STRUCTURE AND SYNTHESIS OF *GUNNERA PERPENSA* SECONDARY METABOLITES

By

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Pietermaritzburg

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DECLARATION

I hereby certify that this research is a result of my own investigation, which has not already been accepted in substance for any degree and is not being submitted in candidature for any other degree.

Signed.....

Xolani Kevin Peter

I hereby certify that this statement is correct

Signed .....

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January 2007

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ABSTRACT

The project focused on the isolation, characterization and synthesis of secondary metabolites of *Gunnera perpensa* L. (Gunneraceae), a South African medicinal plant used by many South African women to induce or augment labour and as an antenatal medication to tone the uterus. From the methanol extracts of the rhizomes we have isolated the compounds *Z*-venusol, methyl lespedezate, 4-*O*-*β*-D-glucopyranosyl-3,3',4'-tri-*O*-methylellagic acid and punicallagin. Structural elucidation of the compounds was performed using NMR spectroscopy. The presence of ellagic acid derivatives and hydrolysable tannins have not previously been reported from the family Gunneraceae.

The study also focuses on the development of an HPLC analytical method to fingerprint the crude extracts of *G. perpensa*. This method was used to determine the chemical composition of the rhizomes of the *G. perpensa* collected in different parts of South Africa. It is clear from the HPLC study that the rhizomes contain large concentrations of the hydrolysable tannin punicalagin and the second most abundant metabolite was *Z*-venusol. However, it was observed from plants collected in different regions that the ratio between punicalagin and *Z*-venusol differs substantially in the different extracts.

An ellagic acid derivative isolated from *G. perpensa* contains a biaryl structure derived from gallic acid. The synthesis described in this thesis focused on reaction methods to access unsymmetrical biaryls and two synthetic routes were investigated - one that relies on the Ullmann reaction and the second that uses the Heck coupling reaction. Success of this coupling reaction towards the formation of ellagic acid derivatives was accomplished by the Heck coupling reaction method. One of the most important considerations towards the synthesis was the manipulation of hydroxyl groups of gallic acid by selective protection reactions that provide entry to the aforementioned preparation of unsymmetrical ellagic acid derivatives.

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LIST OF ABBREVIATIONS

Ac	acetyl
APT	attached proton test
aq	aqueous
Ar	aryl
br t	broadened triplet
CDMT	2-chloro-4,6-dimethoxy-1,3,5-triazine
COSY	correlated spectroscopy
DMSO	dimethylsulfoxide
d	doublet
dd	doublet of doublets
DEAD	diethyl azodicarboxylate
DEPT	distortionless enhancement polarization transfer
DMA	*N,N*-dimethylacetamide
DMAP	4-dimethylaminopyridine
DME	dimethoxyethane
DMF	*N,N*-dimethylformamide
DMTMM	4-(4,6-dimethoxy-1,3,5-triazen-2yl)-4-methylmorpholinium chloride
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
ESI-TOF	electrospray ionization-time-of-flight
EtOAc	ethyl acetate
EtOH	ethanol
HETCOR	heteronuclear correlation
HHDP	hexahydroxydipenoyl
HIV	human immunodeficiency virus
HMQC	heteronuclear multiple quantum coherence
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
Hz	Hertz
Lit.	literature
MeOH	methanol
m.p.	melting point
MS	mass spectrometry

NBS	*N*-bromosuccinimide
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance spectroscopy
NOE	nuclear Overhauser effect
Ph	phenyl
s	singlet
sept	septet
t	triplet
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMAD	*N,N',N''*-tetramethylazodicarboxamide
UKZN	University of KwaZulu-Natal

CHAPTER ONE

INTRODUCTION

1.1 BIOACTIVE NATURAL PRODUCTS

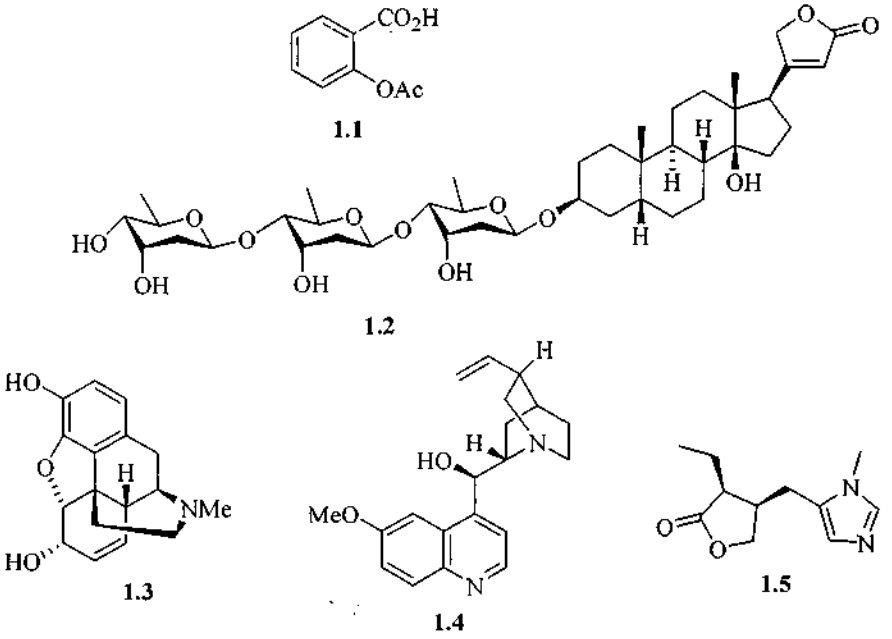
The journey of natural product chemists began in the 19th century when they took up the challenge of determining the structures of complicated natural products. Encouraged by a society who values many natural products for their life-giving properties, chemists have applied their skills and intellect to many tens of thousands of molecules made by living organisms. By the 20th century natural products began to attract the attention of the biological disciplines such as clinical biochemistry, pharmacology, toxicology, microbiology and cell biology. The discovery of antibiotics gave the study of natural products a great boost and ensured that natural products remained central to growing pharmaceutical companies.

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years.[1] The use of plants in traditional medicine of many cultures has been extensively documented. The first records, written on hundreds of clay tablets in cuneiform, are from Mesopotamia and date from about 2600 BC. Amongst the approximately 1000 plant-derived substances which they used were oils of *Cedrus* species (cedar) and *Cupressus sempevirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh), and *Papaver somniferum* (poppy juice), all of which are still used today for the treatment of ailments, ranging from coughs and colds to parasitic infections and inflammation.

Chinese medicine has been documented extensively with the first record dating from about 110 BC. The Indian Ayurvedic [*Ayus* (r), meaning life, and *Veda* meaning knowledge] system dates to about 1000 BC and it takes a holistic view of man, his health and illness.[2,3] Plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organisation that approximately 80% of Africa's

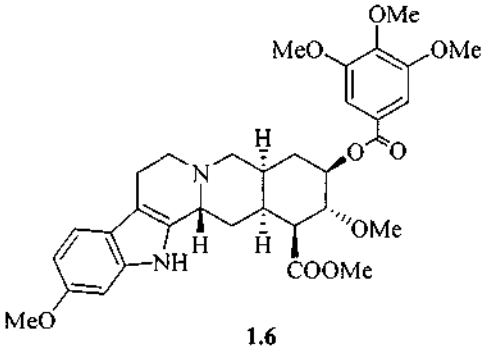
inhabitants rely mainly on traditional medicines for their primary health care.[4] At least 119 chemical substances derived from 90 plant species can be considered as important prescription drugs that are used by one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of active substances from plants used in traditional medicines.[5]

Clinical, pharmacological and chemical studies of traditional medicines derived from plants led to early drugs such as aspirin (**1.1**), digitoxin (**1.2**), morphine (**1.3**), quinine (**1.4**) and pilocarpine (**1.5**). The discovery of antibacterial penicillin by Fleming in 1928 and subsequent re-isolation and clinical studies by Chain, Florey and co-workers in the early 1940s, and the commercialization of synthetic penicillin revolutionized drug discovery research.[6]



Analysis of Ayuverdic remedies resulted in discovery of the drug reserpine (**1.6**), occasionally prescribed for hypertension, from the root of the climbing shrub *Rauwolfia serpentina*.[7,8,9] Some important chemotherapeutic agents currently in use for the treatment of certain types of cancers are plant based. Vinblastine (**1.7**) and vincristine (**1.8**) both isolated from *Catharanthus roseus*, are used for the treatment of Hodgkin's disease, lymphosarcoma and leukaemia.[10,11] Irenotecan (**1.9**), first isolated from the Chinese tree *Camptotheca acuminata* but now obtained mostly from the Indian tree *Nothapodytes*

nimmoniana, is used for the treatment of lung, ovarian and cervical cancers.[12,13] Taxol (**1.10**) was isolated from a Pacific yew *Taxus brevifolia* and is used for the treatment of cancer.



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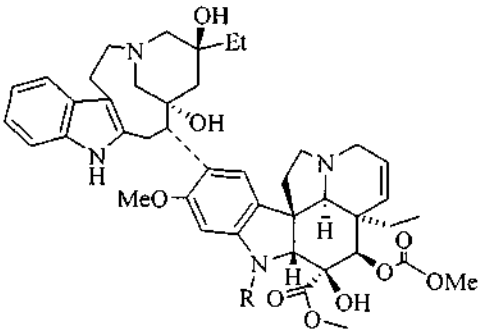
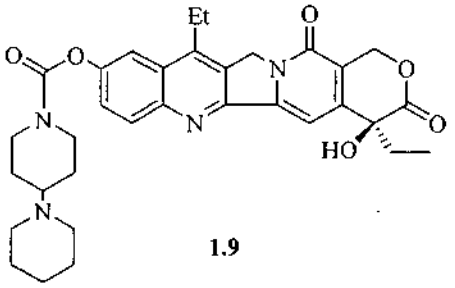


1.7 R=Me
1.8 R=CHO



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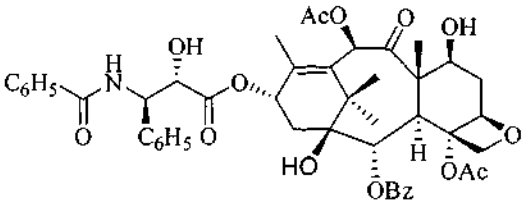
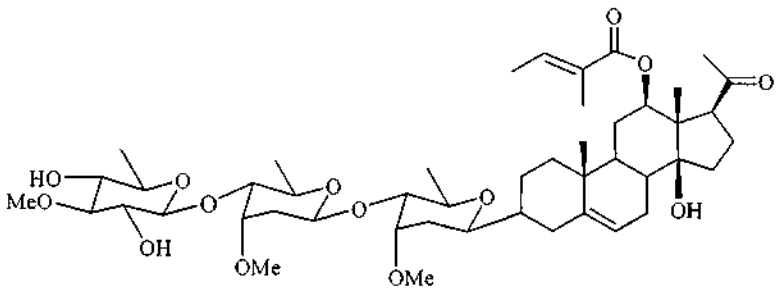


1.10

Southern Africa has a rich botanical diversity with approximately 24 000 species in this region. Of these, about 4 000 plant species are used in traditional medicine to remedy different diseases. The chemistry and pharmacology of many of these medicinal plants have not been investigated yet. South Africa can benefit from the scientific evaluation of this indigenous knowledge base.

Recently, P59 (**1.11**), a compound with appetite suppressant activity, was isolated from the South African plants *Hoodia pilifera* and *H. gordonii* (Apocynaceae: Asclepiadaceae).[14,15] Obesity is increasingly associated with health problems and a natural appetite suppressant may play an important role in future. However, despite the success story of *Hoodia*, there are many other local medicinal plants that need to be investigated.



1.11

1.2 AIM OF THE STUDY

The only South African *Gunnera* species is *Gunnera perpensa* L. known as uGobho in isiZulu and river pumpkin in English. *G. perpensa* rhizomes are used by many South African women to induce labour and as an antenatal medication to tone the uterus. South African indigenous people have also used it to treat other illnesses like rheumatic fever, swellings, menstrual pain and stomach bleeding. Preliminary screening of the crude extract of this plant for uterotonic effects have been reported.[16] However, prior to this investigation, no information was available on the phytochemistry of this plant.

The aims of the current study are:

- The isolation and characterisation of the secondary metabolites in the rhizome of *G. perpensa*
- The development of analytical methods to determine chemical markers present in *G. perpensa*
- The investigation of chemical variation of plants collected in different geographical regions
- The synthesis of an ellagic acid derivative and related compounds isolated from the rhizomes.

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CHAPTER TWO

PLANTS WITH UTEROACTIVITY (OXYTOCIC PLANTS)

2.1 INTRODUCTION

The use of traditional herbal medicines related to pregnancy has been documented throughout the world. Indigenous people in different parts of the world have and still are using decoctions made from plants to facilitate labour in childbirth. The chemical properties and/or the potential toxicity of the plants known to be used during pregnancy have been explored and in some plants the active chemical constituent is known. Plants that produce uterine contraction have similar action to that of the hormone oxytocin.

Oxytocin is the principal uterine-contracting and milk ejecting hormone of the posterior pituitary produced in the hypothalamus.[1] It is released in pulses into the circulation systems to the targeted sites during child labour. In 1909, Sir Henry Dale discovered and described the uterine contractile effect of oxytocin and after analysis came to the conclusion that oxytocin came from the pituitary gland.[2] In 1954 V. du Vigneaud described oxytocin as a polypeptide. Many studies showed that the use of oxytocin may help to optimize delivery without disastrous effects.[3] In 1992, a nocturnal peak in the concentrations of plasma oxytocin was found to correlate with the nocturnal peak in the uterine activity in late pregnancy. Oxytocin acts on the uterine myometrial cells that are responsible for contraction of the uterus and is therefore important in medical control of labour in modern obstetrics. One should not lose sight of the risks associated with mother or fetus with induction i.e. fetal distress, uterine dysfunction and hyperstimulation. By careful use of oxytocins, these risks can be reduced and to avoid adverse effects while using oxytocin, the correct dose is important.[4,5] The first drugs to be used for their uterotonic activity were ergot alkaloids, followed by oxytocin and later prostaglandins.

2.2 SOUTH AFRICAN OXYTOCIC PLANTS

A review compiled by Steenkamp[6] revealed that one hundred and fifty-six plant species distributed in seventy-three plant families are used by indigenous people of South Africa to treat gynaecological problems. The majority of these plants are used to treat infertility (ninety species). Plants are also used to treat menstrual disorders: amenorrhoea (fifteen species), dysmenorrhoea (forty-four species), menorrhagia (twenty-nine species) and irregular menstruation (four species). Many of the plants are prepared as decoctions and taken orally.

In the Vhavenda tribe, the plant part is burnt and the smoke directed into either the vagina [*Barleria randii* (Acanthaceae)] or the vulva [*Tabernaemontana elegans* (Apocynaceae), *Acokanthera oppositifolia* (Apocynaceae), *Asparagus buchananii* (Asparagaceae), *Capparis tomentosa* (Capparaceae), *Maerua cafra* (Capparaceae), *Berchemia discolor* (Rhamnaceae), *Osyris lanceolata* (Santalaceae)]. For the treatment of amenorrhoea, powdered plant material is applied to underwear as in the case of *Albizia brevifolia* Schinz (Fabaceae), *Brackenridgea zanguebarica* (Ochnaceae) and *Pterocarpus angolensis* (Fabaceae).[7]

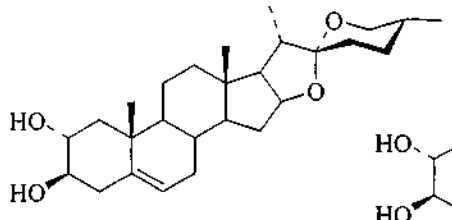
Veale *et al.*[8] has identified fifty-seven plant species that are used by South African traditional people during pregnancy and chiidbirth, and important plants in this respect are: *Clivia miniata* (Amaryllidaceae), *Asclepias fruticosa* (Asclepiadaceae), *Callilepis laureola* and *Vernonia tigna* (Asteraceae), *Gunnera perpensa* (Haloragaceae), *Gladiolus sericeovillosus* (Iridaceae), *Bowiea volubilis* (Hyacinthaceae), *Eulophia clavicornis* and *Eulophia tenella* (Orchidaceae), *Pentanisia prunelloides* (Rubiaceae), *Grewia occidentalis* (Tiliaceae), *Typha capensis* (Typhaceae) and *Rhoicissus tridentate* (Vitaceae).

Decoctions of *Agapanthus africanus* and *C. miniata* of South Africa have been reported to have uterotonic activity on the isolated uterus preparation. Since the uterus comprises of myometrial and an endometrial layer, their mode of action towards different oxytocins could be different. Veale *et al.*[9] compared the mode of action of two plant extracts on a stripped myometrium preparation to acetylcholine and atropine. Both extracts inhibited uterotonic

activity in the myometrium to acetylcholine. Atropine inhibited response of the myometrium only to the *A. africanus* extracts.

Preliminary studies by Kaido *et al.*[10] of plants used in South Africa as herbal remedies investigated the activity of the crude decoction of each plant on the isolated rat and ileum preparation. The oxytocic plants investigated by the authors were *A. africanus*, *P. prunelloides* and *G. perpensa*. Their results showed that aqueous extracts of all three plants initiated contractions in the isolated rat uterus and that the *G. perpensa* extract showed no response on the rat ileum.

Veale *et al.*[11] has investigated the effects of the extract of *A. africanus* (Agapanthaceae) on the isolated rat uterus. The plant is used by South African women as a decoction (in *isihlambezo*) during pregnancy and also in *inembe*, a decoction used to induce labour. It was also revealed that *Agapanthus* was one of the five plants used to treat complication associated with child labour. Preliminary studies reported that the extract of the leaves induces the initial response of the uterus to oxytocin. Compounds isolated from the plant includes yuccagenin (**2.1**), sapogenin agapanthagenin (**2.2**), 7-dehydroagapanthagenin (**2.3**), 8(14)-dehydroagapanthagenin (**2.4**), and 9(11)-dehydroagapanthagenin (**2.5**).[12,13]



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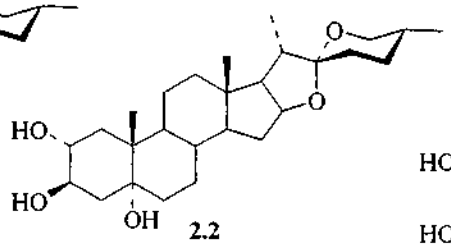

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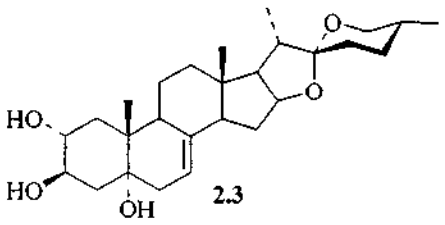

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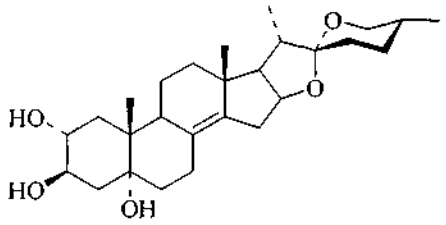

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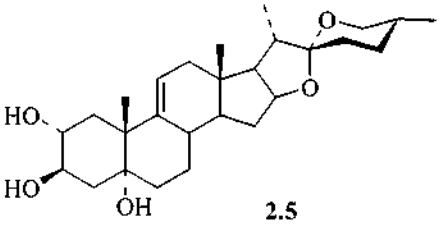
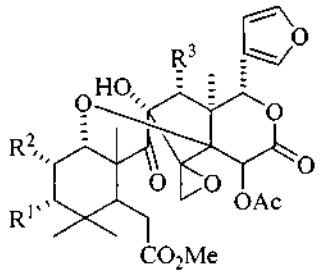
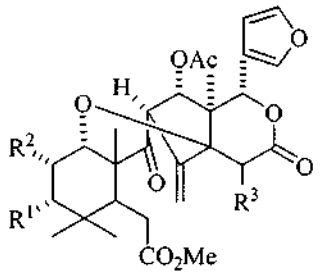


2.5

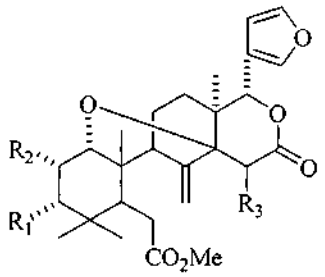
Sewram *et al.*[14] has reported a fast, safe and sensitive method for determining the uterotonic activity of medicinal plants. The South African plants used to evaluate and validate the method were *C. miniata, Ekebergia capensis, Grewia accidentalis* and *Asclepias fruticosa.* These plants were chosen for their current use by some black South African women to either to aid the fetus growth or to act as uterotonic agents to facilitate labour. The extract of *C. miniata, A. fruticosa* and *E. capensis* showed maximum uterotonic activity and the extract of *G. occidentalis* only induced uterine muscle contraction. Compounds isolated from the hexane extract of seeds of *E. capensis* tree are limonides: capensolactones (**2.6** to **2.11**) and methyl 3-α-hydroxy-3-deoxyangolensate (**2.12**).[15,16]



2.6 R^1= OH, R^2= $(CH_3)_2CHCO_2$, R^3= OH
2.7 R^1= ONicotinyl, R^2= $CH_3CH_2CH(CH_3)CO_2$, R^3= OAc
2.8 R^1= $CH_3CH_2CH(CH_3)CO_2$, R^2= ONicotinyl, R^3= OAc



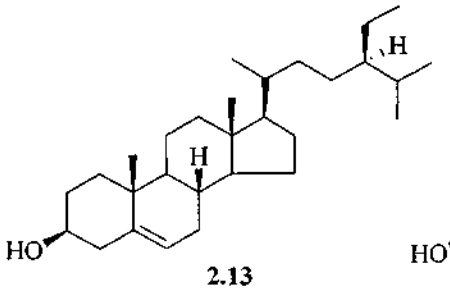
2.9 R^1= ONicotinyl, R^2= $(CH_3)_2CHCO_2$, R^3= OAc
2.10 R^1= $(CH_3)_2CHCO_2$, R^2= ONicotinyl, R^3= OAc



2.11 R^1= OH, R^2= $(CH_3)_2CHCO_2$, R^3= OAc
2.12 R^1= H, R^2= OH, R^3= H

In the supercritical fluid extract of *E. capensis*, five compounds were isolated by Sewram *et al.*[17] and the uterotonic effects of each was determine. These compounds were identified as β-sitosterol (**2.13**), oleanolic acid (**2.14**), 3-*epi*-oleanolic acid (**2.15**), 2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetra-cosatetraene (**2.16**) and 7-hydroxy-6-

methoxycoumarin (**2.17**). The two compounds oleanolic acid and 3-*epi*-oleanolic acid displayed uterotonic activity.



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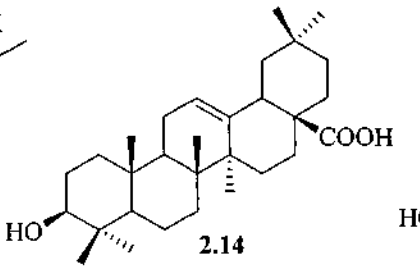

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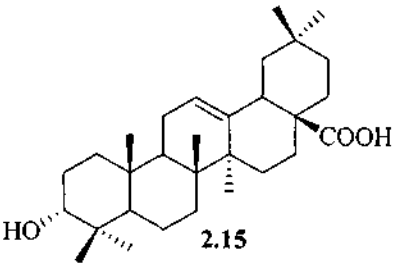

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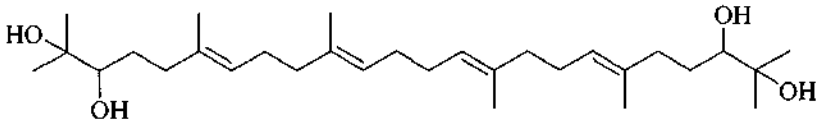

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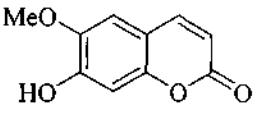
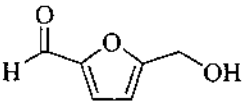


2.17

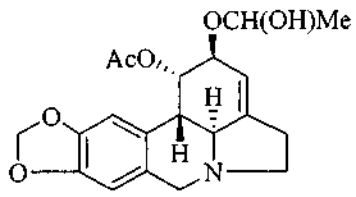
Supercritical fluid extraction of the roots of *C. miniata* resulted in the isolation of two uteroactive compounds. The active compounds that were found to individually induce muscle contractions were identified to be linoleic acid (**2.18**) and 5-hydroxymethyl-2-furancarboxaldehyde (**2.19**). The mode of action of compound **2.19** was assessed with the use of two receptor agonists and antagonists.[18]

2.18

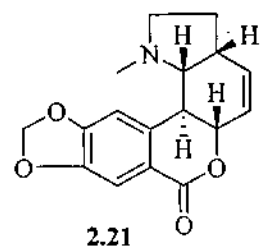


2.19

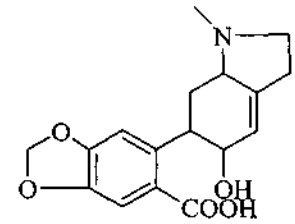
The presence of alkaloids in the roots and leaves of *C. miniata* were reported. The isolated alkaloids are *O*-acetyl-*O*-α-(hydroxyethyl)lycorine (**2.20**)[19], clivonidine (**2.21**)[20], cliviahaksine (**2.22**)[21], and two antiviral agents clivimine (**2.23**) and lycorenan-7-one (**2.24**)[22] but none of these compounds were assayed for activity.



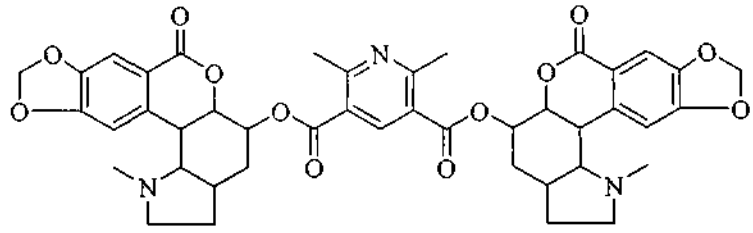
2.20



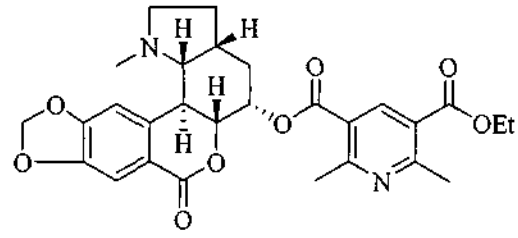
2.21



2.22



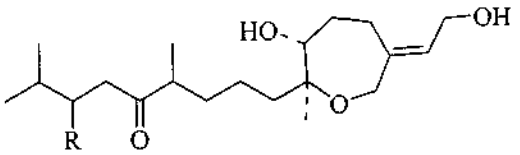
2.23



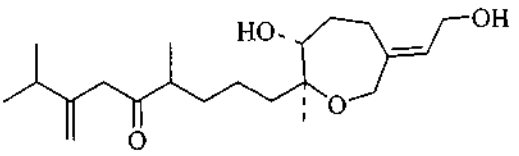
2.24

2.3 NON-SOUTH AFRICAN OXYTOCIC PLANTS

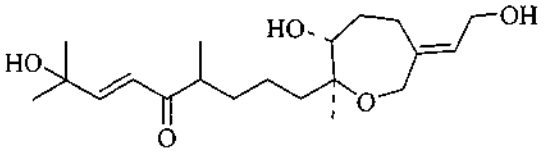
The two Mexican plants *Montanoa tomentosa* and *M. frutenscene* (Heliantheae) are commonly known as 'zoapatle' and have been used since ancient times for their oxytocic properties. From the active extract, a putative oxytocic fraction was isolated as a complex mixture. In the mixture four oxepane diterpenoids, zoapatanol (**2.25**), montanol (**2.26**), tomexanthin (**2.27**), tomentol (**2.28**) and a sesquiterpene lactone, tomexanthol (**2.29**) were identified. Of all the compounds isolated in the uterotonic-active mixture, zoapatanol (**2.25**) and tomentol (**2.28**) were as active as the component mixture.[23]



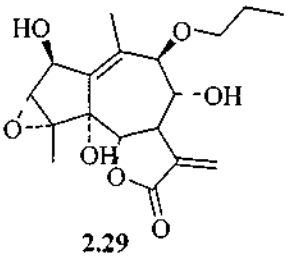
2.25 R = H
2.26 R = Me



2.27



2.28



2.29

Monechma ciliatum (Acanthaceae) is traditionally used in the Plateau State of Nigeria to induce labour. The hot methanolic extract of the plant was found to have potent oxytocic and oestrogenic activities.[24] A lignan, lariciresinol dimethyl ether, has been isolated from *M. ciliatum*.[25] However, bioassay-guided fractionation indicated that the oxytocic principle is very polar and the putative active compound was identified as a small peptide made up of tyrosine, leucine and a third component which is probably serine.[26]

Oldenlandia affinis (Rubiaciae) is a medicinal plant of the northern Congo and south-western Central African Republic. The decoction of the plant is used in Central African Republic to facilitate labour. People of Congo use an aqueous decoction called 'Kalata-Kalata' as a native drug during labour and the source plant was discovered to be *O. affinis*. The earliest report on the uteroactive effect of the plant dates back to 1965 by Sandburg.[27] When the decoction of *O. affinis* was tested on the oestrogenised rat uteri, it showed a very strong uterine activity.[28] Based on those reports and observations Gran *et al.*[29] isolated and identified a uteroactive cyclic peptide, kalata-B1 (Fig 2.1), which consists of 29 amino acids.

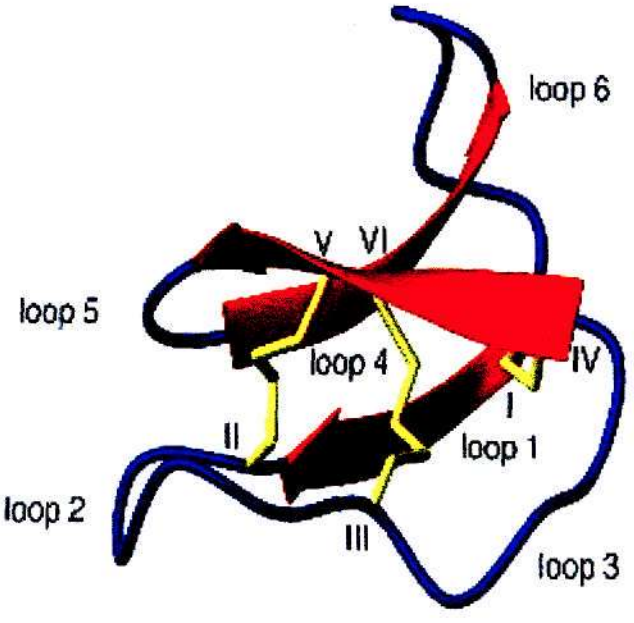
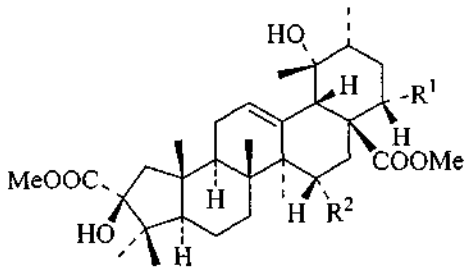
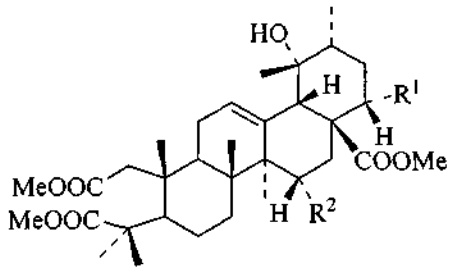


FIGURE 2.1: Three-dimensional structure of the cyclotide kalata B1[30]
Kalata-B1 sequencing:
Cyclo(L-arginyl-L-asparaginylglycyl-L-leucyl-L-prolyl-L-valyl-L-cysteinylglycyl-L-α-glutamyl-L-threonyl-L-cysteinyl-L-valylglycylglycyl-L-threonyl-L-cysteinyl-L-asparaginyl-L-threonyl-L-prolylglycyl-L-cysteinyl-L-threonyl-L-cysteinyl-L-seryl-L-tryptophyl-L-prolyl-L-valyl-L-cysteinyl-L- threonyl),cyclic(7→21),(11→23),(16→28)-tris(disulfide).

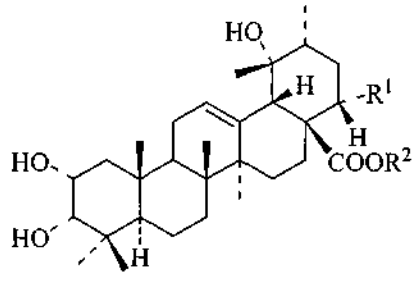
Another medicinal plant used in tropical parts of Africa for its oxytocic activity is *Musanga cecropioides* (Cecropiaceae). The tree is found in the tropical forest of Africa from Guinea to Congo and is used by indigenous people to induce labour. The uterotonic effect of the leaf on the rat uterus was reported by Kamanyi *et al.*[31] and recently the uterotonic effect of the water extract of the stem bark was reported by Ayinde *et al.*[32] Phytochemical studies of the leaf, stem bark and the root wood had been reported.[33,34] Compounds isolated from the plant are triterpenes: methyl musancropate A and B (**2.30** and **2.31**), methyl cecropiacic (**2.32**), methyl musangate A (**2.33**), methyl musangate B (**2.34**), methyl euscaphate A and B (**2.35** and **2.36**) and musangicic acid (**2.37**).



