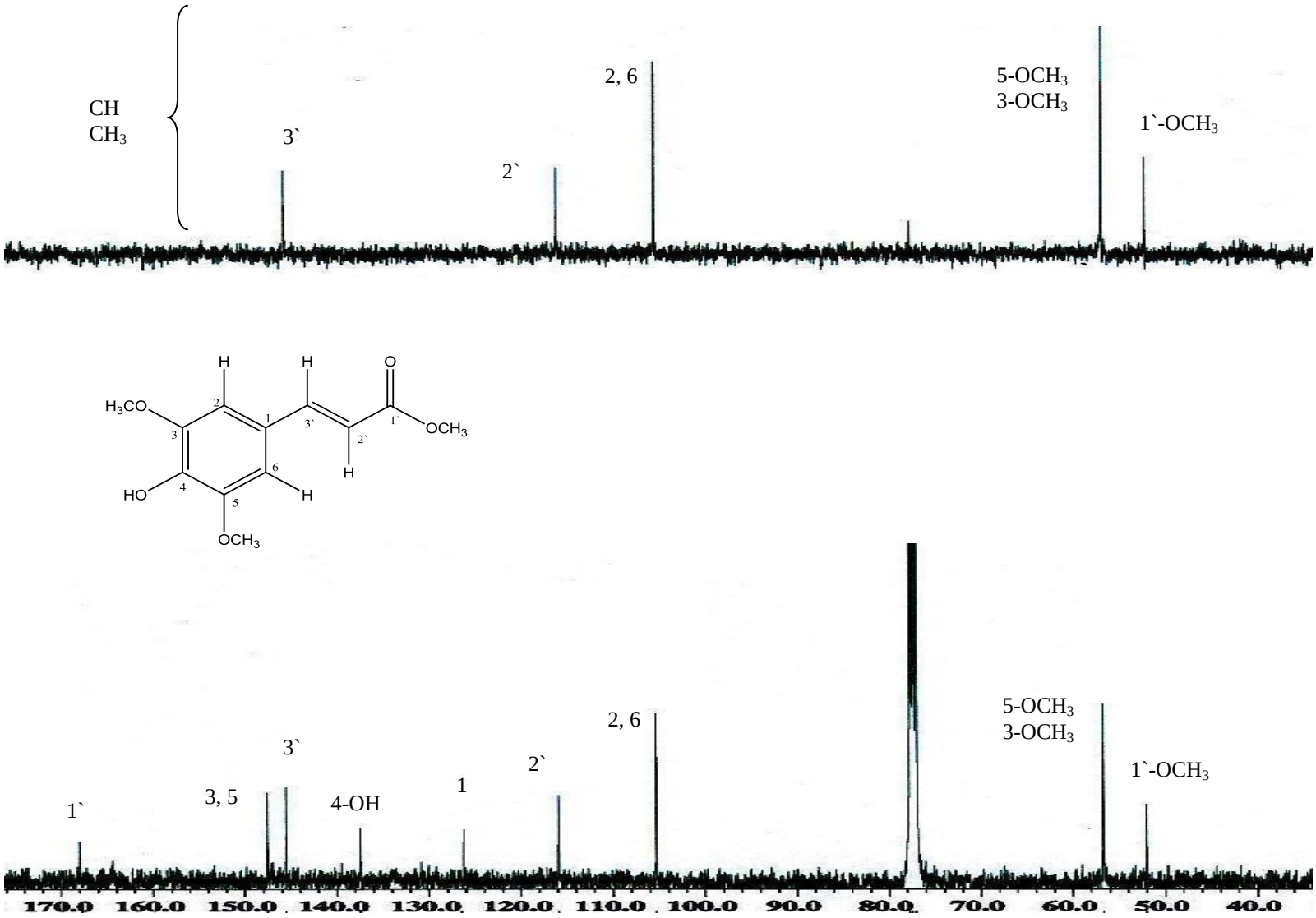


Figure 3.3: DEPT Spectrum of Compound **A**

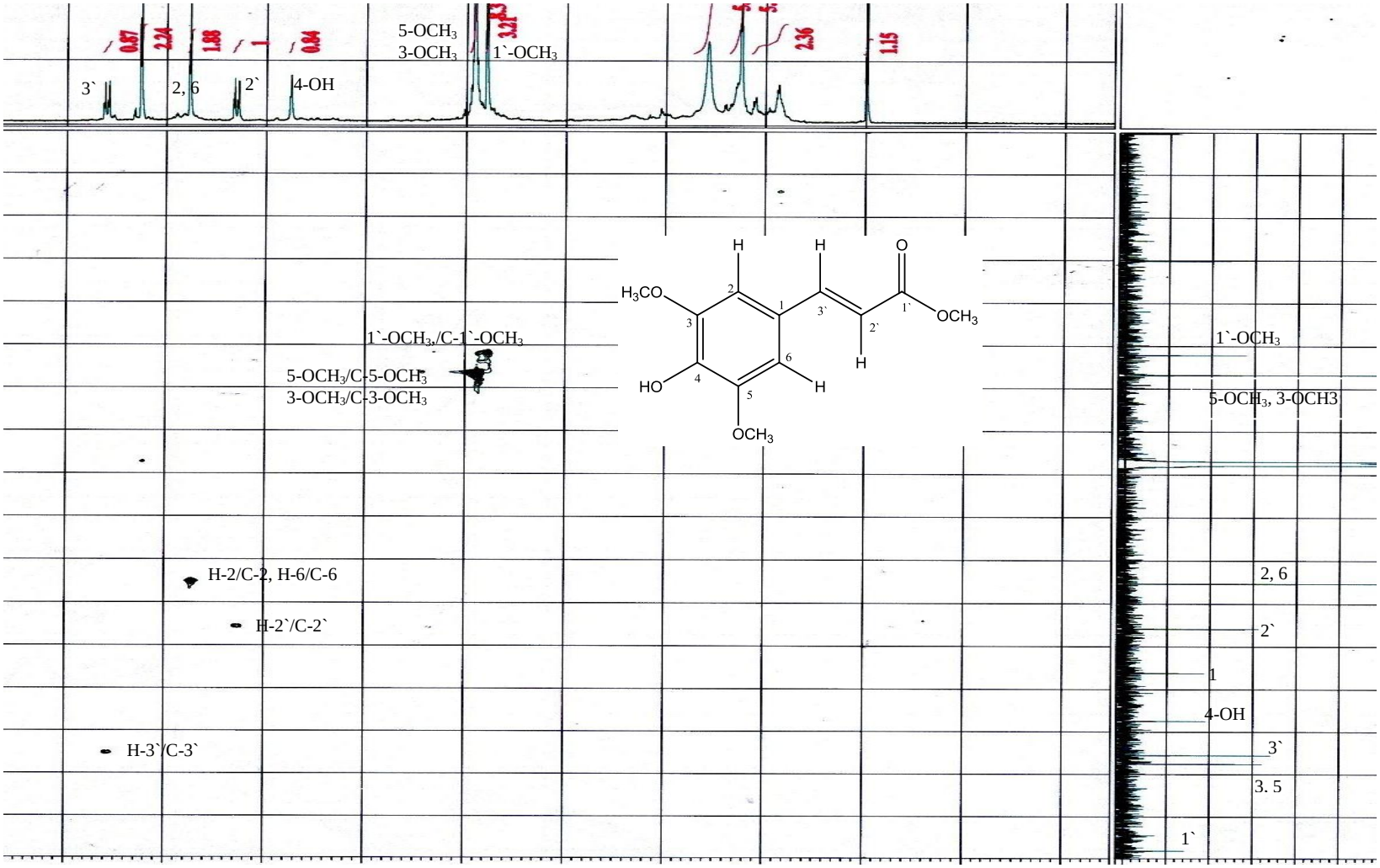


Figure 3.4: HSQC Spectrum of Compound **A**

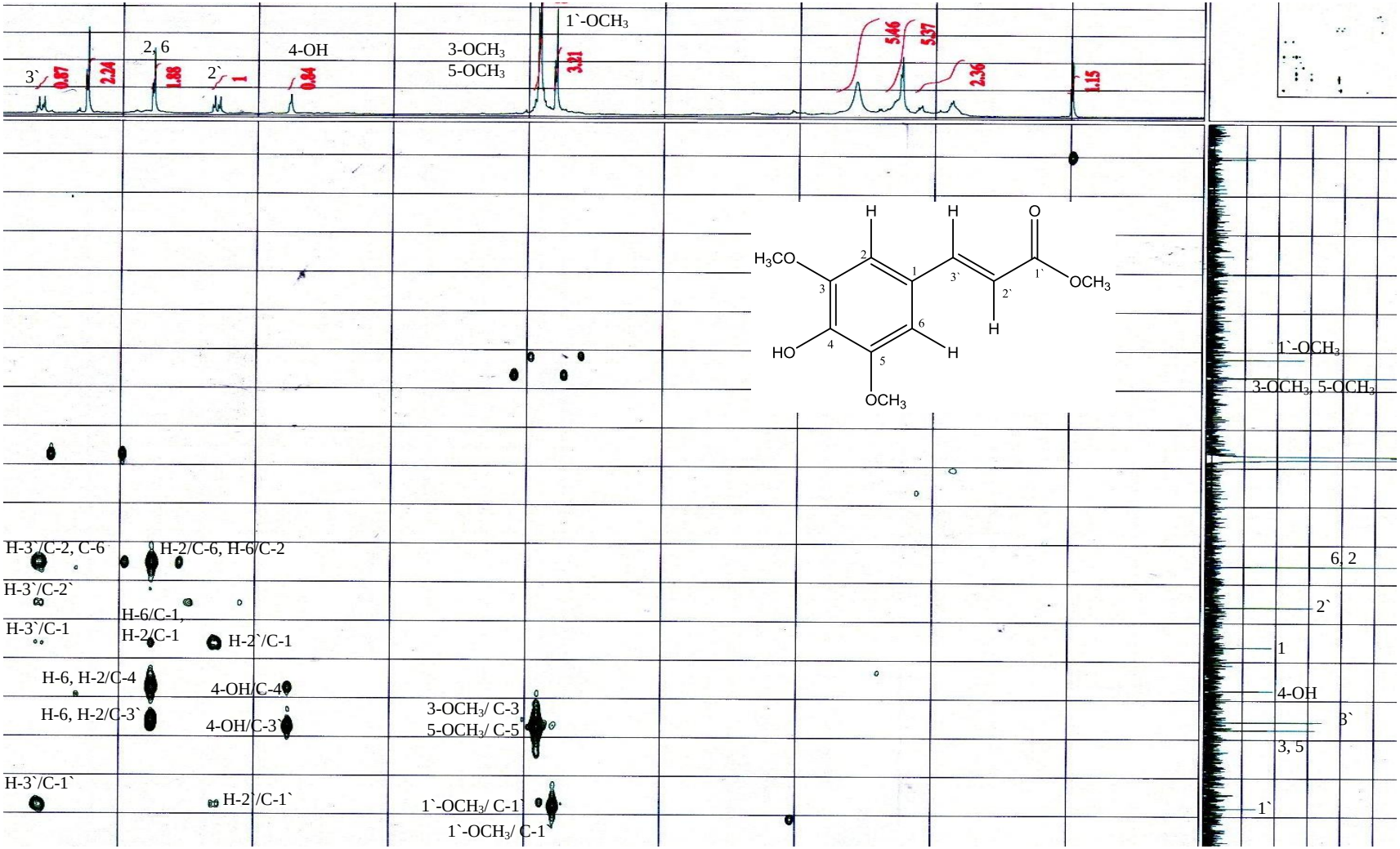
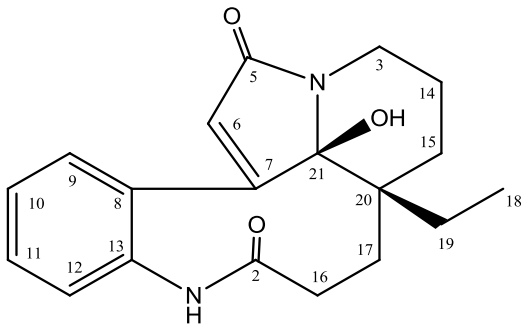


Figure 3.5: HMBC Spectrum of Compound **A**

3.2 **COMPOUND B;** leuconolam **83**



Compound **B**, was isolated as a yellowish amorphous. The HREIMS showed the pseudo-molecular ion peak at m/z 325.1461 $[M-H]^+$ which matched with the molecular formula $C_{19}H_{22}N_2O_3$. The IR spectrum revealed absorption at 1689 cm^{-1} indicating the presence of a lactam carbonyl group.[44,47]

The ^{1}H-NMR spectrum (Figure 3.6) revealed a number of important features, including the presence of ethyl group which was evidenced by the appearance of a CH_2-19 multiplet at δ 1.45 and 1.35 and a triplet of CH_3-18 at δ 0.77. The four aromatic proton signals appeared at δ 7.45 (*dd, J* = 7.5, 1.1Hz), δ 7.12 (*dt, J* = 7.5, 1.1 Hz), δ 7.33 (*dt, J* = 8.2, 1.1 Hz) and δ 8.15 (*d*, 8.2 Hz). Two sets of ddd corresponding to H-3a and H-3b were observed at δ 3.22 and δ 4.46. They are deshielded since they are adjacent to the nitrogen (N_2), an electronegative atom. Other methylene proton (H-14, H-15, H-16, H-17) signals were apparent between δ 1.13 to δ 3.09.

The ^{13}C-NMR (Figure 3.7) and DEPT spectrum of compound **B** showed nineteen carbon signals corresponding to nineteen carbon atoms; five methines at δ 116.2 (C-12), 118.4 (C-

6), 121.8 (C-9), 124.6 (C-10) and 131.8 (C-11), six methylenes at δ 17.0 (C-14), 26.3 (C-15), 30.7 (C-17), 33.4 (C-16), 34.4 (C-19) and 37.2 (C-3), one methyl signal at δ 8.5 (C-18) and seven quaternary carbons in which two are carbonyl carbons (C-2, δ 173.7) and (C-5, δ 176.4) in agreement with the presence a broad and strong IR absorption at 1689 cm^{-1}.[44,47]

In addition, other quaternary carbon resonances were observed at δ 164.5 (C-7), δ 123.8 (C-8), δ 148.8 (C-13) and δ 48.8 (C-20). The observed three-bond correlation from H-6 to C-21 in the HMBC (Figure 3.10) spectrum not only confirmed the assignment of H-6 but also defined the location of the five membered ring lactam groups. The complete assignments of ^{1}H and ^{13}C were aided by the HMBC, HSQC and COSY experiments and were listed in Table 3.2.

In conclusion, comparison with the literature values confirmed that compound **B** is leuconolam.[48-50,47]

Table 3.2: ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of compound **B** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
2		173.7
3	3.22 (*ddd*, 15.5, 9.6, 6.4 Hz)	37.2
	4.46 (*ddd*, 15.5, 11.8, 2.9 Hz)	
4		
5		176.4
6	6.19 (*s*)	118.4
7		164.5
8		123.8
9	7.45 (*dd*, 7.5, 1.1Hz)	121.8
10	7.12 (*dt*, 7.5, 1.1 Hz)	124.6
11	7.33 (*dt*, 8.2. 1.1 Hz)	131.8
12	8.15 (br *d*, 8.2 Hz)	116.2
13		148.8
14	1.77 (*m*)	17.0
	2.03 (*m*)	
15	1.13 (*m*)	26.3
	1.64 (*m*)	
16	2.65 (*ddd*, 16.0, 5.0, 1.8 Hz)	33.4
	3.09 (*dt*, 16.0, 5.9 Hz)	
17	1.67 (*m*)	30.7
	2.09 (*m*)	
18	0.77 (*t*, 7.3 Hz)	8.5
19	1.35 (*m*)	34.4
	1.45 (*m*)	
20		44.8
21-OH		93.6
NH	3.64 (br *s*)	

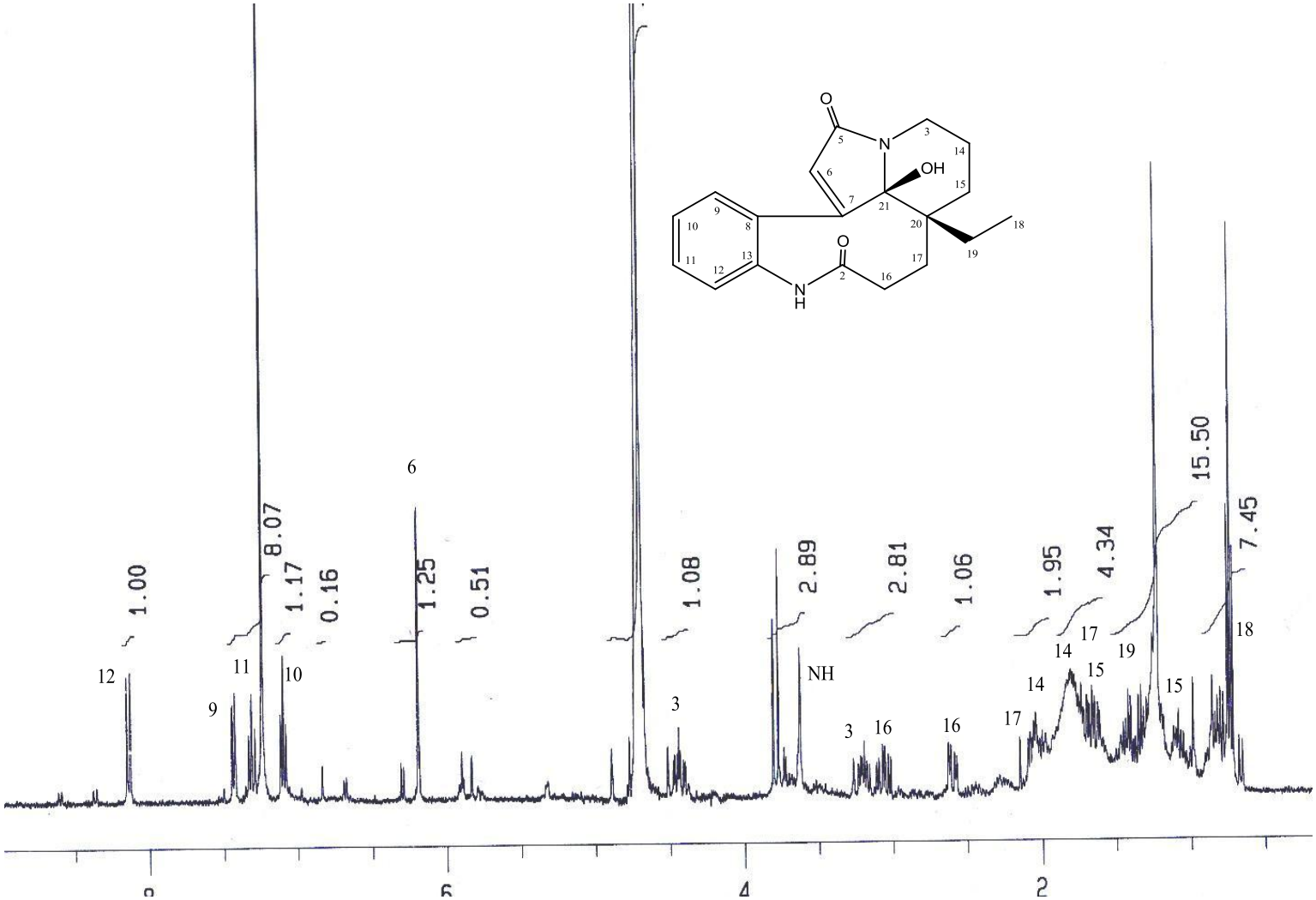


Figure 3.6: ^{1}H-NMR Spectrum of Compound **B**

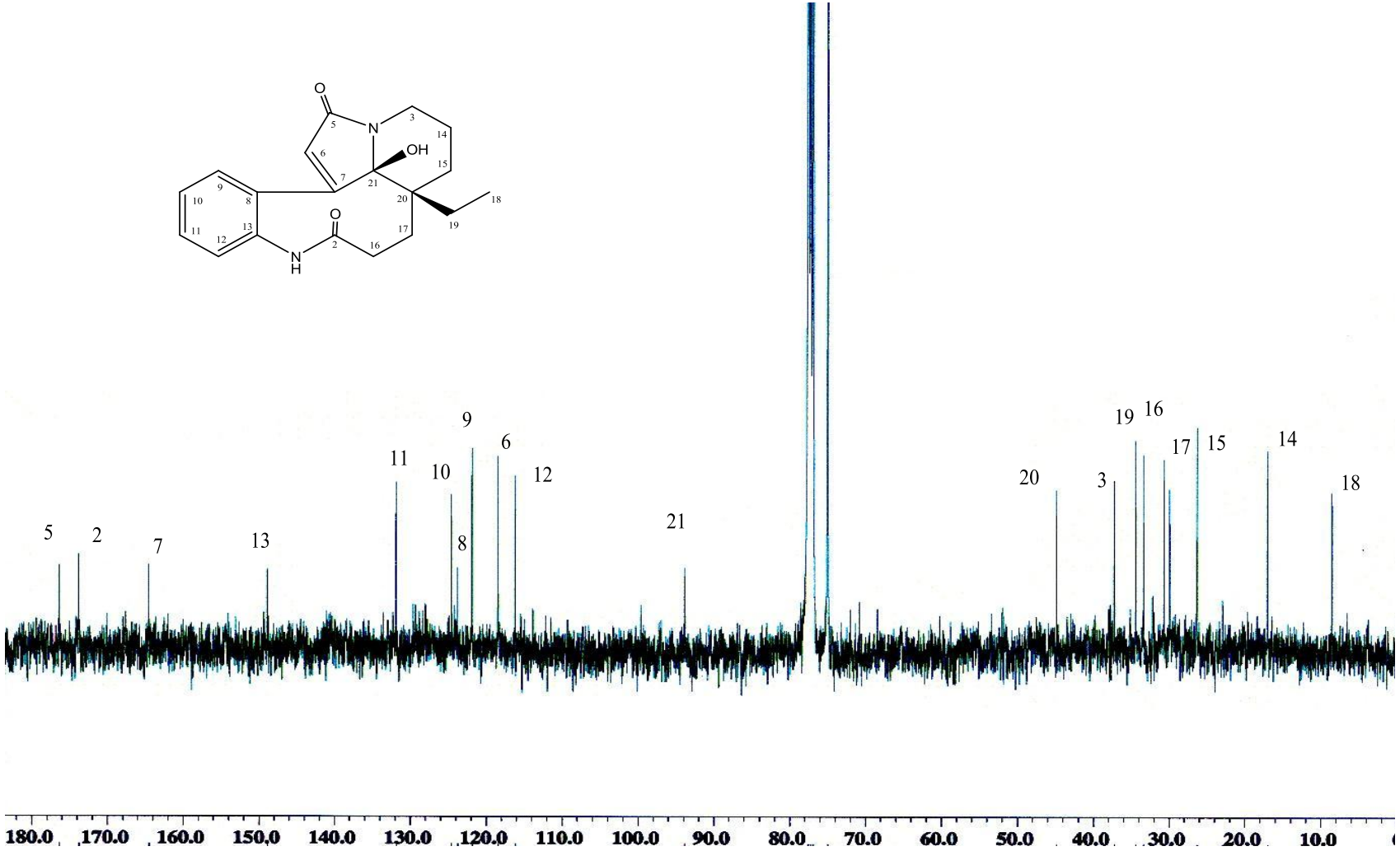


Figure 3.7: ^{13}C-NMR Spectrum of Compound **B**

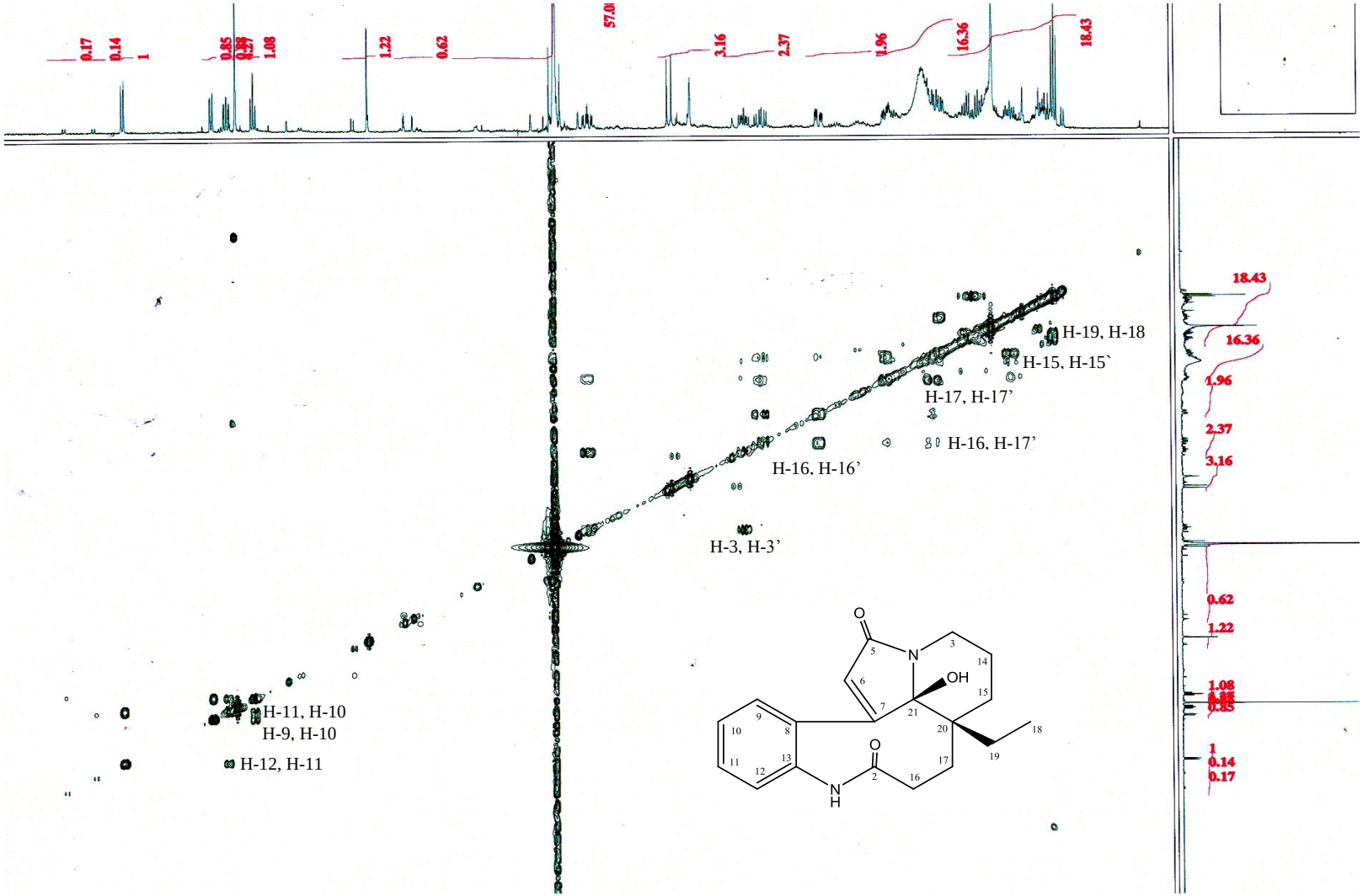


Figure 3.8: COSY Spectrum of Compound **B**

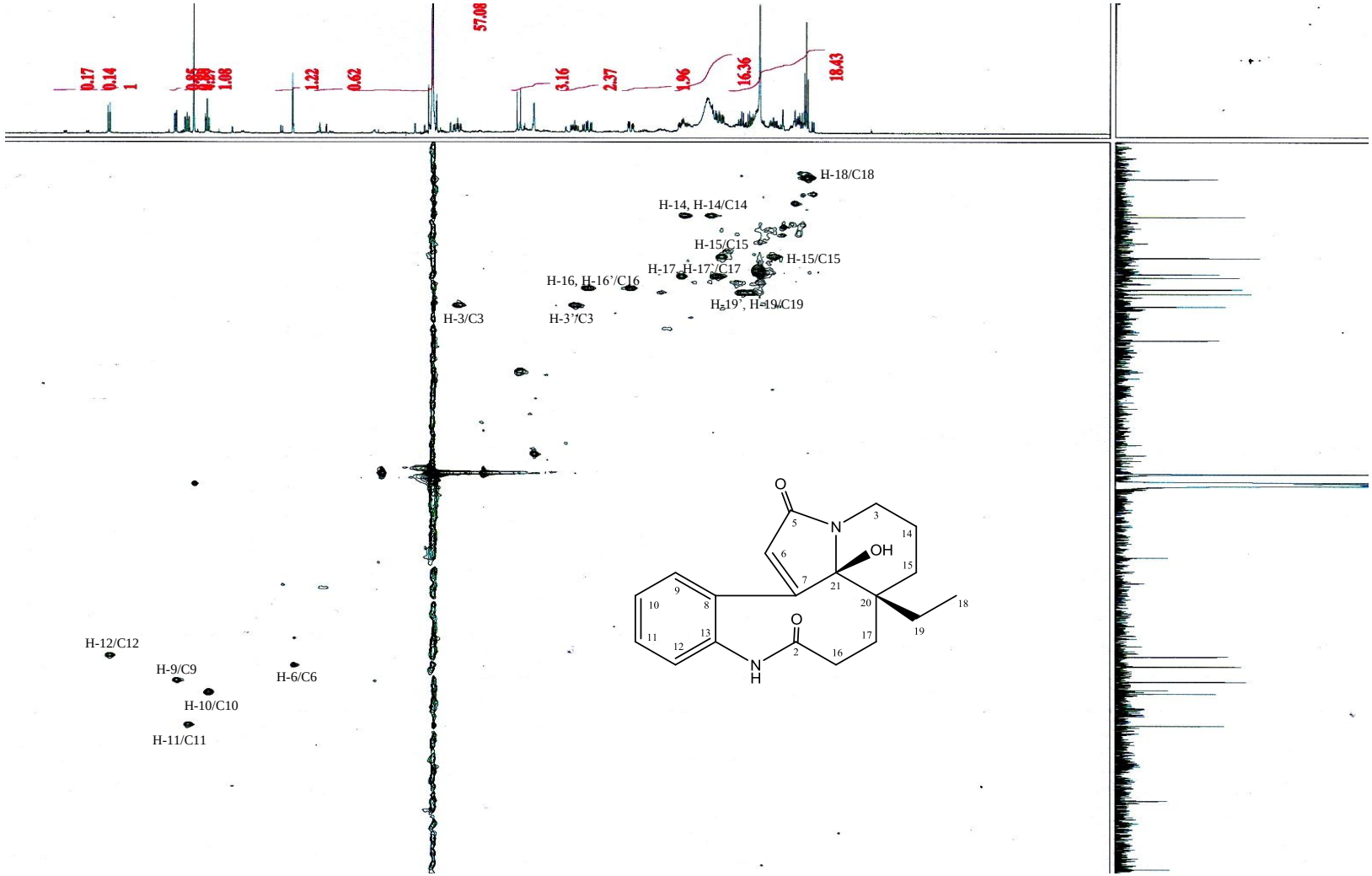


Figure 3.9: HSQC Spectrum of Compound **B**

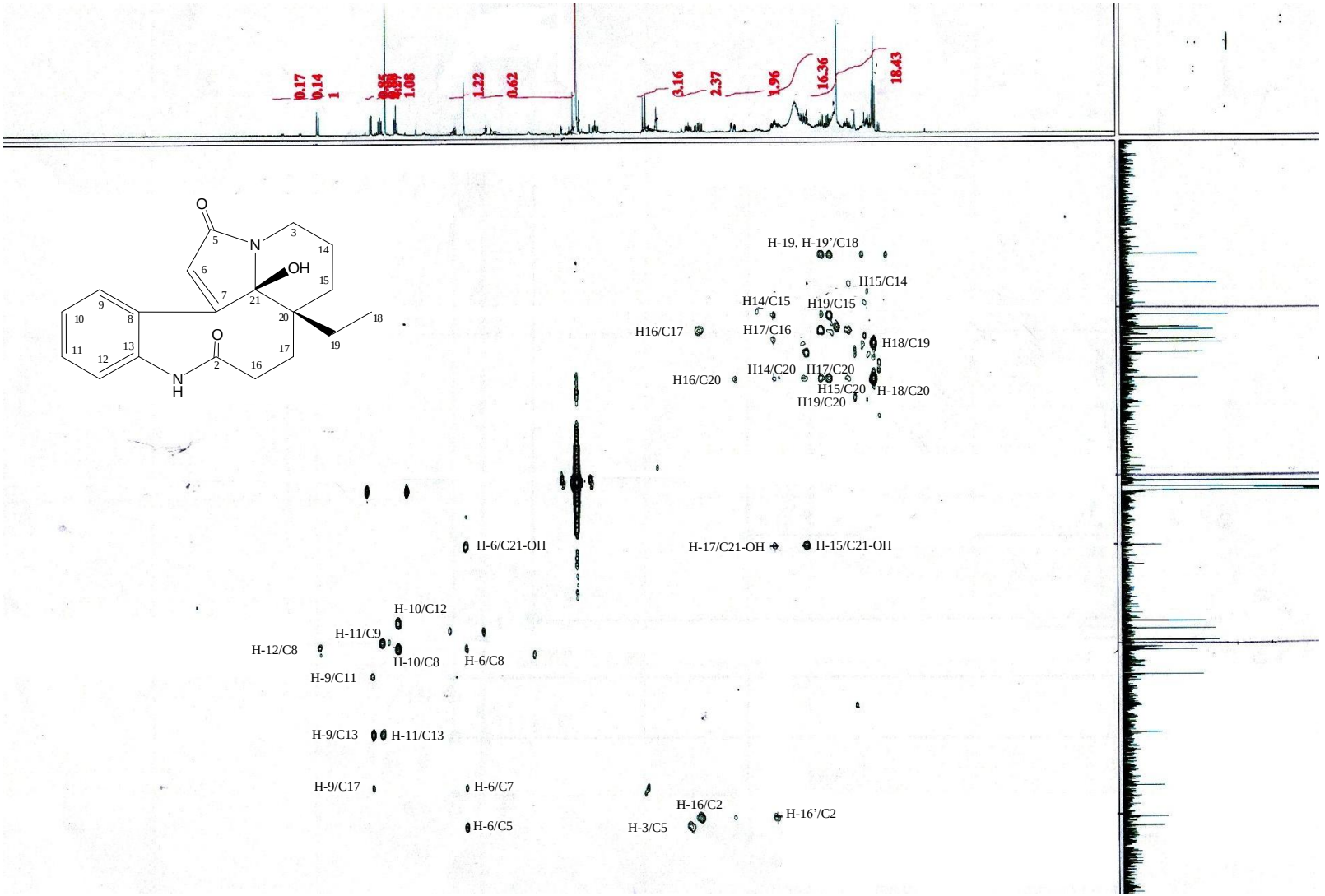
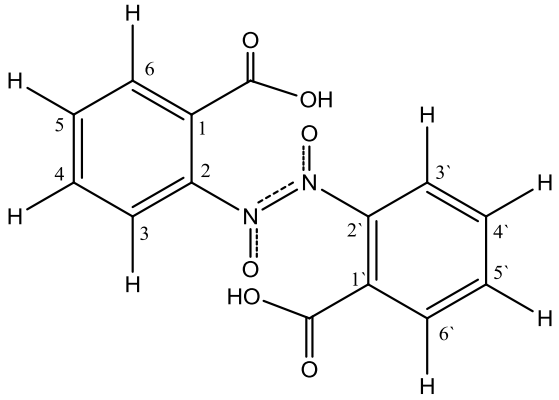


Figure 3.10: HMBC Spectrum of Compound **B**

3.3 COMPOUND C; *trans*-2, 2`- dicarboxyazobenzene dioxide **84**



Compound **C** was obtained as greenish amorphous solid. The mass spectrum of this alkaloid revealed a pseudo-molecular ion peak at m/z 325.1311 $[M+Na]^+$ which corresponded to the molecular formula $C_{14}H_{10}N_2O_6$. The IR spectrum showed a broad band at 3350 cm^{-1} and 1623 cm^{-1} – 1653 cm^{-1} indicating the presence of a conjugated carboxylic acid moiety and nitroso group respectively.[44] In addition, the *trans* nature of the dimer aromatic nitroso compounds is implied from the IR absorption at 1254 cm^{-1}.[51]

The ^{1}H-NMR spectrum (Figure 3.11) and (Table 3.3) showed a singlet at δ 12.14 which may be attributed to the proton of COO<u>H</u>. The four aromatic protons appeared at δ 7.01 (*dd*, 8.3, 0.9 Hz, H-3), δ 7.45 (*ddd*, 8.3, 7.3, 1.5 Hz, H-4), δ 6.87(*ddd*, 8.0, 7.3, 0.9 Hz, H-5) and δ 7.38 (*dd*, 8.0, 1.5 Hz, H-6).

The ^{13}C-NMR (Figure 3.12) spectrum of compound **C** indicated the presence of seven carbons. However, the real carbon number is fourteen as this compound is a dimer. Both parts of the compound are linked by the nitroso group.

The DEPT spectrum gave signals of four methines at δ 119.1 (C-3 and C-5), 135.4 (C-4), 126.8 (C-6). The complete assignments of ^{1}H and ^{13}C were assisted by COSY, HSQC and HMBC experiments and were listed in Table 3.3. Thorough examination of the spectral data led to the identification of compound **C** as *trans*-2, 2`-dicarboxyazobenzene dioxide.[51] This is the first report of its occurrence in nature.

Table 3.3: ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of compound **C** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
1		113.5
2		162.5
3	7.01 (*dd*, 8.3, 0.9 Hz)	119.1
4	7.45 (*ddd*, 8.3, 7.3, 1.5 Hz)	135.4
5	6.87 (*ddd*, 8.0, 7.3, 0.9 Hz)	119.1
6	7.38 (*dd*, 8.0, 1.5 Hz)	126.8
1`		113.5
2`		162.5
3`	7.01 (*dd*, 8.3, 0.9 Hz)	119.1
4`	7.45 (*ddd*, 8.3, 7.3, 1.5 Hz)	135.4
5`	6.87 (*ddd*, 8.0, 7.3, 0.9 Hz)	119.1
6`	7.38 (*dd*, 8.0, 1.5 Hz)	126.8
^{1}COOH	12.14 (*s*)	172.0
^{2}COOH	12.14 (*s*)	172.0