2.30 R^1= OH, R^2= H
2.31 R_1= H, R^2= OH

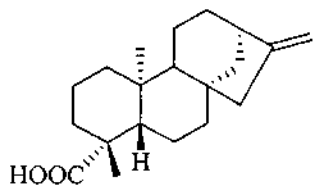


2.32 R^1= R^2= H
2.33 R^1= H, R^2= OH
2.34 R^1= OH, R^2= H

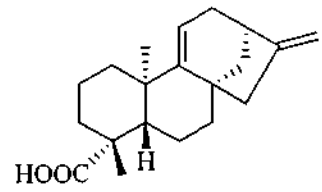


2.35 R^1= OH, R^2= Me
2.36 R^1= OH, R^2= H
2.37 R^1= H, R^2= Me

Aspilia mossambicensis, a medicinal plant used by chimpanzees contains two uteroactive diterpenes. In Tanzania the leaves of the plant are consumed by chimpanzees in an unusual manner for self medication. It has been observed that young leaves of this plant are consumed more often by female chimpanzees than males. In East and West Africa *A. mossambicensis* is used by women to alleviate menstrual cramps and to treat pregnancy-related complications. Driven by its use by human beings and the consumption behavior by chimpamzees, the authors decided to investigate the bioactive constituents of dried plant leaves. The bioassay-guided fractionation led to the isolation of two diterpenes, kaurenoic acid (**2.38**) and grandiflorenic acid (**2.39**).[35] Both these compounds exhibited uterostimulatory activity. These compounds have also been isolated as the active constituents of *Montanoa tomentosa*, a Mexican plant used to induce labour.

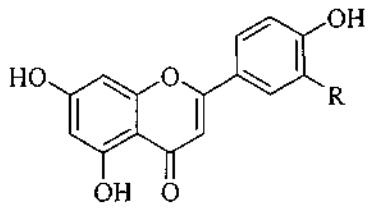


2.38

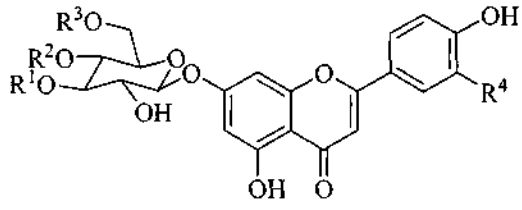


2.39

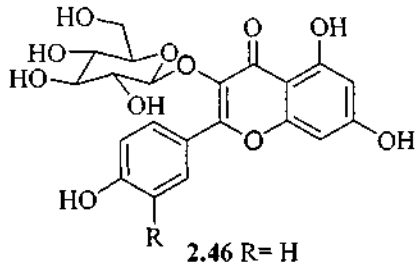
Some plants used in the Dominican Republic to treat women's conditions such as uterine fibroids, menorrhagia, endometriosis and hot flushes have been reviewed.[36] The bark and roots of *Gossypium barbedense* (Malvaceae) are used to treat uterine hermorrhaging and to regulate menses. The leaves of *Lippa micromera* (Verbenaceae) are used to regulate menses. A plant considered to be a woman's plant *Matricaria chamomilla* (Asteraceae) is used to treat uterine colic. Two frequently reported plants in the Republic are *Persea americana* (Lauraceae) and *Momordica charantia* (Cucurbitaceae). *P. americana* was classified by Browner as a plant for uterine expulsion and retention and is also used for menorrhagia as it is claimed to stop bleeding.[37] *P. americana* (avocado leaves) contain flavonoids; apigenin (**2.40**), luteolin (**2.41**), luteolin 7-*O*-glucoside (**2.45**), astragalin (**2.46**) and two glycosylated abscisic acids (**2.48** and **2.49**).[38,39] Phytochemical studies on the flowers of *M. chamomilla* revealed they contain the flavonoid apigenins (**2.40**), luteolin (**2.41**) and the glycosides **2.42** - **2.45**.[40]



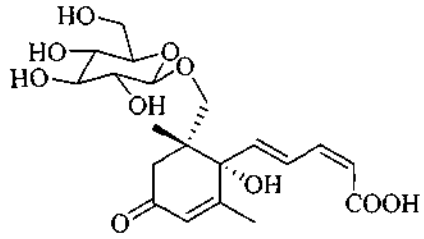
2.40 R= H
2.41 R= OH



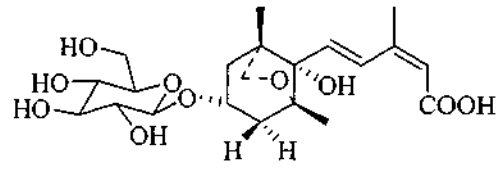
2.42 R= R^1= R^2= R^3= R^4= H
2.43 R= R^1= R^2= R^4= H, R^3= Ac
2.44 R= R^1= Ac, R^2= R^3= R^4= H
2.45 R= R^1= R^2= R^3= R^4= OH



2.46 R= H
2.47 R= OH



2.48

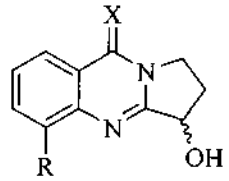


2.49

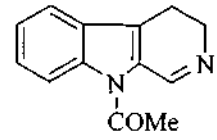
Bourdy *et al.*[41] looked at the oestrogenic activity of five plants reported to be used as traditional medicines in Vanuati. The young fronds of *Aspelenium nidius* (Aspleniaceae) are used as a contraceptive and to reverse sterility. The leaves of *Dysoxylum gaudichaudianum* (Meliaceae) are used as an early abortifacient. A decoction of leaves of *Hemigraphis reptants* (Acanthaceae) are claimed to reverse sterility and the fruits of *Homalanthus nutans* (Euphorbiaceae) are used as an abortifacient and the infusion of the bark of *Pemphis acidula* (Lythraceae) is used as an abortifacient. All five plants tested showed no significant activity.

In rural western Uganda it has been reported that 80% of pregnant women give birth at home and are helped by traditional birth attendants. Herbal medicines are used by birth attendants to induce labour, remove the retained placenta and for the management of post-partum bleeding. Seventy-five medicinal plant species of Uganda and one fungus species were reported as being used to induce labour during child birth. The documented plant species include the herbal drug *Cleome gynandra* (Capparidaceae) which is used to hasten child birth, the seeds of *Luffa cylindrical* (Cucurbitaceae) and an infusion of the leaves from *Vernonia amygdalina* which are used as abortifacients and the dried and fresh leaves of *Erlangia cordifolia* which are used to induce labour.[42,43]

Adhatoda vasica L. Nees is a well-known medicinal plant in Ayuverdic and Unani medicine. It has been used in the indigenous system of medicine in India for more than 2000 years. It has also been used to treat various diseases, specifically respiratory tract ailments. Recent reports on the use of the plant in connection with child labour have appeared. Jain *et al.*[44] observed that the Neterhat people in Bihar used a decoction of leaves to stimulate and heal before and after delivery. It was also observed that people in the Gora village of Lucknow, Utta Pradesh use *A. vasica* as an abortificient.[45] Phytochemical studies on the plant revealed the presence of several alkaloids, steroids and alkanes.[46] The major biologically active compound found in leaves, roots and flowers of the plant is an alkaloid vasicine (**2.50**). Vasicine is a compound that was reported to possess uterine-stimulating activity with a similar effect to oxytocin.[47] Other alkaloids isolated from the leaves includes vasinone (**2.51**), methoxy vasicine (**2.52**), 9-acetamido-3,4-dihydropyrido[3,4-b]indole (**2.53**) and vasicol (**2.54**).[48,49,50]



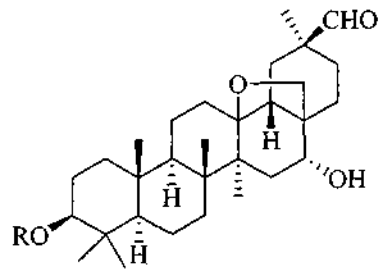
2.50 R= H, X= H_2
2.51 R= H, X= O
2.52 R= OMe, X= H_2



2.53

2.54

Two uterocontracting saponins ardisiacrispin A (**2.55**) and ardisiacrispin B (**2.56**) were isolated from the roots of *Ardisia crispa.* The plant is used in combination with other plants in Thai traditional medicines to remove dirty blood in women who suffer from menstrual pains. 2% Aqueous acetic acid extract of the roots was found to cause contractions of the isolated rat uterus and that lead to the isolation of these two compounds.[51]



2.55 R=β-D-Xyl*p*(1→ 2)-β-D-Glc*p*-[-D-Glc*p*(1→ 4)]-α-L-Ara*p*(1→ 3)

2.56 R=α-L-Rha*p*(1→ 2)-β-D-Glc*p*-[-D-Glc*p*(1→ 4)]-α-L-Ara*p*(1→ 3)

2.4 CONCLUSION

It is clear from this review that plants are used substantially during child birth. In some plants the phytochemistry has been explored to identify the active uteroactive compound(s) and in some plants the uteroactivity could be by a synergistic effect. However, the phytochemistry of a large number of plants with uterotonic activity has not yet been investigated.

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CHAPTER THREE

PHYTOCHEMICAL STUDY OF *GUNNERA PERPENSA* L.

3.1 REVIEW OF GUNNERACEAE

Gunnera L. is a genus of herbaceous flowering plants and is the only member of the family Gunneraceae. *Gunnera* was previously placed within the family Haloragaceae due to its vegetative habit and the endosperm-rich seeds, but later studies on the morphology, anatomy, embryology and palynology accentuate the differences between *Gunnera* and the Haloragaceae taxa.[1,2,3]

The genus was named in honour of the Norwegian bishop and botanist Johan Ernst Gunnerus (1718-1773). The plant has existed for at least 95 million years and there are approximately 50 species mostly distributed in the southern hemisphere (Fig. 3.1). Their natural populations are restricted to super humid areas with heavy rainfalls, on riverbanks, near waterfalls and close to wells.[4,5,6] The characteristic features of the species are long, slender and fleshy stems, rhubarb-like leaves and spikes with greenish flowers. Another characteristic feature of *Gunnera* shared by only few angiosperms is that it contains a number of separate vascular strands in the stems and petioles, which is indicative that *Gunnera* evolved from aquatic ancestors.[7]

The genus is interesting on an ecological and physiological basis for its remarkable symbiosis with cyanobacteria of the genus *Nostoc*.[8] Cyanobacteria are a large, diverse and ancient group of prokaryotic autotrophs with an oxygenic photosynthesis and also perform biological nitrogen fixation, thereby using atmospheric N_2 as their sole nitrogen source. The cyanobacterium infects specialized gland organs located on the stems of the host and once it has passed into the interior of the gland the cyanobacterium also enters the *Gunnera* cells where it starts to differentiate the highest frequency of nitrogen-fixing cells recorded for a cyanobacterium population. A good knowledge of this symbiotic relationship may be helpful in the development of nitrogen-fixing plants.

Taxonomical studies of *Gunnera* have been performed with the most complete morphological revision by Schindler in 1905.[3] He divided *Gunnera* into five subgenera based on modes of propagation and geographic distribution, i.e. *Perpensum*, *Pseudogunnera*, *Milligania*, *Misandra* and *Panke*.

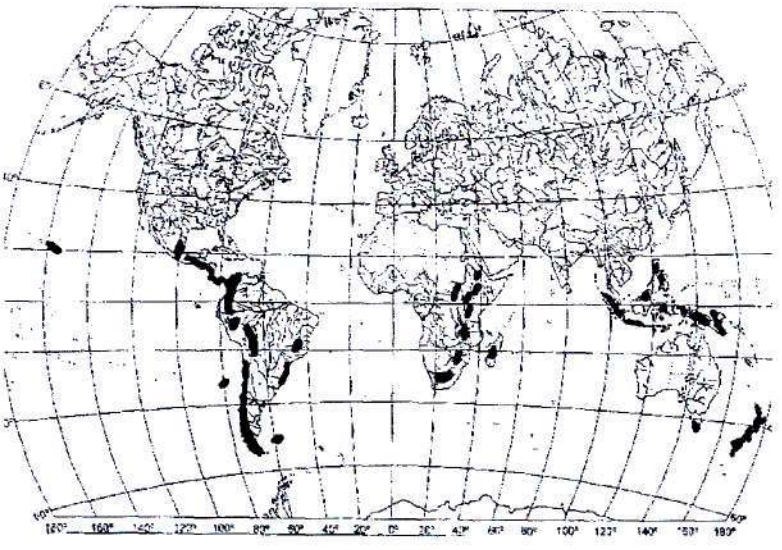
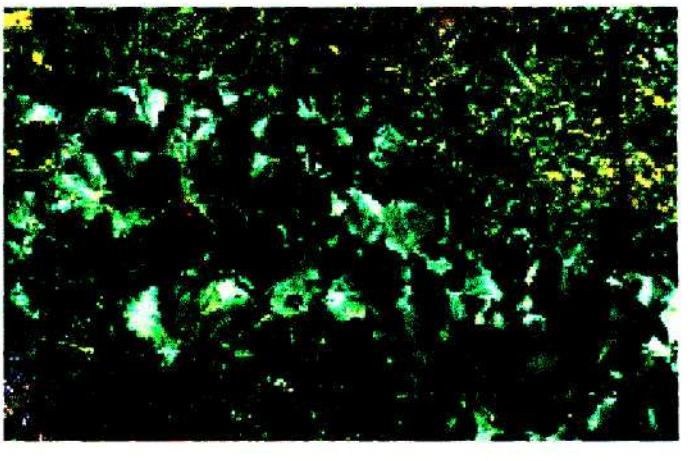


FIGURE 3.1: Distribution of *Gunnera* [9]

The subgenus *Perpensum* consists of the single African species, *G. perpensa* L. This was the first species of the genus to be described and occurs in the mountains of southern Africa and along the Great Rift to Ethiopia and also in Madagascar. It is a medium-sized plant characterized by stout creeping rhizomes and reniform leaves. The inflorescences are large with hermaphroditic flowers (Fig 3.2).



www.plantzafrica.com/medmonographs/gunnerapers

FIGURE 3.2: *Gunnera perpensa*

The subgenus *Pseudogunnera* includes a single species, *G. macrophylla* Blume occurring on the mountains of New Guinea, Solomons Islands and Sulawesi, Wallace's Line of Java, Sumatra, Borneo and in south-eastern Asia. It is a medium-sized plant with reniform leaves. It forms slender axillary stolons carrying paired opposite bracts. It is monoecious and the inflorescences are bisexual, with male flowers on the upper branches and female flowers on the lower branches.

The subgenus *Milligania* includes five to seven species from New Zealand as well as the single Australian species, *G. cordifolia* Hook. f. from Tasmania (Fig 3.3). These are all small, mat-forming stoloniferous plants lacking scales on the stem. They are monoecious, with mostly unisexual, occasionally bisexual inflorescences.

www.utas.edu.au/dicotkey/DicotKey/OTHERS/zGunnera.htm

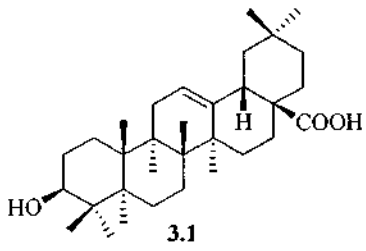
FIGURE 3.3: *Gunnera cordifolia*

The subgenus *Misandra* includes two small, stoloniferous species *G. magellanica* Lam. (Fig 3.4) from South America, characterized by a conical ochrea covering the apical bud. The plants are dioecious.

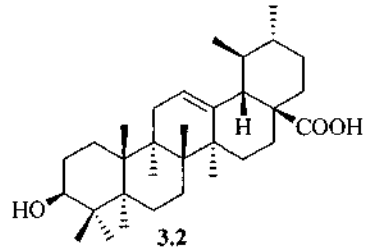
TABLE 3.1: Compounds isolated from selected *Gunnera* species.

Numb.	compound	*G. peltata*	*G. masafuerae*	*G. tintoria*	*G. magellanica*
3.1	oleanolic acid	+	+	+	+
3.2	ursolic acid	-	-	+	+
3.3	lupeol	+	+	+	+
3.4	erythrodiol	+	+	+	+
3.5	urs-12-ene-2α,3α-diol	+	+	+	+
3.6	vomifoliol	+	+	+	+
3.7	liliolide	+	+	-	+
3.8	β-sitosterol	+	+	+	+
3.9	daucosterin	+	+	+	+
3.10	uvaol	-	-	+	-
3.11	ionone derivatives	+	+	+	-
3.12	pinoresinol	+	+	+	+

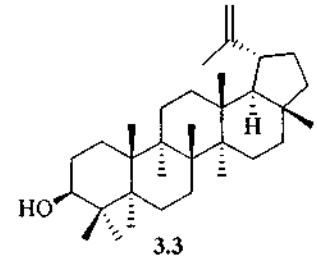
It has been reported that an extract from the New Zealand species *G. hamiltonii* possesses strong lipoxygenase activity and that antithrombin activity was observed from an extract of *G. tinctoria* from the Azores.[13,14]



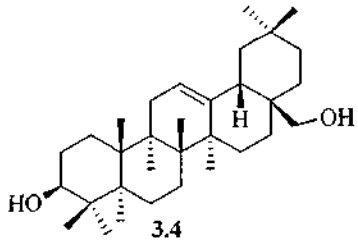
3.1



3.2



3.3



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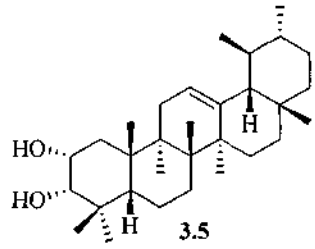

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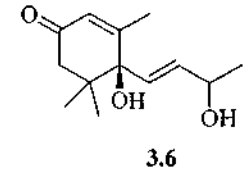

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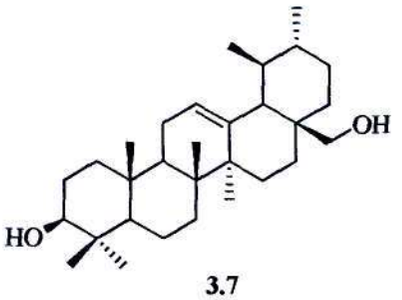

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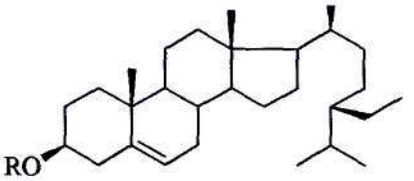
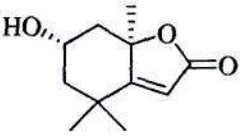


3.8 R = H
3.9 R = β-D-glucoside



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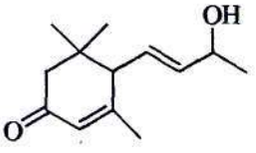

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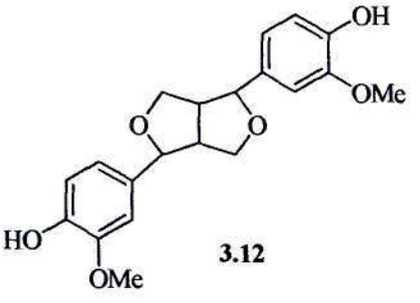
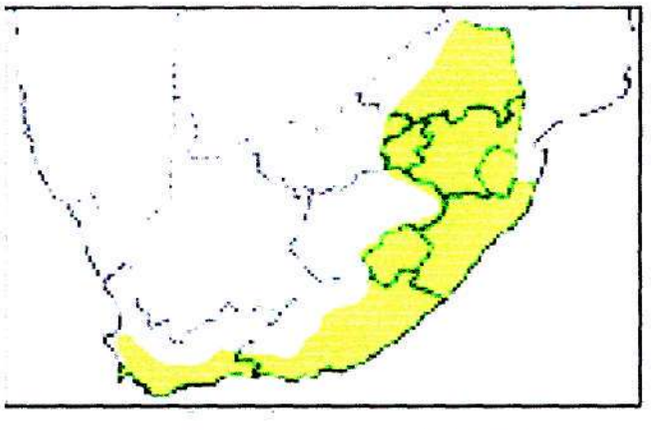


3.12

3.2 BACKGROUND ON *GUNNERA PERPENSA*

The only South African *Gunnera* species is *Gunnera perpensa,* L. known as uGobho in 'isiZulu' and river pumpkin in English. It is widespread in Africa from Sudan, Ethiopia, Zaire, Rwanda, Uganda, Kenya, Tanzania, Zimbabwe, Mozambique and along the central and eastern areas of Southern Africa.[15] It grows around the edges of the rivers and streams and is intolerant to frost. It grows in all types of soils.



E.J. Mendes, Haloragaceae, *Flora Zambeziaca* 1978, 4, 74.

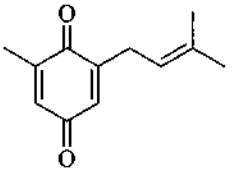
FIGURE 3.6: Geographical distribution of *G. perpensa* in South Africa

It is a rhizomatous, thinly perennial herb to 1 m in height with palmate leaves, tufted, near the apex of rhizome. The green flower spikes (2-10 cm long) appear in August and the

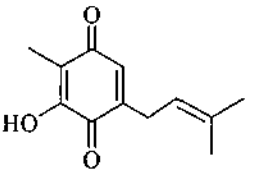
G. perpensa is sometimes used as an ingredient in the medicine called *isihlambezo* (to ensure the health of a mother and a child during pregnancy) and in medicine called *Inembe* (taken regularly during pregnancy to ensure easy childbirth).[20]

Water, hexane and 100% ethanol extracts of dried root were assessed for *in vitro* antibacterial activity against *Staphyloccocus aureus, Klebsiella pneumoniae, Bacillus subtilis* and *Escherichia coli* and were found to be inactive in the concentrations used.[21] The aqueous extract have also been tested and showed the best antibacterial activity, which is the added advantage of its use for the treatment of wound infections.

Motivated by the distinct red color of the stems and skin irritation on contact with the leaves of the plant, Drewes *et al.*[22] looked at the chemical constituents of stems and leaves. They isolated the benzoquinones (**3.13** and **3.14**), a benzopyran (**3.15**) and a phytol (**3.16**). Compound **3.16** is reported as a mild irritant isolated from other plants, hence the skin irritation on handling of the plant by the authors.[23] In comparing the antibacterial activity of the crude extracts of stems and leaves, the leaves showed higher activity than the stems. Amongst the compounds isolated, the benzoquinones showed good antibacterial activity. The leaves of the plant are used by the rural people of Eastern Cape in wound dressing.[24]



3.13



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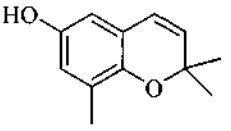

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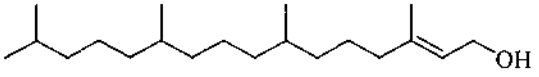


3.16

3.3 RESULTS AND DISCUSSION

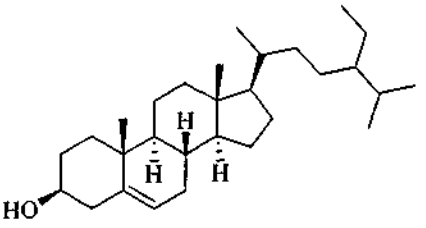
3.3.1 Introduction

The rhizomes of *G. perpensa* were collected in a wetland area in the Johannesburg suburb Montgomery Park. After drying and milling, the ground plant material was extracted with chloroform-methanol (1:1). The solvent was removed and the crude extract was subjected to column chromatography using solvents ranging from non-polar (hexane-ethyl acetate, 8:2) to polar (chloroform-methanol, 9:1 to 7:3) and highly polar (ethyl acetate-water-acetic acid, 8:1.5:0.5). Repeated chromatography led to the isolation of β-sitosterol (**3.8**), *Z*-venusol (**3.17**), methyl lespedezate (**3.18**), 3,3',4'-tri-*O*-methylellagic acid 4-*O*-*β*-D-glucopyranoside (**3.19**), and punicalagin (**3.20**). Apart from these compounds we have also isolated sucrose and another sugar which we did not identify.*

3.3.2 Structural elucidation of β-sitosterol (3.8)

The first compound isolated exhibited twenty-nine signals in the ^{13}C NMR spectrum (Plate 2), which indicated that this compound might be a steroid / triterpene. The ^{1}H NMR spectrum (Plate 1) had peaks characteristic of an alkene proton (δ_H 5.32, br. d, J = 5 Hz), an axial proton on an oxygen-bearing carbon (δ_H 3.49, broad multiplet), two tertiary methyl groups (δ_H 0.64 and 0.93) and four secondary methyl groups (δ_H 0.81, 0.83, 0.84 and 0.93; all doublets with J 7.0 Hz). These data were in agreement with the structure of the ubiquitous β-sitosterol (**3.8**). The NMR data of **3.8** (Table 3.2) is in agreement with published values.[25]

* This project was started at Rand Afrikaans University, Johannesburg. After we have isolated the compounds and elucidated the structures, we became aware that Prof. S.E. Drewes from the University of KwaZulu-Natal, PMB was also investigating the phytochemistry of this plant and had isolated the same compounds as we did. A combined paper was published on the results (see Appendix). Punicalagin was not isolated by the then PMB group. The research group of Prof. van Heerden has relocated in 2005 to UKZN PMB.



3.8

TABLE 3.2: ^{1}H- and ^{13}C-NMR data of β-sitosterol (3.8) in $CDCl_3$

Experimental			Literature[25]	
Position	**δ_C**	**δ_H**	**δ_C**	**δ_H**
1	37.2		37.2	
2	31.6		31.6	
3	71.8	3.49 (m)	71.8	3.53 (m)
4	42.4		42.5	
5	140.7		140.7	
6	121.7	5.32 (br. d, J = 5 Hz)	121.7	5.36 (br. d, J = 5 Hz)
7	31.8		31.8	
8	31.7		31.9	
9	50.1		50.1	
10	36.5		36.5	
11	21.1		21.1	
12	39.8		39.7	
13	42.3		42.3	
14	56.8		56.7	
15	24.3		24.3	
16	28.2		28.2	
17	56.1		56.0	
18	11.9	0.64 (s)	11.9	0.68 (s)
19	19.4	0.93 (s)	19.4	1.01 (s)
20	36.1		36.1	
21	19.4	0.93 (d, J = 7 Hz)	18.8	0.92 (d, J = 7 Hz)
22	34.0		33.9	
23	26.1		26.0	
24	45.9		45.8	
25	29.2		29.2	
26	19.8	0.83 (d, J = 7 Hz)	19.8	0.83 (d, J = 7 Hz)
27	19.0	0.81 (d, J = 7 Hz)	19.1	0.81 (d, J = 7 Hz)
28	23.1		23.0	
29	12.0	0.84 (t, J = 7 Hz)	12.0	0.84 (t, J = 7 Hz)

3.3.3 Structural elucidation of *Z*-venusol (3.17)

The NMR data of compound **3.17** is collated in Table 3.3. The ^{1}H NMR spectrum (Plate 3) of this compound displayed signals with chemical shifts and coupling constants

characteristic of a β-D-glucopyranoside moiety (δ_H 3.48, 3.58, 3.72, 3.79, 4.05 and 5.11). The splitting pattern of anomeric proton (δ_H 5.11, d, J = 7.8 Hz) confirmed a β-*O*-glycosidic linkage. Apart from the sugar protons, a 1,4-disubstituted benzene ring (δ_H 6.77, d, J = 8.7 Hz; δ_H 7.66, d, J = 8.7 Hz) and an alkene proton resonating as a singlet (δ_H 6.84) were also identified.

The ^{13}C NMR spectrum (Plate 4) displayed a total of twelve carbon signals of which the multiplicities were derived from an APT experiment. One CH_2 carbon, eight CH carbons, and four non-protonated carbons were identified. In the ^{13}C NMR spectrum six signals (δ_C 62.1 to 96.2) characteristic of a glucopyranoside moiety, four aromatic signals [δc 125.9, δ_C 133.7 (2C), δ_C 116.4 (2C), δ_C 159.9], a non-protonated carbon at δ_C 136.6 (C-2), an olefinic CH at δ_C 120.9 and an additional non-protonated carbon δ_C 162.3 were observed. A HSQC experiment allowed the correlation of all protonated carbon atoms with well-resolved protons.

In the HMQC experiment, ^1H-^{13}C long range correlations were observed between two aromatic carbons (δ_C 133.7) and the olefinic proton (δ_H 6.84), which indicated that the olefinic proton is localized near the aromatic ring. From this information we could construct the structural fragment given in Fig. 3.8.

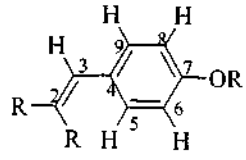


FIGURE 3.8

The chemical shift of the non-protonated olefinic carbon together with its observed long-range correlation with H-1' of the carbohydrate moiety indicates that this carbon is linked to the sugar through a glycosidic bond, thus leading to the proposed fragment given in Fig. 3.9.

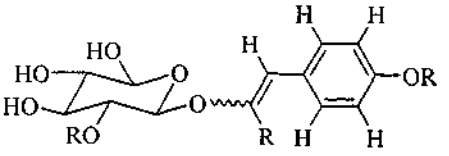
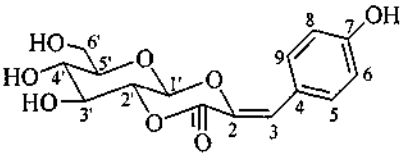


FIGURE 3.9

The ^{1}H NMR chemical shift of H-2' of glucopyranoside has a downfield shift, which may be due to the presence of an acyloxy substituent on this position. By incorporating the unaccounted carbon atom (δ_C 162.5), the structure of this compound was assigned as venusol (**3.17**). NOE experiments did not give any conclusive results to assist with the determination of the geometry of the double bond.

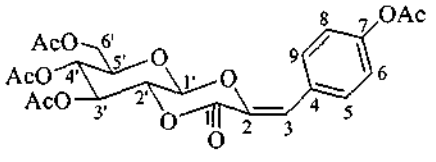


3.17

TABLE 3.3: ^{1}H- and ^{13}C-NMR data of *Z*-venusol (3.17) in CD_3OD (500 MHz)

Experimental				Literature
Position	**δ_C**	**δ_H**	**$^{>1}J_{CH}$ correlations (HMQC)**	**δ_C**
1	162.5		H-3	160.3
2	136.6		H-3	136.0
3	120.9	6.84, s	H-5/9	117.8
4	125.9			124.0
5	133.7	7.66, d, J = 8.7 Hz	H-3	132.2
6	116.4	6.77, d, J = 8.7 Hz	H-5	115.2
7	159.9		H-5/9, H-6/8	158.3
8	116.8	6.77, d, J = 8.7 Hz	H-9	115.2
9	133.7	7.66, d, J = 8.7 Hz	H-3	132.2
β-glucose				
1'	96.2	5.12, d, J = 7.8 Hz	H-2'	95.8
2'	80.1	4.05, t, J = 8.6 Hz	H-1'	78.7
3'	74.6	3.72, t, J = 9.2 Hz	H-2'	73.8
4'	70.9	3.58, t, J = 4.8 Hz		70.6
5'	79.8	3.48, t, J = 9.3 Hz		78.7
6'	62.1	a. 3.79, dd, J = 5.3 Hz b. 3.95, d, J = 12.0 Hz		61.6

Unambiguous proof for the structure of *Z*-venusol (**3.17**) was provided by acetylation of the compound to yield venusol tetraacetate (**3.21**). The NMR data are given in Table 3.4. In the ^{1}H NMR spectrum (Plate 10), resonances of **3.21** are well resolved and the large coupling constants for the carbohydrate protons indicate all coupled proton pairs have a diaxial relationship, thereby confirming that the carbohydrate is glucose. Upon acetylation, a substantial shift to lower field was observed for H-3' and H-4' in the ^{1}H NMR spectrum thereby confirming that the hydroxy on C-2' of the glucosyl was derivatised in venusol (**3.17**). The chemical shift of the aromatic acetyl group (7-OAc) is clearly observed in the spectrum and is separated from the acetyl groups of the sugar.



3.21

TABLE 3.4: NMR data of *Z*-venusol tetraacetate (3.21) in CD_3OD.

Position	δ_C	δ_H
1	159.0	
2	136.2	
3	120.3	6.98, s
4	130.0	
5	132.1	7.12, d, J = 8.7 Hz
6	121.9	7.77, d, J = 8.7 Hz
7	151.3	
8	121.9	7.77, d, J = 8.7 Hz
9	132.1	7.12, d, J = 8.7 Hz
3'-OAc	20.5	2.05, s
4'-OAc	20.6	2.09, s
6'-OAc	20.7	2.12, s
7-OAc	21.1	2.30, s
β-glucose		
1'	94.6	5.09, d, J = 7.9 Hz
2'	75.6	4.32, dd, J = 9.9, 7.9 Hz
3'	70.9	5.39, dd, J = 9.9, 9.2 Hz
4'	67.8	5.13, J = 10.1, 9.2 Hz
5'	73.6	3.95, ddd, J = 10.1, 4.4, 2.2 Hz
6'	61.3	a. 4.21, dd, J = 12.7, 2.2 Hz b. 4.32, dd, J = 12.8, 4.4 Hz

The proposed structure of venusol (**3.17**) was confirmed by X-ray diffraction (Prof O Munro, UKZN) of a single crystal provided by Prof Drewes (UKZN). The X-ray structure (Fig. 3.10) was in full agreement with our proposed structure and indicated that the geometry of the double is *Z*. *Z*-Venusol (**3.17**) was first isolated by Proliac *et al.*[26] from the leaves of *Umbilicus pendulinis*.

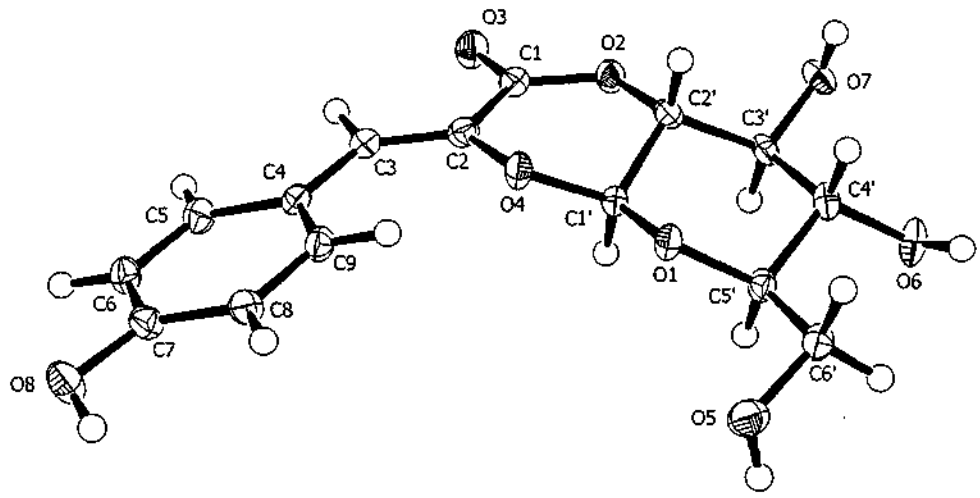


FIGURE 3.10: X-ray structure of *Z*-venusol (**3.17**).