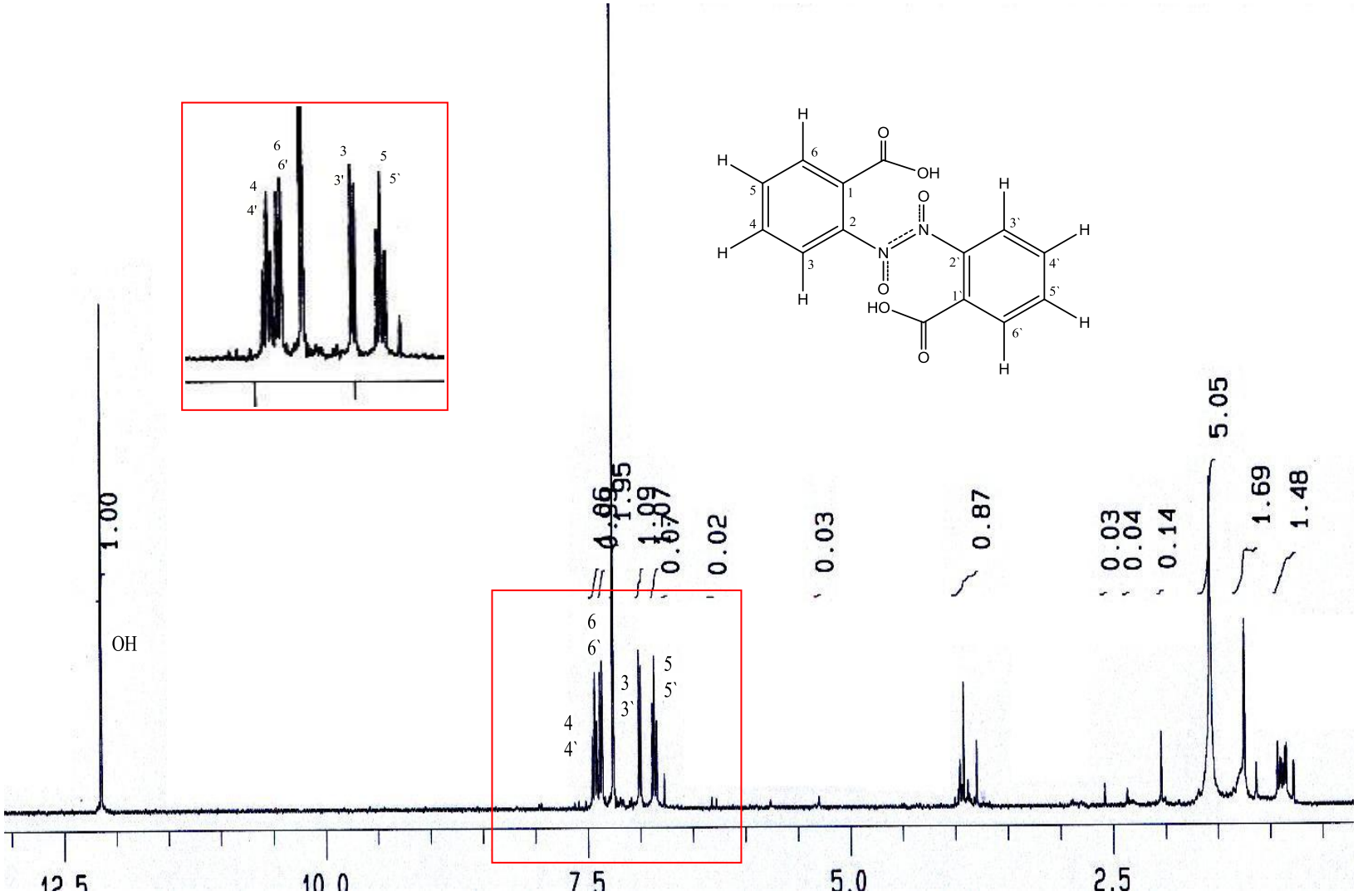


Figure 3.11: ^{1}H-NMR Spectrum of Compound **C**

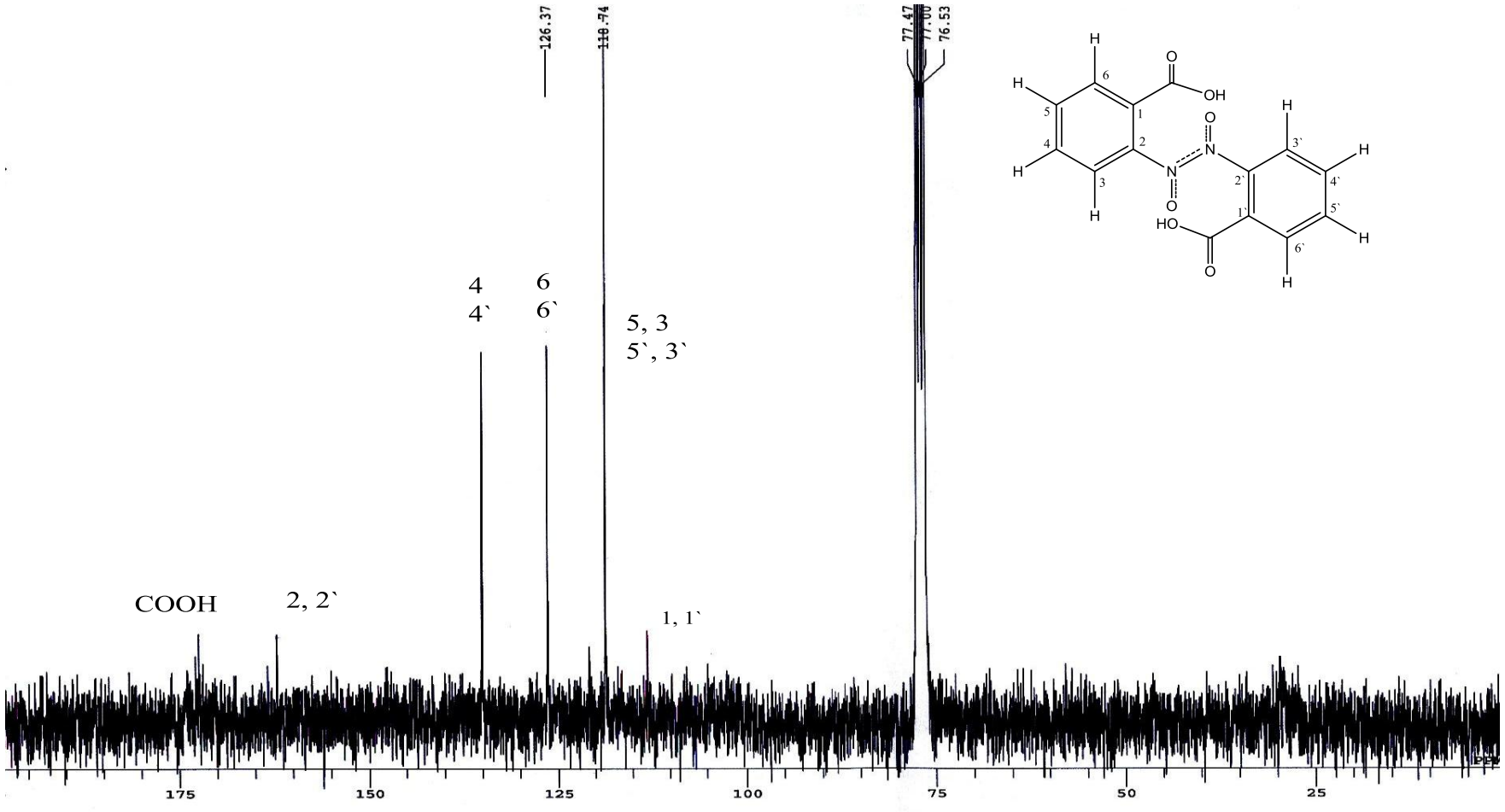


Figure 3.12: ^{13}C-NMR Spectrum of Compound **C**

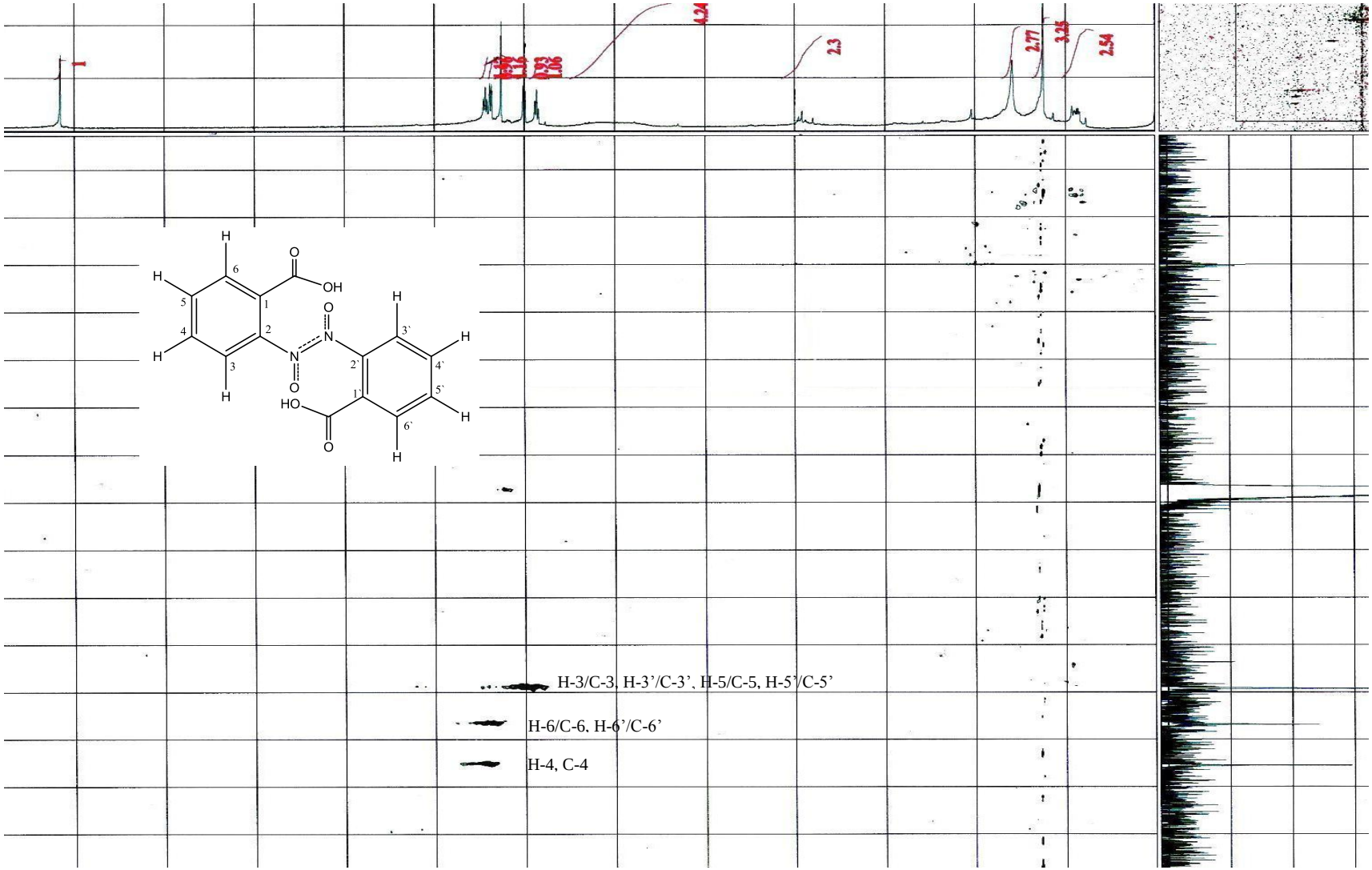


Figure 3.13: HSQC Spectrum of Compound **C**

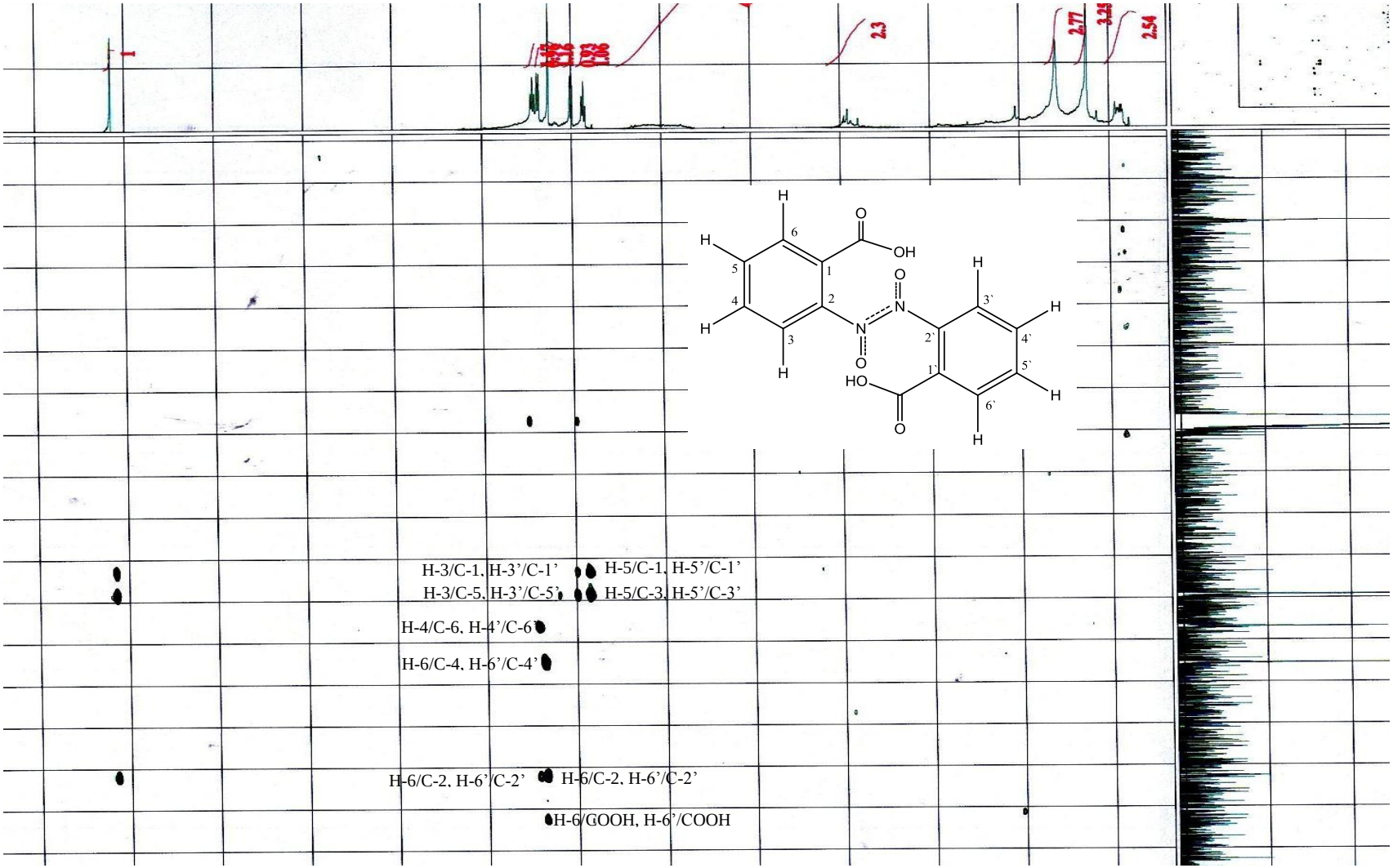
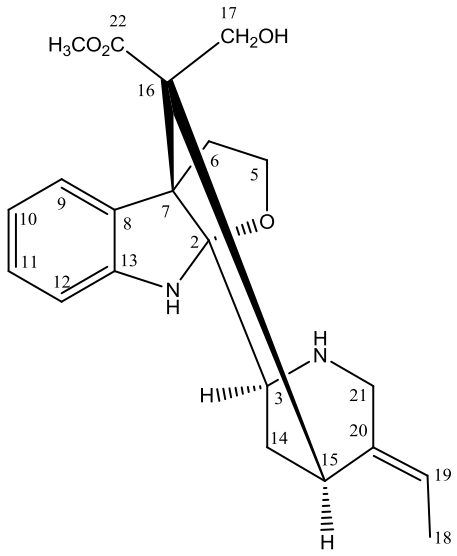


Figure 3.14: HMBC Spectrum of Compound **C**

3.4 **COMPOUND D;** lonicerine **85**



Compound **D** – lonicerine was obtained as yellowish oil. The mass spectrum of this alkaloid revealed a pseudo-molecular ion peak at m/z 371.1706 $[M+H]^+$ which corresponded to the molecular formula of $C_{21}H_{26}N_2O_4$. The IR spectrum showed a broad band at 3320 cm^{-1} indicating the presence of hydroxyl group. The UV spectrum showed absorptions characteristic of an unsubstituted dihydroindole chromophore; 204, 245 and 294 nm.[52]

The ^{1}H-NMR spectrum (Figure 3.15) showed the presence of signals for four aromatic protons at δ 7.07 (*d, J* = 7.8 Hz, H-9), δ 6.68 (*t, J* = 7.8 Hz, H-10), δ 6.99 (*t, J* = 7.8 Hz, H-11) and δ 6.53 (*d, J* = 7.8 Hz, H-12). The ^{1}H-NMR also showed the existence of an ethylidene group (δ 1.59, *d, J* = 6.6 Hz, H_3-18; δ 5.85, *q, J* = 6.6 Hz, H-19), one methoxyl at δ 3.05 (s, 22-OCH_3). The unusual shielded chemical shift value of the methoxyl may be due to the anisotropic effect since the methoxyl protons were oriented on top of the

benzene[53], a high electron density area. In addition, two doublets appeared at δ 3.23 and δ 3.59 or a coupling constant value of 13.9 Hz which can be assigned to the methylene protons of C-21. A pair of an AB doublet corresponding to H-17 was observed at δ 3.77 - δ 3.85.

The ^{13}C-NMR spectrum (Figure 3.16) showed 21 signals corresponding to the 21 carbons; seven methines at δ 53.2 (C-3), 124.5(C-9), 119.0 (C-10), 128.2(C-11), 107.5 (C-12), 33.5 (C-15) and 122.1 (C-19), five methylenes at δ 66.7 (C-5), 37.5 (C-6), 28.2 (C-14), 65.3 (C-17) and 48.7 (C-21), one methyl at δ 13.1 (C-18) and one methoxy signal at δ 51.6 (C-22 OCH_3). In addition there are seven quartenary carbon in which one is a carbonyl of an ester at δ 176.9 (C-22) (Table 3.4).

Thorough analysis of the COSY, HSQC and HMBC spectra allowed the complete assignment of all signals (Table 3.4). In conclusion, comparison with the literature values confirmed that compound **D** is lonicerine.[53,18]

Table 3.4: ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of lonicerine **D** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
2		102.5
3	3.23 (*m*)	53.2
4		
5	4.03 (*br t*, 7.5 Hz)	66.7
	3.41 (*m*)	
6	2.88 (*m*)	37.5
	2.52 (*dd*, 12.8, 4.6 Hz)	
7		55.3
8		134.4
9	7.07 (*d*, 7.8 Hz)	124.5
10	6.68 (*t*, 7.8 Hz)	119.0
11	6.99 (*t*, 7.8 Hz)	128.2
12	6.53 (*d*, 7.8 Hz)	107.5
13		148.6
14	2.79 (*m*)	28.2
	1.75 (*m*)	
15	2.83 (*m*)	33.5
16		57.5
17	3.77 (br *d*, 12.1 Hz)	65.3
	3.85 (br *d*, 12.1 Hz)	
18	1.59 (*d*, 6.6 Hz)	13.1
19	5.85 (*q*, 6.6 Hz)	122.1
20		133.6
21	3.23 (*d*, 13.9 Hz)	48.7
	3.59 (*d*, 13.9 Hz)	
22		176.9
22-OCH_3	3.05 (*s*)	51.6
NH	4.26 (*s*)	

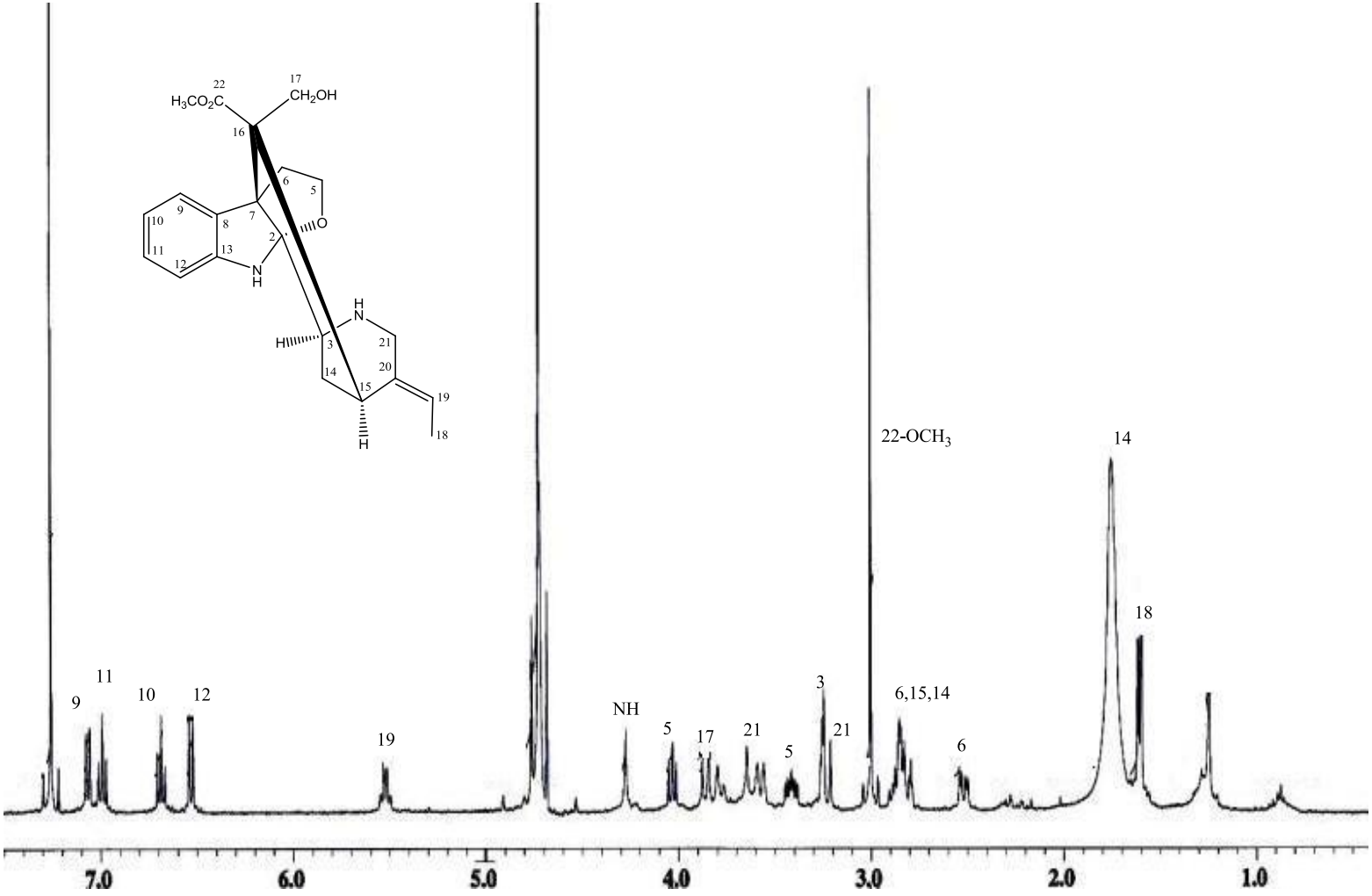


Figure 3.15: ^{1}H-NMR Spectrum of compound **D**

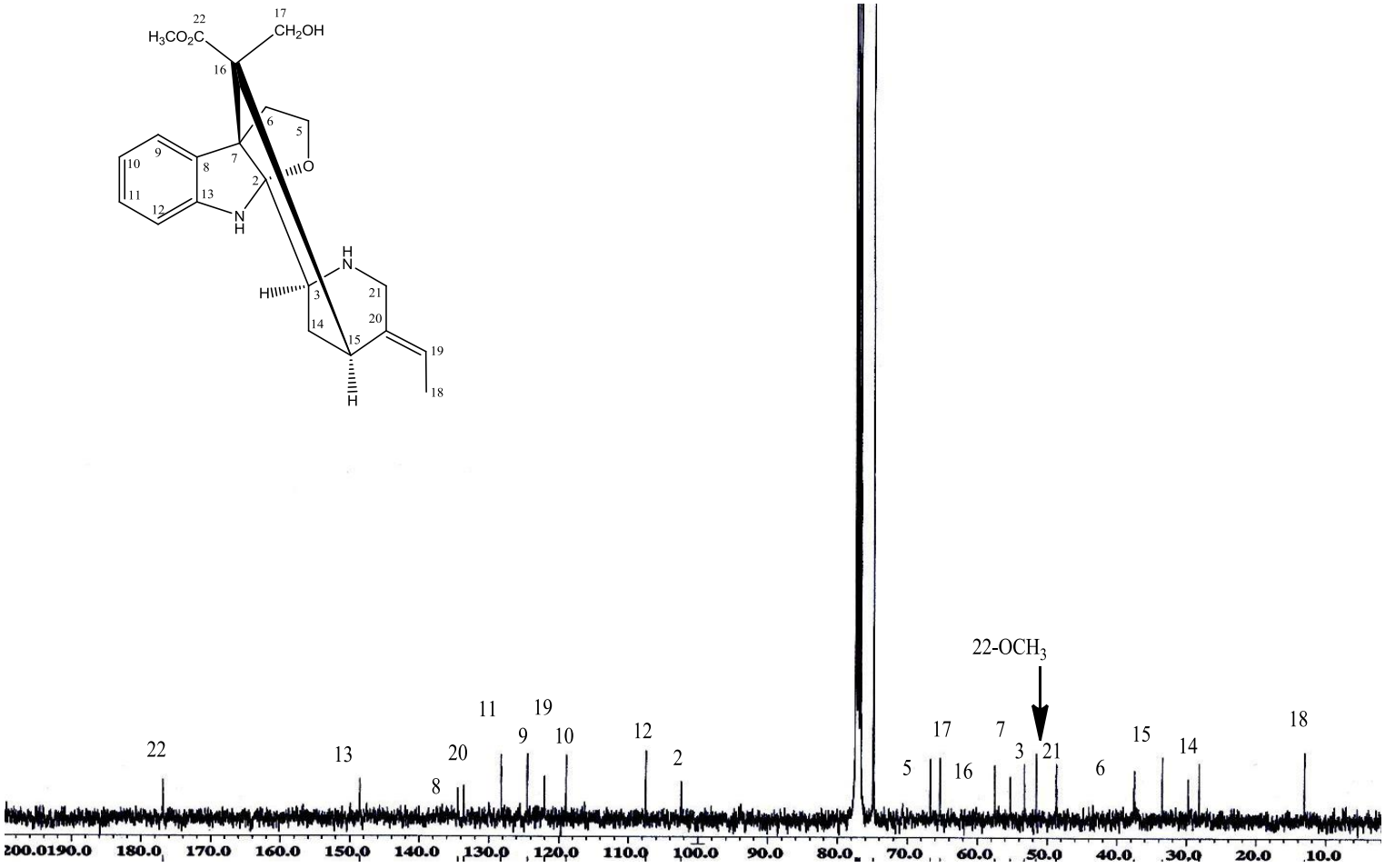


Figure 3.16: ^{13}C-NMR Spectrum of compound **D**

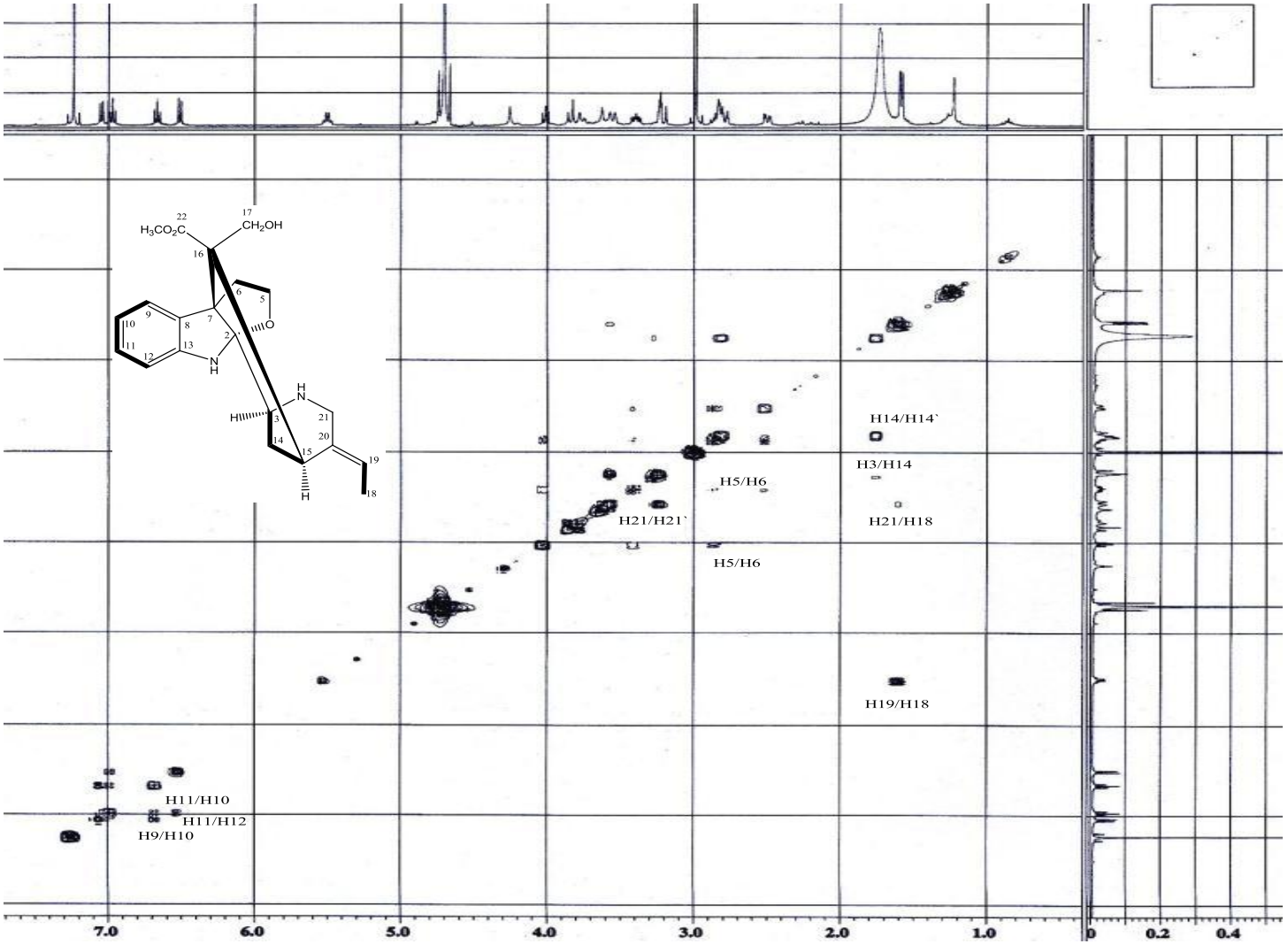


Figure 3.17: COSY Spectrum of compound **D**

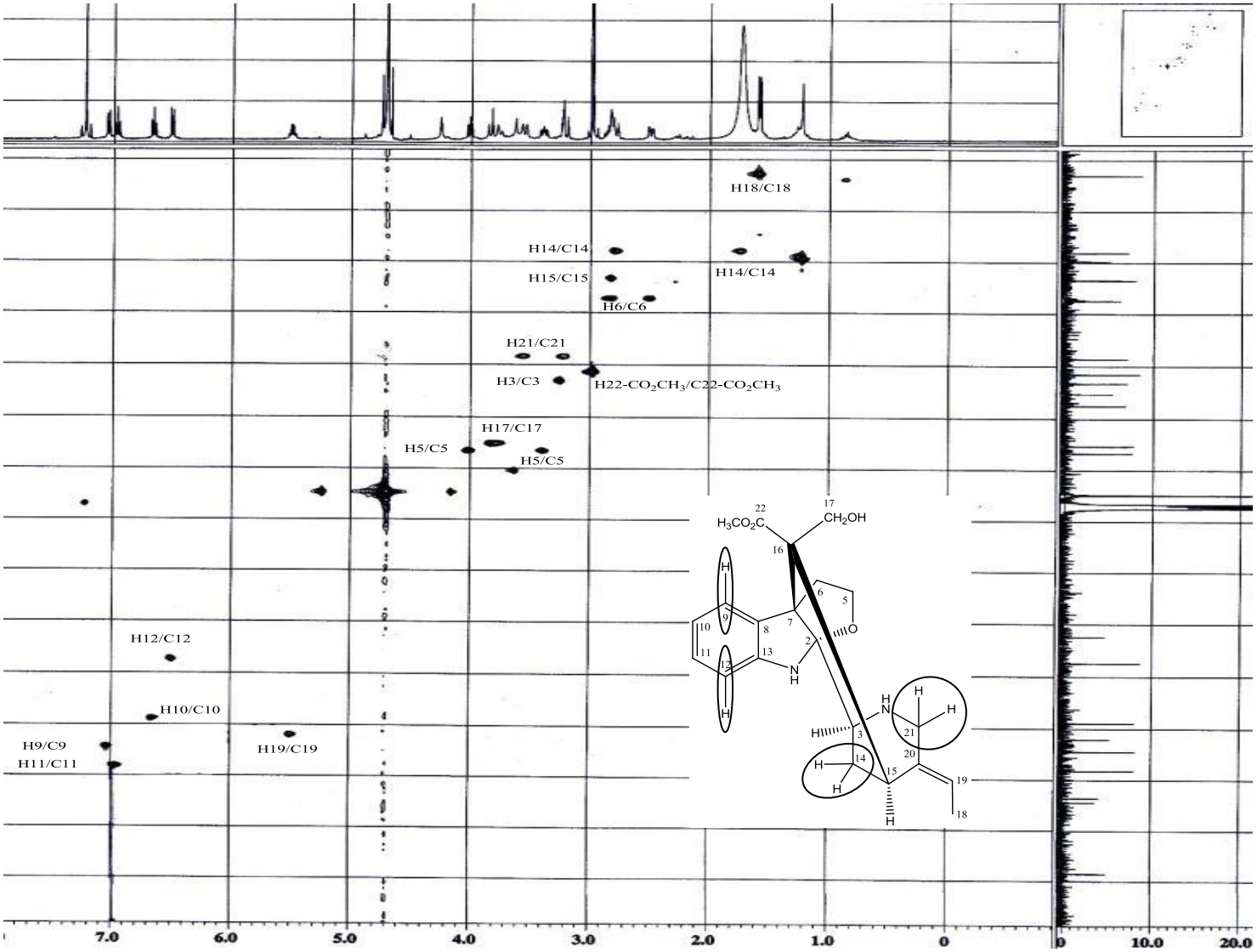


Figure 3.18: HSQC Spectrum of compound **D**

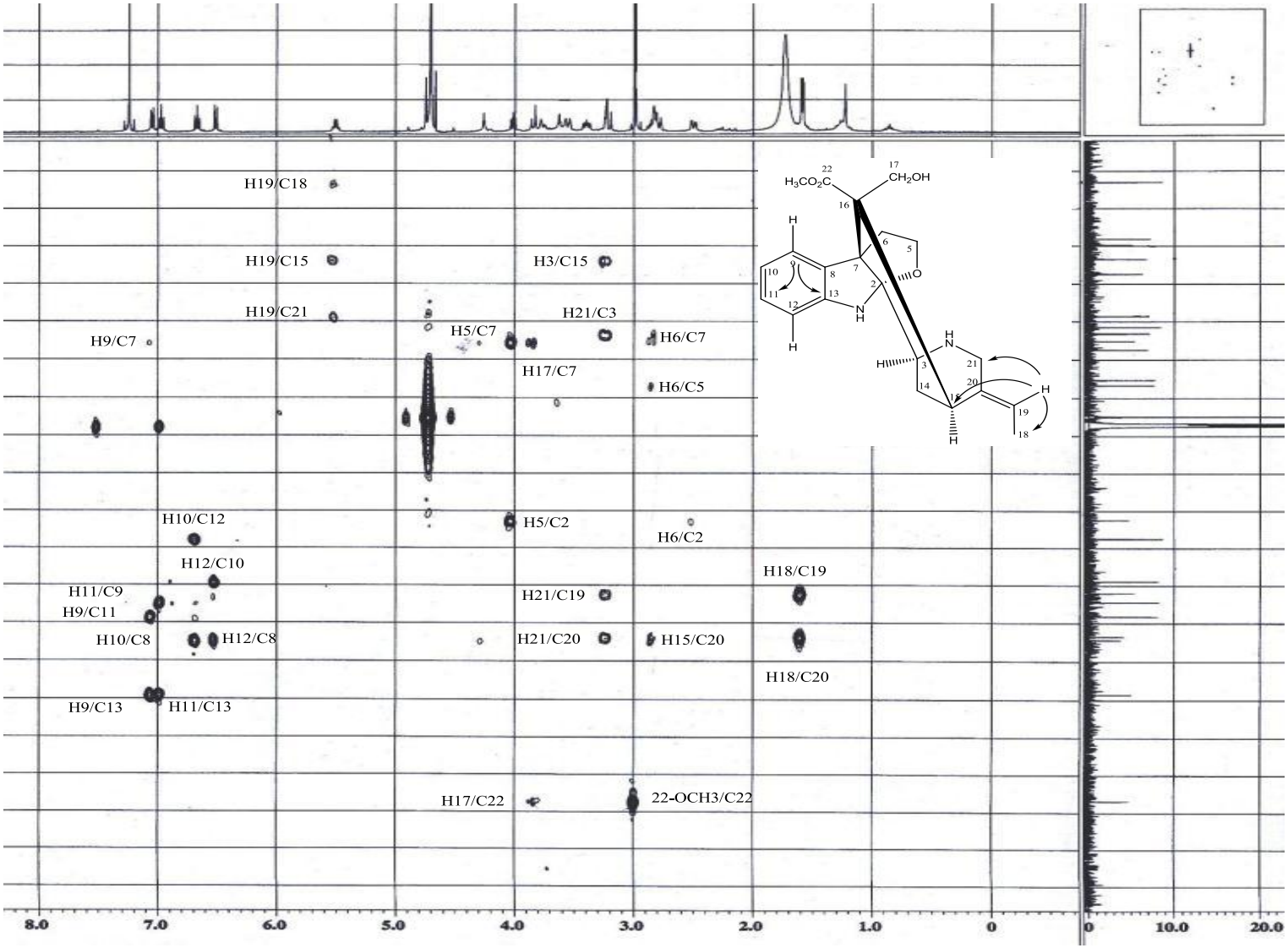
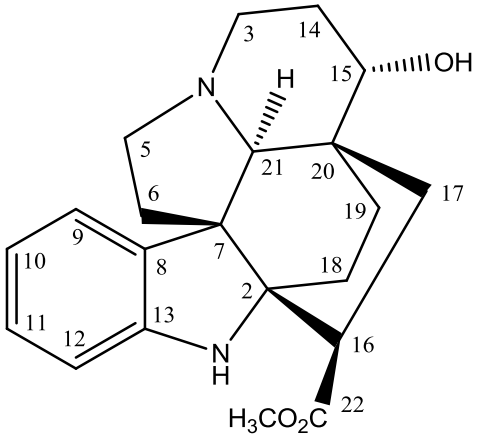


Figure 3.19: HMBC Spectrum of compound **D**

3.5 COMPOUND E ; 15-hydroxykopsinine **86**



Compound **E**, was isolated as a brownish amorphous solid. It`s mass spectrum showed a pseudo-molecular ion peak at m/z 355.1760 $[M+H]^+$ which corresponded to the molecular formula of $C_{21}H_{26}N_2O_3$ as indicated by its mass spectrum. The IR spectrum exhibit absorption at 3336 cm^{-1} and 1724 cm^{-1} indicating the presence of a hydroxyl group and a carbonyl ester group respectively. The UV spectrum showed absorptions characteristic of dihydroindole chromophore.[52]

The ^{1}H-NMR spectrum (Figure 3.20) showed four aromatic proton signals at δ 7.23 (*m*), δ 6.74 (*t*, 7.3Hz), δ 6.97 (*t*, 7.7 Hz) and δ 6.63 (*d*, 7.7 Hz) which can be attributed to H-9, H-10, H-11 and H-12 respectively. The ^{1}H and ^{13}C NMR spectra of this compound were nearly identical of venalstonine[54,55] except for the absence of the two olefinic proton signals at C-14 and C-15. Instead a downfield proton signal at δ 3.65 was observed which is assignable to H-15 which is in proximity with a hydroxyl group. The methoxyl protons at C-22 resonated as a singlet at δ 3.75. H-21 which is close to N_4 resonated at δ 3.66. Other

aliphatic protons CH_2, H_2-5 and H_2-3 whose carbons are attached to N_4 resonated at δ 3.06 / δ 3.35 and δ 2.90 / δ 3.45 respectively. Other proton signals (H_2-6, H_2-14, H-16, H_2-17, H_2-18, H_2-19) appeared in the upfield region between δ 1.24 to δ 2.89 (Table 3.5).

The ^{13}C-NMR spectrum (Figure 3.21) showed a total of twenty one carbon signals; four aromatic carbons, three methines, seven methylenes, one methoxyl carbon and six quartenary carbons. The carbonyl quartenary carbon resonated at δ 174.7. The aromatic carbon (C-9, C-10, C-11 and C-12) appeared between δ 121.9 to δ 110.8 (Table 3.5). The assignments of the position of each carbon were also confirmed with ^{1}H-^{1}H COSY, DEPT, HSQC and HMBC. Complete spectral data for ^{1}H and ^{13}C NMR were listed in Table 3.5. Analysis of all spectra data obtained and comparison with the literature value[18] led to the conclusion that compound **E** is 15-hydroxykopsinine.

Table 3.5: ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of 15- hydroxykopsinine **E** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
2		66.9
3	2.90 (*m*)	40.7
	3.45 (*m*)	
4		
5	3.06 (*m*)	51.3
	3.35 (*dd*, 15.9, 7.6 Hz)	
6	1.57 (*m*)	34.8
	2.64 (*m*)	
7		58.1
8		140.3
9	7.23 (*m*)	121.9
10	6.74 (*t*, 7.3 Hz)	120.1
11	6.97 (*t*, 7.7Hz)	126.1
12	6.63 (*d*, 7.7 Hz)	110.8
13		148.9
14	1.45 (*m*)	24.4
	2.20 (*m*)	
15	3.65 (*m*)	71.7
16	2.89 (*m*)	43.8
17	1.40 (*m*)	31.9
	2.66 (*m*)	
18	1.25 (*m*)	33.5
	1.90 (*m*)	
19	1.24 (*m*)	27.5
	1.89 (*m*)	
20		36.2
21	3.66 (br *s*)	62.8
22		174.7
22-OCH_3	3.75 (*s*)	52.2