3.3.4 Structural elucidation of *Z*-methyl lespedezate (3.18)

During the extraction of fresh plant material, it was noticeable that when the crude extract was exposed to methanol for longer periods, compound **3.18** was isolated as a major compound with only traces of **3.17**.

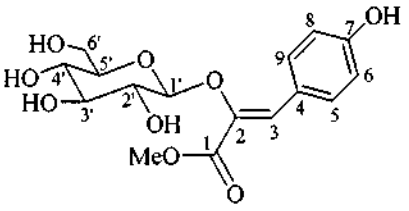
The NMR data of **3.18** is summarized in Table 3.5. In the ^{1}H NMR spectrum (Plate 14) of **3.18**, the *p*-disubstituted aromatic ring (δ_H 6.77, d, J = 8.6 Hz, H-6/8; δ_H 7.74, d, J = 8.1 Hz, H-5/9), an anomeric carbohydrate signal (δ_H 5.10, H-1', d, J = 12.5 Hz), a methoxy group at (δ_H 3.81, 3H, s) and an olefinic proton (δ_H 6.98) were observed. With the exception of the methoxy signal, the resonances were similar to that of venusol (**3.17**), but with slightly different chemical shifts.

The ^{13}C NMR spectrum (Plate 15) of **3.18** displays a total of thirteen carbons with an extra carbon compared to **3.17**, which was assigned to the methyl carbon of a methyl ester at δ_C

52.6. Other than that, signals characteristic of an *O*-glucose moiety at δ_C 62.5 to 102.8, a carbonyl carbon at δ_C 166.6, four aromatic CH carbons at δ_C 116.2 (C-6/8) and 133.7 (C-5/9), three quaternary carbon at δ_C 139.9 (C-2), δ_C 126.7 (C-4) and δ_C 159.9 (C-7) and an olefinic carbon at δ_C 125.9 (C-3), were observed.

The ^{1}H-^{13}C long-range correlations observed in an HMBC experiment showed that the methyl signal at δ_H 3.81 was strongly correlated to the carbonyl carbon signal at δ_C 166.6 (C-1) suggesting the presence of a COOMe functionality. The correlation of H-3 at δ_H 6.98 with aromatic carbons C-5/9 demonstrates that the H-3 proton is localized near the aromatic ring. The same H-3 proton is further correlated to a quaternary carbon C-2 at δ_C 139.9. The fact that the C-2 carbon is further correlated to H-1' at δ_C 5.10 and is shifted downfield indicating that the carbon C-2 is linked to the glucose moiety through an *O*-glycosidic bond. The structure of compound **3.18** was assigned as methyl lespedezate. Methyl lespedezate was first described by Viornery *et al.*[27]

We have observed that venusol (**3.17**) was transformed into methyl lespedezate (**3.18**) upon standing in methanol and the methyl lespedezate that we have isolated, is most likely an artifact formed during the extraction process.



3.18

TABLE 3.5: ^{1}H- and ^{13}C-NMR data of methyl lespedezate (3.18) in CD_3OD (500 MHz)

position	δ_C	δ_H	$^{>1}J_{CH}$ correlations (HMQC
1	166.6		H-3, OMe
2	139.9		H-3
3	125.9	6.98, s	H-5/9
4	126.7		H-6/8
5	133.7	7.74, d, J = 8.1 Hz	H-3, H-9
6	116.2	6.77, d, J = 8.6 Hz	H-8
7	159.9		H-5/9, H6/8
8	116.2	6.77, d, J = 8.6 Hz	
9	133.7	7.74, d, J = 8.2 Hz	H-3
OMe	52.6	3.81, s	
β-glucose			
1'	102.8	5.10, d, J = 12.5 Hz	H-2'
2'	75.7	3.48, t, J = 7.9 Hz	
3'	78.0	3.41, t, J = 9.0 Hz	H-2', H-4'
4'	71.4	3.34, t, J = 9.0 Hz	
5'	78.4	3.21, ddd, J = 1.9, 2.4, 3.3 Hz	
6'	62.5	a. 3.59, dd, J = 5.4, 5.5 Hz b. 3.73, dd, J = 1.7, 1.8 Hz	

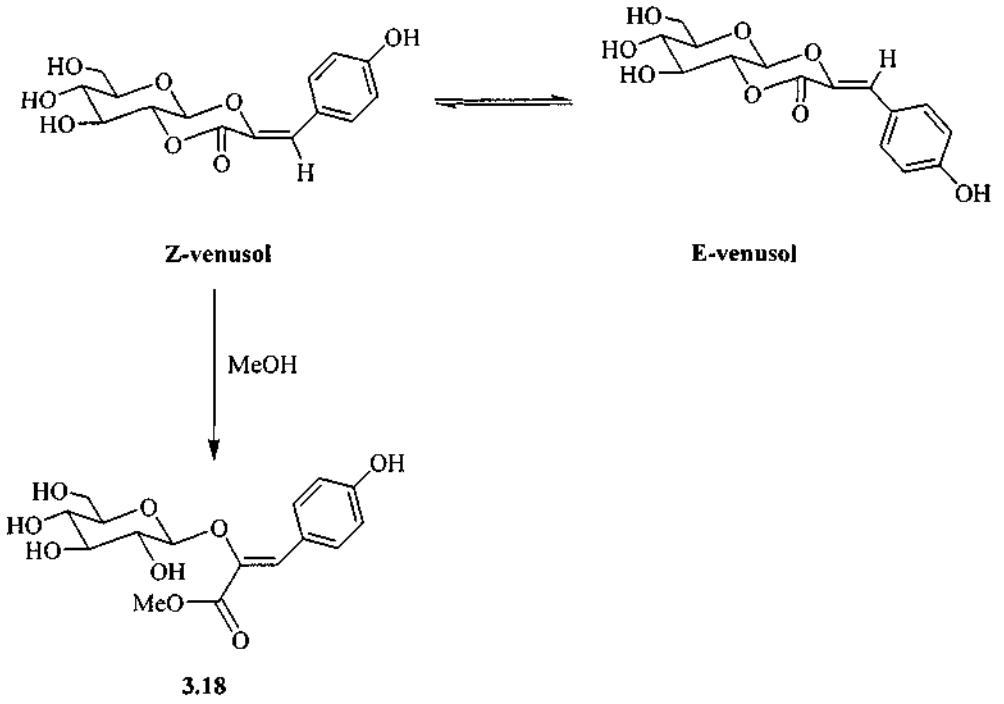
3.3.5 Discussion of phenyl propanoids

Z-Venusol (**3.17**) was first isolated by Proliac *et al.*[26] and later by Pagani[28] from leaves of *Umbilicus pendulinis*. More recently, the *E*-isomer was also obtained and described by Vionery *et al.*[27] from the same plant source. The authors were able to distinguish between the two isomers of venusol (**3.17**) using the NOE and molecular modeling experiments.

The *E*- and *Z*- isomers of these cinnamoyl derivatives have been reported to coexist in nature but the *Z*-isomer is the more stable of the two isomers. It is believed that this coexistence of these isomers is probably induced by light.

We have observed that prolonged exposure of the *Z*-Venusol (**3.17**) in MeOH results in a lactone methanolysis to form a methyl lespedezate **3.18** (Scheme 3.1). The relationship between methyl lespedezate (**3.18**) and lespedezic acid is a close one. Potassium lespedezate was a compound isolated by Viornery *et al.*[27] from *Lespedeza cuneata* and the

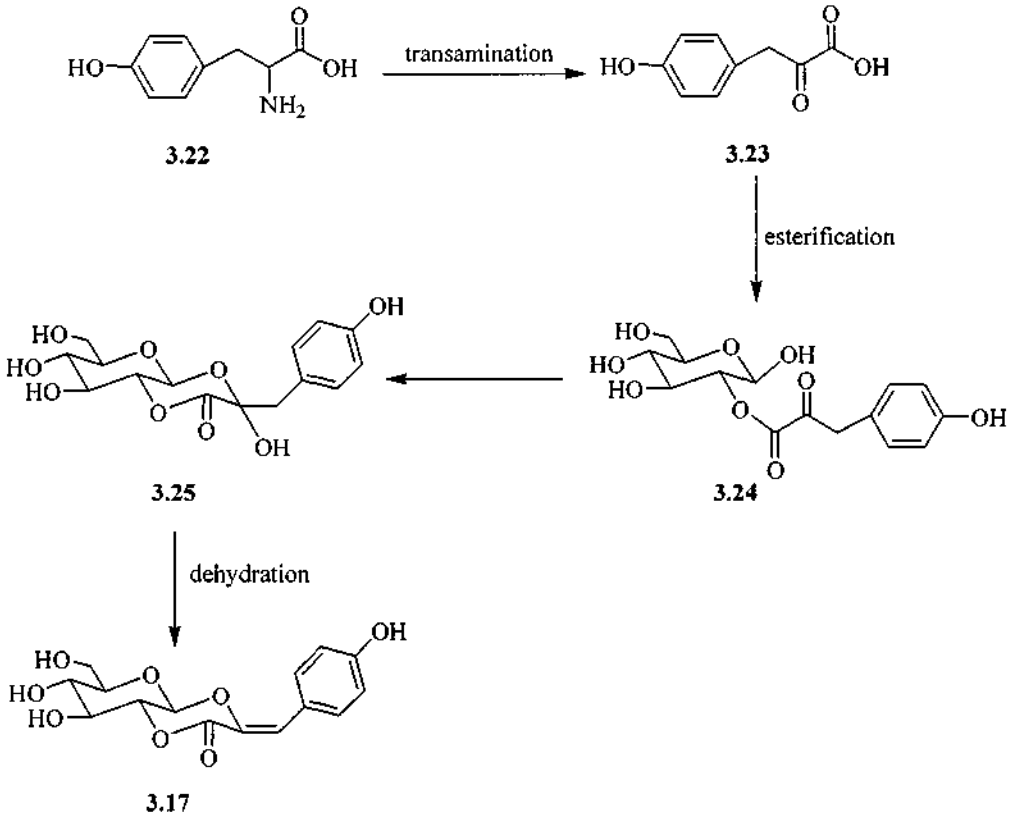
authors were able to isolate both geometric isomers of potassium lespedezate. These two isomers were identified as being leaf-opening substances involved in the leaf-opening and leaf-closing mechanisms at a molecular level in nystinastic plants.



SCHEME 3.1

3.3.6 Biosynthesis of venusol (3.17)

No biosynthetic studies have been done on venusol (**3.17**). However, taking into account the formation of closely related compounds, a biosynthetic scheme can be proposed (Scheme 3.2). Transamination of tyrosine (**3.22**) to *p*-hydroxyphenylpyruvic acid (**3.23**) is followed by selective esterification between acid **3.23** and the 2-OH group of glucose to form **3.24**. Subsequent hemiacetal formation and dehydration of the hemiacetal **3.25** will lead to venusol (**3.17**).



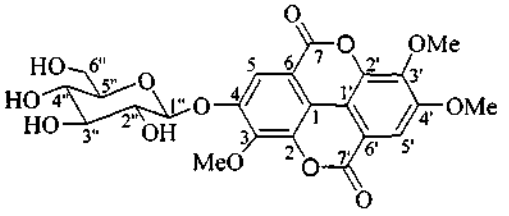
SCHEME 3.2

3.3.7 Structural determination of 3,3',4'-tri-*O*-methylellagic acid 4-*O*-β-D-glucopyranoside (3.19)

The NMR data of **3.19** is collated in Table 3.6. The ^{1}H NMR spectrum (Plate 20) showed the presence of two sharp aromatic signals as singlets at δ_H 7.82 and 7.61 assignable to H-5 and H-5' of the ellagic acid moiety and six signals characteristic of the glucose moiety. The spectrum also revealed the presence of three aromatic methoxyl signals as singlets at δ_H 4.10, 4.03 and 3.99.

The ^{13}C NMR spectrum (Plate 21) displayed 22 distinct carbon resonances and some were separated by only few tenth of a ppm. The two most downfield resonances at δ_C 158.3 and 158.2 were assigned to the non-equivalent lactone carbonyl carbons C-7 and C-7', respectively. Carbons resonating at δ_C 60.5, 61.3, 56.7 were attributed to the methoxyl carbons 3-, 3'- and 4', respectively and the signals at δ_C 141.1, 140.8 and 154.2 were

assigned to carbons carrying methoxyl groups C-3, C-3' and C-4', respectively. The rest were assigned to the remaining aromatic carbons with the help of a HETCOR experiment.



3.19

TABLE 3.6: NMR data of 3,3',4'-tri-*O*-methylellagic acid 4-*O*-β-D-glucopyranoside (3.19)

	Experimental	
position	δ_C	δ_H
1	119.9	
2	141.1	
3	141.1	
4	151.8	
5	119.9	7.82, s
6	112.7	
7	158.2	
1'	112.2	
2'	141.6	
3'	140.8	
4'	154.2	
5'	107.5	7.61, s
6'	113.5	
7'	158.2	
3-OMe	60.5	4.10, s
3'-OMe	61.3	4.03, s
4'-OMe	56.7	3.99, s
β-Glucose		
1''	101.2	5.05, d, J = 5.1 Hz
2''	77.2	5.48, dd, J = 8.0, 10.1 Hz
3''	73.3	5.17, t, J = 11.1 Hz
4''	69.4	4.57, t, J = 11.7 Hz
5''	76.4	3.67, ddd, J = 10.5, 5.7, 3.9 Hz
6''	60.5	a. 3.50, dd, 11.7, 5.7 Hz b. 4.20, dd, 11.6, 4.5 Hz

The high resolution mass spectrum of compound **3.19** revealed a molecular ion peak at $[M]^+$ at m/z = 344.05434. Comparison with the spectral data reported in the literature led to the identification of compound **3.19** as 3,3',4'-tri-*O*-methylellagic acid 4-*O*-β-D-

glucopyranoside, which was previously isolated from stem bark of *Diplopanax stachyanthus*.[28]

3.3.8 Discussion on ellagic acid derivatives

Ellagic acid derivatives exist widely in the plant kingdom. Their biological activities have been studied extensively. They were reported to have aldose reductase (AR) inhibitory activity, DNA-gyrase inhibitory properties, inhibitory activity on HIV-1 protease, antihermorragic, hypotensive, sedative, antimutagenic and antioxidant properties.[29,30,31] These compounds are also unique in that they possess a highly substituted biaryl nucleus with only two protonated carbons among a total of twelve aromatic carbon atoms.

3.3.9 Structural elucidation of punicalagin (3.20)

The rhizomes of *G. perpensa* have a distinct yellow colour. A MeOH extract of the rhizomes was chromatographed on Sephadex LH-20 with an EtOH:H_2O (9:1, 8:2 and 7:3) gradient solvent system. The fraction eluted with 7:3 EtOH:H_2O was rechromatographed on the Sephadex LH-20 with EtOH to give fraction **3.20** as a yellow amorphous powder.

The ^{1}H NMR spectrum (Plate 23) of **3.20** indicates the presence of a number of aromatic protons, but at a first glance the rest of the spectrum was not informative, partly because the solvent peaks observed obscured part of the spectrum.

The ^{13}C NMR spectrum (Plate 24) revealed the presence of eight ester carbonyl carbons at δ_C 169.5-170.7, a large number of aromatic carbons and two carbohydrate moieties, which was identified as an α- and a β-sugar. On closer inspection of the ^{13}C NMR spectrum (Plate 24), we came to the conclusion that fraction **3.20** was in fact a mixture of two compounds, which were the α- and β- anomers of an acylated monosaccharide. Further chromatography of fraction **3.20** led to the isolation of the α-anomer (**3.26**).

The NMR data of compound **3.26** are collated in Table 3.8. In the ^{1}H NMR spectrum (Plate 25) of **3.26** in acetone-d_6, four singlets were observed in the aromatic region. In the region δ_H 2.11-5.20 seven resonance with splitting patterns characteristic of an α-glucose derivative were observed ($J_{1,2}$ = 3.4 Hz, $J_{2,3}$ = 9.7 Hz, $J_{3,4}$= 9.4 Hz, $J_{4,5}$= 10.1 Hz). The

chemical shifts of H-2, H-3, and H-4 (δ_H 4.82, 5.20 and 4.78, respectively) indicate that these hydroxy groups of glucose are acylated. However, the most characteristic feature of the carbohydrate signals was the major difference in chemical shifts of the *geminal* 6-methylene protons (δ_H 4.23 and 2.11). From the chemical shift of H-1, it was clear that C-1 of the carbohydrate was not derivatised. The coupling constant observed for the anomeric carbon (J = 3.4 Hz) is characteristic of an α-anomer.

The ^{13}C NMR spectrum (Plate 26) of **3.26** contained four ester carbonyls (δ_C 170.0, 169.4, 169.0 and 168.5), two carbon signals at δ_C 158.7 and δ_C 158.2 of which the chemical shifts were similar to C-7 and C-7' of ellagic acid derivative **3.19** described on p. 39, thirty-six aromatic carbon atoms and six glucose carbon atoms. The chemical shift of the anomeric carbohydrate carbon atom (δ_C 90.9) is characteristic of an α-anomer.

Taking these data into account, we realized that this compound was most likely a hydrolysable tannin, which has a glucose moiety esterified with gallic acid, ellagic acid or derivatives thereof. A large number of these hydrolysable tannins have been described in the literature.[32]

The correlations observed in an HMQC experiment (Table 3.7) enabled us to assign the structure of the ellagitannin.

TABLE 3.7: Correlation observed in HMQC experiment

δ_H	δ_C
7.01	145.9 (C-5), 145.7 (C-3), 137.1 (C-4), 124.9 (C-1), 124.4 (C-2)
6.58	168.5 (C-7'''), 144.8 (C-2'''), 138.4 (C-5'''), 137.6 (C-3'''), 122.6 (C-1'''), 137.5 (C-4'''), 109.6 (C-6'')
6.59	169.4 (C-7), 145.8 (C-4), 136.8 (C-5), 127.5 (C-2), 115.6 (C-1)
6.50	170.7 (C-7'), 146.2 (C-4'), 145.5 (C-6'), 137.0 (C-5'), 127.4 (C-2'), 118.9 (C-1')
4.82 (H-2 gluc.)	169.4 (C-7')
5.20 (H-3 gluc.)	170.7 (C-7')
4.78 (H-4 gluc.)	169.0 (C-7)
3.27 (H-6 gluc.)	168.5 (C-7''')

With the exception of H-1 and H-5, each of the carbohydrate protons correlated with one of the ester carbonyl carbons. Furthermore, each of these four ester carbonyl carbons were also correlated with one of the four aromatic protons. The ellagic acid carbonyls (δ_C 158.7, 158.2) are not coupled to any of the aromatic hydrogens, indicating that this moiety is fully substituted. From the chemical shifts, it was clear that all the aromatic rings were derived from gallic acid. All this data enabled us to construct the following fragments (Fig 3.11):

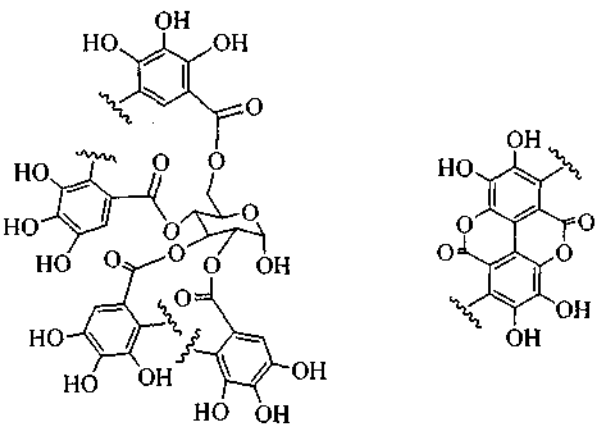


FIGURE 3.11

The gallic acid-derived ester moieties can be arranged into a hexahydroxydiphenyl (HHDP) moiety (Fig. 3.12) and a tetraphenyl moiety (Fig. 3.13). The long range ^{1}H, ^{13}C-couplings observed in the HMQC experiment for these two moieties are indicated in Fig. 3.12 and Fig. 3.13.

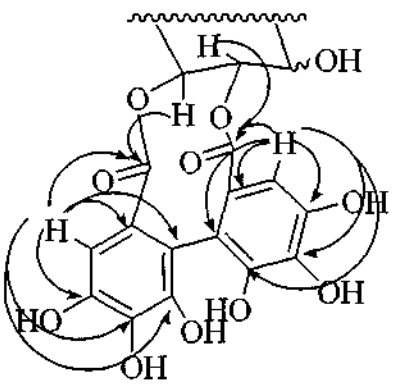


FIGURE 3.12: HMQC correlations of the HHDP moiety

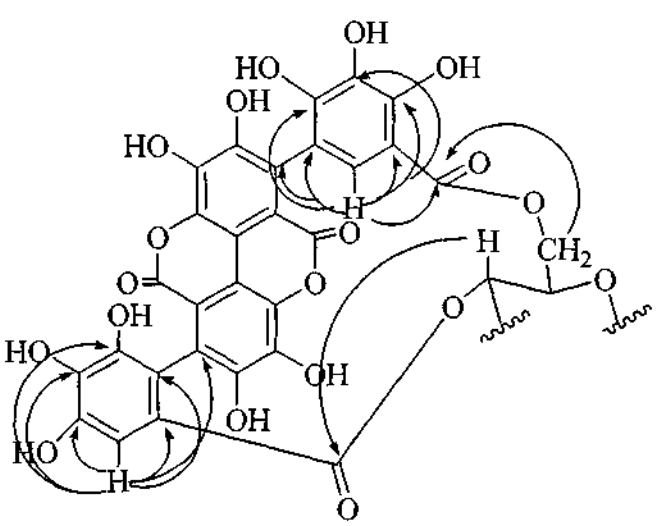


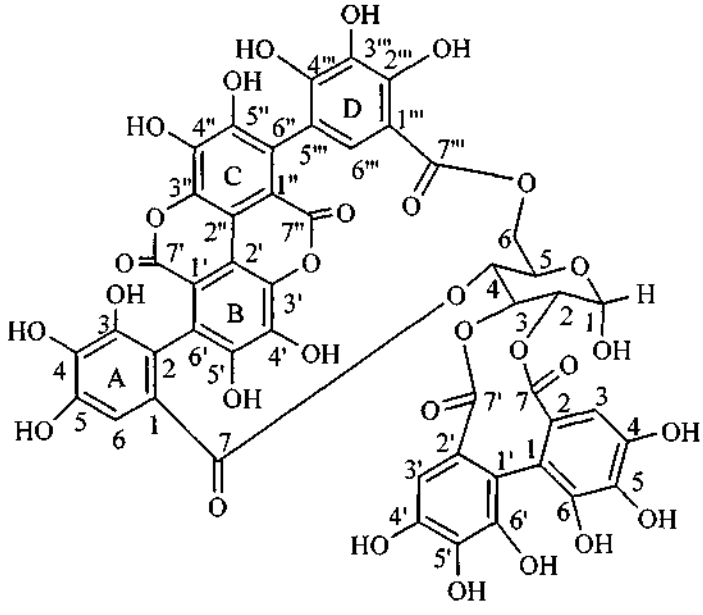
FIGURE 3.13: HMQC correlations of the tetraphenyl moiety

It is physically impossible to link the tetraphenyl moiety to any other but C-4 and C-6 hydroxy groups of glucose. This structural assignment was confirmed by a set of correlations in the HMQC experiment, where the aromatic proton that is correlated to the

ester carbonyl on C-6 (gluc), is also correlated to one of the ellagic acid carbons [H-6(Gluc)→ester carbonyl → aromatic proton →ellagic acid carbon].

This information allowed us to assign the structure of compound **3.26**, which is that of the known ellagitannin punicalagin. The NMR data of α-punicalagin **3.26** is given in Table 3.8. The NMR data obtained correlates well with those published for punicalagin,[33] although some of the assignments we made in the ^{13}C NMR spectrum are different to the reported assignments.

In an ESI-TOF mass spectrum obtained in the ESI negative mode, a peak with *m/z* 1083.02 $[M-H]^-$ corresponding to a molecular formula of $C_{48}H_{28}O_{30}$ confirmed the structural assignment of the compound **3.26**.



3.26

TABLE 3.8: NMR data of α-punicalagin (3.26) in acetone d_6

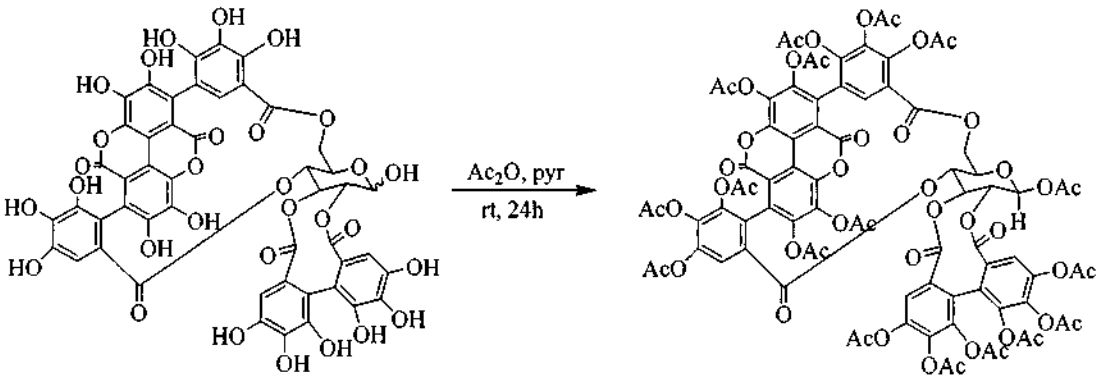
position	δ_C	δ_H	position	δ_C	δ_H
α-Glucose					
1	90.9	5.11, d, J = 3.4	6	112.4	7.01, s
2	71.8	4.82, dd, J = 3.6, 9.7	7	169.0	
3	77.1	5.20, t, J = 9.4,	1'	109.6	
4	75.1	4.78, t, J = 9.4	2'	115.2	
5	67.6	3.27, td, J = 10.0	3'	137.1	
6	64.9	a. 4.23, t, J = 10.7 b. 2.11, dd, J = 10.1	4'	139.7	
HDDP			5'	146.2	
1	115.6		6'	126.3	
2	127.5		7'	158.2	
3	108.3	6.59, s	1''	115.0	
4	145.8		2''	114.7	
5	136.8		3''	139.0	
6	144.9		4''	144.6	
7	169.4		5''	148.5	
1'	118.6		6''	109.6	
2'	127.4		7''	158.7	
3'	108.3	6.50, s	1'''	122.6	
4'	146.2		2'''	144.8	
5'	137.0		3'''	137.6	
6'	145.5		4'''	137.5	
7'	170.7		5'''	138.4	
tetraphenyl unit			6'''	109.6	6.58, s
1	124.9		7'''	168.5	
2	124.4				
3	145.7				
4	137.1				
5	145.9				

3.3.10 Structural elucidation of β-punicalagin acetate (3.27)

In order to confirm the structure of punicalagin, peracetate and permethyl derivatives of the polyphenolic compound were prepared. The anomeric mixture **3.20** was acetylated with acetic anhydride in the presence pyridine for 24 h at room temperature. After work up, the crude reaction mixture was then subjected to chromatography (hexane:EtOAc, 7.5:2.5), to afford a light yellow powder (**3.27**) in 79% yield (Scheme 3.3). The mass was established

to be ESI MS obtained in ESI negative mode in which *m/z* 1757.2 $[M-H]^-$ corresponding to a molecular formular of $C_{82}H_{62}O_{46}$ was observed.

In the ^{1}H-NMR spectrum (Plate 30) seventeen acetyl signals at δ_H 1.84-2.40, sugar protons at δ_H 2.51-6.04 and four aromatic singlets at δ_H 7.24-8.06 were observed. In the ^{13}C-NMR spectrum (Plate 31), 23 signals are observed at δ_C 154.57-168.19 corresponding to 23 carbonyl carbons (seventeen acetate carbonyl carbons and six other ester carbonyl carbons), six sugar carbons δ_C 63.9-90.0 and the rest of the carbons were assigned to the aryl carbons. The NMR data is collated in Table 3.9. The NMR data obtained for the acetate corroborated the structure that we have assigned to the polyphenolic compound **3.26**.



3.20 **3.27**

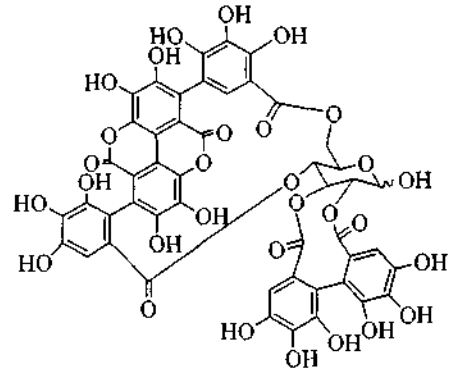
SCHEME 3.3

TABLE 3.9: NMR data of β-punicalagin acetate (3.27) in CD_3OD

position	δ_C	δ_H	position	δ_C	δ_H
β-Glucose					
1'	90.1	6,03, d, J = 7.5	6	119.7	8.06, s
2'	75.7	5.10, dd, J = 7.0, 10.5	7	164.6	
3'	72.8	5.19, dd, J = 6.5, 10.5	1'	119.5	
4'	73.6	4.63, d, J = 6.4	2'	123.2	
5'	75.2	3.19, d, J = 10.9	3'	137.3	
6	63.9	a. 4.61, t, J = 6.4 b. 2.54, d, J = 11.5	4'	142.6	
HDDP			5'	144.3	
1	124.8		6'	129.0	
2	130.8		7'	154.5	
3	116.4	7.48, s	1''	123.5	
4	143.6		2''	123.1	
5	136.2		3''	142.5	
6	143.5		4''	142.7	
7	164.8		5''	144.7	
1'	126.5		6''	117.5	
2'	131.2		7''	155.0	
3'	116.2	7.31, s	1'''	127.0	
4'	143.7		2'''	142.9	
5'	136.6		3'''	141.9	
6'	143.5		4'''	139.1	
7'	165.3		5'''	142.0	
tetraphenyl unit			6'''	116.7	7.22, s
1	128.4		7'''	163.2	
2	128.1				
3	125.7				
4	138.4				
5	143.8				

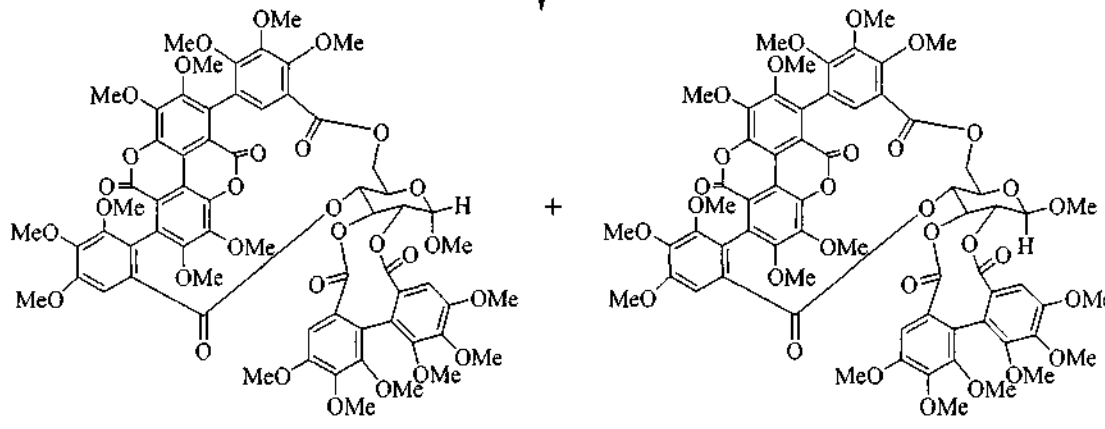
3.3.11 Structural elucidation of α- and β-punicalagin permethyl ether (3.28 and 3.29)

Methylation of fraction **3.20** with methyl iodide and a base in acetone afforded two separable components which were partitioned on silica with hexane:EtOAc (8:2) to afford two light yellow compounds, **3.28** and **3.29**, in 76% and 24% yields, respectively (Scheme 3.4). The molecular mass of both compounds was established by ESI^--MS where $[M-H]^-$ was observed at *m/z* 1323.3130 each, corresponding to a molecular formular of $C_{65}H_{62}O_{30}$.



3.20

MeI, K_2CO_3,
acetone



3.28 + 3.29

SCHEME 3.4

In the ^{1}H-NMR spectum (Plates 35 and 40) seventeen methoxy signals are observed at δ_H 3.17-4.28 for α-anomer (**3.28**) and δ_H 3.26-4.35 for β-anomer (**3.29**), respectively. In both spectra the anomeric methoxy signal is shifted upfield from the aromatic ones as expected. The sharp aromatic singlets corresponding to four protons are clearly observed in the spectra of the two compounds indicating the presence of four tetrasubstituted aromatic rings. The sugar moiety proton signals are also well resolved.

In the ^{13}C NMR spectra of the two compounds, it was clearly observed that **3.28** differ from **3.29** in anomeric carbon chemical shifts with α-anomeric carbon at δ_C 96.27 and the β-anomeric carbon resonated at δ_C 100.14. In both spectra two aromatic γ-lactone rings were also observed at δ_C 155.8 (α)/ 156.43 (β) and δ_C 156.9 (α)/ 156.9 (β). The observation of four carboxyl carbonyl carbon signals with normal chemical shifts (δ_C 168.2/ 167.2, 168.2/ 167.4, 167.5/ 167.1 and 167.2/ 165.9) is consistent with the 2,3,4,6 tetraacylation pattern of the hexopyranose moiety for each.