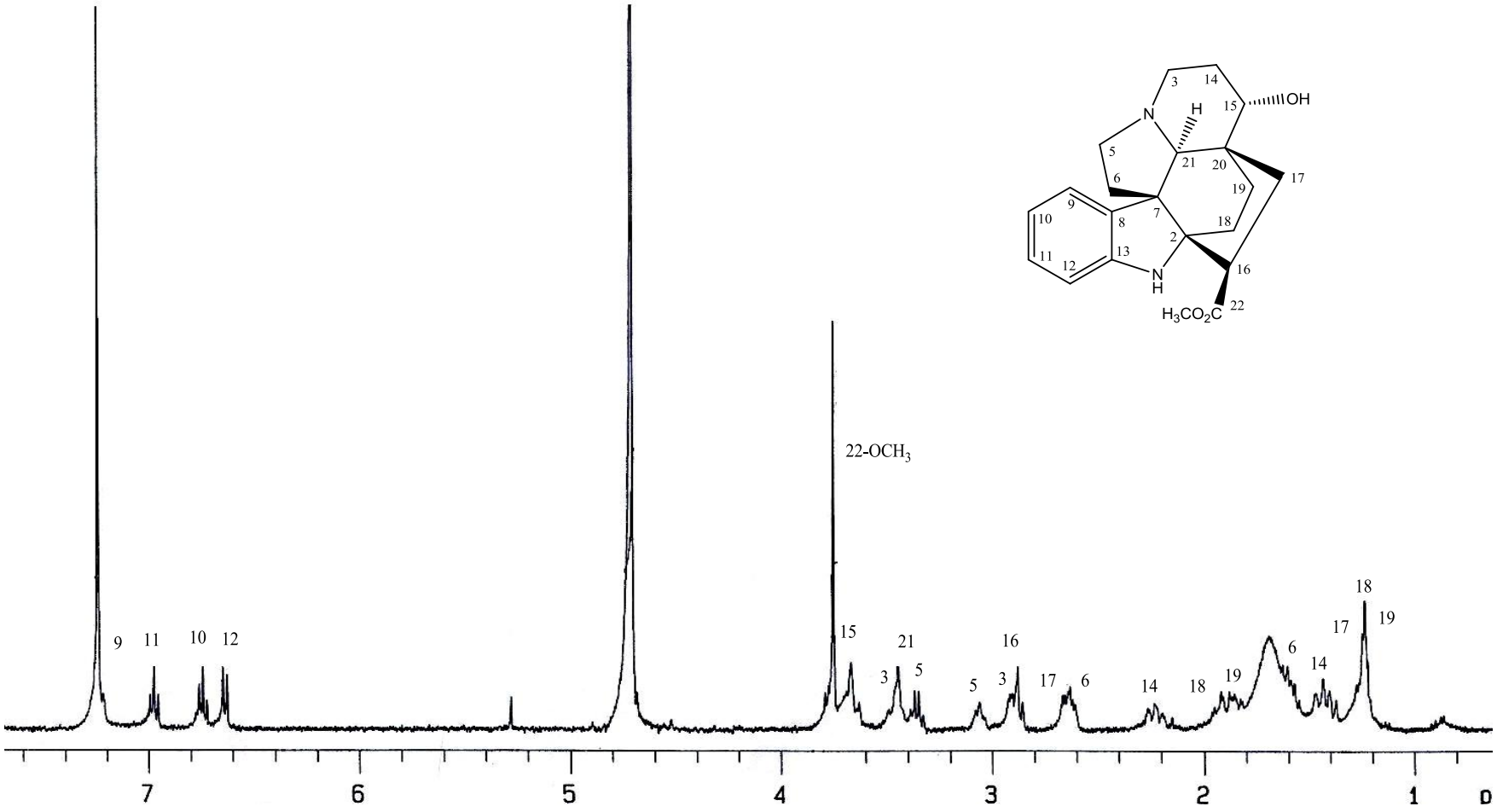


Figure 3.20: ^{1}H-NMR Spectrum of compound **E**

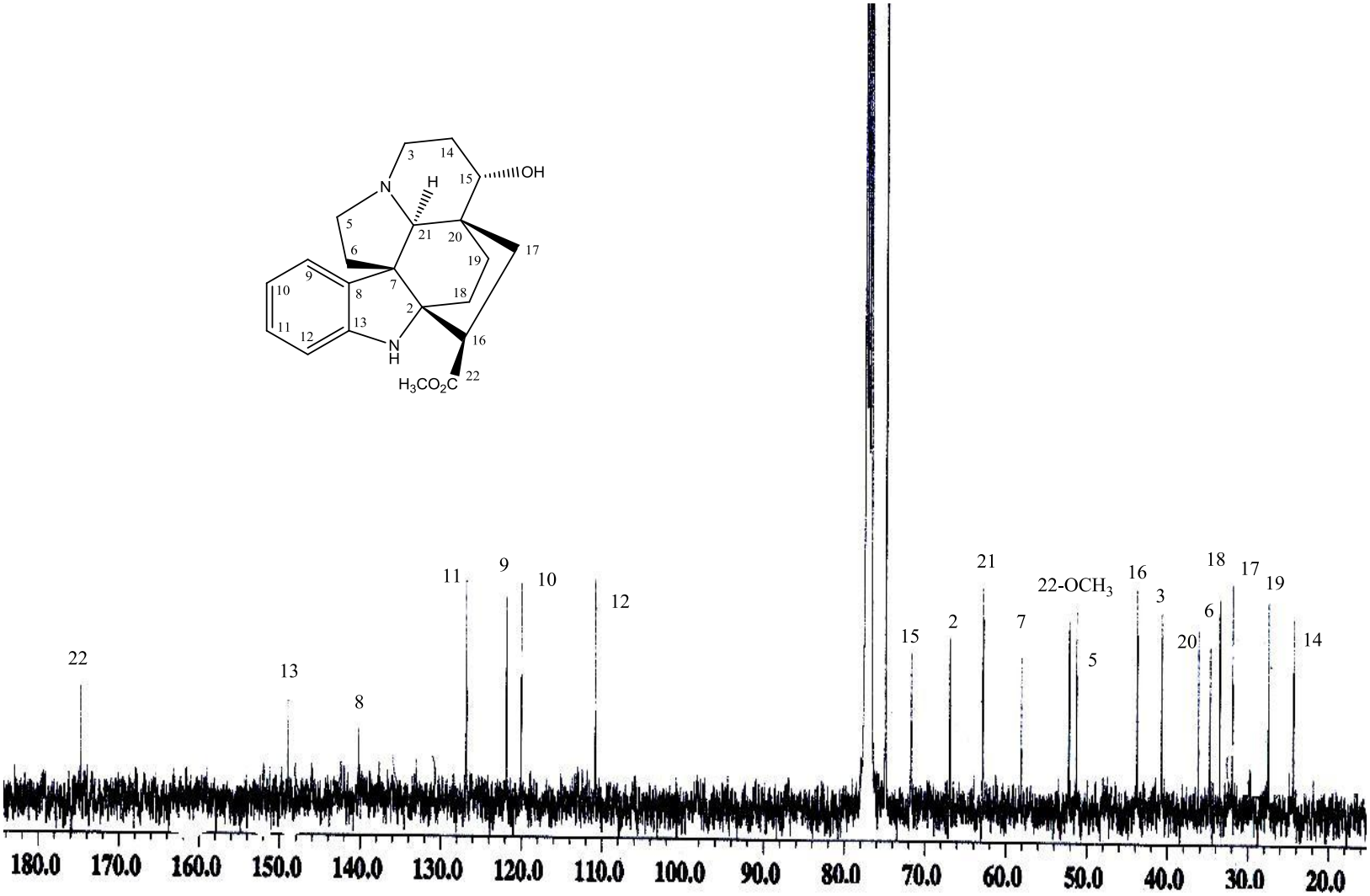


Figure 3.21: ^{13}C-NMR Spectrum of compound **E**

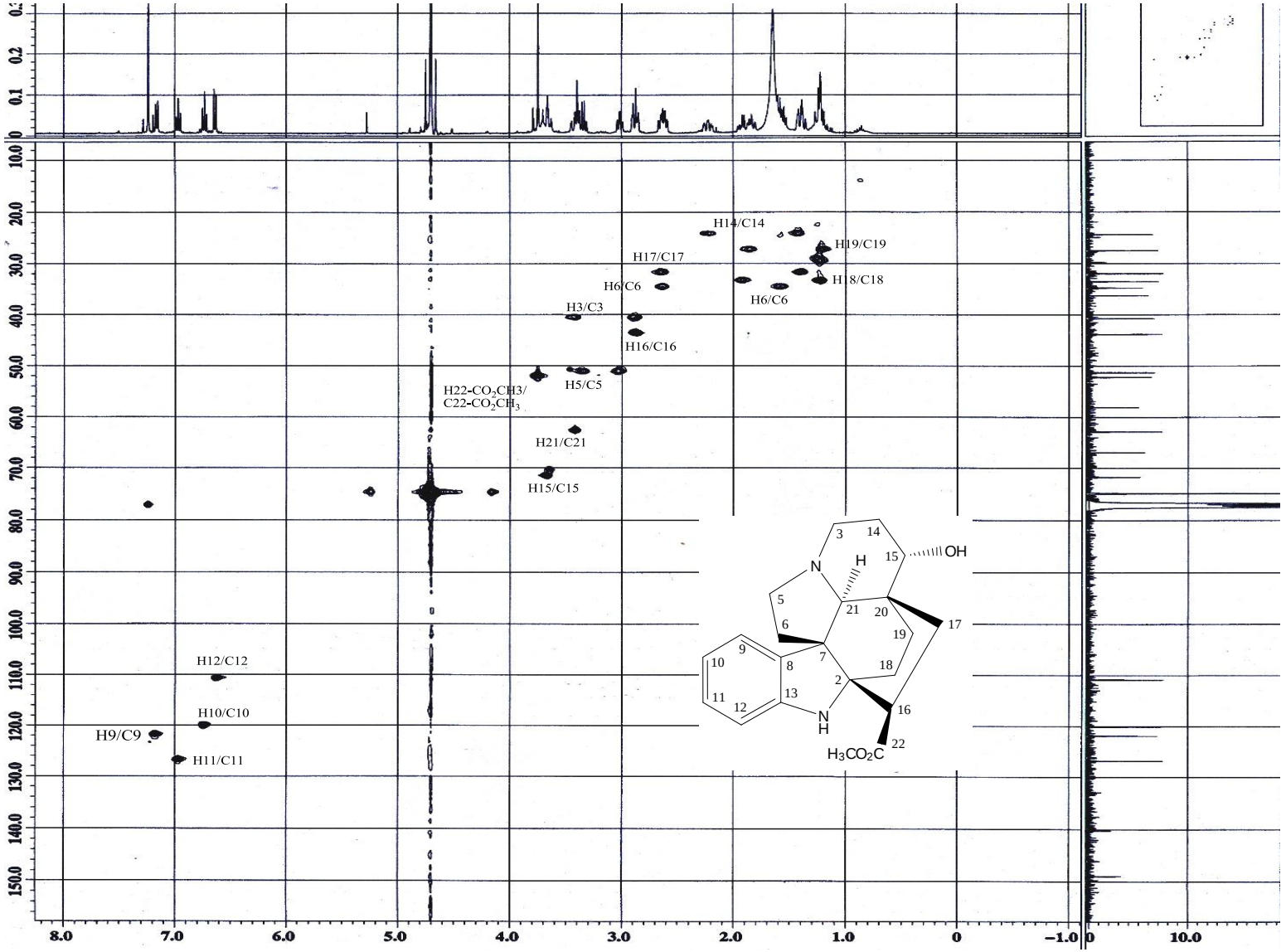


Figure 3.22: HSQC Spectrum of compound **E**

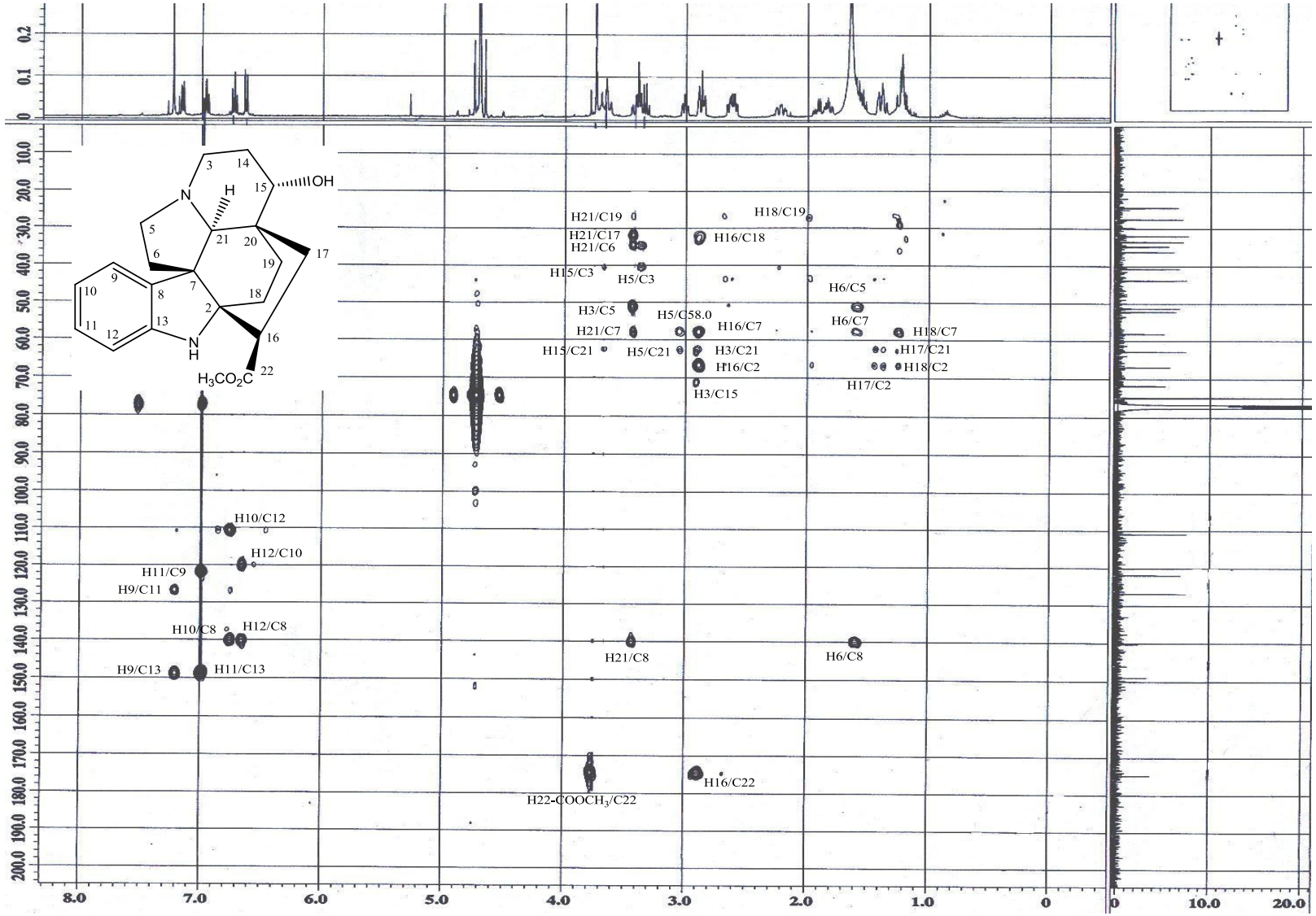
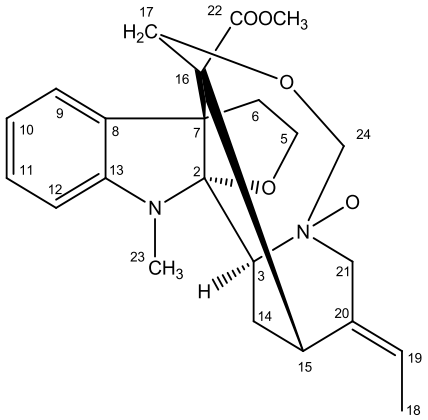


Figure 3.23: HMBC Spectrum of compound **E**

3.6 **COMPOUND F**; singapurine **87**



Compound **F** was obtained as yellowish oil. The IR spectrum showed a broad band at 3358 cm^{-1} representing a hydroxyl group. The ^{1}H-NMR (Figure 3.24) showed the existence of an ethylidene group (δ 1.55, *d*, *J* = 6.8 Hz, H_3-18; δ 5.80, *q*, *J* = 6.80 Hz, H-19), one deshielded methylene (δ 3.99, *d*, *J* = 8.2 Hz and δ 3.61, *d*, *J* = 8.2 Hz , H-5) and one methoxyl signal at δ 3.82 (s) corresponding to 22-OCH_3. These signals are characteristic of an aspidodasycarpine type of alkaloids. ^{1}H-NMR spectrum also showed the presence of four aromatic protons at δ 7.09 (*d*, 7.3 Hz, H-9), δ 6.85 (*dt*, 7.3, 1.1Hz, H-10), δ 7.19 (*dt*, 7.8, 1.1 Hz, H-11) and δ 6.69 (*d*, 7.8 Hz, H-12). An N-methyl singlet appeared at δ 2.82. In addition a pair of deshielded doublet was apparent at δ 5.90 and δ 6.40 due to the resonances of the C-24 oxo methylene protons.

The ^{13}C-NMR spectrum (Figure 3.25) showed 23 signals corresponding to the 23 carbons; seven methines, six methylenes, one methoxyl, two methyls and seven quartenary carbons. (Table 3.6)

The HMBC (Figure 3.28) spectrum of compound F showed the correlations of H-9/C-9, H-10/C-10, H-11/C-11, H-12/C-12, C-12, C-2, C-7, C-8, C-13 and 23-N-CH_3 which established the substructure of the indole alkaloid. The HMBC correlations of H-24a CH_2OH with C-17 and H-24b CH_2OH with C-3 were observed thus implying the connection between C-17 and C-24 through an ethereal linkage forming a new ring into the aspidodasycarpine skeleton.

Thorough analysis of ^{1}H-^{1}H COSY (Figure 3.26) and C-H correlations from HSQC (Figure 3.27) and HMBC (Figure 3.28) led us to propose the structure of alkaloid **F** as depicted in **87**. The proposed structure is based only on ^{1}H-NMR and ^{13}C-NMR (1D and 2D) due to its very minute quantity.

Table 3.6: ^{1}H-NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of compound **F** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
2		102.3
3	4.69 (br *s*)	63.1
4		
5	3.99 (*d*, 8.2 Hz)	74.4
	3.61 (*d*, 8.2 Hz)	
6	3.74 (m)	27.2
	1.83 (m)	
7		51.4
8		136.7
9	7.09 (*d*, 7.3 Hz)	122.7
10	6.85 (*dt*, 7.3, 1.1 Hz)	121.7
11	7.19 (*dt*, 7.8, 1.1 Hz)	128.9
12	6.69 (*d*, 7.8 Hz)	111.6
13		150.6
14	2.98 (*d*, 15.5 Hz)	26.2
	2.59 (*d*, 15.5 Hz)	
15	3.60 (*m*)	38.5
16		56.2
17	4.36 (*m*)	56.7
	3.68 (*m*)	
18	1.55 (*d*, 6.8 Hz)	13.5
19	5.80 (*q*, 6.8 Hz)	126.0
20		129.8
21	4.58 (*d*, 15 Hz)	64.1
	5.12 (*d*, 15 Hz)	
22		170.7
23-N-CH_3	2.82 (*s*)	30.32

Continued....

Table 3.6: ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of compound **F** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
24a-CH_2OH	6.40 (*d*, 9.8 Hz)	72.12
24b-CH_2OH	5.90 (*d*, 9.8 Hz)	
22-OCH_3	3.82 (*s*)	52.67