The hexahydroxydiphenyl (HHDP) substructure was represented in the ^1H NMR spectrum by the aromatic protons H-3 at δ_H 6.75 (α)/ 6.77 (β) and H-3' at δ_H 6.68 (α)/ 6.68 (β). Carbons C-3 and C-3' were identified by the HSQC at δ_C 104.7 and δ 104.2/104.3 respectively. Each of the protons H-3 and H-3' of the HHDP moiety exhibited ^1H-^{13}C long-range correlation to an ester carbonyl carbon (C-7: δ_C 168.2/167.2; C-7': δ 168.2/167.4), two carbon corresponding to aromatic C-C bonds (C-1, C-1': δ 120.8/120.6, 121.1/122.5; C-2: δ 128.3/128.1, C-2': δ_C 129.8/128.1) and to three oxygen-bearing aromatic carbons (C-4, C-4': δ153.1/152.6, 153.2/153.1; C-5: δ_C 131.9/130.2; C-5': δ 140.9/132.2; C-6, C-6': δ_C 152.2/152.5, 152.5/151.4. The ^1H-^{13}C long range (Plates 39 and 44) between the low field glucose protons H-2 (α: δ_H 4.98; β: 4.91) and the HHDP carbonyl carbon C-7 as well as the H-3 (α: δ_H 5.37, β: δ_H 5.17) and C-7' indicated that the HHDP unit is attached to *O*-2 and *O*-3 of the central glucose core by ester linkages.

For the tetraphenyl unit the ^1H-^{13}C long range correlations between the ester carbonyl carbon C-7 (δ_C 167.57/167.12) and the low field glucose protons H-4 (α: δ_H 4.99; β: δ_H 4.62) along with the aromatic proton H_A-6 [δ_H 7.34(α)/7.26(β); C-6: δ_C 112.2/112.2 by the HSQC] indicated that the aromatic ring A is linked to the *O*-4 of the glucose core. The remaining five ring-A carbons were identified by the HMQC at δ_C 153.2/153.2, 152.9/120.8, 127.8/126.7, 127.8/126.6 and 112.2/112.2. The aromatic ring system D was represented in the ^1H NMR by the proton H_D-6''' at δ_H 6.71 (α)/ 6.70 (β) (C-6''': δ_C 107.1/107.6 by HSQC).

In the HMQC spectrum H_D-6''' was correlated with an ester carbonyl carbon C-7''' at δ_C 167.2/165.9 and the low field glucose protons H-6 at δ_H 4.40/4.42 and 2.07/2.84 similarly to ring A indicated that the ring D is linked to the *O*-6 of the glucose core. Once again the HMQC was used to identify the remaining ring-D carbons. The NMR data of the two

permethyl ether derivative is also in full agreement with the structure assigned to punicalagin.

Although at first the structure of this hydrolysable tannin seemed to be very complex, we have shown here that analysis of high-field NMR spectra of punicalagin and its derivatives allowed us to assign an exact structure to this very complex compound.

TABLE 3.10: NMR data of β-punicalagin permethyl ether (3.29) in $CDCl_3$

position	δ_C	δ_H	position	δ_C	δ_H
β-Glucose					
1'	100.1	4.55, d, J = 6.9	6	112.2	
2'	74.3	4.91, dd, J = 6.9,10.4	7	167.5	
3'	75.7	5.17, dd, J = 8.3, 10.2	1'	110.8	
4'	72.1	4.62, dd, J = 8.1, 13.1	2'	116.0	
5'	74.6	2.57, bt, J = 4.6	3'	141.2	
6	64.4	a. 4.42, t, J = 11.6 b. 2.84, d, J = 10.6	4'	146.0	
HDDP			5'	153.2	
1	120.6		6'	128.2	
2	128.1		7'	155.8	
3	104.7		1''	116.1	
4	152.6		2''	113.0	
5	130.2		3''	145.6	
6	152.5		4''	150.6	
7	167.2		5''	153.5	
1'	122.5		6''	112.2	
2'	128.1		7''	156.9	
3'	104.3		1'''	124.3	
4'	153.1		2'''	151.3	
5'	132.2		3'''	144.2	
6'	151.4		4'''	144.2	
7'	167.4		5'''	145.6	
tetraphenyl unit			6'''	107.1	
1	126.7		7'''	167.2	
2	124.5				
3	120.8				
4	141.3				
5	153.2				

TABLE 3.11: NMR data of α-punicalagin permethyl ether (3.28) in $CDCl_3$

position	δ_C	δ_H	position	δ_C	δ_H
α-Glucose					
1'	96.2	4.70, d, J = 3.4	6	112.2	
2'	73.1	4.98, dd, J = 3.4	7	167.1	
3'	75.7	5.37, t, J = 9.3	1'	110.8	
4'	70.6	4.99, d, J = 9.3	2'	115.9	
5'	66.8	3.03, t, J = 9.8	3'	141.1	
6	63.9	a. 4.40, t, J = 10.9 b. 2.07, d, J = 10.2	4'	145.9	
HDDP			5'	153.4	
1	120.8		6'	128.1	
2	128.3		7'	156.4	
3	104.7		1''	116.2	
4	153.1		2''	112.5	
5	131.1		3''	145.7	
6	152.6		4''	146.1	
7	168.2		5''	153.5	
1'	121.1		6''	109.4	
2'	129.8		7''	156.9	
3'	104.2		1'''	124.3	
4'	153.2		2'''	150.7	
5'	140.9		3'''	144.2	
6'	152.5		4'''	144.0	
7'	168.2		5'''	144.8	
tetraphenyl unit			6'''	107.6	
1	127.8		7'''	165.9	
2	125.0				
3	152.9				
4	144.1				
5	153.2				

3.3.12 Discussion on punicalagin (3.20)

Tannins are widely distributed in higher plants and are divided into two groups, condensed and hydrolysable. Tannins gained originally popularity in the commercial tanning industry where animal hides were converted into leather by using plant extracts but have attracted much attention recently due to their numerous biological activities and implications in

potential benefits to human health. Punicalagin (**3.20**), a hydrolysable tannin, belongs to a group of polyphenolic compounds called ellagitannins.

Ellagitannins have numerous intramolecular C-C linked ester groups that have been located in the monomers and various intermolecular linking esters groups that have been defined in the formation of the oligomeric structures (Scheme 3.5). The principal members of these two classes of ester groups are (*R*)- and (*S*)-hexahydroxydiphenoyl (**3.30**), flavogallonyl (**3.31**) and gallagyl (**3.32**) groups.

β-1,2,3,4,6-Pentagalloyl-D-glucose

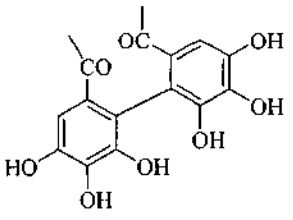
-[H] intramolecular C-C coupling

hexahydroxydiphenoyl and dehydrohexahydroxydiphenoyl esters (monomers)

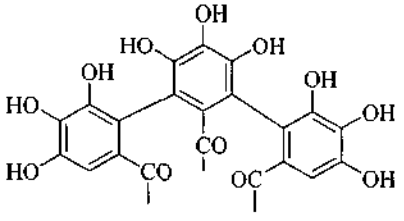
-[H] intermolecular C-O coupling

[hexahydroxydiphenoyl and dehydrohexahydroxydiphenoyl esters] (oligomers, n = 2,3,4)

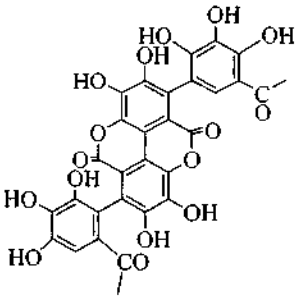
SCHEME 3.5: Overall pattern of oxidative metabolism of β-1,2,3,4,6-pentagalloyl-D-glucose



3.30



3.31



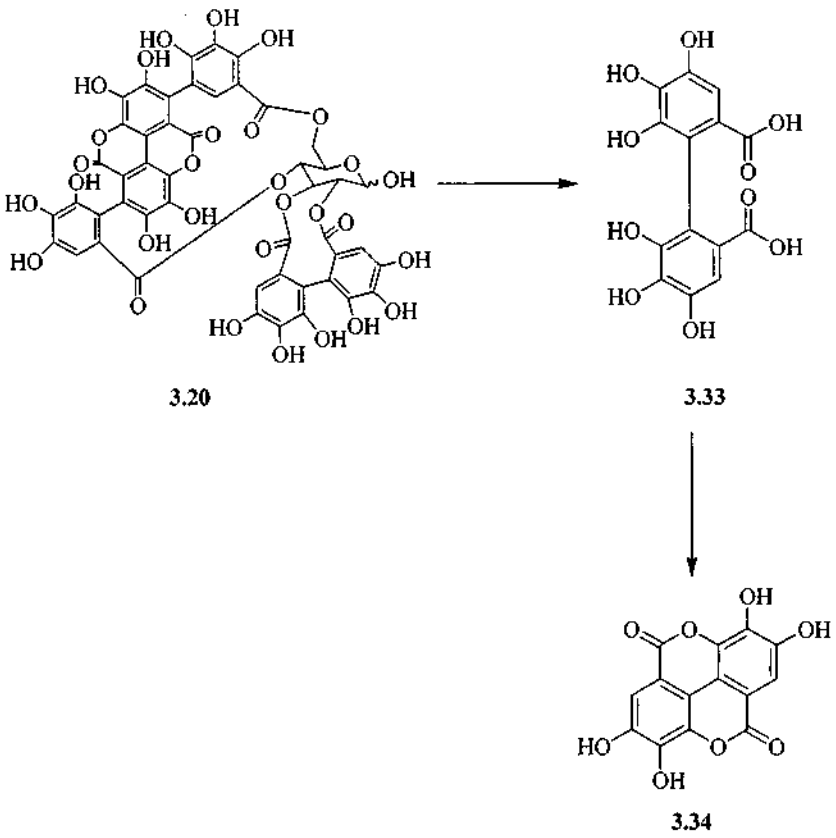
3.32

Punicalagin is the major polyphenolic compound present in pomegranate and has attracted much recent attention due to its numerous biological activities. Pomegranate fruits are widely consumed fresh and in beverage forms as juice and wines. The pomegrenate juice has been reported to show potent antioxidant and anti-atherosclerotic properties attributed to its high content of polyphenols including punicalagin. Punicalagin was also found to be an active substance in pomegranate fruits against methicillin-resistant *Staphylococcus aureus* strains which implies antimicrobial activity. [34,35] A recent review by Lansky and Newman[36] highlighted the potential of plants rich in punicalagin (**3.20**) for the prevention and treatment of inflammation and cancer.

In Taiwan the leaves of *Terminalia catappa* (Combretaceae) have been used as a folk medicine for preventing hepatoma and treating hepatitis.[37] In the leaves of this plant punicalagin is present as one of the hydrolysable tannins. The compound has also been reported to possess chemo-preventive effect on H-*ras*-transformed NIH3T3 cells.[38]

The ellagitanins and their hydrolysis product, ellagic acid have been reported to induce apoptosis in tumor cells.[39] They are not absorbed *in vivo* but reach the colon and release ellagic acid that is metabolized by the human microflora. Punicalagin is one of the hydrolysable tannins that can be hydrolyzed producing ellagic acid via spontaneous lactonisation of hexahydrodiphenic acid (Scheme 3.6). In this study punicalagin and ellagic acid were assayed on both human colon cancer Caco-2 and colon normal CCD-112CoN cells.

Larrosa *et al.*[40] assayed urolithins, metabolites of punicalagin in the human body (see Chapter 4, p.72) to evaluate their capacity to induce cell proliferation on the estrogen-sensitive human breast cancer MCF-7 cells as well as the ability to bind the α- and β-estrogen receptors and their findings were that both urolithins displayed both estrogenic and antiestrogenic activities.



3.20 3.33 3.34

SCHEME 3.6

3.3.13 HPLC study of the chemical variation of *G. perpensa*

Although secondary metabolites are normally associated with specific plant species, chemical variation of the compounds present in a specific species is sometimes observed for plants collected from different geographical areas. This variation may be ascribed to different climatic conditions, but most often it is as a result of different genetic types of this species. When working with medicinal plants that grow in the wild, it is important to investigate the chemical variation of plants collected in different areas before making a general statement on the composition and activity of the plants. Therefore, we have decided to investigate the chemical variation of *G. perpensa.*

The rhizomes of *G. perpensa* were collected near the streams of water in Gauteng (Montgomery Park, Johannesburg), two parts of KwaZulu Natal (Underberg and

Pietermaritzburg) and in Western Cape (Murrayburg). After drying and milling, the ground plant material was extracted using MeOH.

Four extract (Underberg, Johannesburg, Pietermaritzburg and Murraysburg) and four compounds (**3.17**, **3.18** and **3.19** and **3.20**) were analyzed with the reversed-phase high-performance liquid chromatography with UV detection. The mobile phase consisted of ultra-pure H_2O and MeOH. Fig. 3.14 to 3.21 shows the HPLC chromatograms of the four isolated compounds and the crude extracts.

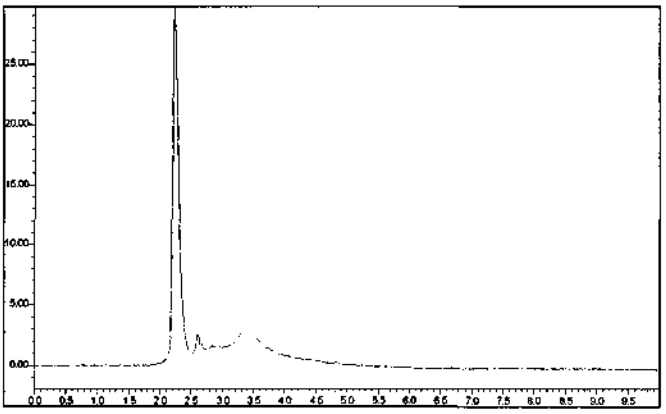


FIGURE 3.14: HPLC chromatogram of punicalagin (**3.20**)

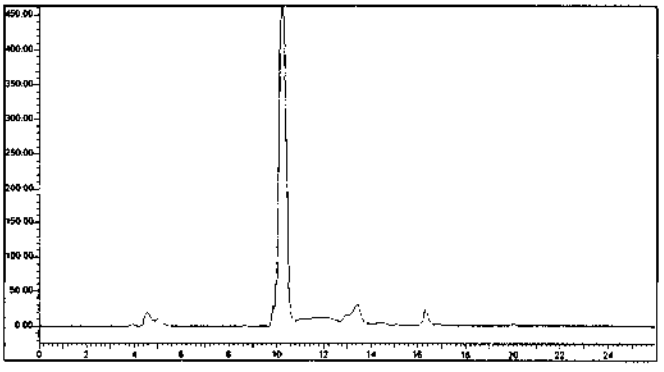


FIGURE 3.15: HPLC chromatogram of methyl lespedezate (**3.18**)

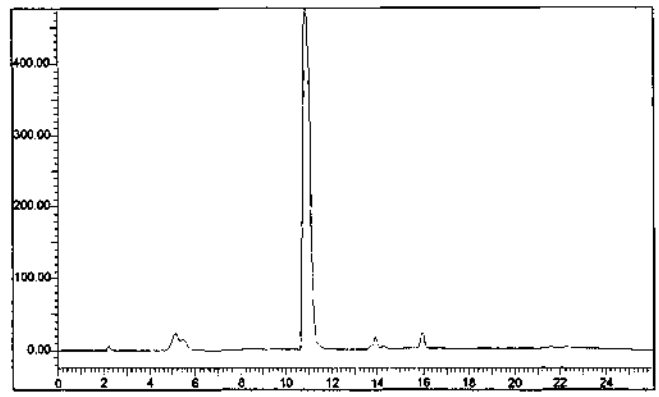


FIGURE 3.16: HPLC chromatogram of Z-venusol (**3.17**)

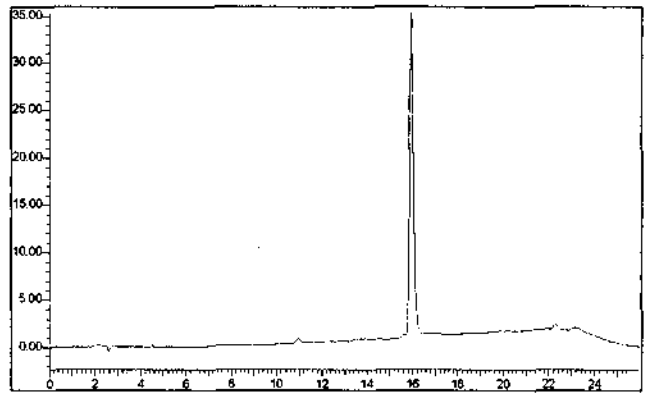


FIGURE 3.17: HPLC chromatogram of 3,3',4'-tri-*O*-methylellagic acid 4-*O*-β-D-glucopyranoside (**3.19**)

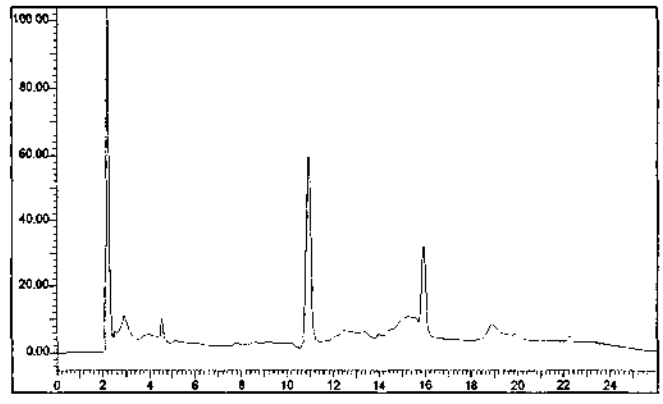


FIGURE 3.18: HPLC chromatogram of a *G. perpensa* extract from Johannesburg

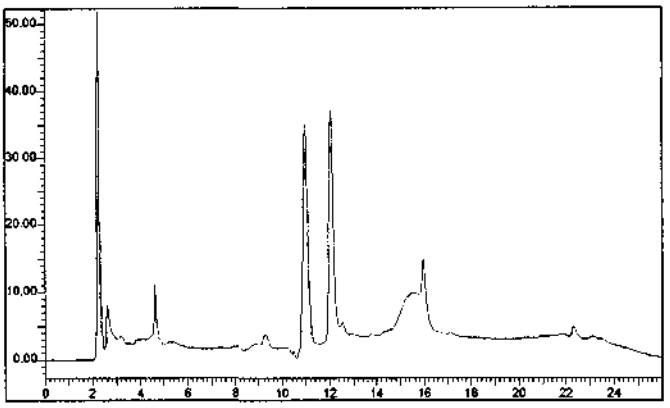


FIGURE 3.19: HPLC chromatogram of a *G. perpensa* extract from Underberg

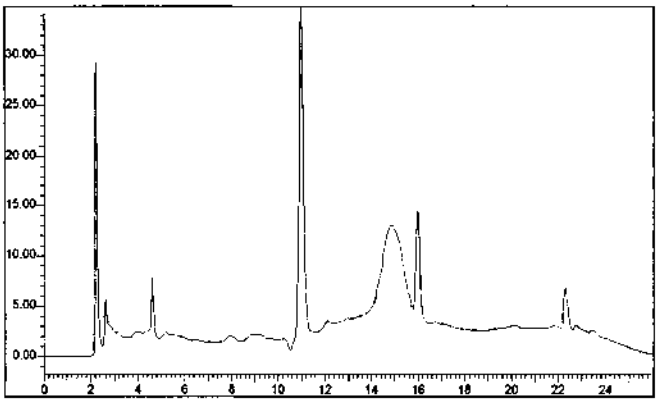


FIGURE 3.20: HPLC chromatogram of a *G. perpensa* extract from Pietermaritzburg

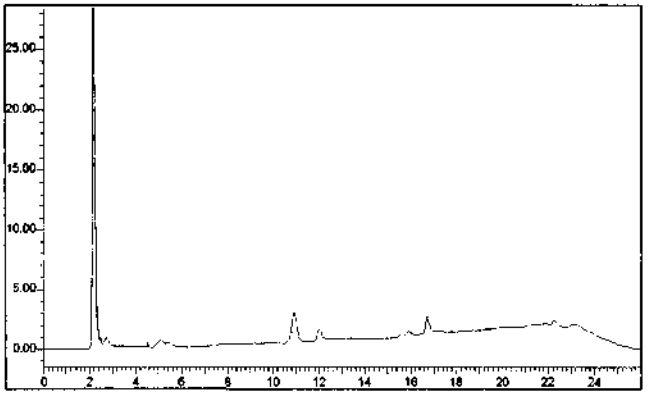


FIGURE 3.21: HPLC chromatogram of a *G. perpensa* extract from Murraysburg

In the HPLC chromatograms all the extracts (Fig. 3.18 to Fig. 3.21) of *G. perpensa* contain peaks at 2.2, 11.0, and 16.2 min. which were assigned to the isolated compounds **3.17**, **3.19**

and **3.20**. However the presence of methyl lespedezate (**3.18**) was not observed in any of the crude extracts. This confirmed our earlier hypothesis that methyl lespedezate (**3.18**) isolated from the bulk extract was an artifact from the extraction procedure and chromatographic procedures.

The chromatograms of the extracts from Underberg (Fig. 3.19) and Murraysburg (Fig. 3.21) show the presence of an extra peak at 12.2 min. which is absent in the chromatogram of the extracts from the Johannesburg and Pietermaritzburg (Fig. 3.18 and 3.20). In the Murraysburg extract, the relative concentration of punicalagin (**3.20**) compared to the other metabolites, is much higher. It is clear that there is a degree of chemical variation in the rhizomes of *G. perpensa* collected in different geographical areas.

3.3.14 Uterotonic activity of crude extract and *Z*-venusol (3.17)

The uterotonic effect of the aqueous, ethyl acetate and ethyl acetate-methanol extracts of *G. perpensa* and pure venusol (**3.17**) were evaluated using the uterine and ileal muscle obtained from Sprague-Dawley rats. These experiments were done by Dr Gehring in collaboration with Prof. S.E. Drewes. Unfortunately Dr Gehring emigrated before conclusive results on the active compound in *G. perpensa* were achieved and the investigation on the biological activity was not continued. What is known is that the aq. extracts exhibited uterotonic contraction to both the ileum and the uterus muscle when the extract and the added acetylcholine were rinsed from the organ bath. The contractile response was reduced when acetylcholine was replaced with atropine. The organic extracts and pure venusol (**3.17**) did not induce contraction but when the test substance was flushed from the organ bath, venusol (**3.17**) did induce a state of spontaneous contractility. From these observations it is possible that the venusol exerts its action in conjunction with substances present in the whole extract.

In this experiment, punicalagin (**3.20**) and ellagic acid were not evaluated for their activity. It is known that the active principle of *Oldenlandia affinis*, another African plant with uterotonic effect, is a cyclic peptide consisting of 29 amino acids (a cyclotide) (see Chapter 2, p. 12). We have made an aqueous extract of *G. perpensa* using a procedure specific for the extraction of cyclotides, but did not obtain any peptide in this extract.

3.3.15 HIV-RT inhibitory activity of the crude extract and punicalagin (3.20)

In a related project in our research group, the inhibitory activity of plant extracts on the HIV-reverse transcriptase enzyme was investigated and it was observed that at a concentration of 500 µg/ml the methanol extract of *G. perpensa* inhibited the enzyme by 97%. Bioassay-guided fractionation of the crude extracts led to the isolation of punicalagin (**3.20**) as the active compound. What is known is that tannins bind non-selectively to proteins and that false positive results are obtained in most *in vitro* biological assays when tannins are present in the extracts that are being assayed.

It is of interest to note that a patent on the use of *G. perpensa* for the treatment of immune system disorders (HIV) was registered recently.[41] This patent describes different methods for the processing and extracting of the plant material, but does not give any details on the activity observed for the plant.

3.4 CONCLUSION

As can be seen from the literature review in the beginning of the Chapter, *G. perpensa* is still used widely by the indigenous people in South Africa. Prior to this study, nothing was known on the phytochemistry of this plant and very little was reported on the chemistry of the other *Gunnera* species. Herewith, we have presented the isolation and structural elucidation of secondary metabolites present in *G. perpensa*. All compounds isolated are known compounds but have not previously been reported from the genus *Gunnera*. The compounds isolated, venusol (**3.17**), 3,3',4'-tri-*O*-methylellagic acid 4-*O*-β-D-glucopyranoside (**3.19**) and punicalagin (**3.20**), all have interesting biological activities. In the past, natural product chemists have ignored tannins in plant extracts. However, with the current interest in the biological activity of punicalagin,[36] phytochemists need to pay more attention to the hydrolysable tannins when investigating medicinal plants.

It is clear that the aqueous crude extract has an uterotonic effect but at this stage it is not clear which compound(s) is the active principle. Some activity have been observed for venusol (**3.17**) and it is most likely that the activity observed for the crude extract is due to a synergistic effect.

We have developed an HPLC method for the analysis of *G. perpensa* extracts. The chromatographic results obtained with extracts of plants collected in different geographical regions indicated that there is some chemical variation in this plant species.

3.5 EXPERIMENTAL

3.5.1 General

Mass spectra were obtained with a Waters API Q-TOF Ultima LCMS or a Kratos MS 80 RF double-focussing magnetic sector instrument. Nuclear magnetic resonance spectroscopy (NMR) of the isolated compounds was performed on either a Varian Unity-Inova 300 MHz or Varian Unity-Inova 500 MHz spectrometer. All NMR spectra were recorded at 25 °C and the chemical shifts were recorded in ppm referenced to the solvent shift. Deuterated methanol (CD_3OD), dimethyl sulfoxide (DMSO-d_6) and acetone-d_6 were used for the polar compound and deuterated chloroform ($CDCl_3$) was used for non-polar and acetylated compounds.

Optical rotations of the isolated compounds were determined with the appropriate spectroscopic solvent with Perkin-Elmer 241 polarimeter.

Thin-layer chromatography (TLC) of the crude extracts was performed with aluminum backed, 0.2 mm silica gel plates (Merck 0.20 mm or Machery-Nagel 0.20 mm). The TLC-plates were visualized with the aid of UV radiation, iodine or anisaldehyde spray reagent (6 g anisaldehyde in 250 ml ethanol containing 2.5 ml concentrated sulfuric acid). Once the plates were sprayed they were heated in an oven to allow for the colour development.

Normal and flash chromatography were performed with silica gel 60 (Merck 0.040-0.063 mm) and on columns with diameters 4 cm and 1 cm. The extracts and mixed fractions were dissolved in a small amount of solvent and loaded onto the column with pipettes. Vaccum-liquid chromatography was carried out using Merck silica gel 60 G on columns with diameters of 10 cm, 4 cm and 2.5 cm. The extracts or mixed fractions were adsorbed onto silica and were then packed onto dry columns. The mobile phases were comprised of different ratios of chloroform-methanol or hexane-ethyl acetate. Both isocratic and gradient mobile phases were employed.

HPLC chromatography was performed on the Perkin Elmer Series 200 system using a Luna C18 (250 x 4 mm) column and a flow rate of 3 min/ml. The following gradient system was used:

Minutes	MeOH	H_2O
0	30	70
2	30	70
17	70	30
20	70	30
23	30	70
28	30	70

3.5.2 Acetylation Procedure

Acetylation of samples was performed by the addition of pyridine-acetic anhydride (1:1) to the sample in the round-bottomed flask. The reaction mixture was then stirred for 48 h at room temperature. Ethanol (3 ml) was added to react with the excess acetic anhydride. The resultant mixture was poured into ice-chloroform mixture and the acetylated product was extracted two to three times by liquid-liquid partitioning in a separating funnel. The organic phases were combined and the solvent removed by rotovapor evaporation.

3.5.3 Methylation Procedure

To a sample solution (300 mg) in dry acetone methyl iodide (1.5 ml) and K_2CO_3 (3.0 g) were added. The mixture was then refluxed in an oil bath for 5 h. The inorganic precipitates were filtered; the filtrate was concentrated *in vacuo* to remove the solvent. The residue was then subjected to TLC chromatography.

3.5.4 Extraction of *G. perpensa*

The rhizomes of *Gunnera perpensa* were collected near a stream of water in Montgomery Park in Johannesburg. The plant material (2 kg) was semi dried, blended with a mixture of 1:1 $CHCl_3$:MeOH and filtered. The filtrate was evaporated to remove the organic solvents

and then freeze-dried to give a light-yellow powdered crude fraction PET-1-5A (42.42 g). The remaining plant material was once again subjected to a blender using the same solvent system, filtered, organic solvents removed and freeze-dried to give a second yellow powdered fraction PET-1-5B (3.72 g). The plant material was then extracted twice with a mixture of $CHCl_3$:MeOH (1:1) for a period of 18 h. Removal of the solvent by rotary evaporation and drying under vaccum yielded two sticky dark brown residues PET-1-5C (58.6 g) and PET-1-5D (14.1 g). PET-1-5C crude extract (20 g) was fractionated by vaccum liquid chromatography with hexane:EtOAc (9:1) as an initial eluent. The polarity of solvents was increased gradually and five fractions [(Fr1, 400 mg), (Fr2, 74 mg), (Fr3, 332 mg), (Fr 4, 830 mg), (Fr5, 11.69 mg)] were obtained

3.5.5 Isolation of β-sitosterol (3.8)

A non-polar fraction (Fr 1, 330 mg) was subjected to liquid chromatography with hexane:EtOAc (9.5:0.5). Three fractions were collected and one of the fractions was a single compound identified as β-sitosterol (**3.8**) (6 mg).

3.5.5.1 Physical data for β-sitosterol (3.8)

Description	white solid
Emperical formula	$C_{29}H_{50}O$
Melting point	137-139 °C (lit.[42] 139-142)
^{1}H NMR (CD_3OD)	Plate 1, Table 3.2
^{13}C NMR	Plate 2, Table 3.2

3.5.6 Isolation of *Z*-venusol (3.17)

The crude extract (20 g) was fractionated with chloroform:MeOH (9:1) using a vaccum-liquid chromatography to yield four fractions, one of which was *Z*-venusol (**3.17**), 424 mg.

3.5.6.1 Physical data for *Z*-venusol (3.17)

Description	white solid
Emperical formula	$C_{15}H_{16}O_8$

MS *m/z*	324.28
Melting point	259 °C [lit.[26] 259-260 °C]
Optical rotation	$[\alpha]_D^{20}$ +84.5 (*c* = 1, MeOH)
^{1}H NMR (CD_3OD)	Plate 3, Table 3.3
^{13}C NMR	Plate 4, Table 3.3
APT	Plate 5
COSY	Plate 6
HSQC	Plate 7, Table 3.3
HMQC	Plate 8
NOESY	Plate 9

3.5.7 Isolation of *Z*-venusol tetracetate (3.21)

Z-venusol (100 mg) was acetylated according to the acetylation procedure described in § 3.4.2 and after workup afforded compound **3.21** (63 mg).

3.5.7.1 Physical data for *Z*-venusol tetracetate (3.21)

Description	courless oil
Emperical formula	$C_{23}H_{24}O_{12}$
Optical rotation	$[\alpha]_D^{20}$ +25 (MeOH)
MS *m/z*	492.12
^{1}H NMR (CD_3OD)	Plate 10, Table 3.4
^{13}C NMR	Plate 11, Table 3.4
COSY	Plate 12
HSQC	Plate 13

3.5.8 Isolation of methyl lespedezate (3.18)

A crude fraction of 300 mg containing compound **3.17** was chromatographed (9:1, $CHCl_3$:MeOH) to afford compound **3.18** (36 mg).

3.5.8.1 Physical data for methyl lespedezate (3.18)

Description	white solid
Emperical formula	$C_{16}H_{20}O_8$
MS *m/z*	340.33
Melting point	234 °C
Optical rotation	$[\alpha]_D^{20}$ 84.5 (*c* = 1, MeOH)
^{1}H NMR (CD_3OD)	Plate 14, Table 3.5
^{13}C NMR	Plate 15, Table 3.5
COSY	Plate 16
HSQC	Plate 17
HMQC	Plate 18
NOE	Plate 19

3.5.9 Isolation of 3,3',4'-tri-*O*-methylellagic acid 4-*O*-β-D-glucopyranoside (3.19)

Crude extract (6.23 g) was chomatographed (hexane:EtOAc, 8:2; $CHCl_3$:MeOH, 9:1, 8:2, 7:3) to give four fractions. During the elution, a white precipitate was observed and was filtered to give compound **3.19** (5 mg).

3.5.9.1 Physical data for -3,3',4'-tri-*O*-methylellagic acid 4-*O*-β-D-glucopyranoside (3.19)

Description	cream white solid
Emperical formula	$C_{23}H_{22}O_{13}$
EI-MS *m/z*	343.66
Melting point	210-211 °C
^{1}H NMR (CD_3OD)	Plate 20, Table 3.6
^{13}C NMR	Plate 21, Table 3.6
HETCOR	Plate 22

3.5.10 Isolation of punicalagin (3.20)

5 g Crude extract was chromatographed on Sephadex LH-20 (120 x 4 cm) with gradient solvent system of EtOH-H_2O (9:1-7:3) to afford 3 crude fractions: Fr1 (2.36 g); Fr2 (1.23 g) and Fr3 (1.40 g). Following qualitative TLC analysis on silica gel (EtOAc-H_2O-HCO_2H, 7:1.5:0.5). Fr3 was rechromatographed on Sephadex LH-20 using a EtOH-H_2O gradient system (9:1 - 8:2) to afford punicalagin (**3.20**) as a yellow amorphous powder (900 mg).

3.5.10.1 Physical data for α,β-punicalagin (3.20)

Description	light yellow powder
Emperical formula	$C_{48}H_{28}O_{30}$
MS *m/z*	1083.02
Melting point	236-238 °C (amorphous material)
Optical rotation	$[\alpha]_D^{20}$ -143.4 (MeOH)
^{1}H NMR (CD_3OD)	Plate 23, Table 3.7
^{13}C NMR	Plate 24, Table 3.7

3.5.10.2 Physical data for α-punicalagin (3.26)

Description	light yellow powder
Emperical formula	$C_{48}H_{28}O_{30}$
MS *m/z*	1084.02
Melting point	234-236 °C (amorphous material)
^{1}H NMR (CD_3OD)	Plate 25, Table 3.8
^{13}C NMR	Plate 26, Table 3.8
COSY	Plate 27
HSQC	Plate 28
HMQC	Plate 29

3.5.11 Isolation of β-punicalagin acetate (3.27)

Punicalagin (**3.20**, 100 mg) was acetylated according to acetylation procedure described in § 3.4.2. After work up, a yellow mixture was obtained, which was chromatographed (hexane:EtOAc, 8:2) to afford compound **3.27** (18 mg).

3.5.11.1 Physical data of β-punicalagin acetate (3.27)

Description	light yellow powder
Emperical formula	$C_{82}H_{62}O_{47}$
MS *m/z*	1757.2
Melting point	198-199 °C
^{1}H NMR (CD_3OD)	Plate 30, Table 3.9
^{13}C NMR	Plate 31, Table 3.9
COSY	Plate 32
HSQC	Plate 33
HMQC	Plate 34

3.5.12 Isolation of α- and β-punicalagin permethyl ester (3.28 and 3.29)

Punicalagin **3.20** (220 mg) was methylated according to methylation procedure described in § 3.4.3. After work up, the mixture was flash chromatographed (hexane:EtOAc, 9:1) to afford light yellow compounds **3.27** (87 mg) and **3.29** (36 mg).

3.5.12.1 Physical data for β-punicalagin permethyl ether (3.29)

Description	light yellow powder
Emperical formula	$C_{65}H_{62}O_{30}$
MS *m/z*	1323.3130
Melting point	201-203 °C
^{1}H NMR (CD_3OD)	Plate 35, Table 3.10
^{13}C NMR	Plate 36, Table 3.10
COSY	Plate 37
HSQC	Plate 38
HMQC	Plate 39

3.5.12.2 Physical data for α-punicalagin permethyl ether (3.28)

Description	light yellow powder
Emperical formula	$C_{65}H_{62}O_{30}$

MS *m/z*	1323.3130
Melting point	202-203 °C
^{1}H NMR (CD_3OD)	Plate 40 Table 3.11
^{13}C NMR	Plate 41 Table 3.11
COSY	Plate 42
HSQC	Plate 43
HMQC	Plate 44

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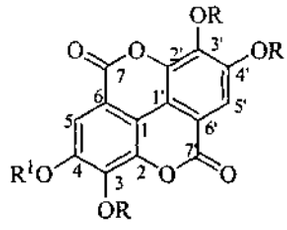
CHAPTER FOUR

SYNTHESIS OF ELLAGIC ACID AND UROLITHIN DERIVATIVES

4.1. INTRODUCTION

Ellagic acid (**4.1**) and its derivatives exist widely in the plant kingdom and their biological activities have been studied extensively. Some of the derivatives were reported to have aldose reductase (AR) inhibitory activity, DNA-gyrase inhibitory properties, HIV-1 protease inhibitory activity, antihermorragic, hypotensive, sedative, antimutagenic and antioxidant properties.[1,2,3] These compounds are unique in that they possess a highly-substituted biaryl nucleus with only two protonated carbons of a total of twelve aromatic carbon atoms.

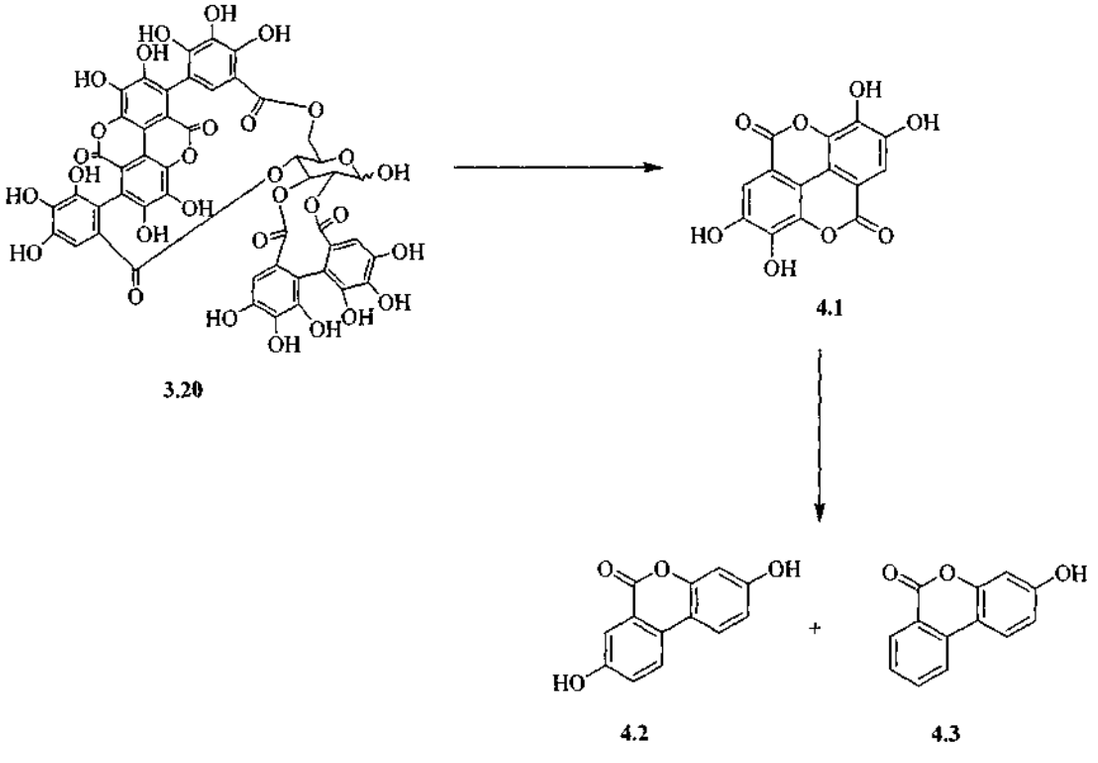


4.1 R = R^1 = H
3.19 R = Me, R^1 = gluc.

3,3',4'-tri-*O*-methylellagic acid 4-*O*-β-D-Glucopyranoside (**3.19**) was first isolated from *Diplopanax stachyanthus* (Cornaceae) of Viet Nam.[4] The authors isolated the compound as its acetate after acetylation of the crude plant extract. Recently it was also isolated from *Alangium javanicum* (Alangiaceae)[5] and now we have isolated it from *Gunnera perpensa* (Gunneraceae).[6]

Ellagic acid is also part of many hydrolysable tannis called ellagitannins. Punicalagin (**3.20**), which we have isolated from *G. perpensa*, is one such tannin that contains ellagic

acid. These tannins are present in many food plants, for example, punicalagin is the major tannin in pomegranate. It has been shown that punicalagin (**3.20**) from different sources is not absorbed in humans but is hydrolysed to yield ellagic acid (**4.1**), which is further metabolized by human colonic microflora to yield the bioavailable urolithins A (**4.2**) and B (**4.3**) (Scheme 4.1).[7] These metabolites reach concentrations of micromolar levels in the blood and are excreted in the urine of humans. Urolithins have been reported to show very poor antioxidant activity and nothing is yet known about other (if any) potential biological activities in the human body.[8] It was recently proposed that urolithins A (**4.2**) and B (**4.3**) could be used as biomarkers of human exposure to dietary ellagic acid derivatives.[8]



SCHEME 4.1. Metabolism of punicalagin (**3.20**)

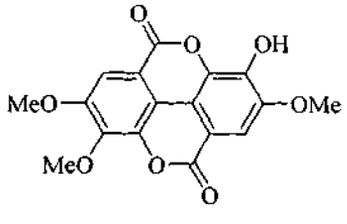
Ellagic acid (**4.1**) and its derivatives are strong antioxidants. The biologial activities of punicalagin (**3.20**), ellagic acid (**4.1**) and its derivatives and the ellagic acid metabolites have not been explored extensively. This may be partly due to the fact that these compounds are normally available only in small amounts from natural sources. The need for larger quantities of ellagic acid and its derivatives for biological studies, prompted us to investigate the synthesis of these compounds. In the next section of this chapter we give a

brief overview of simple ellagic acid derivatives that are widely distributed in the plant kingdom.

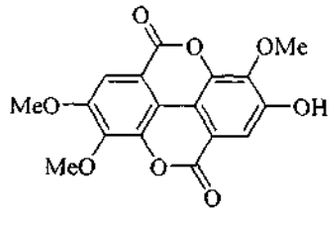
4.2 NATURALLY OCCURING ELLAGIC ACID DERIVATIVES

Many natural ellagic acid derivatives occur as the methyl and glycosyl analogues. Ellagic acid is also part of many hydrolysable tannins. In this section we will limit ourselves to the discussion of a number of representative methyl ethers and glycosides of ellagic acid.

A trimethyl ether of ellagic acid was isolated from the trunk bark of *Eugenia maire* (Myrtaceae), a tree found in the lowland of New Zealand.[9] Two structures (**4.4** and **4.5**) were possible for the compound and since, at that time, the authors did not have advanced NMR techniques available, they have used the expected ionic behavior of the free hydroxy groups of ellagic acid (**4.1**) to distinguish between these two isomers.[10] The 3,3'-hydroxy groups of ellagic acid have a greater acidity than those in 4,4'-positions and it has been shown that it is possible to ionize the two pairs of hydroxy groups selectively by the use of bases of varying strength and obtaining the characteristic shifts of the absorption peaks in the ultraviolet spectrum. When the 3,3'-hydroxy groups are free, a change of the short wavelength peak is observed in the presence of sodium acetate but ionization of the 4,4'-hydroxy groups requires a stronger base such as sodium ethoxide to obtain a bathochromic shift of the long wavelength band. The authors noticed that their phenol was unchanged in the presence of sodium acetate and then came to a conclusion that the free hydroxy group was in the 4-position.



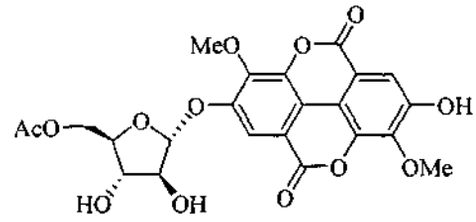
4.4



4.5

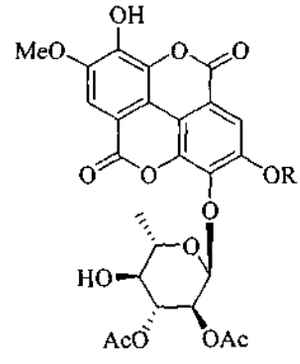
An ellagic acid derivative, 3,3'-di-*O*-methylellagic acid 4-(5''-*O*-acetyl)-α-L-arabinofuranoside (**4.6**), was isolated by Tanaka *et al.*[11] from the roots of *Cornus capitata*

(Cornaceae). The plant genus is well known to contain a large amount of polyphenols such as tannins.



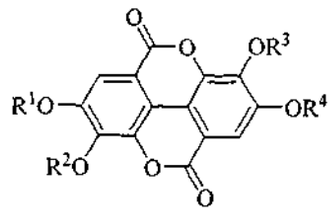
4.6

Fractionation of a bark extract of *Elaeocarpus masterii* (Elaeocarpaceae) that exhibits cytotoxicity towards a human oral epidermoid carcinoma cell line led to the isolation of two ellagic acid derivatives, 4'-O-methylellagic acid 3-*O*-2'',3''-di-*O*-acetyl-α-L-rhamnoside (**4.7**) and 4,4'-*O*-dimethylellagic acid 3-*O*-2'',3''-di-*O*-acetyl-α-L-rhamnoside (**4.8**). The two derivatives were evaluated for their cytotoxicity but they did not exhibit any strong cytotoxic effects.[12]



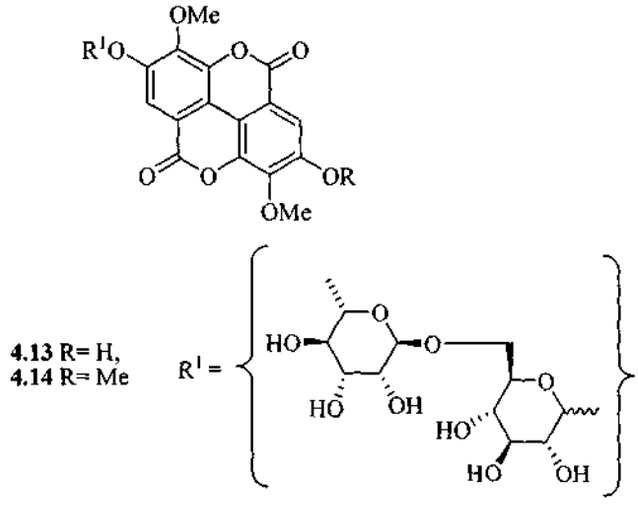
4.7 R= H
4.8 R= Me

Dionaea muscipula (Droseraceae) is a carnivorous plant considered to be a source of anticancer drugs. The methanolic extract led to the isolation of ellagic acid (**4.1**), 3-*O*-methylellagic acid (**4.9**), 3,3'-di-*O*-methylellagic acid (**4.10**) and 3,3'-di-*O*-methylellagic acid 4-*O*-β-glucoside (**4.11**) and 4,4'-di-*O*-β-glucoside (**4.12**).[13]



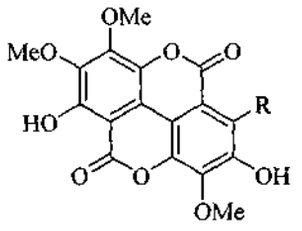
4.1 $R^1 = R^2 = R^3 = R^4 = H$
4.9 $R^1 = R^3 = R^4 = H$, $R^2 = Me$
4.10 $R^1 = R^4 = H$, $R^2 = R^4 = Me$
4.11 $R^1 = Glc$, $R^2 = R^3 = Me$, $R^4 = H$
4.12 $R^1 = R^4 = Glc$, $R^2 = R^3 = Me$

The rhizomes of *Euphorbia acaulis* (Euphorbiaceae) are used by tribes of central India for curing various inflammatory disorders. They are also recommended by the tribal medicine men for the treatment of gout and rheumatic disorder by external application. From this species, Bindra *et al.*[14] isolated three ellagic acid derivatives, 3,3'-di-*O*-methylellagic acid 4'-rutinoside (**4.13**), 3,3',4-tri-*O*-methylellagic acid 4'-rutinoside (**4.14**) and 3,3',4-tri-*O*-methylellagic acid (**4.5**).



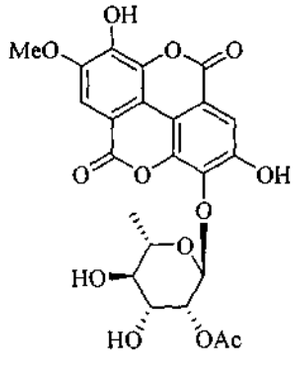
4.13 R= H,
4.14 R= Me

A phytochemical study on *Rhabdodendron macrophyllum* (Rhabdodendraceae) revealed that the roots contains 3,3',4-tri-*O*-methylflavellagic acid (**4.15**) and 3,3',4-tri-*O*-methyl-5'-methoxyflavellagic acid (**4.16**), besides other compounds isolated.[15]



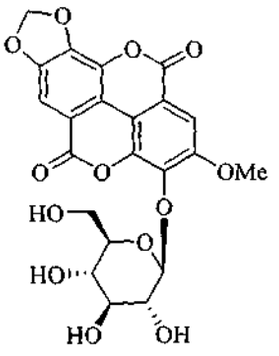
4.15 R= H,
4.16 R= OMe

Qing-Mei and Xiu-Wei[16] reported the isolation of a new ellagic acid derivative, 3-*O*-methylellagic acid 4'-*O*-α-L-2''-*O*-acetylrhamnopyranoside (**4.17**) and other known ellagic acid derivatives from the fruits of *Eucalyptus globules* (Myrtaceae).

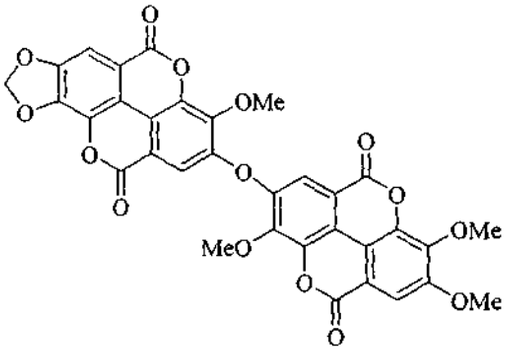


4.17

Bioassay-guided fractionation led to the isolation of two antioxidant and antimicrobial ellagic acid derivatives, 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-glucoside- (**4.18**) and pteleoellagic acid derivative **4.19** along with two other known ellagic acid derivatives from the stem bark of *Pteleopsis hylodendron* (Combretaceae). Compound **4.18** was found to have significant antioxidant activity whilst all four compounds showed antibacterial activity against different pathogenic bacteria.[17]

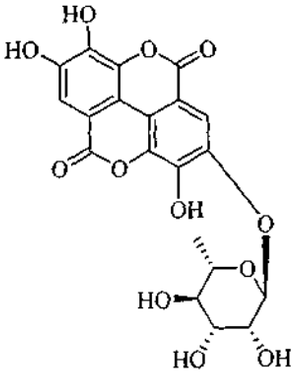


4.18

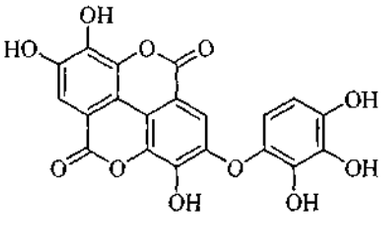


4.19

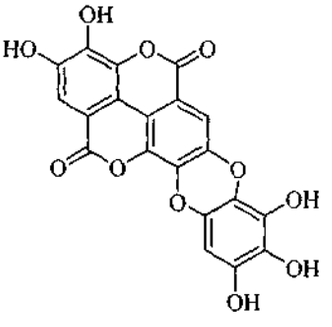
Extraction and bioassay-guided fractionation of the bark of *Eschweilera coriacea* (Lecythidaceae) by Young *et al.*[18] led to the isolation of three ellagic acid derivatives, eschweilenol A (**4.20**), eschweilenol B (**4.21**) and eschweilenol C (**4.22**).



4.20

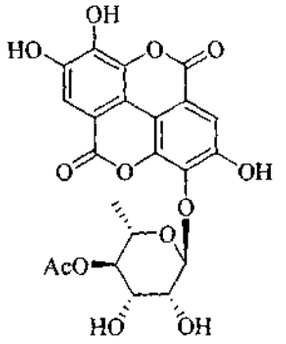


4.21



4.22

During the screening of new bioactive products from Chinese plants distributed in Yunnan, Yukihiro *et al.*[19] found that a 60% ethanol extract of branches of *Combretum yunnanensis* (Combretaceae) inhibited growth of adriamycin-resistant mouse leukemia cells. Motivated by these findings, they isolated the active ellagic acid derivative, ellagic acid 4-(4''-O-acetyl-α-rhamnopyranoside (**4.23**).

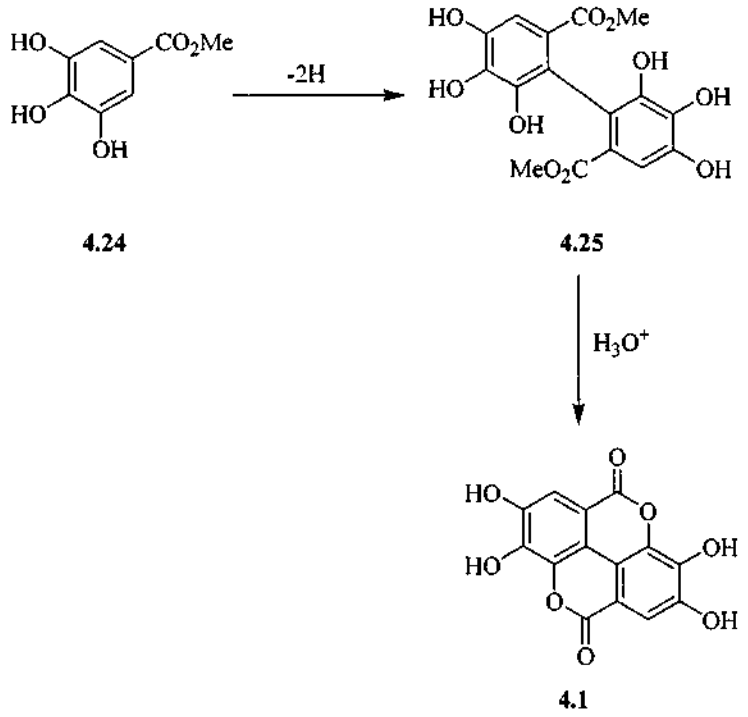


4.23

It is clear that ellagic acid derivatives are widespread in nature and that some of them have pronounced biological activity.

4.3 BIOSYNTHESIS OF ELLAGIC ACID

Naturally-occurring ellagic acid and its derivatives are formed by the oxidative coupling of galloyl esters with the creation of new C-C and C-O bonds (Scheme 4.2). The biogenetic pathway is initiated by oxidation of methyl gallate (**4.24**) by peroxidase to give dimethyl hexahydroxybiphenoate (**4.25**). The biphenyl ester is then readily transformed to the highly insoluble bis-lactone ellagic acid (**4.1**) on standing in water. This transformation is facilitated by the proximal juxta-position of the phenolic and ester groups on separate aromatic nuclei and by free rotation around the biphenyl ring.[20]



4.24 **4.25** **4.1**

SCHEME 4.2

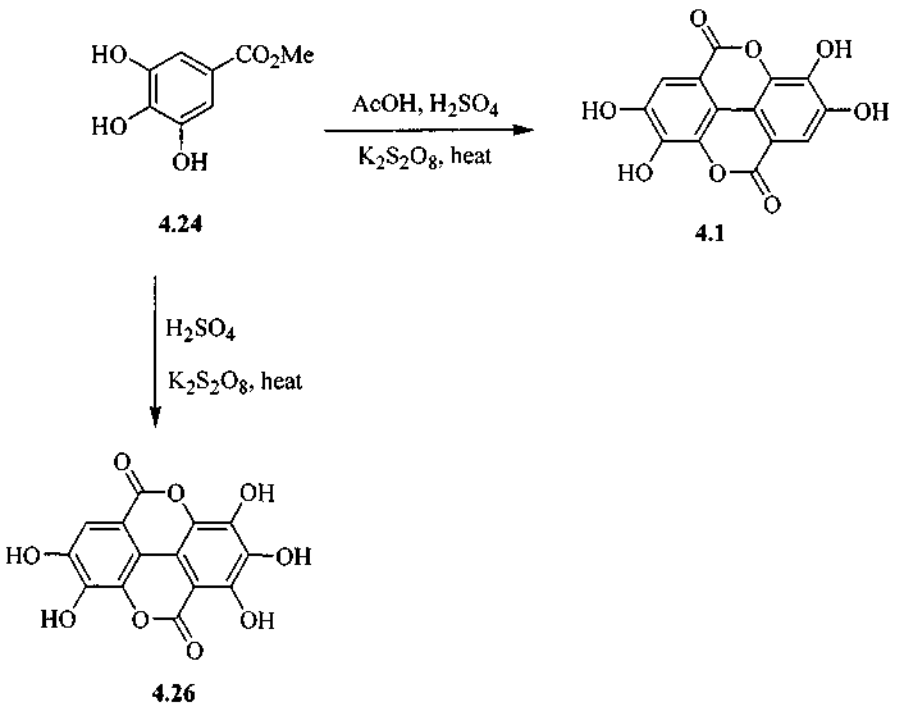
4.4 SYNTHESIS OF ELLAGIC ACID DERIVATIVES AND UROLITHIN A: A LITERATURE REVIEW

4.4.1 Synthesis of ellagic acid derivatives

Although a large number of ellagic acid derivative have been isolated from natural sources, only limited information on the synthesis of these compounds are available. Problems associated with the synthesis are the coupling of highly-substituted electron-rich aryl rings and difficult *O*-glycosidation reactions.

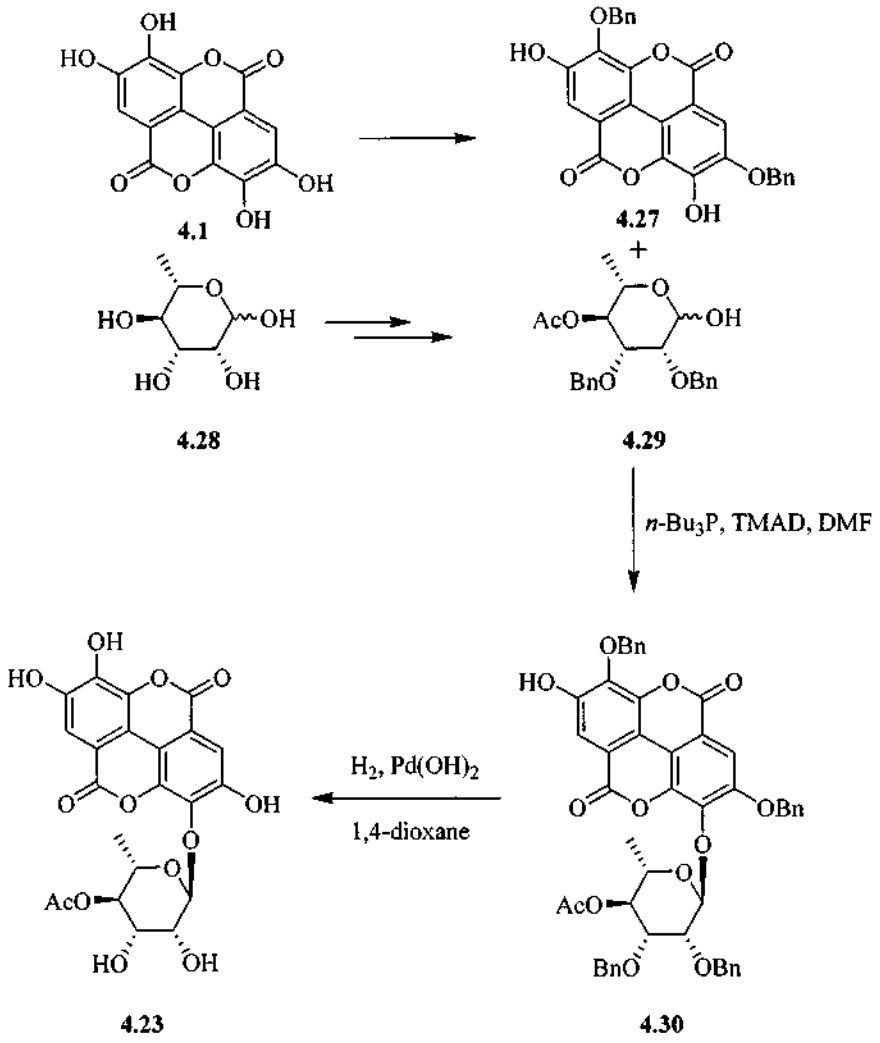
Alkylated gallic and ellagic acid derivatives have become important in studies of the naturally-occurring gallotannins and ellagitannins. Many partial methyl ethers of ellagic acid have been prepared and in some cases the reactions used have not given satisfactory yields of these ethers.[10] The oxidation of gallic acid and its ethers by potassium persulfate in glacial acetic acid / sulfuric acid solution gives ellagic acid (**4.1**), whereas in sulfuric

acid alone, flavellagic acid (**4.26**) is formed (Scheme 4.3). Using this methodology, only symmetrically-substituted ellagic acid derivatives can be formed.



SCHEME 4.3

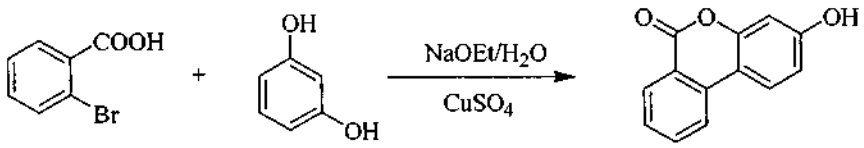
Yukihiro *et al.*[19] reported on the semi-synthesis of a bioactive ellagic acid derivative isolated from *C. yunnenesis* using ellagic acid (**4.1**) as a starting material. Selective benzylation of the more reactive 3,3'-hydroxy groups of **4.1** yielded 3,3'-di-*O*-benzylellagic acid (**4.27**) and subsequent glycosidation with a L-rhamnose derivative **4.29** and debenzylation led to the target compound **4.23** in a good yield (Scheme 4.4).



SCHEME 4.4

4.4.2. Synthesis of urolithins

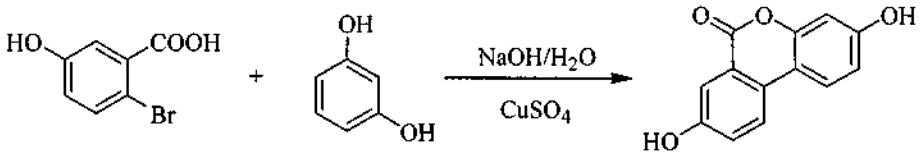
Hurtley[21] reported that the condensation of 2-bromobenzoic acid (**4.31**) with resorcinol (**4.32**) (Scheme 4.5) proceeded smoothly in an ethanol solution in the presence of sodium ethoxide and either copper powder or Cu(II) sulfate as a catalyst to form urolithin B (**4.3**). Bruggink and McKillop[22] re-examined these reaction conditions and used sodium hydroxide as the base; a 1:1 mixture of compound **4.3** and salicylic acid together with small amounts of unchanged 2-bromobenzoic acid (**4.31**) were formed.



4.31 **4.32** **4.3**

SCHEME 4.5

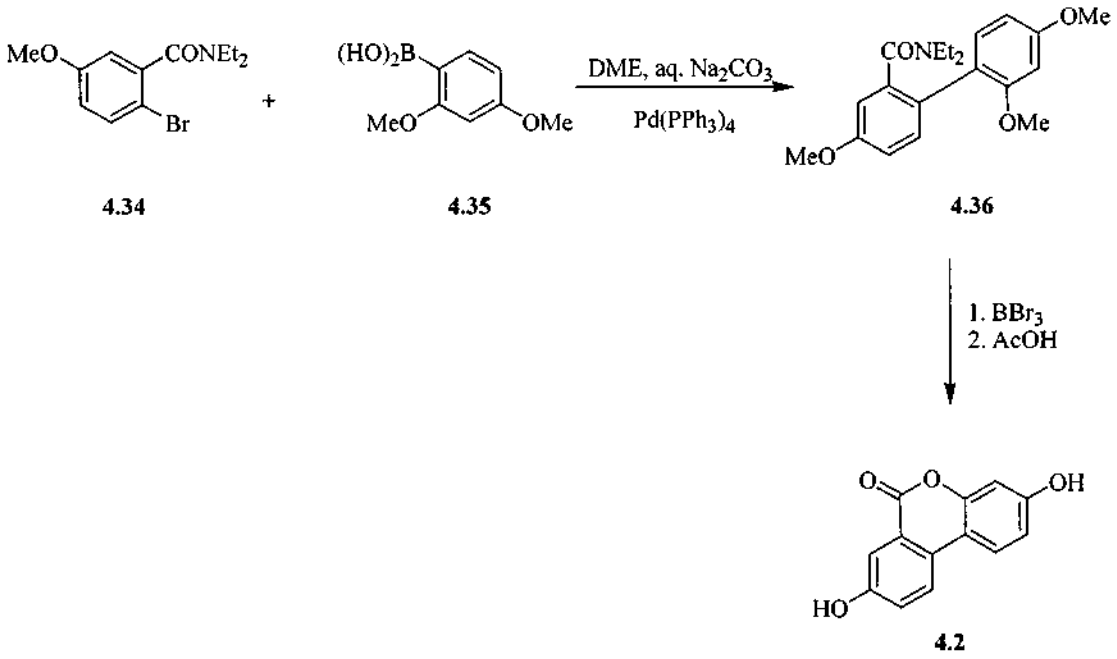
Pandey *et al.*[23] have recently used Hurtley's condensation methodology[21] to synthesize urolithin A (**4.2**) and other substituted benzopyrones. Condensation of resorcinol (**4.32**) with 2-bromo-5-hydroxybenzoic acid (**4.33**) gave urolithin A (**4.2**) in good yield (Scheme 4.6).



4.33 **4.32** **4.2**

SCHEME 4.6

Alo *et al.*[24] used a modified Suzuki cross-coupling to form a C-C biaryl bond in the synthesis of urolithin A (**4.2**) (Scheme 4.7). The aryl bromide **4.34** was subjected to modified Suzuki cross-coupling conditions with boronic acid **4.35** to give a biaryl derivative **4.36**. Demethylation of **4.36** with excess BBr_3 followed by acetic acid mediated cyclization afforded urolithin A (**4.2**) in 89% yield.



4.34 **4.35** **4.36** **4.2**

SCHEME 4.7

4.5 METHODS FOR THE REDUCTIVE COUPLING OF ARYL HALIDES

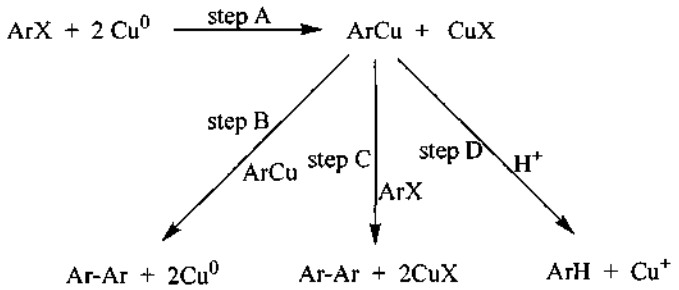
Ellagic acid derivatives often have a non-symmetrical biaryl structure. In general, the two methods that are the most often used for the C-C bond formation between two aryl rings are the Ullmann coupling and the Heck coupling reactions.