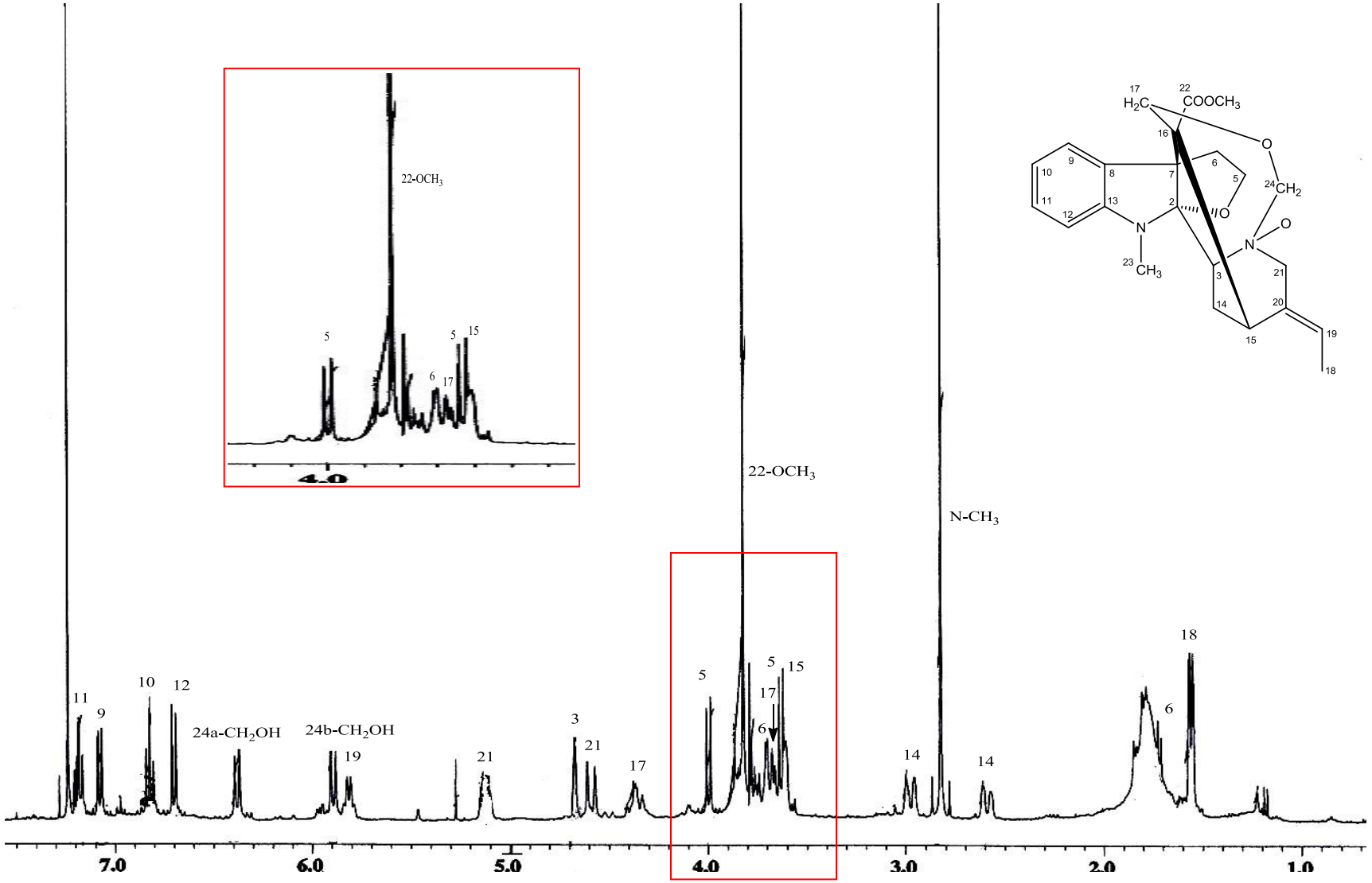


Figure 3.24: ^{1}H-NMR Spectrum of compound **F**

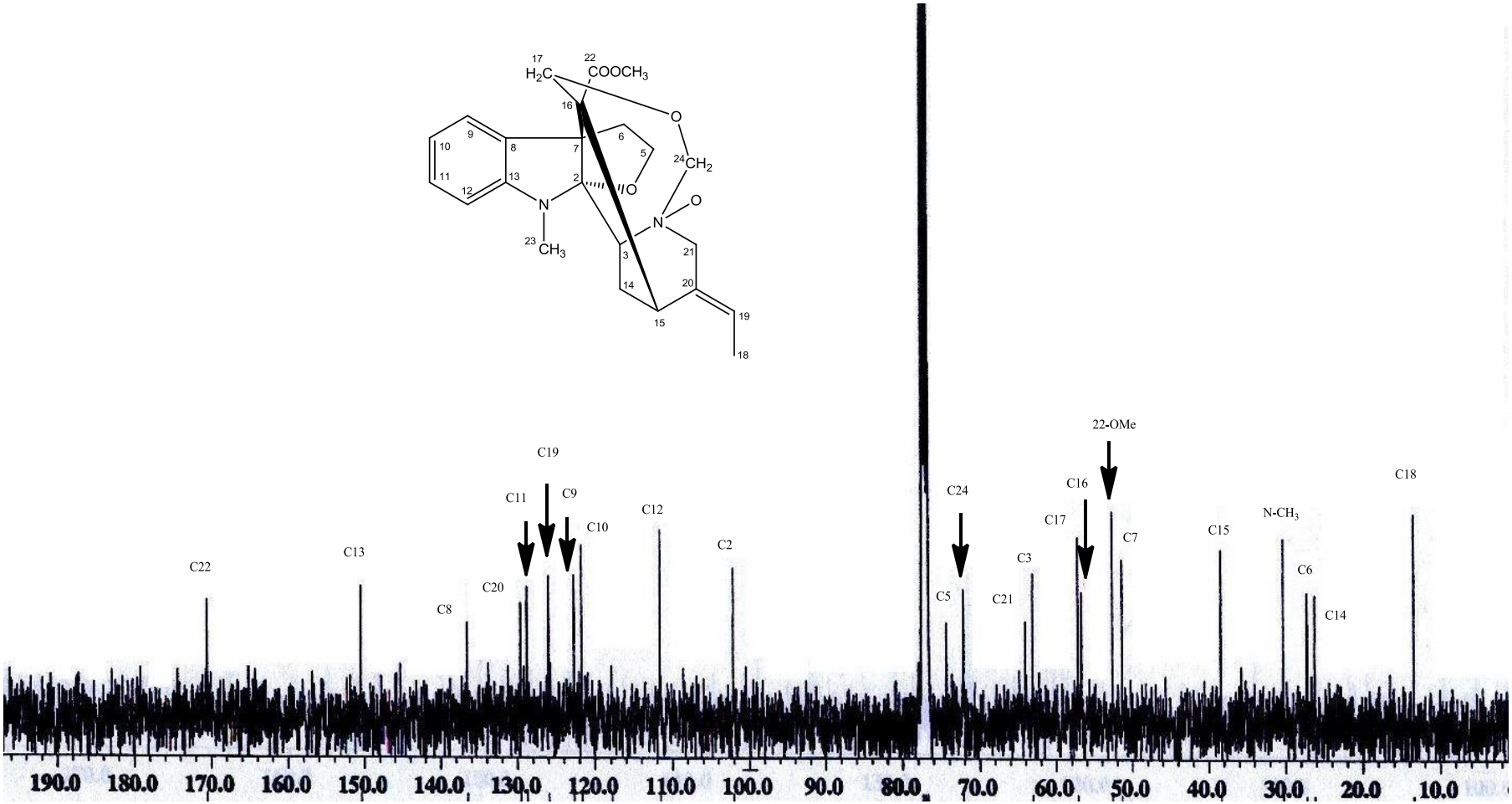


Figure 3.25: ^{13}C-NMR Spectrum of compound **F**

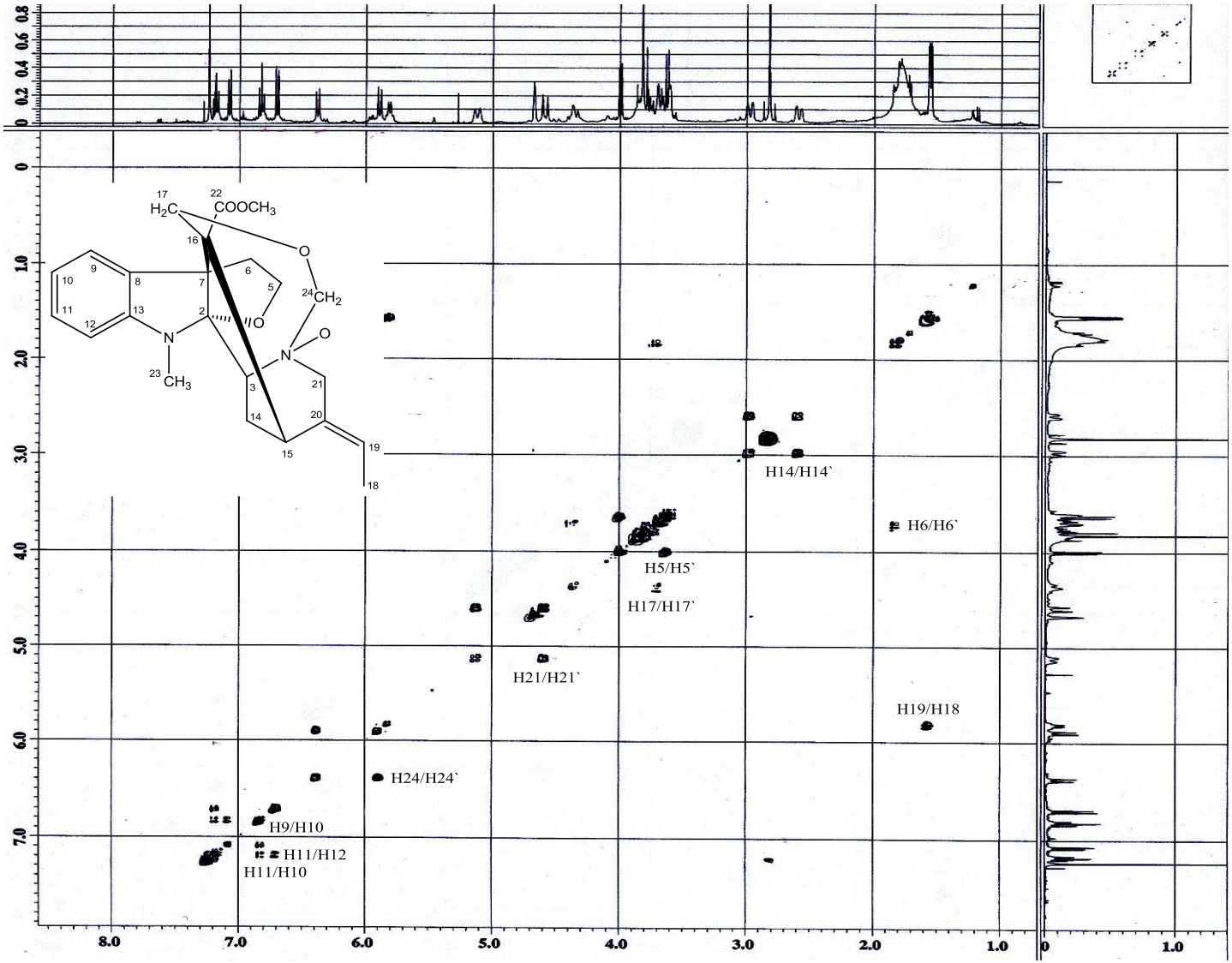


Figure 3.26: COSY Spectrum of Compound **F**

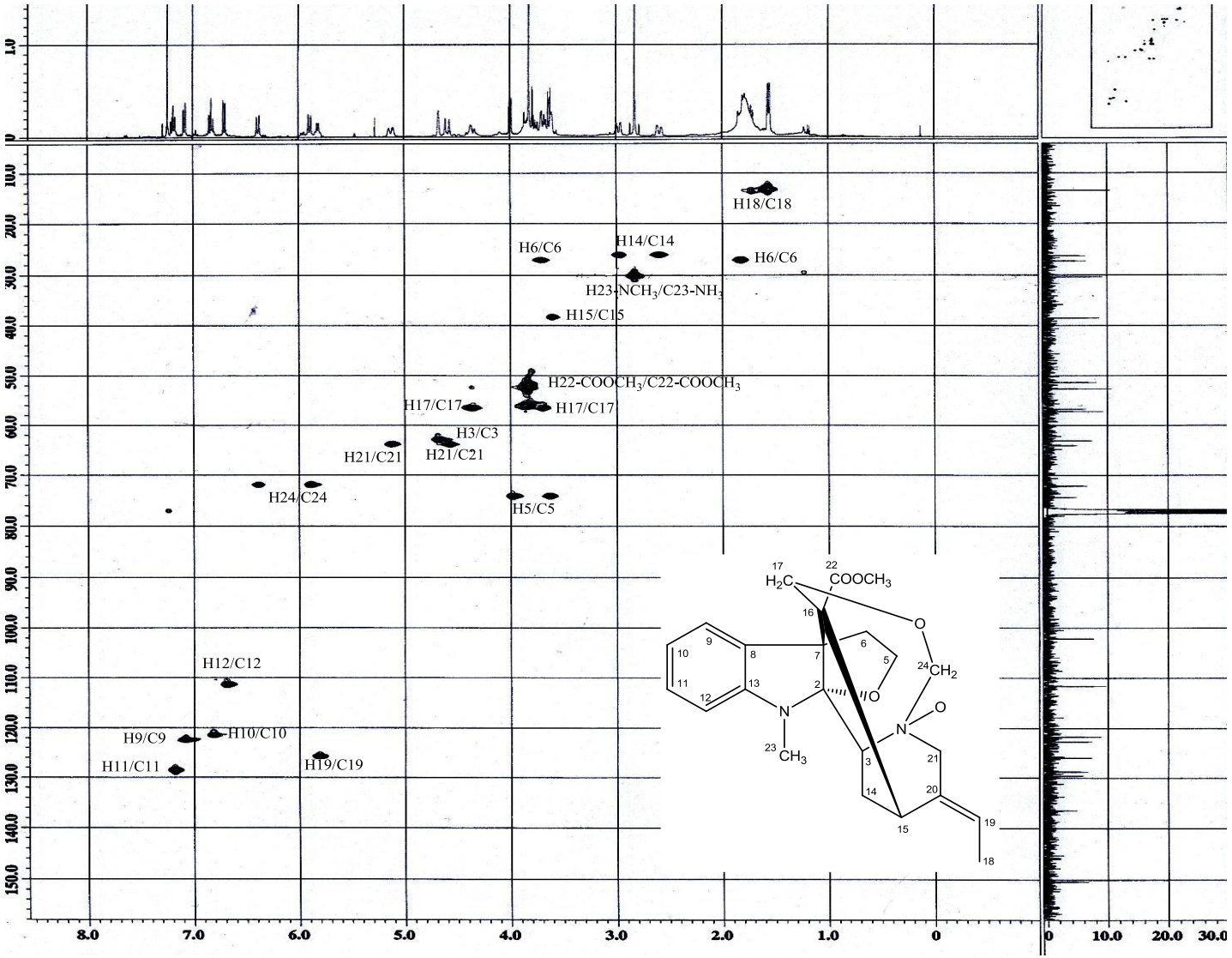


Figure 3.27: HSQC Spectrum of Compound **F**

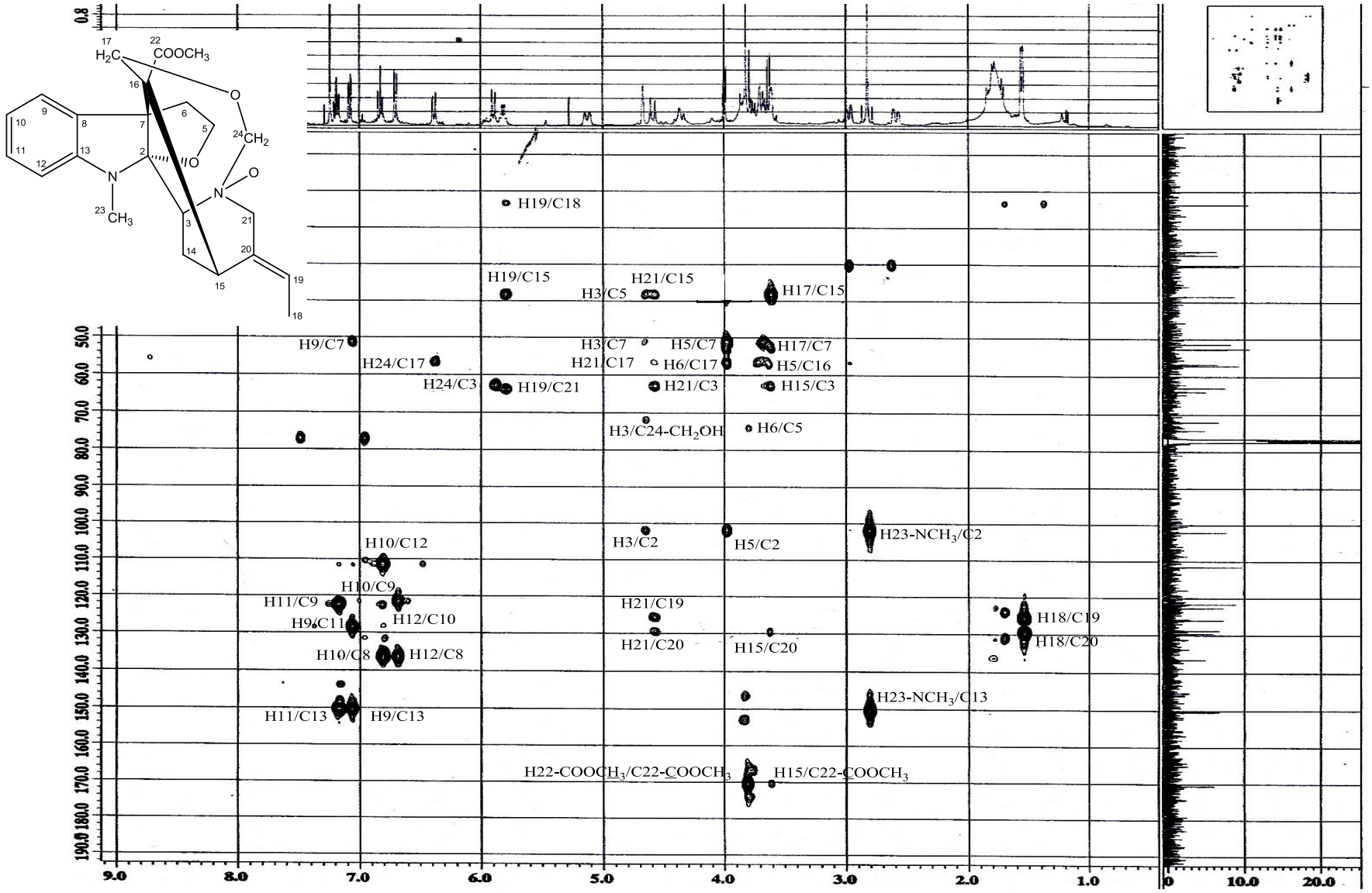
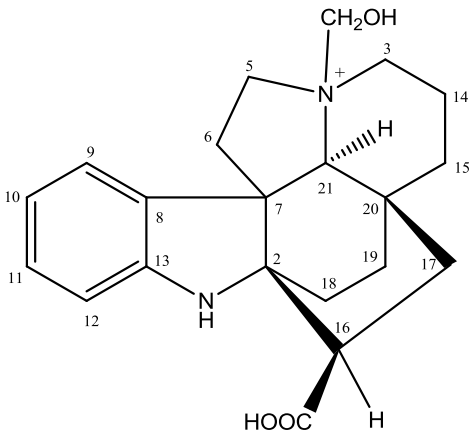


Figure 3.28: HMBC Spectrum of Compound **F**

3.7 COMPOUND G; N_4-hydroxymethyl kopsinic acid **88**



Compound **G** was obtained as pale yellowish oil. The HREIMS showed the pseudo-molecular ion peak at m/z 354.1341[M-H]$^+$ corresponded to the molecular formula of $C_{21}H_{27}N_2O_3$. The IR spectrum showed a broad band at 3391 cm^{-1} indicating the presence of hydroxyl group.[44]

The ^{1}H-NMR spectrum (Figure 3.29) indicated the presence of an unsubstituted indoline ring; (δ 7.07, *m*, 6.71, *t*, *J* = 8.0 Hz, 6.98, *m* and 6.65, *d*, *J* = 8.0 Hz). In addition deshielded signals due to the methylene proton at C-3 and C-5 appeared at δ 3.70 - 4.20. Normally the values of their resonances were in the region of δ 2.90 – 3.45 as obtained in compound **E**. This observation suggested that N-4 is an N_4-hydroxymethyl. Moreover, H-21 which usually appeared at δ 3.66 (*s*),[56] resonated as a singlet at δ 3.89 (^{13}C-NMR; δ 74.9) in this compound. A downfield signal of a set of two doublets appeared at δ 5.56 (*d*, 10.7 Hz) and δ 5.06 (*d*, 10.7 Hz) which could be assigned to the methylene, H-22.

The ^{13}C NMR spectrum (Figure 3.30) indicated the presence of three sp^2 quaternary carbons, four sp^2 methines, nine sp^3 methylenes, two sp^3 methines and three sp^3 quaternary carbons. More deshielded values for the ^{13}C resonances of C-3 and C-5 were observed at δ 55.9 and δ 61.3 respectively. Usually the values are between δ 40 – 55.[56,18] These observations confirmed that N-4 is indeed an N-CH_2OH. The ^1H-^1H COSY spectrum (Figure 3.31) of compound **G** suggested the following fragments; C-3-C-14, C-5-C-6, C-14-C-15, C-16-C-17 and C-18-C19. These correlations indicated that this alkaloid belong to the aspidofractinine skeleton.

In addition the HMBC spectrum showed correlation between H-22 with C-3 and C-21, thus confirming the position of the hydroxymethyl on N_4. The absolute stereochemistry is as indicated based on the absolute chemistry of the known kopsiloscine G, which is also isolated from this plant.[57] Finally thorough analysis of compound **G** spectra, suggested that it is a new alkaloid named N_4-hydroxymethyl kopsininic acid.[18]

Table 3.7: ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of compound **G** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
2		66.3
3	3.84 (*m*)	55.9
	3.70 (*m*)	
4		
5	4.20 (*m*)	61.3
	3.73 (*m*)	
6	3.23 (*m*)	31.1
	1.54 (*m*)	
7		59.2
8		137.1
9	6.99 (*m*)	129.1
10	6.70 (*t*, 7.8 Hz)	120.7
11	6.96 (*m*)	120.8
12	6.64 (*d*, 8.2 Hz)	113.9
13		150.0
14	2.26 (br *d*, 14.6 Hz)	16.7
	1.83 (br *d*, 14.6 Hz)	
15	1.63 (*m*)	32.6
	1.24 (*m*)	
16	2.72 (*m*)	44.1
17	2.74 (*m*)	33.3
	1.49 (*m*)	
18	1.67 (*m*)	35.0
	1.28 (*m*)	
19	1.78 (*m*)	32.0
	1.37 (*m*)	

Continued....