4.5.1 Ullmann coupling methodology

The Ullmann reaction was discovered and first reported by Ullmann and Bieleckie in 1901 and is employed to generate a C-C bond between two aromatic nuclei.[25] Two molar equivalents of aryl halide are reacted with one equivalent of finely divided copper at a high temperature (above 200 °C) to form a biaryl and a copper halide.

The Ullmann biaryl synthesis involves an organocopper reaction intermediate (Scheme 4.8). Its mechanism first involves the formation of an aryl copper intermediate (step A), where electrons are donated from copper to an aryl halide and electron-withdrawing groups on the aryl should stabilize this transition state. A good yield of unsymmetrical biaryls can

be obtained from this reaction if an activated and an unactivated aryl halide at temperatures too low for the latter halide alone to react (steps B and C). It is also evident that a proton source would suppress the Ullmann reaction and lead to reductive dehalogenation of the aryl halide (step D).



SCHEME 4.8

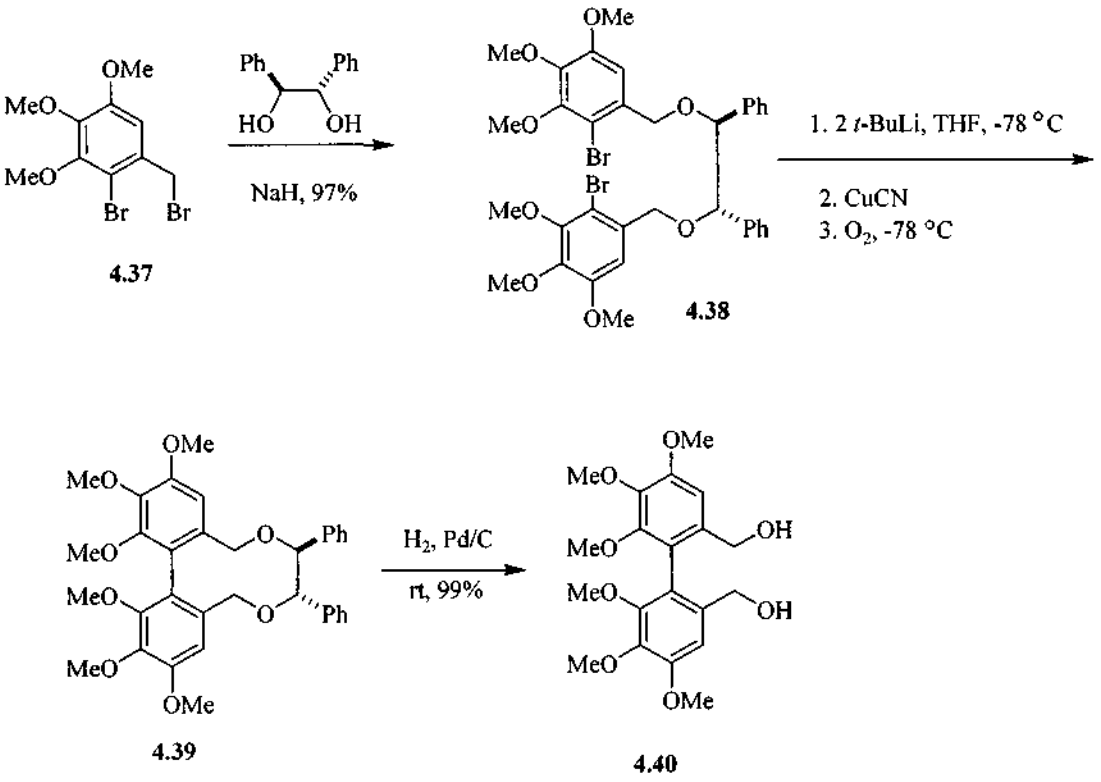
Normally intramolecular Ullmann coupling of an aryl halide in the presence of activated copper at high temperatures will give a symmetrical biaryl. Dimethylformamide is a solvent that permits the use of lower temperatures and a lower molar proportion of copper. When an Ullmann coupling reaction is applied to two different aryl halides, the reaction rarely works well to give the unsymmetrical biaryl in good yield because of the formation of the symmetrically-coupled products which are difficult to separate from the desired one.

Therefore, the use of disposable tethers will set the stage for the intramolecular Ullmann coupling of unsymmetrical alkyl halides and once the reaction has been conducted, the tether, having served its purpose, is removed.[26] The primary requirements for the choice of a disposable tether are:

- that the linkage should be assembled by straight-forward synthetic transformations from readily-available components
- that the intramolecular reaction precursor should be stable to purification and subsequent reaction conditions
- that the temporary tether should be susceptible to easy and selective cleavage.

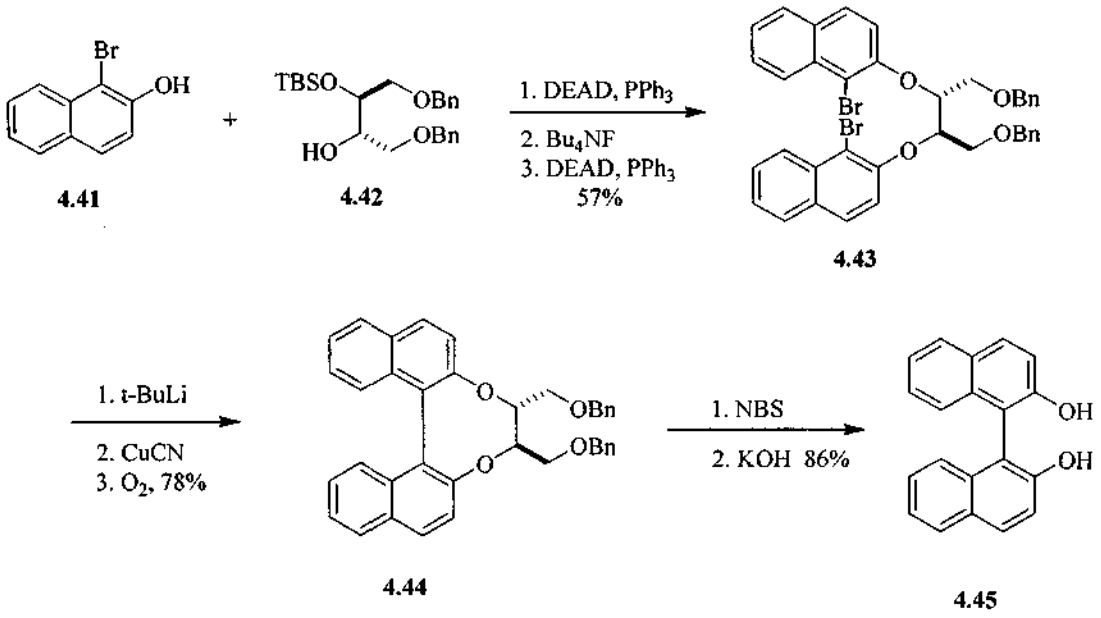
This approach was applied in the synthesis of (+)-*O*-permethyltellium II (**4.40**) in which the key challenge was the preparation of an axially-chiral, non-racemic biaryl.[27] The bis coupling of benzyl bromide **4.37** to (-)-hydrobenzoin afforded dibromide **4.38** in 97%

yield. This was followed by subsequent metal-halogen exchange, CuCN addition and exposure of the reaction mixture to oxygen to yield **4.39**. Debenzylation to remove the tether led to the required product, **4.40** (Scheme 4.9).



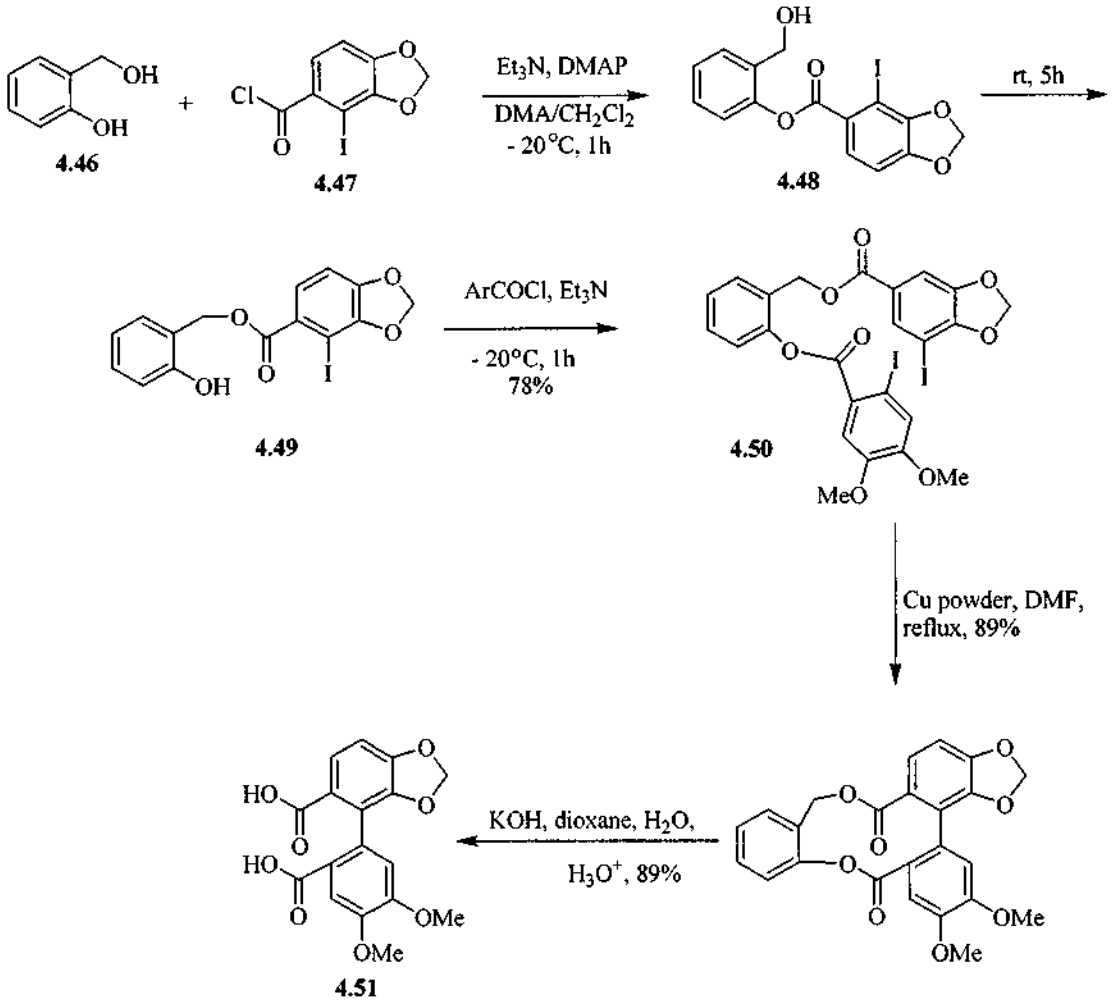
SCHEME 4.9

Lipshutz *et al.*[28] have used tartrate-derived tethers to prepare optically-pure binaphthyls (Scheme 4.10). The approach utilizes 1-bromo-2-naphthol (**4.41**) with a tartrate derivative **4.42** as the tether. Consecutive Mitsonubo reactions of **4.41** with **4.42** yielded **4.43**. Treatment of **4.43** with *t*-BuLi followed by reaction with CuCN, and exposure to oxygen led to enantiomerically pure binaphthol derivative **4.44**. Tether cleavage was achieved by double benzylic oxidation with NBS followed by addition of aqueous KOH to afford **4.45**.



SCHEME 4.10

To effect a coupling reaction of two unsymmetrical aryl iodides, Takahashi *et al.*[29,30] used salicyl alcohol as a disposable tether (Scheme 4.11). The method capitalizes on the differing reactivity of the phenolic and benzyl hydroxy groups. Salicyl alcohol **4.46** was treated with one equivalent of aryl acid halide **4.47** to give an aryl ester **4.48** which rearranges at ambient temperature to benzyl ester **4.49**. The resulting phenol was treated with an aryl acid halide to give the diester **4.50**. The diester was subjected to Ullmann coupling conditions and subsequent ester hydrolysis removed the tether giving the unsymmetrical diphenic acid **4.51**.

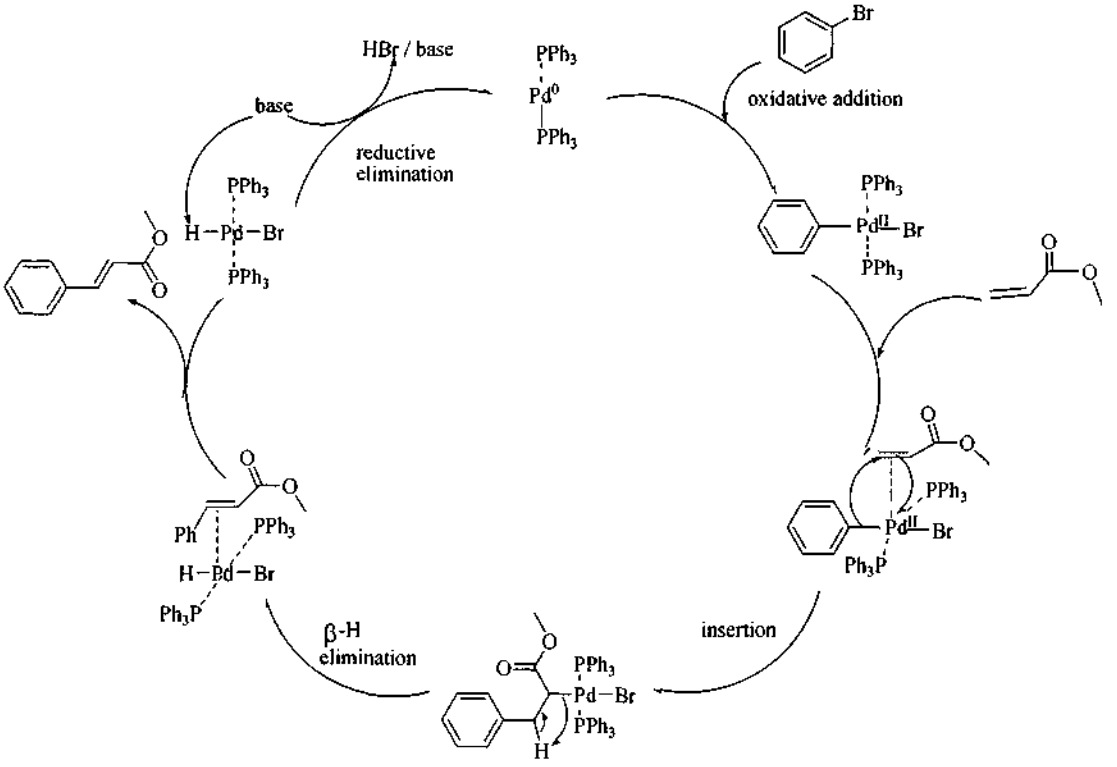


SCHEME 4.11

4.5.2 Heck coupling methodology

The Heck reaction is a palladium-catalyzed C-C coupling reaction between aryl halides or vinyl halides and alkenes in the presence of a base.[31] A number of palladium catalysts are used in the reaction, which are either sources of palladium(0) such as $Pd(PPh_3)_4$, $Pd(dba)_2$ and $Pd(dba)_3$ or sources of palladium(II) such as $Pd(OAc)_2$ and $PdCl_2(MeCN)$. Palladium(0) is the catalytic species in this reaction and when a palladium(II) complex is used, the active palladium(0) species is formed by *in situ* reduction, normally by reduction with PPh_3. A wide range of high-boiling point solvents such as DMF, DMA and toluene are often used. Inorganic bases such as NaOAc and K_2CO_3 are used with polar solvents to

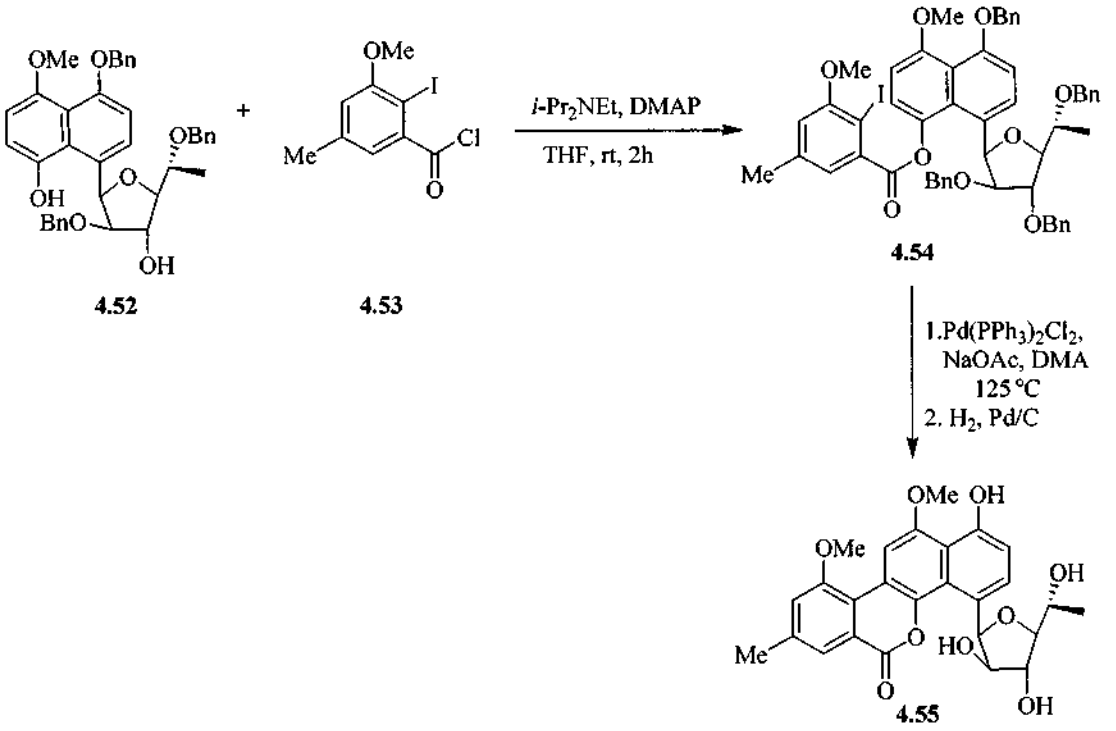
achieve homogeneity. The proposed mechanism for the reaction between an aryl bromide and an activated alkene is outlined in Scheme 4.12.[32]



SCHEME 4.12

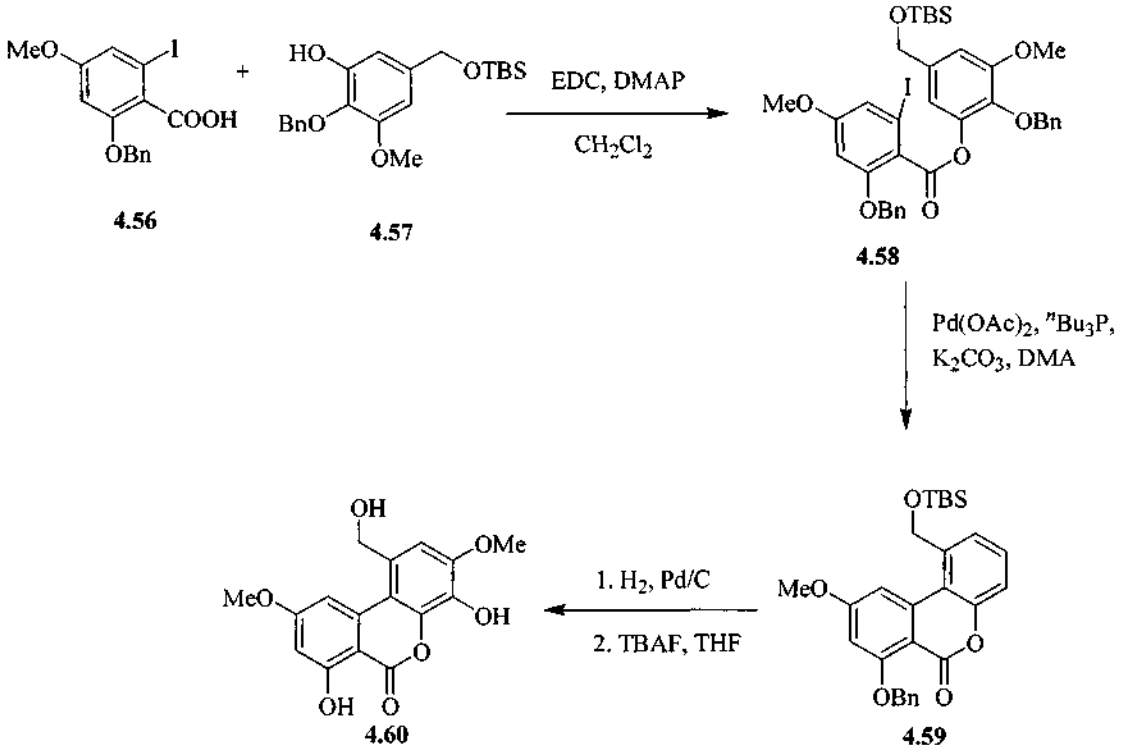
The intramolecular Heck reaction has been established as a powerful tool for the construction of complex polycyclic ring systems in natural product synthesis.[33]

A palladium-catalysed Heck coupling reaction was used to form an intramolecular biaryl in the synthesis of a gilvocarsin metabolite of a *Streptomyces sp.*[34] (Scheme 4.13). In the synthesis of (-)-gilvocarsin M (**4.55**), acylation of **4.52** with acid chloride **4.53** gave an ester **4.54**. Treatment of the ester with $Pd(PPh_3)_2Cl_2$ and sodium acetate in DMA at 125 °C to effect the intramolecular biaryl coupling followed by hydrogenolysis resulted in the formation of **4.55**.



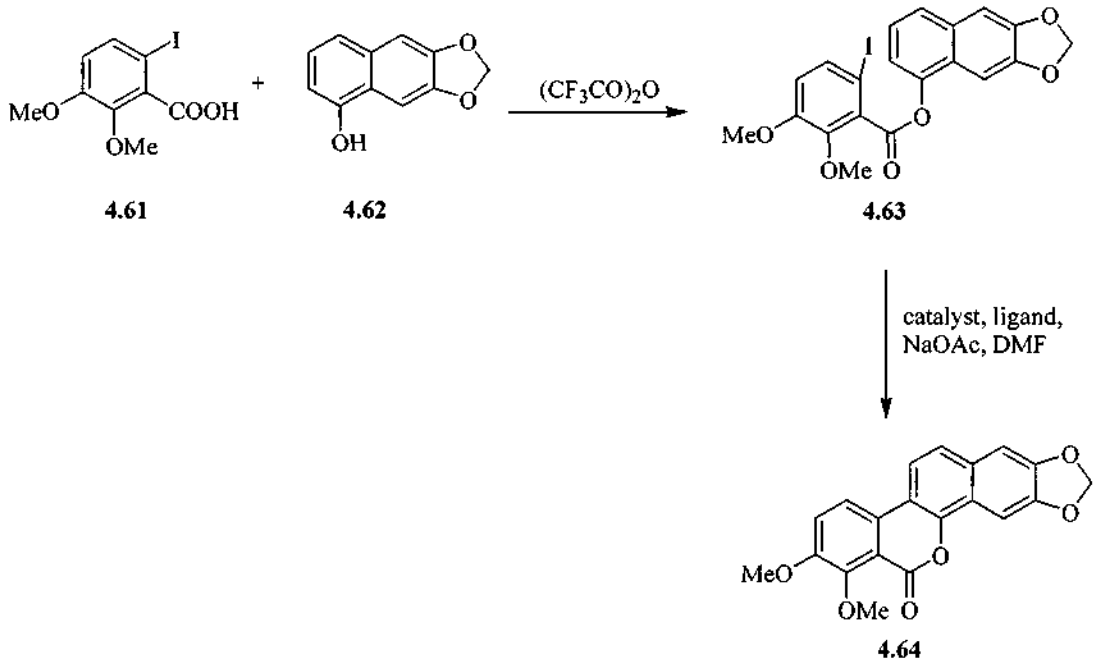
SCHEME 4.13

Graphislactone C (**4.60**) was prepared by Abe *et al.*[35] by a palladium-mediated biaryl coupling reaction of phenyl benzoate derivative **4.58** (Scheme 4.14). Esterification of phenol **4.57** with an acid **4.56** using EDC and catalytic amount of DMAP afforded phenyl benzoate **4.58** in 65% yield. Intramolecular biaryl coupling reaction of **4.58** with subsequent removal of the protecting groups produced **4.60**.



SCHEME 4.14

Harayama and Yasuda[36] synthesized arnottin 1 (**4.64**) isolated from *Zanthoxylum arnottianum* (Rutaceae) via intramolecular biaryl coupling reaction using a palladium reagent (Scheme 4.15). An ester **4.63** was prepared from an acid **4.61** and a naphthol **4.62** using trifluroacetic anhydride. The palladium-assisted internal biaryl coupling reaction of **4.63** resulted in the cyclised product **4.64** in a high yield. To improve the yield the authors examined a variety of Pd(II) reagents with or without a ligand (Table 4.1). From the table it can be seen that better yields can be obtained at high temperature.



4.61 4.62 4.63 4.64

SCHEME 4.15

TABLE 4.1

run	catalyst	equiv.	ligand	time	temp.	Yield of **4.64** (%)
1	$Pd(PPh_3)_2Cl_2$	0.1	—	5 h	130 °C	52
2	$Pd(acac)_2$	0.1	PPh_3	4 h	130 °C	56
3	$Pd(acac)_2$	0.1	PPh_3	2 h	150 °C	72
4	$Pd(PPh_3)_4$	0.1	—	3.5 h	130 °C	58
5	$Pd(PPh_3)_4$	0.1	—	2 h	150 °C	71

In conclusion, it can be seen from the literature review that both the Ullmann coupling and the Heck coupling reactions can be applied in the formation of the crucial biaryl bond of ellagic acid derivatives.

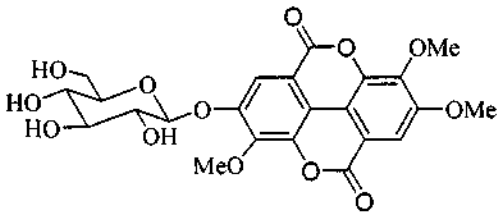
4.6 RESULTS AND DISCUSSION

4.6.1 Retrosynthetic analysis

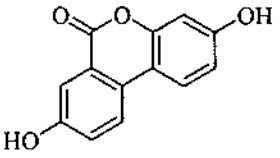
The aim of this project was to synthesize

(i) a non-symmetrical ellagic acid derivative that can be used for the preparation of **3.19**

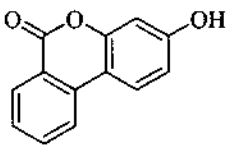
(ii) urolithins derivatives



3.19



4.2: Urolithin A



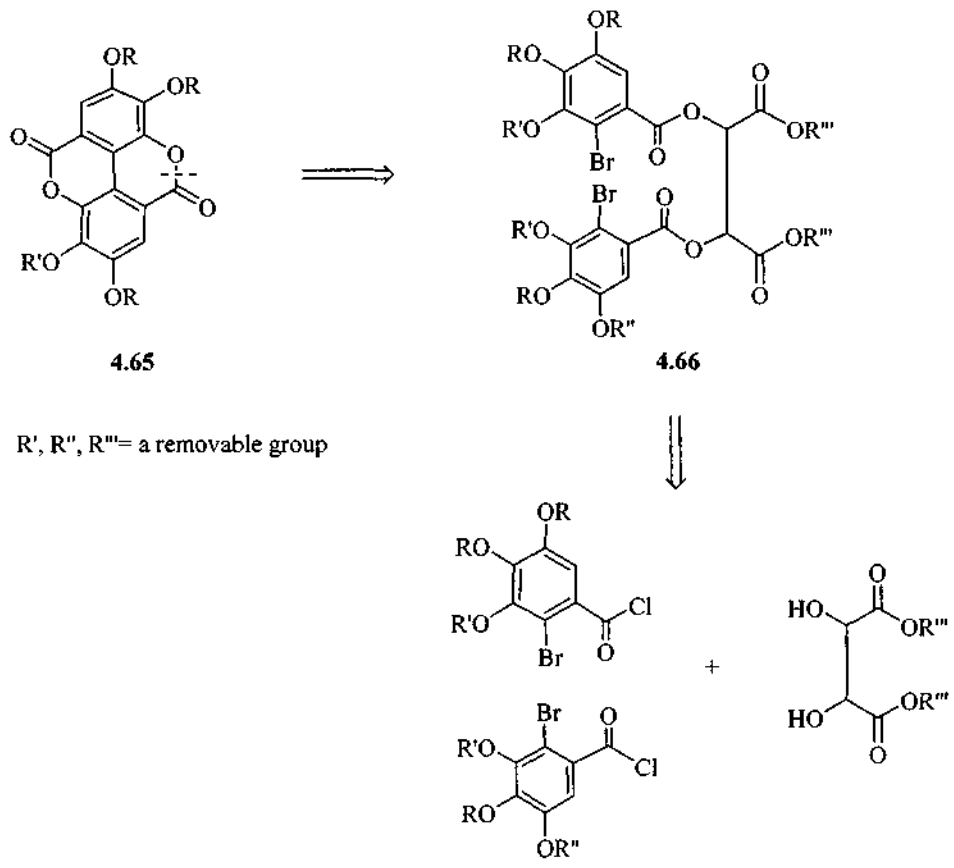
4.3: Urolithin B

We will first discuss the synthesis of the ellagic acid derivatives. Two synthetic routes were followed - one that relies on the Ullmann reaction and the second one that uses the Heck coupling reaction and these two routes will be discussed separately.

As can been seen from our literature overview (§ 4.5.1), the best way to prepare an unsymmetrical ellagic acid derivative is to use a disposable tether. In our initial approach we have chosen the readily-available *meso*-tartaric acid as the tether. The starting benzoic acids needed will depend on whether an Ullmann or a Heck coupling reaction is used. An Ullmann reaction requires two aryl halides, whereas a Heck reaction requires an aryl halide and an unsubstituted aryl as precursors. In both the proposed reactions, aryl bromides are more reactive substrates than aryl chlorides. Although aryl iodes are more reactive that aryl bromides, these substrates are not easily accessible and therefore we concentrated on the use of aryl bromides as substrates in our reactions.

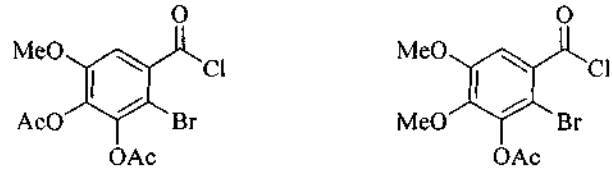
4.6.2 Preparation of starting materials for the Ullmann coupling reaction

A retrosynthetic analysis for this route using an Ullmann coupling is given in Scheme 4.16.

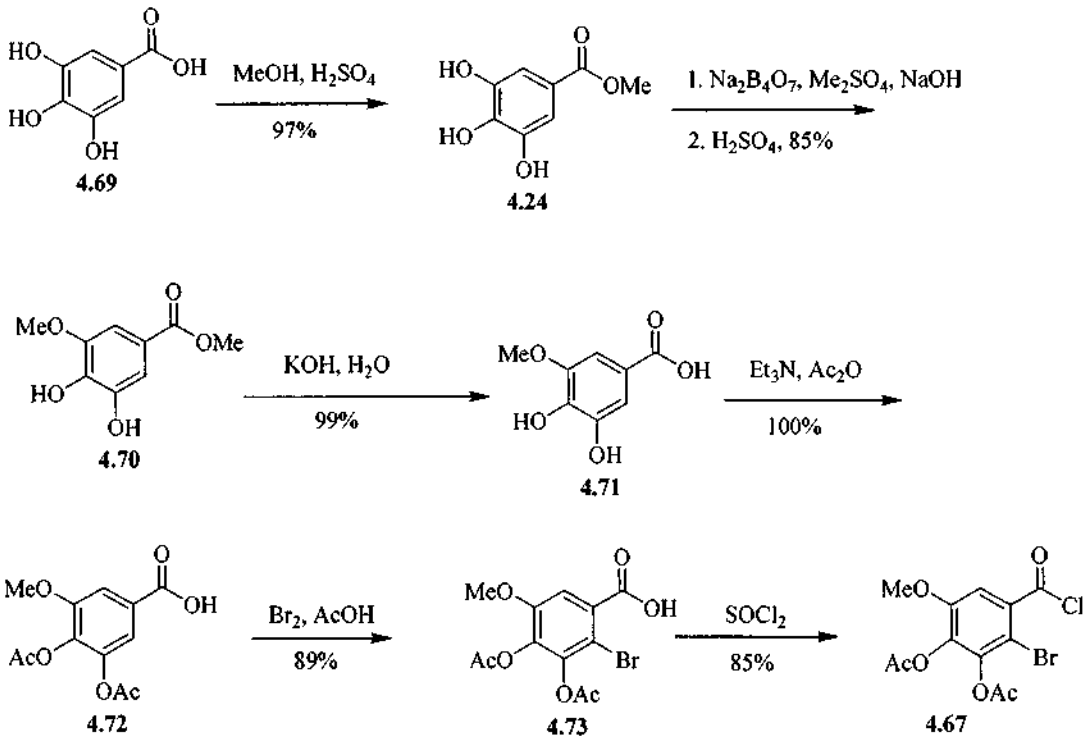


SCHEME 4.16

For the esterification with diethyl tartrate, suitably protected gallic acid derivatives were required. At this stage we have used an acetate as a protecting group and therefore, our first task was to prepare **4.67** and **4.68**.

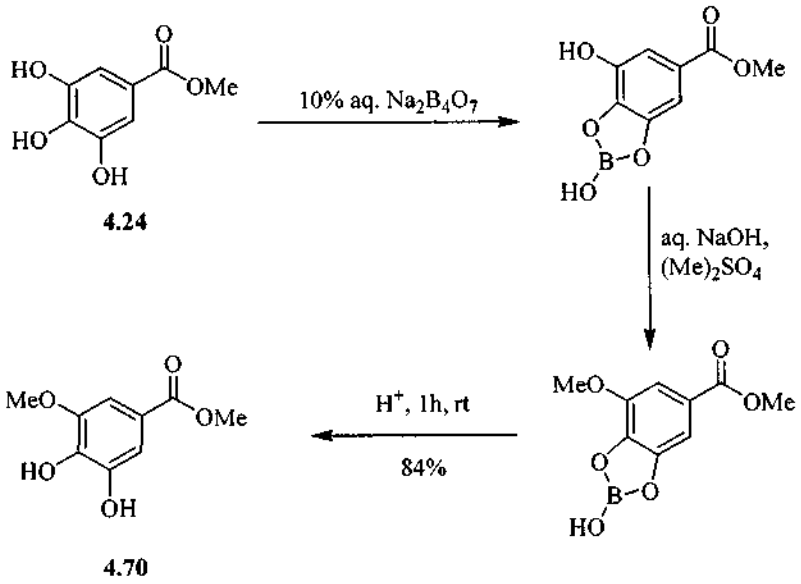


The synthesis of 3,4-diacetoxy-2-bromo-5-methoxygallic acid chloride (**4.67**) is outlined in Scheme 4.17.



SCHEME 4.17

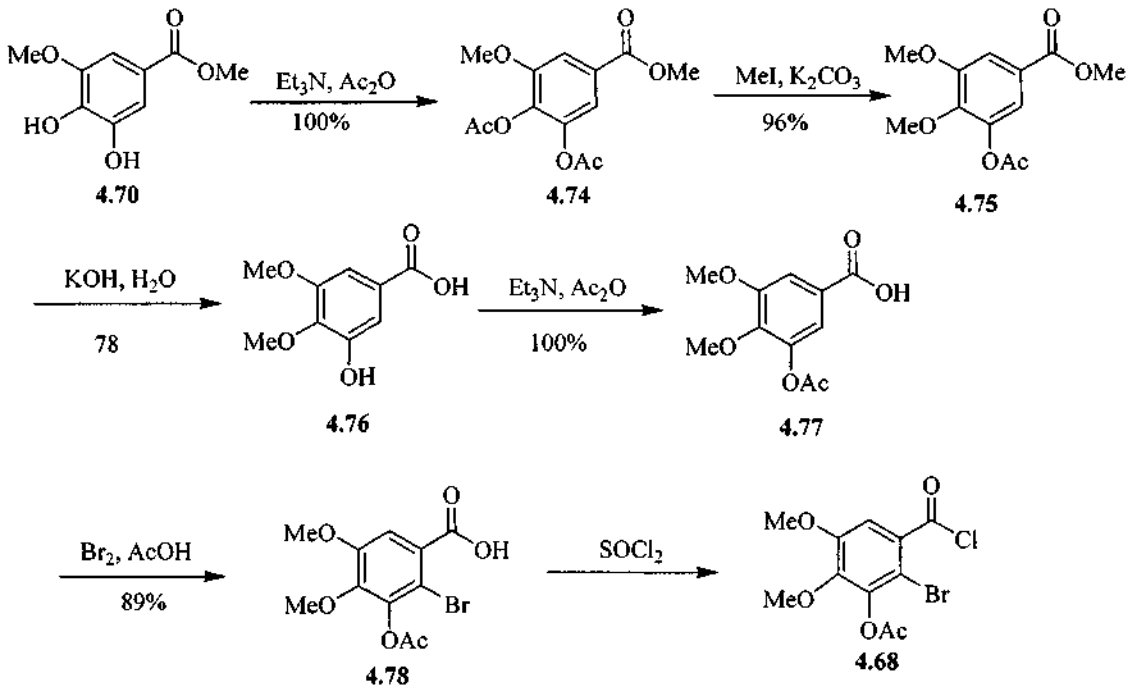
Acid-catalyzed esterification of gallic acid (**4.69**) yielded methyl gallate (**4.24**). Monoalkylation of a gallic acid esters is possible by the *in situ* protection of two *ortho*-hydroxy groups as a borate ester, which leaves the third hydroxy group free for reaction (Scheme 4.18).[37,38] Using this methodology with dimethyl sulfate as the alkylation reagent, we were able to prepare monomethyl ether **4.70**.



SCHEME 4.18

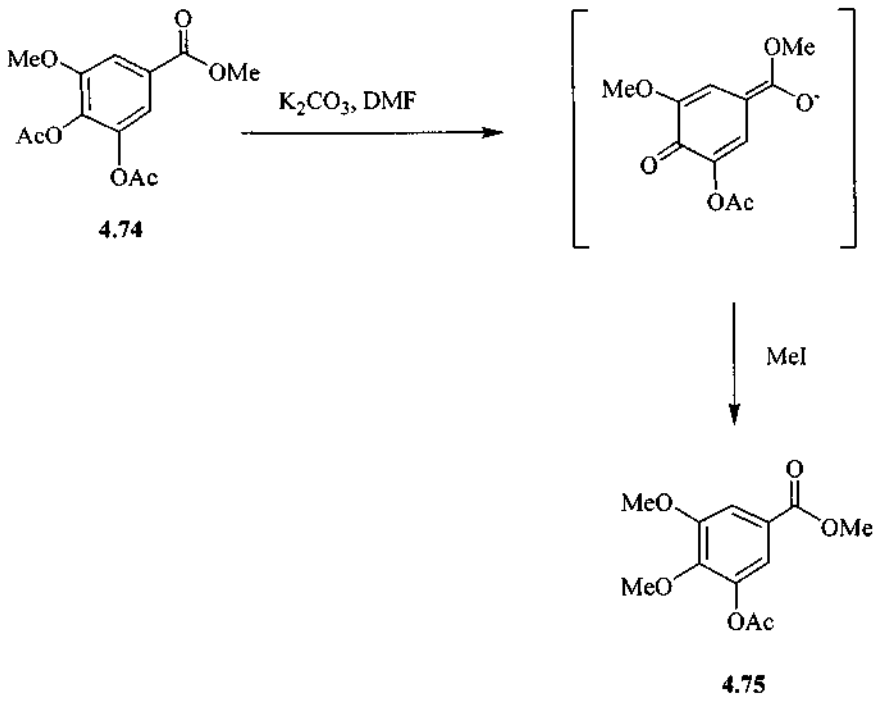
Hydrolysis of the methyl ester **4.70** was followed by acetylation of the free hydroxy groups to yield diacetate **4.72** in quantitative yield. The formation of a monobrominated product was required in this synthesis. In our hands, a mixture of the mono- and dibrominated products was obtained if gallate esters were used as substrates. However, with free gallic acid derivatives as substrate, the exclusive formation of the monobrominated product was possible. Selective bromination of **4.72** afforded **4.73** in 89% yield. The ^{1}H NMR spectrum of **4.73** confirmed the presence of one aromatic proton, one methoxy groups and two acetyl groups. In the ^{13}C NMR spectrum the methoxy group on C-5 resonated at δ_C 57.1 and the two methyl groups of the acetyl appeared at δ_C 19.9 and 20.1. The characteristic carbonyl signal of the acid was observed at δ_C 168.4. To confirm the position of the bromo substituent, an NOE experiment was performed. The results showed a clear NOE effect between the methoxy protons and the aromatic proton, thereby confirming the position of the bromine.

The acid chloride **4.67** was obtained by refluxing **4.73** in $SOCl_2$ and after removal of the excess thionyl chloride, this acid chloride was used immediately in the next step without purification.



SCHEME 4.19

The preparation of the second acid chloride **4.68** is summarized in Scheme 4.19. Our starting material was the monomethylated gallic ester **4.70**. Although the 4-hydroxy group of **4.70** is more reactive than the 3-hydroxy group, selective monomethylation was not possible and, therefore, a method developed by Zhu *et al.*[39,40] and Pearson[41] on selective alkylation of the 4-OH group of gallic acid was followed. This approach involves heating the diacetate in DMF at 40 °C in the presence of K_2CO_3 and MeI to give exclusively the 4-methoxylated compound. The proposed mechanism for this step is given in Scheme 4.20.



4.74 **4.75**

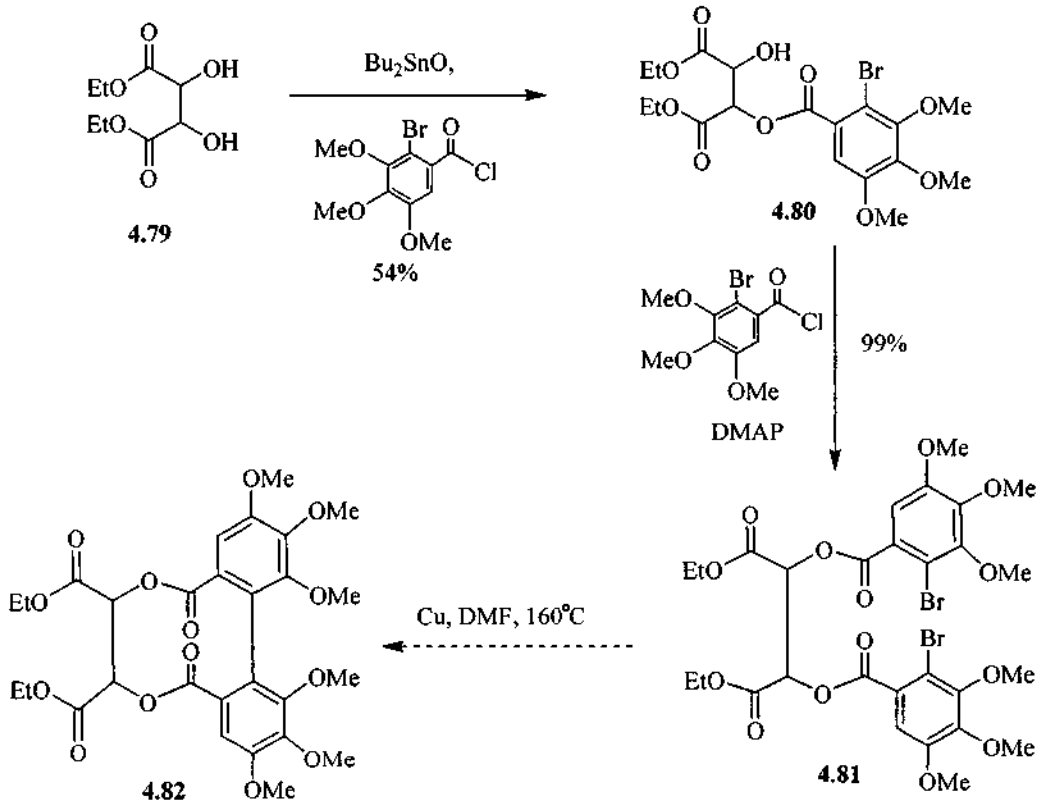
SCHEME 4.20

Hydrolysis of methyl ester followed by acetylation of the 3-hydroxy group and selective bromination at position 2 afforded **4.78** in 67% yield (Scheme 4.19). The ^{1}H NMR spectrum of **4.78** confirmed the presence of one aromatic proton, two methoxy groups and one acetyl group. In the ^{13}C NMR spectrum the two methoxy groups at C-4 and C-5 resonated at δ_C 56.1 and δ_C 61.0, one methyl of acetyl group appeared δ_C 20.4. The characteristic carbonyl signal of the acid was observed at δ_C 168.0. The acid chloride **4.68** was obtained by refluxing **4.78** in $SOCl_2$ and after evaporation of the excess of thionyl chloride, the acid chloride was used immediately in the next step without further purification.

4.6.3 Ullmann coupling reactions

In order to obtain a monoacylated tartrate, a tin-mediated acylation reaction was used.[42,43,44] Several steps are involved in the preparation of the above starting materials **4.67** and **4.68**. (Schemes 4.18 and 4.19) and, therefore, to save starting material we have decided to use a model reaction first to test the effectiveness of our chosen disposable

tether, *meso*-diethyl tartrate (**4.79**). 2-Bromo-3,4,5-trimethoxybenzoyl chloride was obtained by bromination of commercially-available 3,4,5-trimethoxygallic acid followed by treatment of the product with thionyl chloride. Tin-mediated acylation of diethyl tartrate allowed the formation of the monoacylated alcohol **4.80** in 54% (Scheme 4.21). The ^{1}H NMR spectrum confirmed the structure of **4.80**. The two backbone protons of the tartrate moiety had different chemical shifts - one of the protons appeared as a doublet at δ_H 5.61 (J = 2.4) caused by the anisotropic effect of the attached acyl carbonyl hydroxy group and the other as a broadened singlet at δ_H 4.77. In the ^{13}C NMR spectrum the carbon carrying a hydroxy group resonating at δ_C 70.3 and the one carrying acyl group appeared at δ_C 73.6. The characteristic carbonyl signal of the ester was observed at δ_C 164.1.



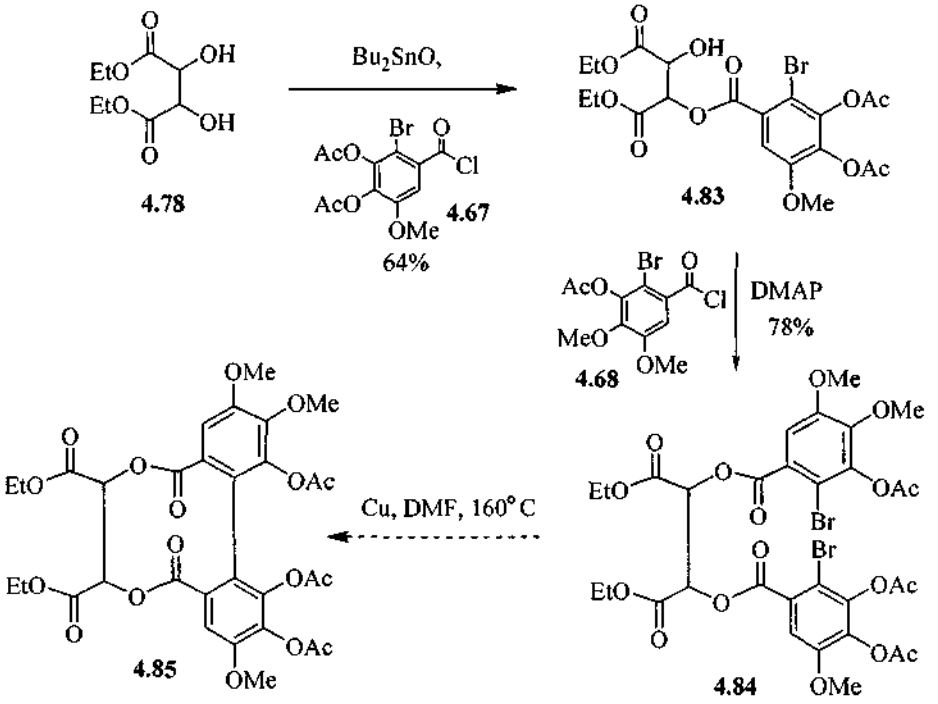
SCHEME 4.21

Subsequent acylation of the free hydroxy group of **4.80** using the same benzoyl chloride in the presence of a catalytic amount of DMAP afforded **4.81**. The ^{1}H NMR spectrum confirmed the presence of diacylated product and it was observed that the two protons in the tartrate moiety now overlap and both appear as a singlet resonated at δ_H 5.99. Since the

compound is symmetrical, only one set of signals representing half the molecule was observed in both the ^{1}H and ^{13}C NMR spectra.

The diester **4.81** was subjected to Ullmann conditions (activated copper in DMF at 160 °C), but no biaryl product could be isolated.

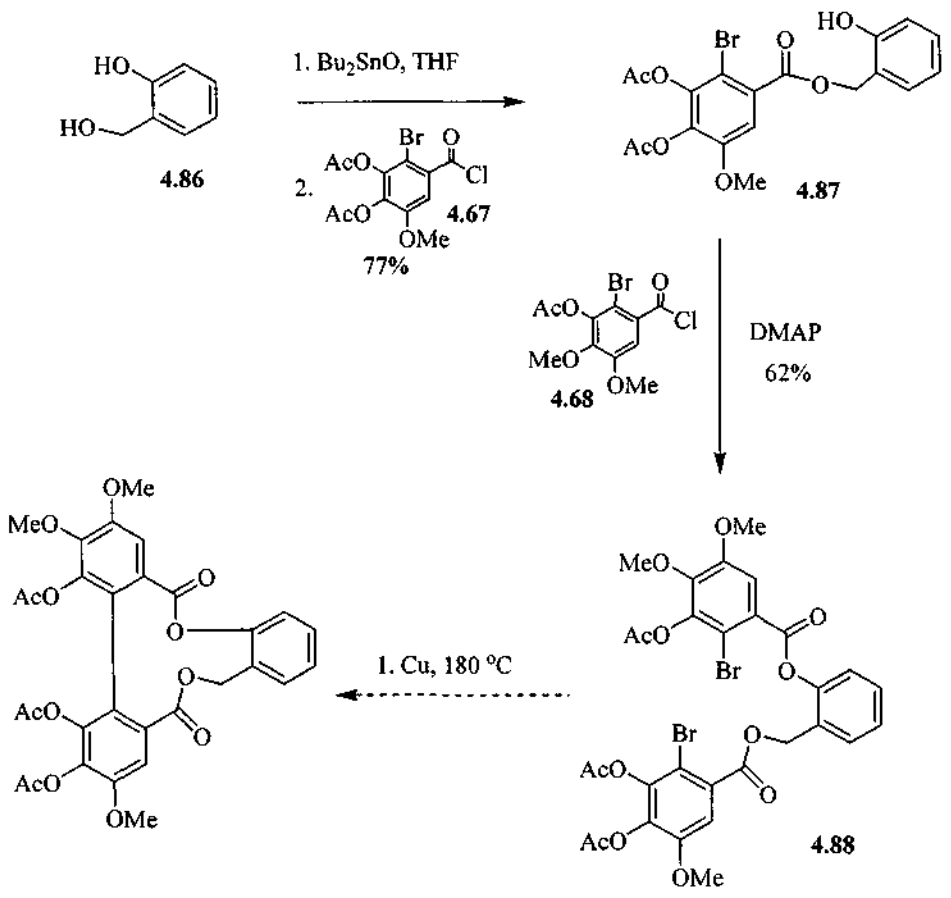
In another attempt to test the effectiveness of the disposable tether under Ullmann conditions, it was decided to use the synthesized aryl halides **4.67** and **4.68** in the assembly of an unsymmetrical biaryl (Scheme 4.22). A monoacylated alcohol **4.83** was obtained by treating diethyl tartrate **4.78** with dibutyltin oxide and subsequent acylation with an acid chloride **4.67**. Compound **4.83** was subjected to esterification with an acid chloride **4.68** and a catalytic amount of DMAP to afford diester **4.84**. The ^{1}H NMR spectrum is in agreement with the presence of the diester. Three acetoxy signals, three methoxy signals and two singlets in the aromatic region were observed in the spectrum. In the ^{13}C NMR spectrum, a total of 12 carbons in the aromatic regions were observed suggesting the presence of two non-identical aromatic rings. Compound **4.84** was also subjected to Ullmann conditions but once again no biaryl product was observed.



SCHEME 4.22

These results confirm that diethyl tartrate may be useful as a tether since it allow the suquential formation of two different aryl esters. However, with aryl bromides and under the reaction conditions that we have followed, the formation of a diaryl bond is not a viable process.

It was decided to change the tether to salicyl alcohol as it had receive attention in the literature.[28] Following a published method,[29] salicyl alcohol (**4.86**) was treated with benzoyl chloride **4.67** in the presence of DMAP. However, we obtained only the diacylated product under these conditions. Therefore, we decided to use the tin-mediated procedure to obtain the monoacylated product. Treatment of salicylalcohol with dibutyltin oxide[44] in THF followed by the addition of benzoyl chloride **4.67** yielded monoester **4.87** in 77% yield (Scheme 4.23). The ^{1}H NMR spectrum is in agreement with the presence of the monoester as two acetoxy (δ_H 2.35, 2.29), one methoxy (δ_H 3.84), and five aromatic protons were observed. The benzylic proton resonances appear at δ_H 5.39, which is in agreement with the chemical shift expected for an acylated benzyl alcohol.[42] In the ^{13}C NMR only three carbonyl carbon signals were observed of which two at δ_C 166.8 and 166.9 can be attributed to the two acetoxy carbonyl carbons and the one at δ_C 167.4 attributed to an ester linker carbonyl carbon, thus confirming that **4.87** is indeed a monoester. According to the literature, [30] the more reactive phenol will be acylated first at low temperature and, upon standing at room temperature, the product will rearrange to form the more stable benzylic ester. We have conducted the tin-mediated acylation reaction at room temperature and only observed the formation of the more stable benzylic ester.



SCHEME 4.23

Addition of acid chloride **4.68** to monoester **4.87** in the presence of catalytic amount of DMAP afforded diester **4.88** in 62% yield. The ^{1}H NMR spectrum confirmed the presence of the diester, three signals in the acetoxy region, three signals in the methoxy region and two singlets in addition to other aromatic resonances were observed. In the ^{13}C NMR spectrum, a total of 18 carbons in the aromatic region suggested the presence of two unsymmetrical aromatic rings with a salicyl tether.

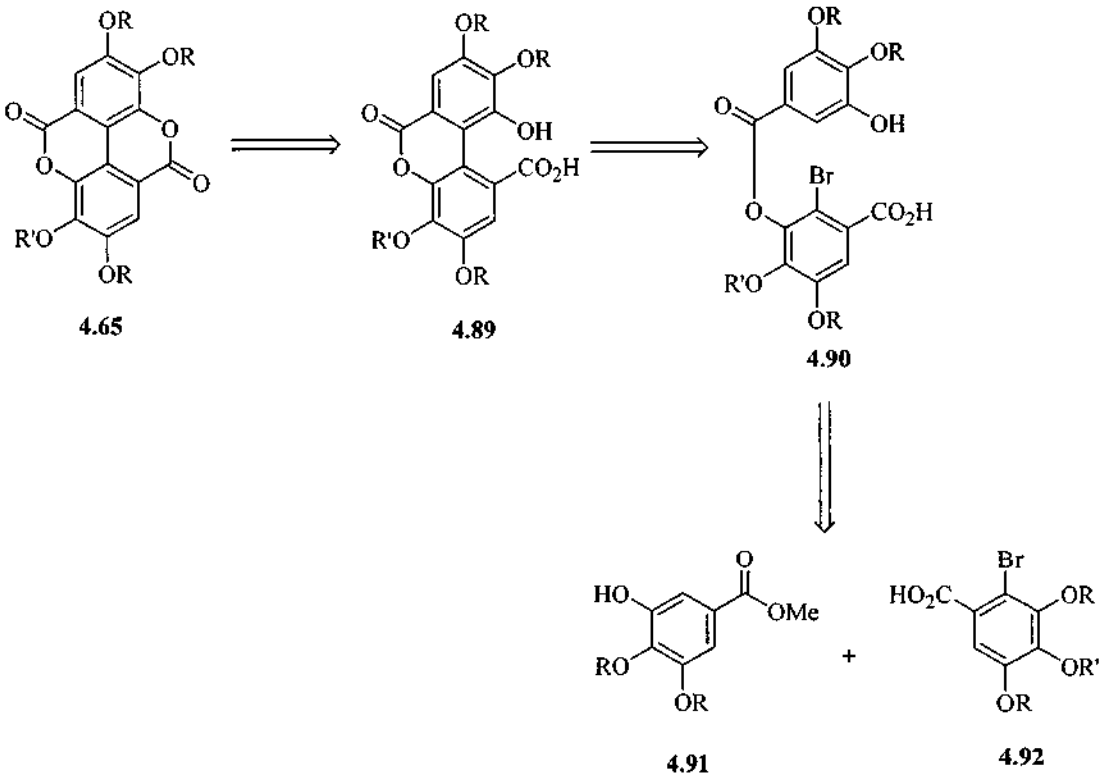
Once again, no coupled product was detected when an intramolecular Ullmann coupling reaction was attempted. Ullmann reactions notoriously require very high temperatures for a reaction to occur and it is possible that the temperature (160 °C) that we have used was not high enough. With the equipment available to us, we were unable to achieve higher temperatures. Our precursors in the attempted Ullmann reactions were aryl bromides. From the results obtained here, we can conclude that aryl bromides are not reactive enough

to be viable starting materials for the synthesis of ellagic acid derivatives by using Ullmann reactions.

4.6.4 Heck coupling reactions

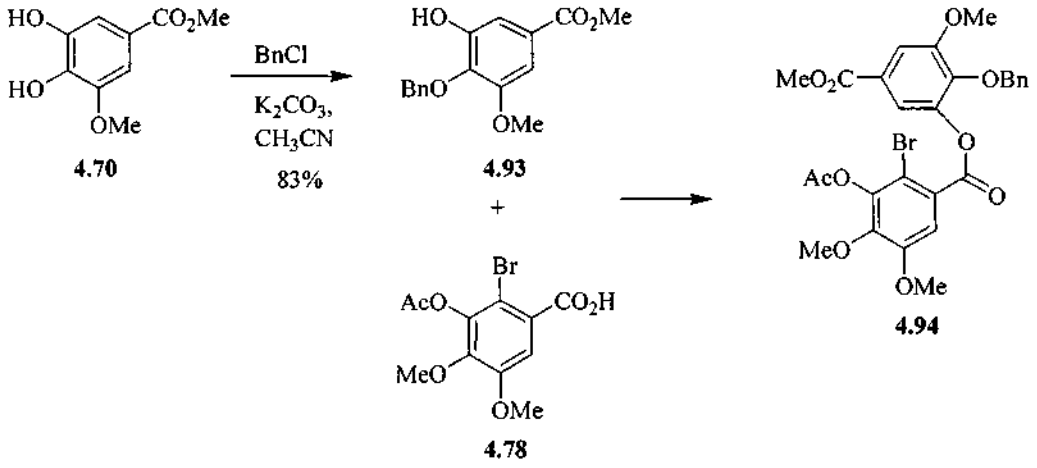
Our second approach to the synthesis of ellagic acid derivatives was based on Heck coupling methodology. This approach requires the preparation of carboxylic esters for the intramolecular biaryl formation between two non-identical aryl rings.

The proposed retrosynthetic analysis for the preparation of ellagic acid derivative **4.65** is given Scheme 4.24. The required product **4.65** can be obtained by intramolecular esterification of lactone **4.89**. The unsymmetrical biaryl **4.89** can be accessed by intramolecular C-C coupling using the Heck coupling reaction of ester **4.90** as substrate. The starting materials **4.91** and **4.92** were required for the preparation of intermediate **4.90**.



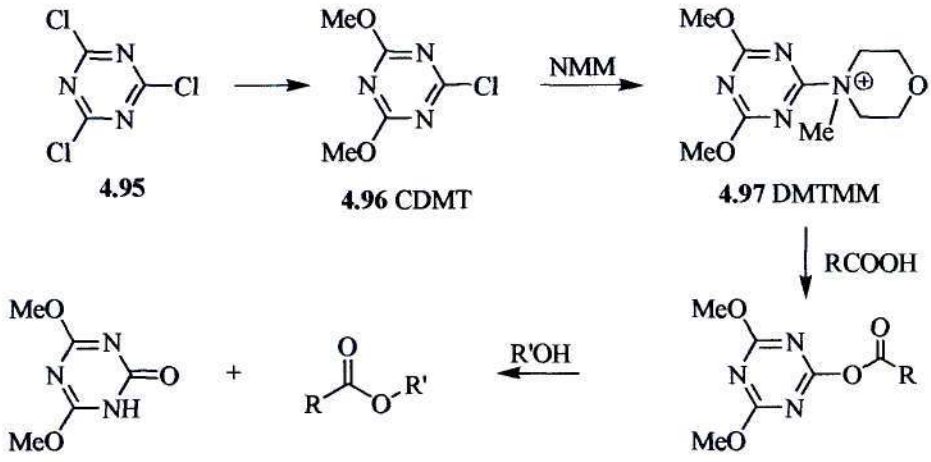
SCHEME 4.24

Our first target was the preparation of ester **4.94** (Scheme 4.25). The preparation of carboxylic acid **4.78** has been described in Scheme 4.19. Phenol **4.93** was prepared by the selective benzylation of methyl 3-*O*-methylgallate (**4.62**). Selective benzylation of **4.93** was achieved with acetonirile as the solvent and at a lower temperature (40 °C) than is normally used for benzylation reactions.



SCHEME 4.25

The esterification of an electron-rich benzoic acid and an electron-rich phenol is not an easy task. Special activating reagents such as 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) (**4.96**) in the presence of N-methylmorpholine (NMM)[45] have been used for the preparation of such esters. In this reaction DMTMM (**4.97**), which is prepared from cyanuric chloride (**4.95**), activates the acid towards the nucleophilic attack by the alcohol (Scheme 4.26).



SCHEME 4.26

In an attempt to prepare the carboxylic ester **4.94** *in situ* by reaction between carboxylic acid **4.78** and phenol **4.93,** an unexpected novel triazine derivative was isolated when using the condensation reagent CDMT (**4.95**) in the presence of *N*-methylmorpholine. The structure of this compound was confirmed by X-ray crystallography (Fig. 4.1). By considering the structure of this product, it can be concluded that in the attempted preparation of the condensation reagent CDMT (**4.96**), we have formed the monomethoxy derivative **4.98**, and that this compound reacted with the phenol to give the undesired product **4.99**. (Scheme 4.27).