Table 3.7: ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of compound **G** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{13}C
20		32.9
21	3.89 (*s*)	74.9
22-CH_2OH	5.56 (*d*, 10.7 Hz)	70.53
	5.06 (*d*, 10.7 Hz)	
COOH		178.33

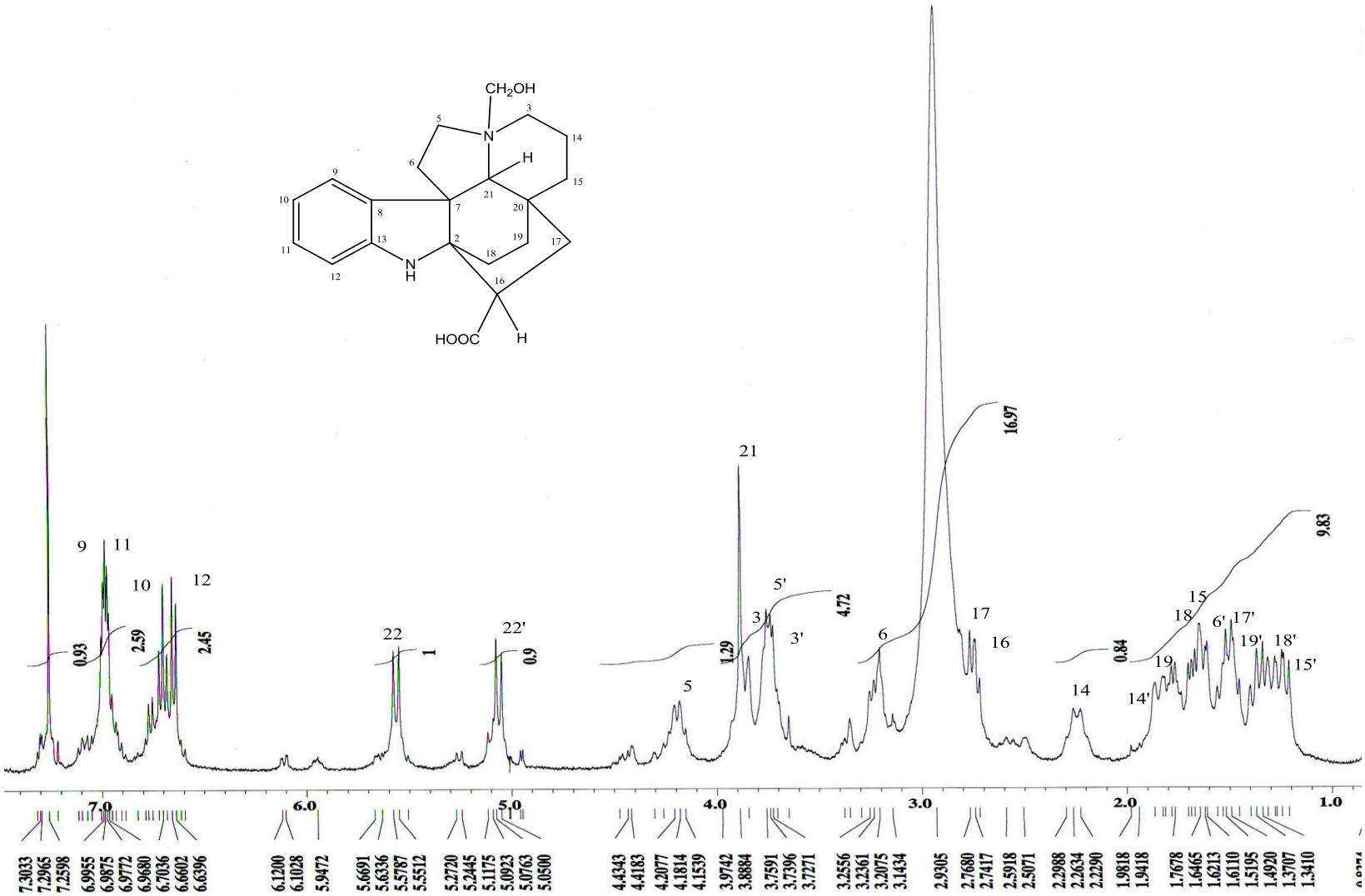
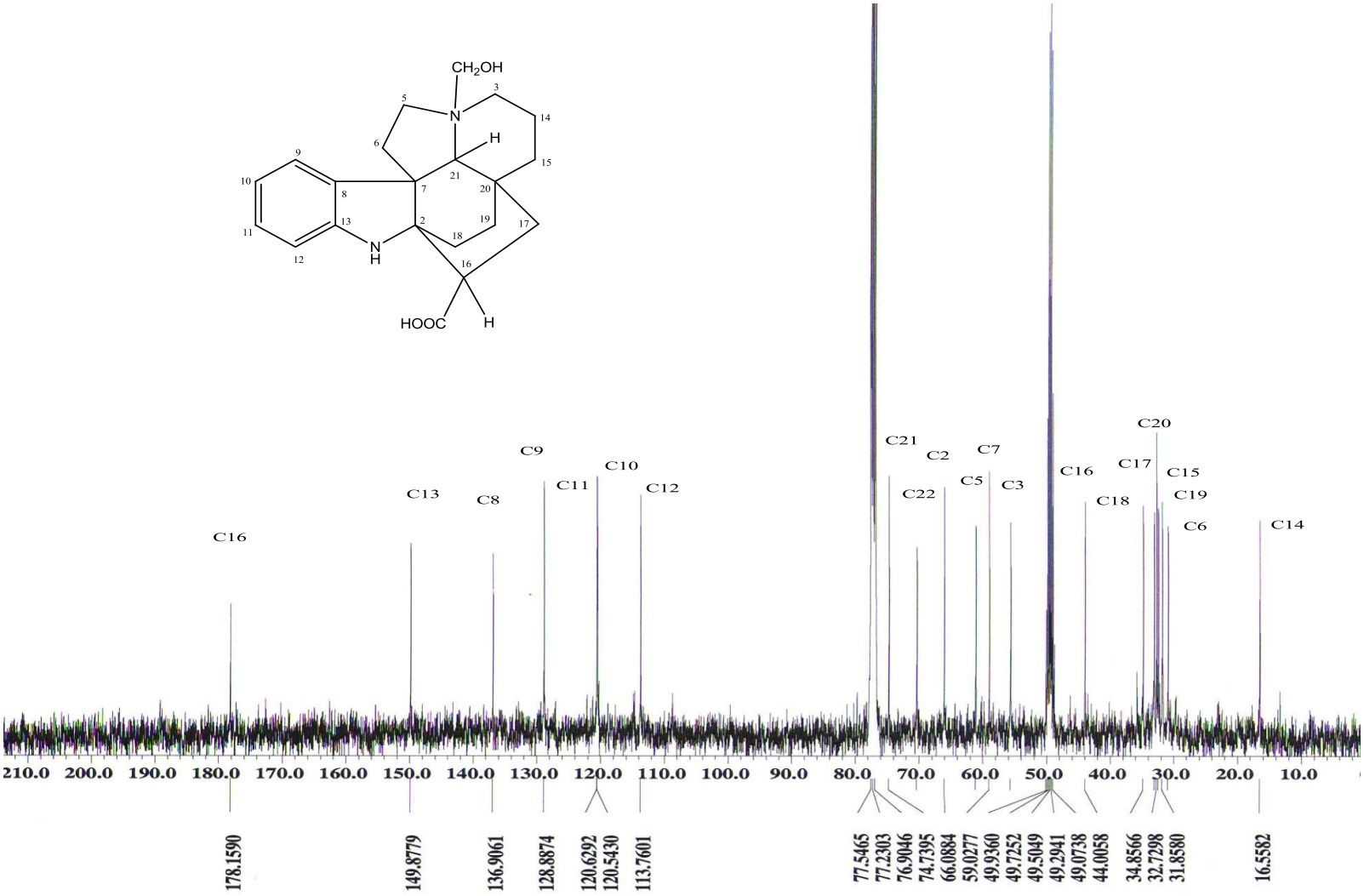
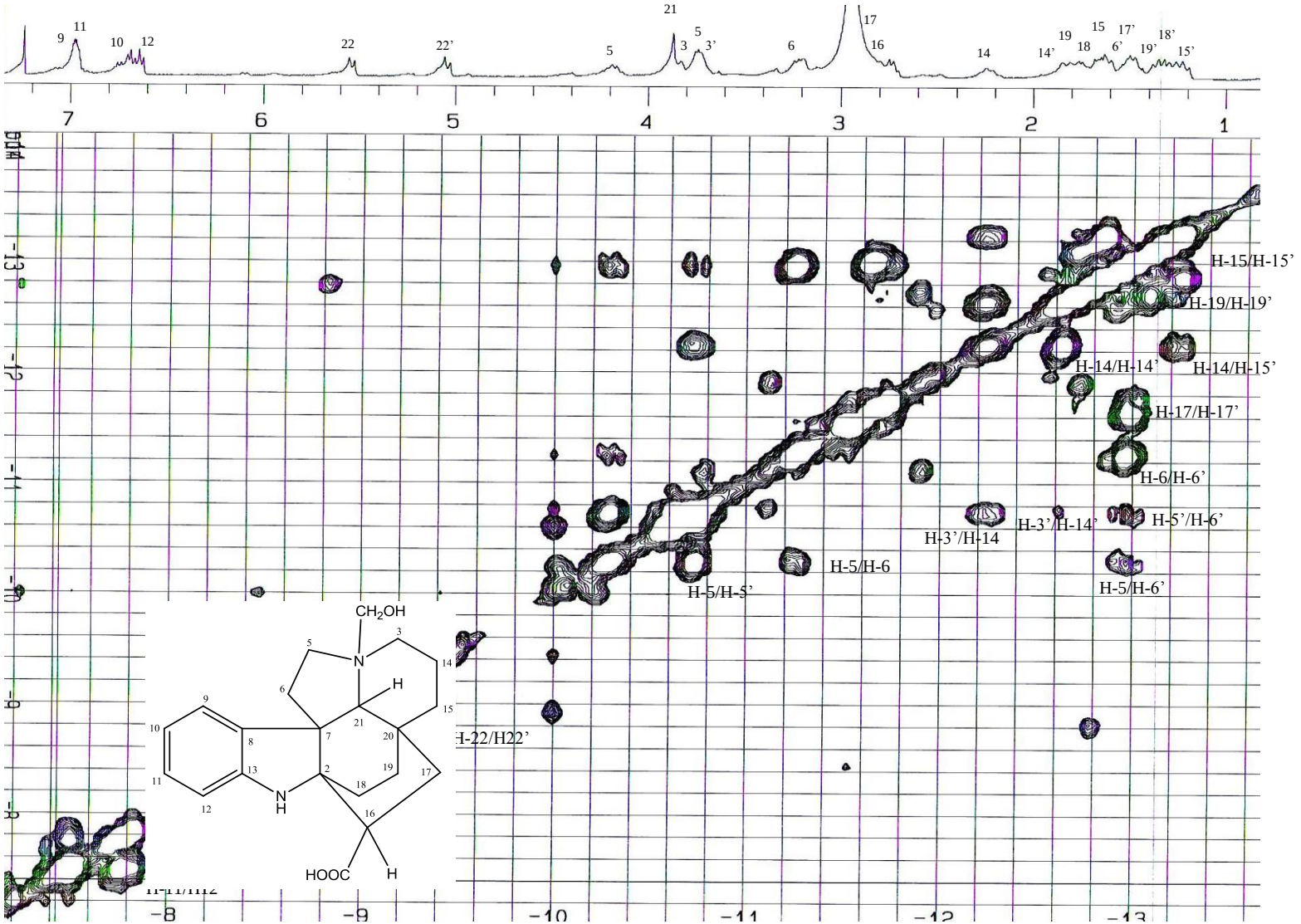
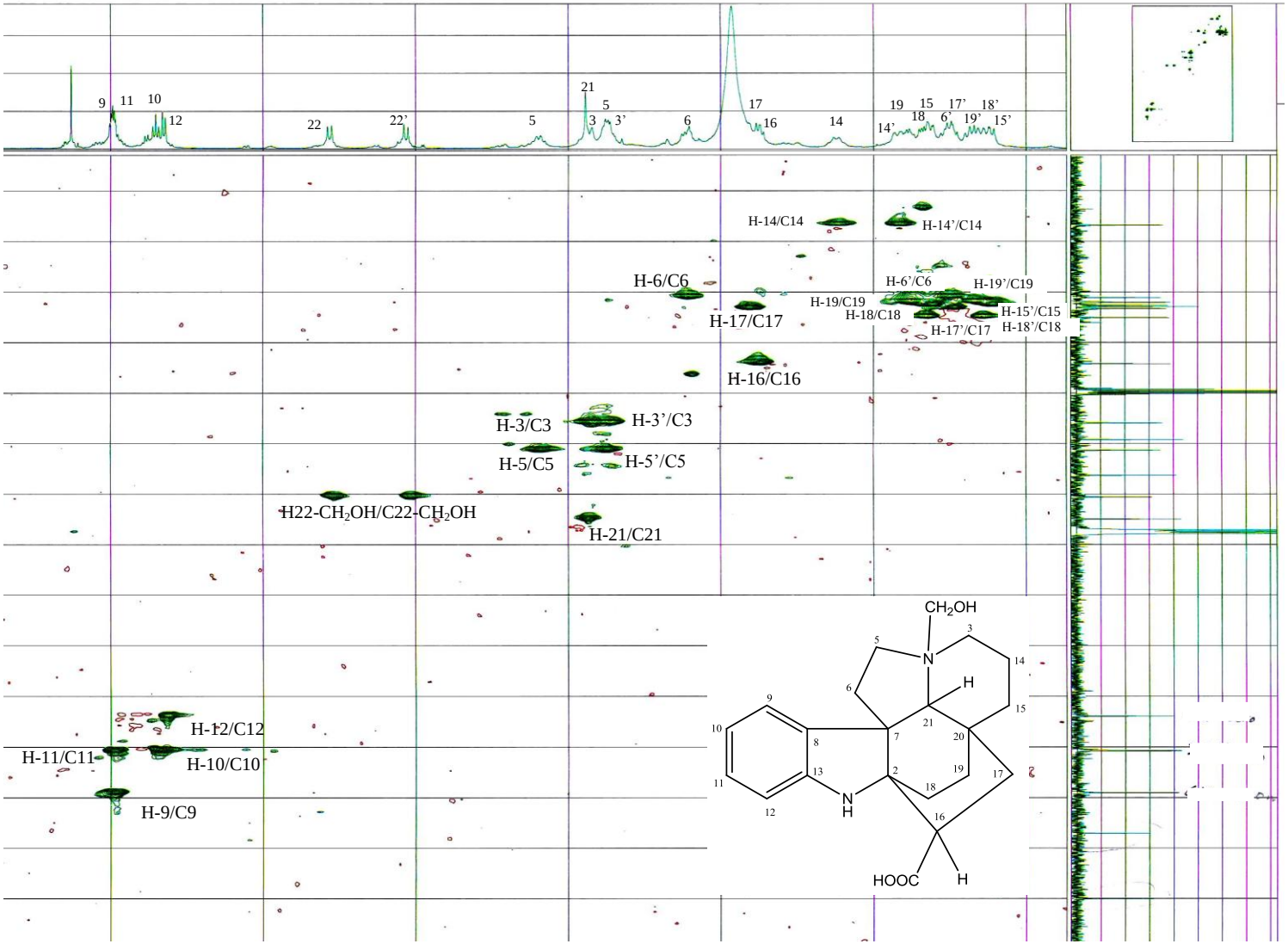


Figure 3.29: ^{1}H-NMR Spectrum of compound **G**


CH2OH
N
H
HOOC
H
N
H
C16
C13
C8
C9
C11
C10
C12
C21
C2
C22
C5
C7
C3
C16
C20
C17
C15
C18
C19
C6
C14
210.0 200.0 190.0 180.0 170.0 160.0 150.0 140.0 130.0 120.0 110.0 100.0 90.0 80.0 70.0 60.0 50.0 40.0 30.0 20.0 10.0
178.1590
149.8779
136.9061
128.8874
120.6292
120.5430
113.7601
77.5465
77.2303
76.9046
74.7395
66.0884
59.0277
49.9360
49.7252
49.5049
49.2941
49.0738
44.0058
34.8566
32.7298
31.8580
16.5582


H-15/H-15’
H-19/H-19’
H-14/H-14’
H-14/H-15’
H-17/H-17’
H-6/H-6’
H-5’/H-6’
H-3’/H-14’
H-3’/H-14
H-5/H-6
H-5/H-5’
H-5/H-6’
H-22/H22’
CH2OH
HOOC


9
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22'
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18
6'
19'
15'
H-14/C14
H-14'/C14
H-6/C6
H-6'/C6
H-19'/C19
H-19/C19
H-18/C18
H-15'/C15
H-18'/C18
H-17'/C17
H-17/C17
H-16/C16
H-3/C3
H-3'/C3
H-5/C5
H-5'/C5
H22-CH2OH/C22-CH2OH
H-21/C21
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CH2OH
N
H
HOOC
H
N
H

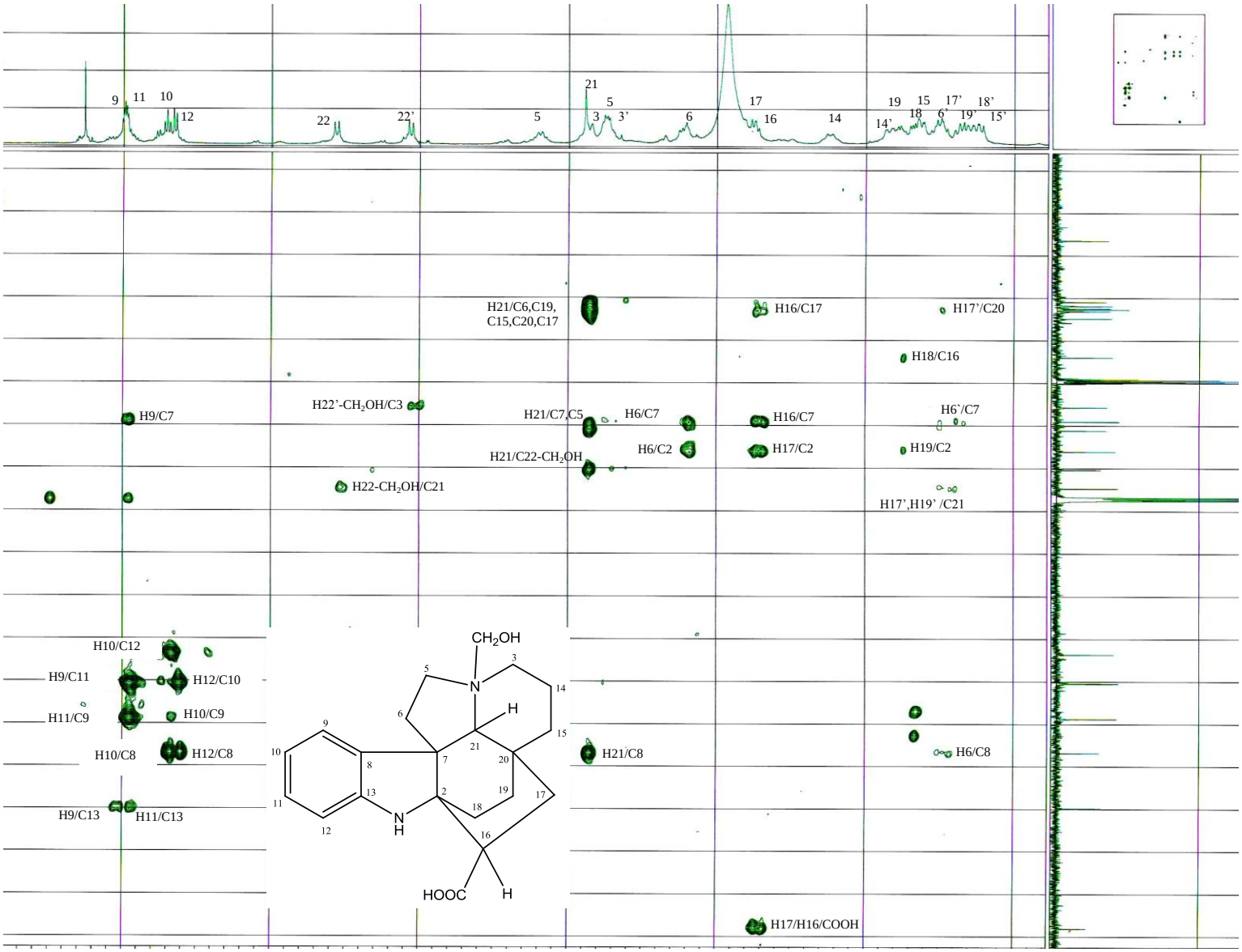
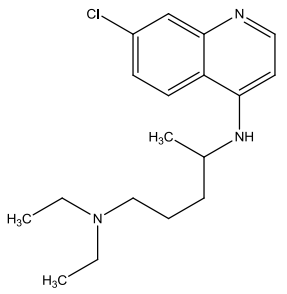


Figure 3.33: HMBC Spectrum of compound **G**

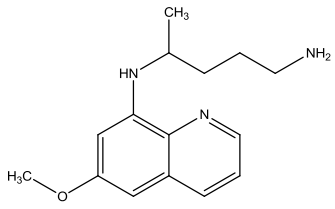
BIOACTIVITY

4.1 INTRODUCTION

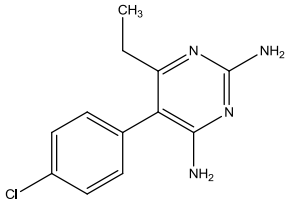
In the 1950s it was thought that the crisis of malaria had been solved.[58] It gave the impression that the syndrome would be eradicated by the use of synthetic drugs such as chloroquine **116**, primaquine **117** and pyrimethamine **118** whilst the use of DDT and other insecticides could be relied upon to eradicate the mosquito host. Indeed, by 1956 the World Health Organization (WHO) had commenced a programmed of worldwide eradication of malaria. By 1966, this programmed was judged as being successful and several hundred million people were then living in former malaria regions without any risk of infection. Unfortunately, since that time, the situation has worsened dramatically because of the resistance of *Plasmodium* to clinically useful drugs and because of the resistance of vector mosquitoes to insecticides.[59]



116



117

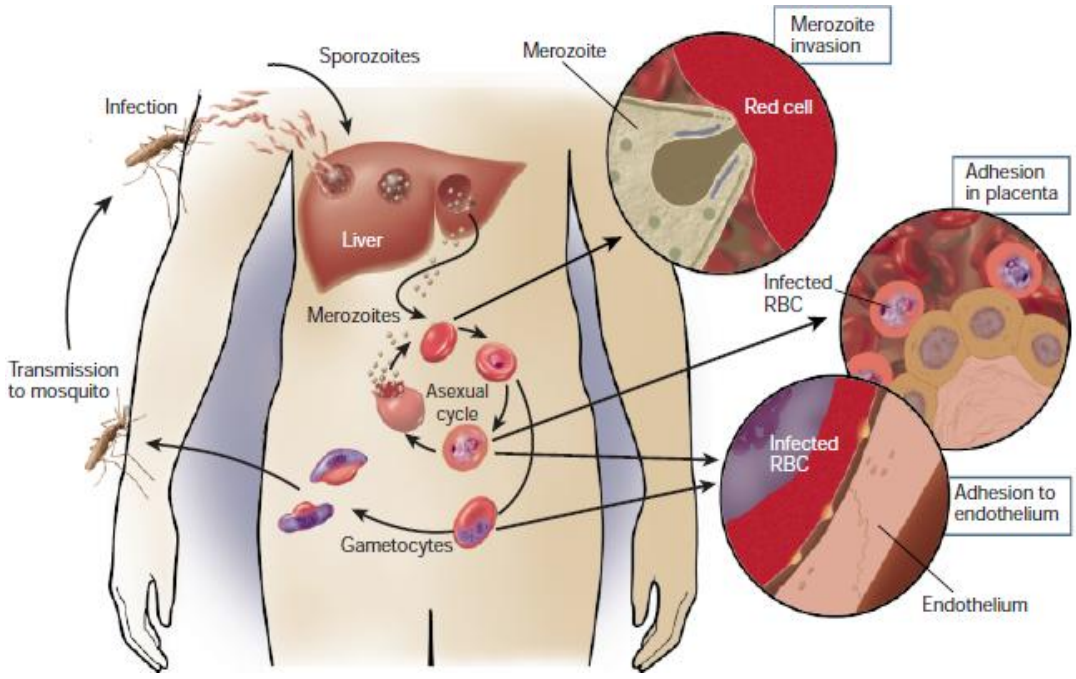


118

Many South-East Asian and South American countries now have increasing numbers of malaria cases per annum and the majority of these are caused by chloroquine-

resistant strains of *Plasmodium falciparum* which causes malignant tertiary malaria. *P. falciparum* often produces fulminating infections in non-immune patients and such infections may prove fatal unless early. *P. vivax* produces milder attacks of benign tertian malaria and has a lower mortality rate in the untreated adults; relapses may well occur for at least 2 years after the primary infection. *P. malariae*, responsible for quartan malaria, may also give rise to relapses several years after the primary infection. The fourth species of *Plasmodium* which produces malaria in human is *P. ovale* and the infections are similar to those of *P. vivax*.

Bites from female anopheline mosquitoes infected with *Plasmodium* sporozoites are responsible for transferring the disease to man (Scheme 4.1). During the symptom-free (pre-erythrocytic stages of the disease) the sporozoites rapidly disappear from the blood and inhabit the liver where they proliferate and sporulate. Merozoites released the liver to enter erythrocytes and start the blood cycle. Some of the parasites may infect tissue cells causing an exoerythrocytic stage but this does not occur with *P. falciparum*. Merozoites in the erythrocytes develop into trophozoites which reproduce asexually to form schizonts, from which many more merozoites burst out and infect more erythrocytes. The bursting of the erythrocytes is accompanied by feelings of intense coldness in patients. Some of the merozoites differentiate into male and female gametocytes which do not develop further unless blood is transferred to the female *Anopheles* mosquito whereupon the sexual phase of the life cycle of the malaria parasites takes place in the mosquito gut.

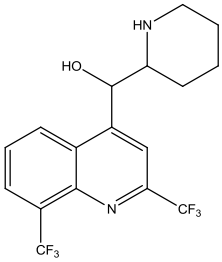


Scheme 4.1: Life Cycle of *Plasmodium falciparum*

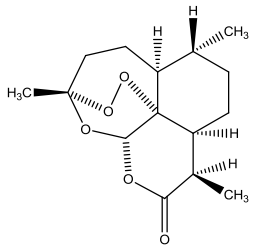
Adapted from *Nature* **415**, 673-679, 2002 doi: 10.1038/415673a

By 1960, resistance of *Plasmodium* to chloroquine **116** was recognized and had been confirmed in Colombia and Venezuela as well as along Thailand and then Cambodian border. Resistance has continued to spread steadily and by 1977 it was confirmed in East Africa. In 1977, it was estimated that the worldwide incidence of malaria was 800 million per annum with 1.2 million deaths per annum.[60] Malaria which is resistant to chloroquine **116** has now been confirmed in more than 40 countries and it creates a huge threat to world health. In retrospect, it has been proposed that resistance to chloroquine **116** was inevitable since the drug had been used in increasing quantities over 40-year period and by 1985 it was estimated that more than 350 000 kg had been used to treat some 234 million doses.[61]

Although the problem of malaria is acknowledged to be a major one it remains difficult to obtain accurate figures for the number of cases. It has, for example, been pointed out that WHO estimate of 100 million cases per annum in 1986 should more realistically have been reported as 489 million clinical cases worldwide and that *P. falciparum* alone accounted for 234 million cases, of which 2.3 million proved to be deadly.[62] Resistance to the more recently introduced mefloquine **119** has also been reported, and resistance has been produced readily, in the laboratory, against artemisinin **120**.[63]



119



120

4.2 Assays for Antiplasmodial Activity

The techniques accessible for the screening of drugs for antiplasmodial activity have been the subject of an extensive and comprehensive review.[64] In 1912, techniques were developed for keeping *P. falciparum* and *P. vivax* alive for a few hours outside of the body and these techniques formed the basis of *in vitro* screening procedures for over half a century.

By 1920s and 1930s avian malarias including *P. relictum* in canaries, *P. gallinaceum* in chicks, *P. cathemerium* and *P. lophurae* in ducklings, were developed for primary screening of antiplasmodial activity. Inadequate use has been made of simian parasites in monkeys but there are obvious limitations of these methods for primary screening.

In 1948, a major breakthrough in screening for antiplasmodial activity came through when the discovery of the rodent malaria parasite *P. berghei* at Kreyberg then Belgian Congo. This parasite, which readily infects laboratory mice and rats, has proved to be valuable for estimation of activity in chemotherapeutic research programmer in which more than 300 000 compounds have been screened.[64]

4.3 Antiplasmodial Test Against *Plasmodium falciparum* Strains

This procedure for evaluating compound effectiveness against *Plasmodium falciparum* (*in vitro*) utilize chloroquine **116** as a marker for inhibition of parasite growth.[65,66]

Various alternative etiquettes exist, including the ones based on microscopic detection of Giemsa-stained slides, assays based on production of parasite lactate dehydrogenase, and the use of flow cytometry.[67]

4.4 Preparation of the Antiplasmodial Test [68-75]

Test involves five (5) major parts which will be discussed in the following subchapters.

4.4.1 Malaria Culture Media

RPMI 1640 medium containing 50 mg/liter hypoxanthine, 25mM HEPES, L-glutamine (Catalog number 31800, Invitrogen), 10 μg/ml gentamicin, 0.225% $NaHCO_3$ and either 10% human serum or 0.5% Albumax I or II (purified lipid-rich bovine serum albumin, Invitrogen). Medium was adjusted to a pH about 7.3 to 7.4.

4.4.2 Parasite Strain

Some well-characterized strains (Table 4.1) are available, either from www.malaria.mr4.org (reagents available to registered users) or academic laboratories. One recommendation would be to test activity against a drug-sensitive line such as 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), 7G8 (Brazil), FCB (SE Asia) or K1 (Thailand).

Table 4.1: Standard *Plasmodium falciparum* Strains.

Name	Clone	Origin	Resistant to	Multiplication Rate
Dd2	Yes (from WR'82)	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 Leone-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.

4.4.3 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 500 x *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 5×10^9 cells/ml.

4.4.4 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.

4.4.5 Parasite Culture Conditions

P. falciparum asexual blood stage parasites are propagated at 37°C in malaria culture media at 3-5% hematocrit in a reduced oxygen environment (e.g. a custom mixture of 5% O_2, 5% CO_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 suffused with the low O_2 gas and maintained 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.

Compounds can often be dissolved in 100% dimethyl sulfoxide (DMSO) and stored at –20°C. Particle 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 3-12 (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 prior to addition of the parasites.

IC_{50} values are determined by linear regression analyses on the linear segments of the curves (IC_{90} values can also be determined by curve-fitting and can provide a 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. Differences 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.

4.4.6 Antiplasmodial Activity Test Result of Crude Extract and Isolated Pure Compounds

The bark of *K. singapurensis* was extracted with DCM. Hexane was then added to the dried extract and the mixture was stirred. The dissolved portion is separated from the undissolved portion of the crude extract. The dichloromethane crude extracts (DH & UDH) and isolated compounds of *Kopsia singapurensis* (bark) was tested against *Plasmodium falciparum* strains. The results were shown in Table 4.2 and Table 4.3. The DCM crude extract dissolved in hexane (DH) gave low value of IC_{50} compared to DCM crude extract undissolved in hexane (UDH).

Table 4.2 Antiplasmodial activity from crude *K. singapurensis*

SAMPLE	IC_{50} (µg/mL)
BARK – UDH	14.30

Notes:

- IC_{-50} < 25 µg/mL , to extract / fraction is considered as a potential anti-malarial

Compound methyl sinapate exhibited the most potent antiplasmodial activity with an IC_{50} of 0.11 followed by compound 15-hydroxykopsinine with an IC_{50} 1.43.

Table 4.3 Antiplasmodial activity from isolation of *K. singapurensis*

COMPOUND	IC_{50} (μg/mL)
chloroquine **Standard**	**0.0069**
methyl sinapate **82**	**0.1100**
trans-2,2`-dicarboxyazobenzene dioxide **84**	-
lonicerine **85**	-
15-hydroxykopsinine **86**	**1.4300**

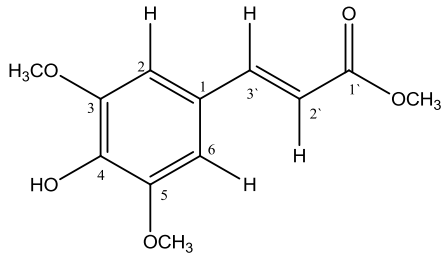
CONCLUSION

The bark of *Kopsia singapurensis* has been studied for their chemical constituents. Their structures have been fully elucidated by conventional spectral methods such as; NMR (^{1}H, ^{13}C and 2D), UV, IR, EIMS and HREIMS.

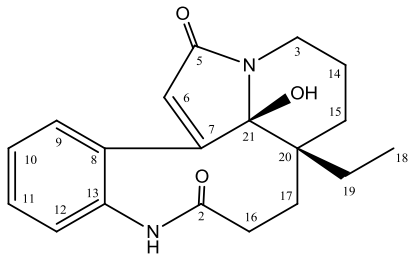
The study on the bark of *Kopsia singapurensis* which belong to the family of Apocynaceae afforded 3 known indole alkaloids, namely leuconolam **83**, lonicerine **85** and 15-hydroxykopsinine **86** while methyl sinapate **82** and *trans*-2,2`-dicarboxyazobenzene dioxide **84** are two new natural compounds in natural products. In addition two new alkaloids; singapurine **87** and N_4-hydroxymethyl kopsinic acid **88** were obtained.

This work has also shown that some of the constituents showed potent antiplasmodial activity against *Plasmodium falciparum* and compound **82** has interesting antiplasmodial activity. Perhaps compound **82** (IC_{50} = 0.1100 μg/mL) can be taken up as a lead compound for further structure activity studies on antiplasmodial activity.

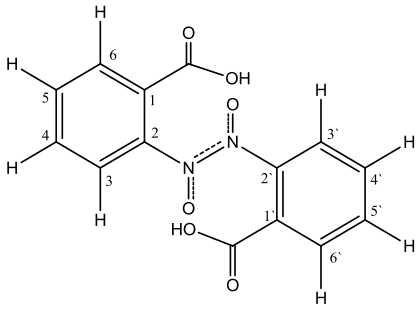
Other further works that can be done on chemicals of this plant is the study mechanism of the active compound **82**(IC_{50} = 0.1100 μg/mL) and **86** (IC_{50} = 1.4300 μg/mL).



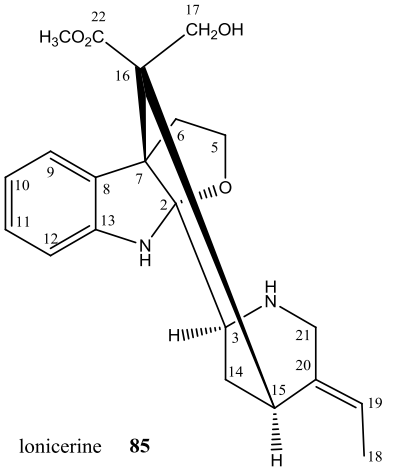
methyl sinapate **82**



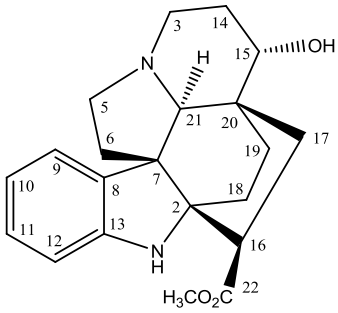
leuconolam **83**



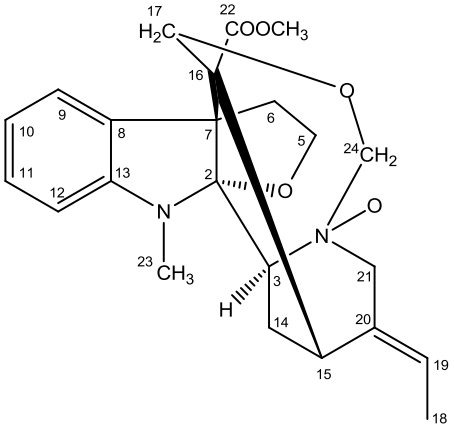
trans-2,2'-dicarboxyazobenzenedioxide **84**



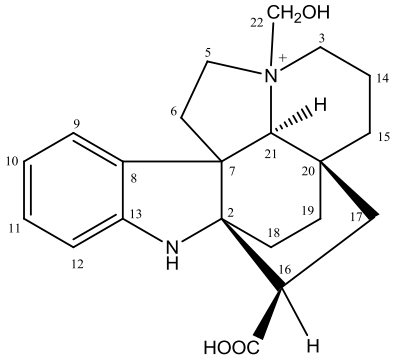
lonicerine **85**



15-hydroxykopsinine **86**



singapurine **87**



N_4-hydroxymethyl kopsininic acid **88**

MATERIAL AND METHODS

6.1 General Methods

Spectra were recorded on the following instruments as follows:

Infrared Spectra (IR)

The infrared spectra were recorded on a Perkin Elmer FTIR (model 1600) spectrophotometer. $CHCl_3$ is added to the sample and then the sample was applied evenly on the surface of the NaCl cell. After that the sample was dried using hair dryer. The NaCl cell was then placed to the sample holder and then data was collected.

Liquid Chromatography Mass Spectrometer (LCMS)

Mass spectra were recorded using Shimadzu Liquid Chromatography Mass Spectrometer Ion Trap Time of Fligth (LCMS-IT-TOF) using methanol as a solvent.

Nuclear Magnetic Resonance (NMR)

NMR spectra were obtained using JEOL JNM-FX100 (400 MHz). Deuterated chloroform ($CDCl_3$) was used as NMR solvent. Chemical shifts were reported in ppm, and coupling constants were given in Hz.

6.2 Reagent

6.2.1 Mayer's Reagent (Potassium mercuric iodide)

- ☞ It is a solution of 1.4g mercuric iodide in 60ml of distilled water mixed with a solution of 5.0g of potassium iodide in 10ml of distilled water.
- ☞ The mixture was then made up to a 100ml solution.
- ☞ A positive test is indicated by formation of a white precipitate when the aqueous layer (acidified) is treated with 2-3 drops of Mayer's reagent.

6.2.2 Dragendorff`s Reagent (Potassium bismuth iodide)

Solution A:	Bismuth (III) nitrate (0.85g) in a mixture glacial acetic acid (10ml) and distilled water (40ml).
Solution B:	Potassium iodide (8.0g) in distilled water (20ml).
Stock solution:	A mixture of equal volumes of both Solution A and B.
Spray Reagent:	The stock solution (20ml) was diluted in a mixture of acetic acid (20ml) and distilled water (60ml).
Dragendorff`s Test:	A positive results yields formation of orange spots.

6.3 Plant Material

Kopsia singapurensis was collected by a team of phytochemist, Department of Chemistry, Faculty of Science, University of Malaya; with the herbarium series number KL 5334, located in Kluang, Johor Darul Takzim.

6.4 Extraction, isolation, purification and bioactivity of *Kopsia singapurensis*

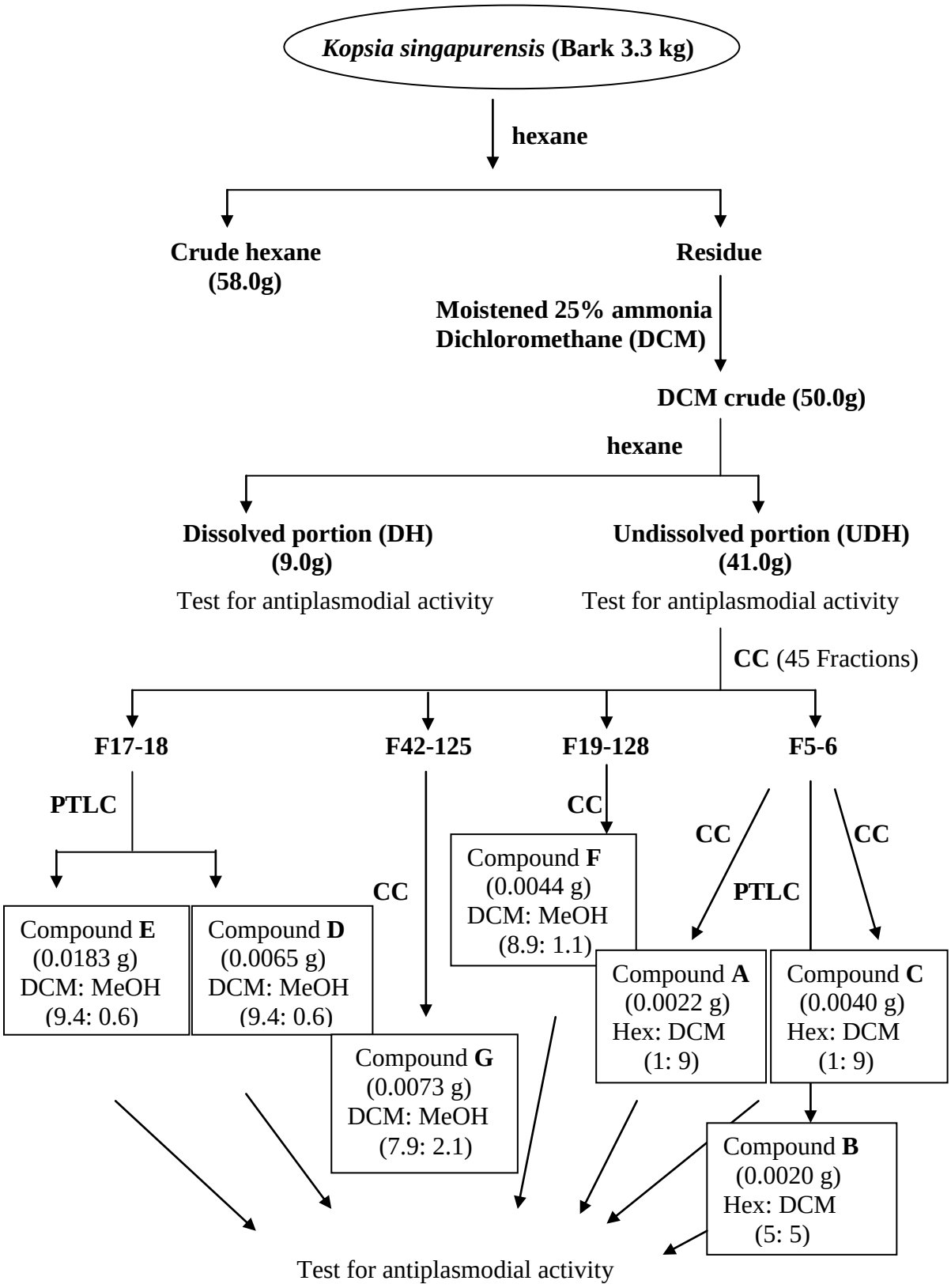
3.3 kg dried sample from the bark of *Kopsia singapurensis* was extracted and first defatted in hexane by soxhlet extractor for 17 hours. Then the extract was dried out on the rotary evaporator. After that, the extract was wetted with 10 % ammonia solution and left for overnight. They were then re-extracted successively with dichloromethane (CH_2Cl_2). After removal of the solvents, the crude extracts of hexane (58.0 g) and dichloromethane (50.0 g) was obtained for the bark.

The dichloromethane (DCM) extract (bark) was partitioned with hexane for three days. The dissolved portion (DH) (9.0 g) was separated from the undissolved portion (UDH) (41.0g). The crude extract (30.0 g) from the plant material was subjected to column chromatography with by silica gel. Then, the column was eluted with solvent mixtures of increasing polarity, hexane, dichloromethane, and methanol. 45 fractions were obtained. Through ^{1}H-NMR analysis, fraction 5 and 6 were pooled and subjected to column chromatography using solvent system with increasing polarity (Hexane:DCM to DCM:MeOH) which resulted compound **A** (Hexane:DCM; 1:9) and compound **C** (Hexane:DCM; 1:9). Whereas, about 220 tubes were generated from fraction 19 and resulted compound **F** (Dichloromethane:Methanol; 8.9:1.1). Fraction 42 was also subjected to column chromatography to afford compound **G** (Dichloromethane:Methanol; 7.9:2.1). Meanwhile, compounds **B** (Hexane:Dichloromethane; 5:5), **D** (Dichloromethane:Methanol; 9.4:0.6) and **E** (Dichloromethane:Methanol; 9.4:0.6) was obtained using preparative thin layer chromatography method.

The fractions which given spots with the same R_f value were grouped into a series of fractions (observed by NMR and TLC). To purify the compound, each one of fractions were then treated individually by extensive column chromatography and preparative TLC

The structural elucidation was carried out by spectroscopic methods (^{1}H-NMR, ^{13}C-NMR, COSY, DEPT, HMBC, HMQC, IR and UV). Then, the crude extract and the isolated compounds were subjected to screening for antiplasmodial activity.

The flow chart of the extraction, isolation, purification and bioactivity process is shown in Scheme 6.4.



Scheme 6.4: Extraction, isolation and purification of compounds *Kopsia singapurensis* (bark)

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