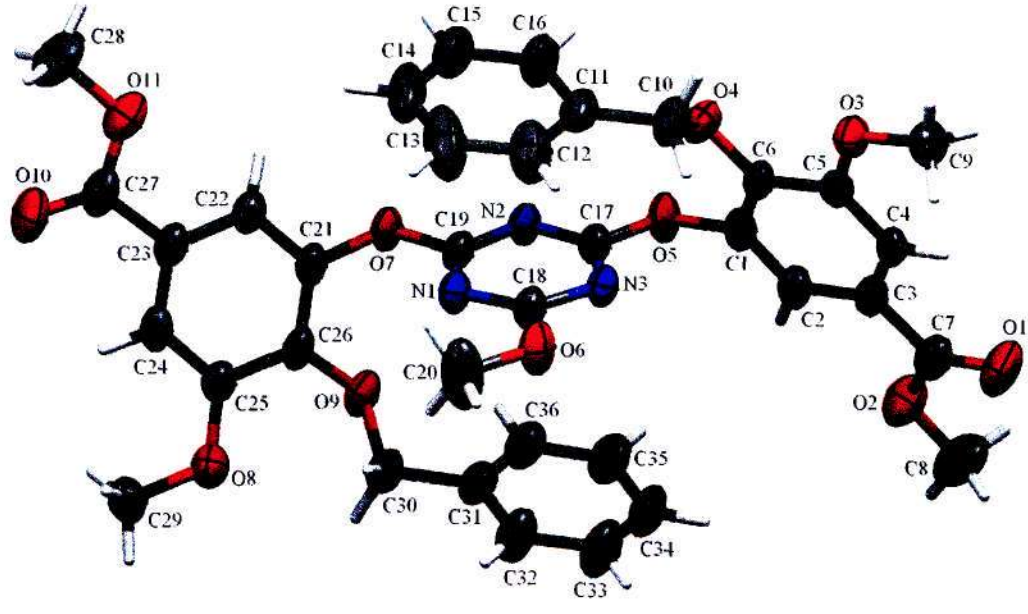
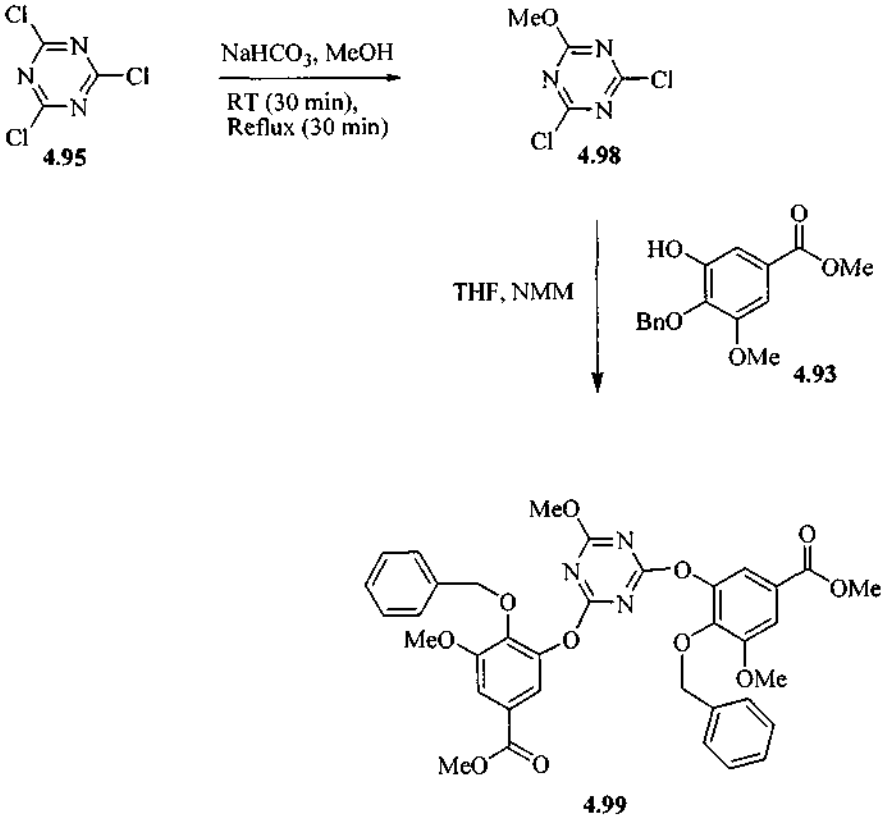


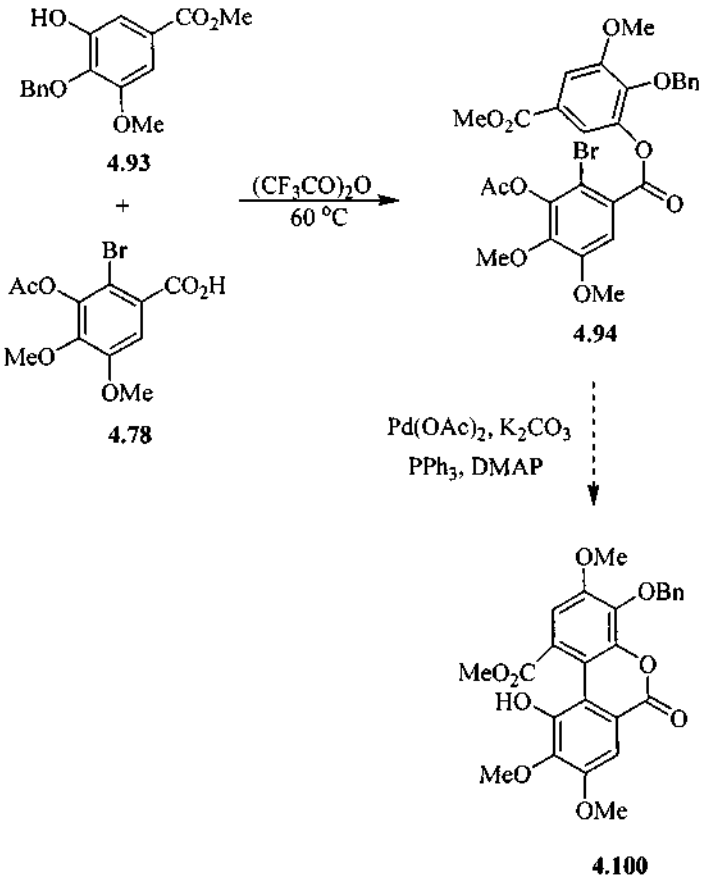
FIGURE 4.1 X-ray crystal structure of compound **4.99**



SCHEME 4.27

In an X-ray crystal structure of **4.99**, it is observed that the benzyloxy groups are positioned above and below the triazine ring of the molecule forming an intramolecular triple-decker sandwich (Fig 4.1). This compound maybe of interest since phenoxytriazine derivatives have been shown to form intriguing supramolecular structures when a suitable hydrogen-bonding substituent is present on the phenoxy ring.[46,47,48]

An ester **4.94** (72%) was prepared from acid **4.78** and phenol **4.93** using trifluoroacetic anhydride (Scheme 4.28). The attempted palladium-assisted intramolecular biaryl coupling of **4.94** resulted in the cleavage of a lactone and the starting materials (**4.78** and **4.93**) were formed. With the benzyl as a protecting group cleavage of the lactone while attempting biaryl formation, was also observed by other authors.[49]

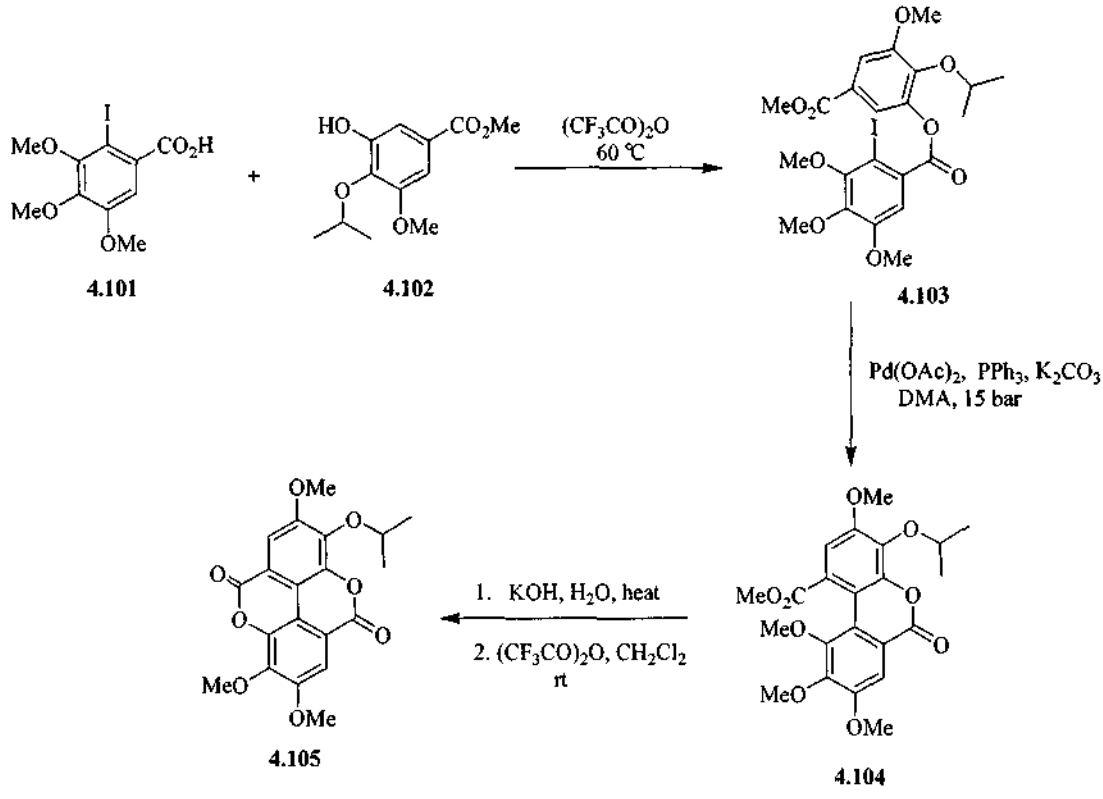


SCHEME 4.28

It was clear to us that, once again, aryl bromides were not reactive enough for these coupling reactions, and for our final reaction we changed to an aryl iodide (Scheme 4.29). Another important aspect that we have considered was the protecting groups in the ring and have decided to use an isopropyl group instead of a benzyl group.

2-Iodo-3,4,5-trimethoxygallic acid (**4.101**) was prepared by adding iodine to a solution of 3,4,5-trimethoxygallic acid and silver trifluoroacetate. Esterification with methyl 4-*O*-isopropyl-5-*O*-methylgallate (**4.102**) gave the required ester (**4.103**) in 92% yield. This ester (**4.103**) was then subjected to palladium-catalysed Heck coupling conditions first by stirring the mixture [ester (**4.100**), $Pd(OAc)_2$, PPh_3 and K_2CO_3] at 60 °C (15 bar) without solvent for 1 h and then with DMA as a solvent for another hour. The process resulted in the formation of the desired biaryl lactone **4.104** in 52% yield. In the ^1H NMR spectrum, two aromatic singlets were observed at δ_H 7.62 and 7.24. All five methoxy signals were well-resolved as compared to the methoxy resonances of ester **4.103**. In the ^{13}C NMR

spectrum, the characteristic carbonyl signals of the lactone was observed at δ_C 160.1 whereas the ester carbonyl resonates at δ_C 169.0.

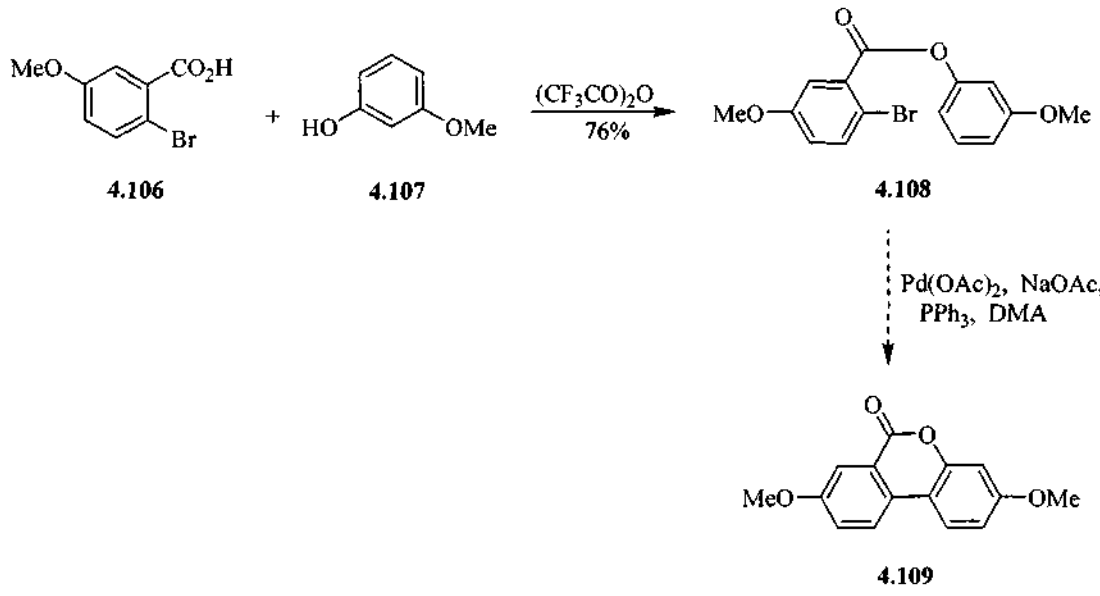


SCHEME 4.29

Hydrolysis of **4.103** in aqueous KOH followed by cyclization using trifluoroacetic anhydride afforded the required ellagic acid derivative **4.105** in quantitative yield. In the ^{1}H NMR spectrum of **4.105,** only three methoxy signals were observed, two overlapping aromatic protons appear at δ_H 7.6 and in the ^{13}C NMR spectrum, the two lactone carbonyl carbons can be observed (δ_C 158.9 and 159.0).

Synthesis of the punicalagin metabolite urolithin A was attempted using the Heck coupling reaction applied in the preparation of biaryl compound **4.104**. The synthesis began with an esterification reaction between 2-bromo-5-methoxybenzoic acid (**4.106**) and 3-methoxy phenol **4.107** to form **4.108** in 76% yield. Attempted intramolecular Heck coupling resulted in a 1:1mixture of products including the desired biaryl product **4.109**. We then thought that adjusting the reaction conditions and also using another palladium catalyst

might result in the formation of only the desired product. It was observed that changing the catalyst from $Pd(OAc)_2$ to $Pd(PPh_3)_2Cl_2$ and decreasing the reaction time from 1h to 30 minutes, the yield of the mixture remained the same but the ratio of the desired product to the undesired product was increased to 2:1.



4.106 **4.107** **4.108** **4.109**

SCHEME 4.30

4.7 CONCLUSION

In the synthesis of an ellagic acid derivative, we were faced with the challenge to accomplish biaryl formation of two unsymmetrical electron-rich rings. In that regard, the Ullmann and the Heck coupling reactions were efficiently explored and it was clear that the Heck coupling provide a better method for the formation of the desired product. The problem with the Ullmann coupling is the high temperature needed to achieve coupling. Furthermore aryl iodides are more reactive than aryl bromides, but the bromides are more accessible than iodides.

The success of the Heck coupling reaction led to the formation of a new ellagic acid derivative **4.105** which can be converted to the desired target molecule. We were able to differentiate between the hydroxy groups of gallic acid by selective protection reactions

and that provided entry to the preparation of unsymmetrical ellagic acid derivatives. The Heck coupling methodology also proved to be a promising method in the coupling of biaryls toward synthesis of the punicalagin metabolite urolithin A (**4.2**).

In this research project we have managed to characterize the secondary metabolites of an important South African medicinal plant and have developed an analytical method to fingerprint the crude extract of this plant.

We do propose that ellagic acid derivatives may play a role in the biological activity of this plant. Therefore, we have developed a methodology for the preparation of unsymmetrical ellagic acid derivatives

4.8 EXPERIMENTAL

4.8.1 General

All the required chemicals or reagents were obtained from FLUKA, ALDRICH or MERCK and used without further purification. All solvents were purified and distilled before use. All reactions requiring anhydrous solvents were performed under nitrogen pressure.

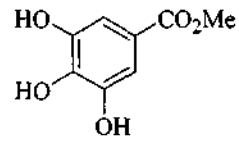
Thin-layer chromatography (TLC) was performed with aluminum-backed, 0.2 mm silica gel plates (Merck 0.20 mm or Machery-Nagel 0.20 mm). Normal and flash chromatography were performed with silica gel 60 (Merck 0.040-0.063 mm) and on columns with diameters 4 cm and 1 cm. The mobile phases were comprised of different ratios of chloroform-methanol or hexane-ethyl acetate. Detection of the developed TLC plate was accomplished using the UV_{254} light or by using spraying reagents.

Nuclear magnetic resonance spectroscopy (NMR) of the isolated compounds was performed on either the Varian 300 MHz or 500 MHz spectrophotometer. All NMR spectra were recorded at 25 °C and the chemical shifts were recorded in ppm referenced to the solvent shift. Where required, nuclear overhauser effect (NOE) spectroscopy were employed to determine the position of substituents. Coupling contstants are calculated as observed in the ^1H NMR spectrum. Deuteriated methanol (CD_3OD) was used for polar

compounds and deuteriochloroform ($CDCl_3$) was used for non polar and acetylated compounds. Mass spectra were obtained with Waters API Q-TOF Ultima LCMS. Optical rotations of the isolated compounds were determined with the appropriate spectroscopic solvent with Perkin-Elmer 241 polarimeter.

4.8.2 Synthetic procedures

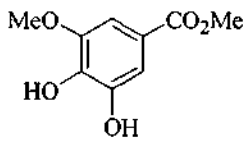
4.8.2.1 Methyl 3,4,5-trihydroxybenzoate (4.24)



3,4,5-Trihydroxybenzoic acid (**4.69**) (10.0 g, 58.8 mmol) was dissolved in absolute CH_3OH and conc. H_2SO_4 (0.3 ml) was added. After refluxing overnight, the solvent was evaporated to give a white solid. The residue was dissolved in ether, washed with sodium bicarbonate solution and brine. The organic phase was dried ($MgSO_4$) and evaporated to afford methyl gallate **4.24** (10.5 g, 97%) as a white solid.

Mp:	94-96 °C (lit.[50] 95-97)
^{1}H NMR (CD_3OD) δ_H:	
	7.03 (2H, s, H-2, H-6, ArH), 3.79 (3H, s, $COC\underline{H}_3$).
^{13}C NMR (CD_3OD) δ_C:	
	52.2 ($CO_2\underline{C}H_3$), 109.9 (2C, C-2 and C-6), 121.3 (C-1), 139.7 (C-4), 146.4 (2C, C-3 and C-5), 169.0 ($\underline{C}O_2CH_3$).
MS *m/z*	184.0438

4.8.2.2 Methyl 3,4-dihydroxy-5-methoxybenzoate (4.70)



Methyl gallate (5.0 g, 27 mmol) was dissolved in 10% aq. sodium tetraborate decahydrate (borax) solution (400 ml) and stirred for 30 min. Dimethyl sulfate (15 ml) and a solution of sodium hydroxide (7.0 g in 50 ml of H_2O) were each added dropwise to a stirred solution over 2.5 h and stirring was continued overnight. Conc. H_2SO_4 (25 ml) was added and stirring continued for an additional hour. The product was extracted with chloroform and the combined extracts were washed with 20% aq. sodium bicarbonate solution, brine, dried ($MgSO_4$) and concentrated to give **4.70** (4.7 g, 85%) as a white solid.

Mp: 131 °C (lit.[51] 110-111)

^{1}H NMR (CD_3OD) δ_H:

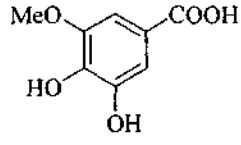
7.32 (1H, s, H-2, H-6, ArH), 7.19 (1H, s, H-2, H-6, ArH) 3.79 (3H, s, $COC\underline{H}_3$), 5.89 (1H, br s, OH), 5.55 (1H, br s, OH), 3.90 (3H, s, OCH_3), 3.86 (3H, s, OCH_3).

^{13}C NMR (CD_3OD) δ_C:

52.1 ($CO_2\underline{C}H_3$), 56.4 (OCH_3), 104.8, 110.9, 121.7, 136.8, 143.3, 146.3 (6ArC), 166.7 ($\underline{C}O_2CH_3$).

MS *m/z* 198.4037

4.8.2.3 3,4-Dihydroxy-5-methoxybenzoic acid (4.71)



To compound **4.70** (300 mg) was added KOH solution (4 g in 20 ml). The mixture was heated to reflux for 5h and concentrated hydrochloric acid (pH = 2) was added to give a white precipitate, which was filtered to give phenol **4.71** and recrystallised from benzene.

Mp: 131 °C (lit.[52] 131-132 °C)

^{1}H NMR (CD_3OD) δ_H:

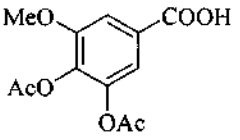
7.22 (1H, d, J = 2.0 Hz, H-2 or H-6, ArH), 7.17 (1H, d, J = 2.0 Hz, H-2 or H-6, ArH), 3.84 (3H, s, $OC\underline{H}_3$).

^{13}C NMR (CD_3OD) δ_C:

56.4 (OCH_3), 106.2, 112.0, 126.8, 141.7, 151.3, 154.2 (6ArC), 168.9 (CO_2H).

MS *m/z* 185.044

4.8.2.4 3,4-Diacetoxy-5-methoxybenzoic acid (4.72)



To a solution of phenol **4.71** (10.0 g, 54 mmol) in acetic anhydride (19.0 ml, 202 mmol) was added triethylamine (42.5 ml, 305 mmol) at 0 °C. The solution was stirred at room temperature for 1h. The excess of acetic anhydride was destroyed by addition of EtOH (3 ml). The reaction mixture was poured into water and extracted with EtOAc. The organic extracts were washed with brine, dried ($MgSO_4$) and evaporated *in vacuo* to give a white solid **4.72** (12.4 g, 100%) of diacetate.

Mp: 104 °C

^{1}H NMR (CD_3OD) δ_H:

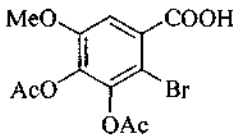
7.59 (1H, d, J = 2.0 Hz, H-2 or H-6, ArH), 7.57 (1H, d, J = 2.0 Hz, H-2 or H=6, ArH), 3.91 (3H, s, $OC\underline{H}_3$), 2.32 (3H, s, OAc), 2.31 (3H, s, OAc).

^{13}C NMR (CD_3OD) δ_C:

20.5 ($OCOC\underline{H}_3$), 20.7 ($OCOC\underline{H}_3$), 56.7 (OCH_3), 111.4, 117.9, 127.5, 136.8, 143.5, 152.7 (6ArC), 168.2 ($OC\underline{O}COCH_3$), 168.8 ($OC\underline{O}COCH_3$)

MS *m/z* 283.0808

4.8.2.5. 2-Bromo-3,4-diacetoxy-5-methoxybenzoic acid (4.73)

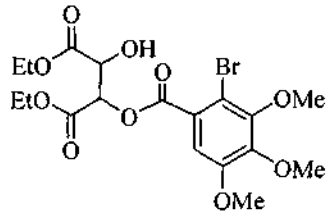


Bromine (11.2 ml, 220 mmol) in glacial acetic acid (25 ml) was added dropwise for a period of 20 min to the stirred solution of diacetate **4.72** (30.0 g, 111mmol) in glacial acetic acid (160 ml). Stirring was continued for 1 h. The precipitate formed during this

process was filtered and dried under vacuum to give 2-bromo-3,4-diacetoxy-5-methoxybenzoic acid **4.73** (43.4 g, 89%).

Mp:	127-129 °C
^{1}H NMR (CD_3OD) δ_H:	
	7.47 (1H, s, H-6, ArH), 3.88 (3H, s, $OC\underline{H}_3$), 2.34 (3H, s, OAc), 2.28 (3H, s, OAc)
^{13}C NMR (CD_3OD) δ_C:	
	19.9 ($OCOC\underline{H}_3$), 20.1 ($OCOC\underline{H}_3$), 57.1 (OCH_3), 108.3, 112.8, 132.9, 136.9, 143.8, 152.8 (6ArC), 168.7 ($O\underline{C}OCH_3$), 168.8 ($O\underline{C}OCH_3$)
MS *m/z*	269.0651

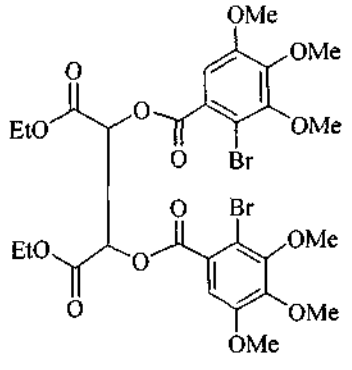
4.8.2.6. Diethyl 2-(2-bromo-3,4,5-trimethoxybenzoyloxy)-3-hydroxysuccinate(4.80)



The acid **4.73** was converted to the acid chloride by boiling in $SOCl_2$ for 5 h. After the reaction the $SOCl_2$ was distilled off under vacuum to give 2-bromo-3,4,5-trimethoxybenzoyl chloride, which was used in the next step without purification. To a solution of diethyl tartrate (1 g, 3.2 mmol) in THF, Bu_2SnO (2 g, 9.6 mmol) was added and the water was separated by azeotropic distillation in a Dean-Stark apparatus for 4 h. 2-Bromo-3,4,5-trimethoxybenzoyl chloride (840 mg, 2.7 mmol) in 1 ml THF was added dropwise with a syringe through a septum cap to the stirred solution. The reaction mixture was then stirred at room temperature for 1 h. The solvent was evaporated and the residue dissolved in 8 ml of anhydrous CH_3CN. The solution was then cooled to 0 °C, 0.75 mmol of oxalic acid in 3.5 ml CH_3CN was added, and the mixture was stirred at room temperature for 1 h. The resulting suspension was filtered under vacuum and solid washed several times with CH_3CN. The residue was flash chromatographed (7:3 hexane:EtOAc) to give monoacylated alcohol **4.80** (0.83 g, 54%).

Mp:	132-133 °C
^{1}H NMR ($CDCl_3$) δ_H:	
	7.16 (1H, s, H-6, ArH), 5.61 (1H, d, J = 2.4 Hz, OHC<u>H</u>CO), 4.77 (1H, s, COCHCO), 4.21 (4H, m, OC<u>H</u>$_2$CH$_3$) 3.84 (3H, s, OCH_3), 3.79 (3H, s, OCH_3) 3.79 (3H, s, OCH_3), 1.15 (6H, m, $OCH_2C\underline{H}_3$)
^{13}C NMR ($CDCl_3$) δ_C:	
	13.88 ($OCH_2\underline{C}H_3$), 13.94 ($OCH_2\underline{C}H_3$), 56.02 (OCH_3), 60.15 (OCH_3), 60.94 (OCH_3), 62.05 ($O\underline{C}H_2CH_3$), 62.48 ($O\underline{C}H_2CH_3$), 70.38 (OH<u>C</u>HCO), 73.68 (CO<u>C</u>HCO), 109.72, 110.55, 125.60, 146.45, 151.38, 152.18 (6ArC), 164.13 (CO_2), 166.18 (CO_2), 170.59 (CO_2)
MS *m/z*	479.0543

4.8.2.7. Diethyl 2,4-bis-(2-bromo-3,4,5-trimethoxy-benzoyloxy)succinate (4.81)



The monoacylated alcohol **4.80** (1 g, 2 mmol), a catalytic amount of DMAP and 2-bromo-3,4,5-trimethoxybenzoyl chloride (840 mg, 2.7 mmol) in THF was stirred at room temperature for 2 h. The reaction mixture was poured into water and extracted with EtOAc. The EtOAc extract was washed several times with sodium bicarbonate, brine and flash chromatographed (8:2 hexane:EtOAc) to give diester **4.81** (1.55g, 99%).

^{1}H NMR ($CDCl_3$) δ_H:

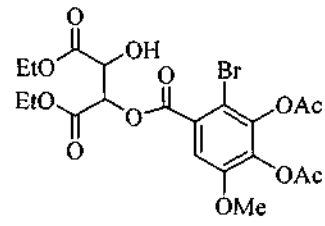
7.29 (2H, s, H-6, ArH), 5.99 (2H, s, COCHCO), 4.29 (4H, m, $OC\underline{H}_2CH_3$) 3.84 (6H, s, OCH_3), 3.79 (6H, s, OCH_3), 3.79 (6H, s, OCH_3), 1.23 (6H, t, $OCH_2C\underline{H}_3$).

^{13}C NMR ($CDCl_3$) δ_C:

14.01 ($OCH_2\underline{C}H_3$), 56.14 (OCH_3), 60.88 (OCH_3), 60.88 (OCH_3), 62.45 ($O\underline{C}H_2CH_3$), 71.56 ($CO\underline{C}HCO$), 110.24, 111.05, 125.23, 146.73, 151.48, 152.19 (6ArC), 164.06 (CO_2), 165.52 (CO_2).

MS *m/z* 751.0227

4.8.2.8 Diethyl 2-(3,4-diacetoxy-2-bromo-5-methoxybenzoyloxy)-3-hydroxysuccinate (4.83)



The acid **4.73** was converted to acid chloride by boiling in $SOCl_2$ for 5 h. After the reaction the $SOCl_2$ was distilled off under vacuum to give benzoyl chloride **4.67**, which was used in the next step without purification. To a solution of diethyl tartrate (1 g, 3.2 mmol) in THF, Bu_2SnO (2 g, 9.6 mmol) was added and the water was separated by azeotropic distillation in a Dean-Stark apparatus for 4 h. Benzoyl chloride **4.67** (900 mg, 2.5 mmol) in 1 ml THF was added dropwise with a syringe through a septum cap to the stirred solution. The reaction mixture was then stirred at room temperature for 1 h. The solvent was evaporated and the residue dissolved in 8 ml of anhydrous CH_3CN. The solution was then cooled to 0 °C, 0.75 mmol of oxalic acid in 3.5 ml CH_3CN was added, and the mixture was stirred at room temperature for 1 h. The resulting suspension was filtered under vacuum and solid washed several times with CH_3CN. The residue was flash chromatographed (7:3 hexane:EtOAc) to give monoacylated alcohol **4.83** (0.86 g, 64%).

Mp: 126 °C

^1H NMR ($CDCl_3$) δ_H:

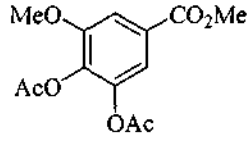
7.42 (1H, s, H-6, ArH), 5.66 (1H, d, J = 1.5 Hz, OHC<u>H</u>CO), 4.82 (1H, d, J = 1.5 Hz, COCHCO), 4.25 (4H, m, OC<u>H</u>$_2$CH$_3$) 3.85 (3H, s, OCH$_3$), 2.32 (3H, s, OAc) 2.28 (3H, s, OAc), 1.24 (6H, m, OCH$_2$C<u>H</u>$_3$).

^{13}C NMR ($CDCl_3$) δ_C:

13.8 (OCH$_2$<u>C</u>H$_3$), 13.8 (OCH$_2$<u>C</u>H$_3$), 19.8 (OCO<u>C</u>H$_3$) 19.9 (OCO<u>C</u>H$_3$), 56.2 (OCH$_3$), 62.0 (O<u>C</u>H$_2$CH$_3$), 62.4 (O<u>C</u>H$_2$CH$_3$), 70.2 (OH<u>C</u>HCO), 73.8 (CO<u>C</u>HCO), 108.5, 112.4, 128.2, 136.3, 142.45, 151.02 (6ArC), 163.22 (CO_2), 165.90 (CO_2), 166.65 (CO_2), 166.84 (CO_2), 170.38 (CO_2)

MS *m/z* 535.0441

4.8.2.9. Methyl 3,4-diacetoxy-5-methoxybenzoate (4.74)



To the solution of phenol **4.70** (10.0 g, 50 mmol) in acetic anhydride (19.0 ml, 202 mmol) at 0 °C, triethylamine (42.5 ml, 305 mmol) was added. The solution was stirred at room temperature for 1 h. The excess of acetic anhydride was destroyed by addition of EtOH (3 ml). The reaction mixture was poured into water and extracted with EtOAc. The organic extracts were washed with brine, dried ($MgSO_4$) and evaporated *in vacuo* to give **4.74** (14.2 g, 100%) of diacetate.

^{1}H NMR (CD_3OD) δ_H:

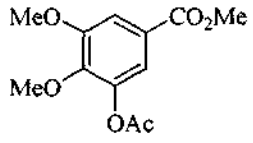
7.54 (1H, d, J = 1.5 Hz, H-2 or H-6, ArH), 7.50 (1H, d, J = 1.5 Hz, H-2 or H-6, ArH), 3.90 (3H, s, OCH$_3$), 3.88 (3H, s, CO_2CH_3), 2.31 (3H, s, OAc), 2.24 (3H, s, OAc).

^{13}C NMR (CD_3OD) δ_C:

20.2 (OCO<u>C</u>H$_3$), 20.5 (OCO<u>C</u>H$_3$), 52.4 (CO_2<u>C</u>H$_3$), 56.4 (OCH$_3$),110.7, 116.9, 128.2, 135.8, 143.1, 152.3 (6ArC), 167.2 (CO_2), 168.0 (CO_2).

MS *m/z* 283.0808

4.8.2.10 Methyl 3-acetoxy-4,5-dimethoxybenzoate (4.75)



The mixture of the diacetate **4.74** (10.0 g, 35 mmol), K_2CO_3 (15 g, 101 mmol) and CH_3I (4.5 ml, 105 mmol) in DMF was heated at 40 °C for 8 h. The inorganic salt was filtered out and the filtrate was diluted with EtOAc. The organic extract was washed several times with water to remove most of the DMF, dried ($MgSO_4$), evaporated and flash chromatographed (9:1 hexane:EtOAc) to afford **4.75** (8.6 g, 96%).

^{1}H NMR (CD_3OD) δ_H:

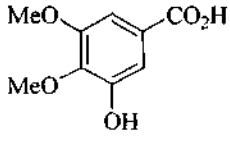
7.39 (1H, d, J = 1.5 Hz, H-2 or H-6, ArH), 7.28 (1H, d, J = 1.5 Hz, H-2 or H-6, ArH), 3.91 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.88 (3H, s, CO_2CH_3), 2.32 (3H, s, OAc).

^{13}C NMR (CD_3OD) δ_C:

20.5 ($OCO\underline{C}H_3$), 52.1 ($CO_2\underline{C}H_3$), 56.1 (OCH_3) 60.7 (OCH_3), 111.2, 117.0, 125.0, 143.4, 145.1, 153.1 (6ArC), 165.9 (CO_2), 168.8 (CO_2).

MS *m/z* 255.0858

4.8.2.11 3-Hydroxy-4,5-dimethoxybenzoic acid (4.76)



Compound **4.75** (2 g, 7.9 mmol) was added to a KOH solution (4 g 20 ml). The mixture was heated to reflux for 5h and hydrochloric acid (pH = 2) was added to give a white precipitate, which was filtered and recrystallised from benzene to give phenol **4.76**.

Mp: 185-186 °C (lit.[52] 184-185 °C)

^{1}H NMR (CD_3OD) δ_H:

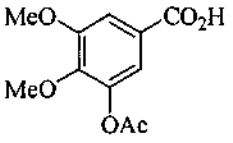
7.20 (1H, d, J = 1.8 Hz, H-2 or H-6, ArH), 7.18 (1H, d, J = 1.8 Hz, H-2 or H-6, ArH), 3.86 (3H, s, $OC\underline{H}_3$), 3.84 (3H, s, $OC\underline{H}_3$).

^{13}C NMR (CD_3OD) δ_C:

56.3 (OCH_3), 60.9 (OCH_3), 106.0, 112.1, 126.9, 141.7, 151.3, 154.1 (6ArC), 168.9 (CO_2H).

MS m/z 199.0596

4.8.2.12 3-Acetoxy-4,5-dimethoxybenzoic acid (4.77)



To the solution of phenol **4.76** (1 g, 5 mmol) in acetic anhydride (1.9 ml, 20.2 mmol) was added triethylamine (4.3 ml, 30.5 mmol) at 0 °C. The solution was stirred at room temperature for 1 h. The excess of acetic anhydride was destroyed by addition of EtOH (3 ml). The reaction mixture was poured into water and extracted with EtOAc. The organic extracts were washed with brine, dried ($MgSO_4$) and evaporated *in vacuo* to give **4.77** as a white solid (1.2 g, 100%) of diacetate.

Mp: 78-79 °C

^{1}H NMR (CD_3OD) δ_H:

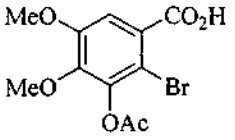
7.25 (1H, d, J = 1.5 Hz, H-2 or H-6, ArH), 7.16 (1H, d, J = 1.5 Hz H-2 or H-6, ArH), 3.84 (3H, s, OCH_3), 3.83 (3H, s, OCH_3).

^{13}C NMR (CD_3OD) δ_C:

20.53 ($OCO\underline{C}H_3$), 55.8 (OCH_3) 60.7 (OCH_3), 105.3, 109.9, 125.3, 139.4, 148.9, 151.8 (6ArC), 166.6 ($\underline{C}O_2H$).

MS *m/z* 241.0668

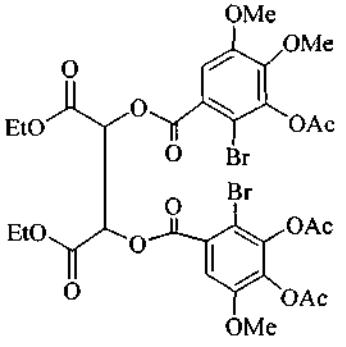
4.8.2.13 3-Acetoxy 2-bromo-4,5-dimethoxybenzoic acid (4.78)



Bromine (11.2 ml, 220 mmol) in glacial acetic acid (25 ml) was added dropwise for a period of 20 min to the stirred solution of monoacetate **4.77** (30.0 g, 111mmol) in glacial acetic acid (160 ml). Stirring was continued for 1 h. The precipitate formed during this process was filtered and dried under vacuum to give **4.78** (43.4 g, 89%).

Mp:	165-166 °C
^{1}H NMR (CD_3OD) δ_H:	
	10.17 (1H, brs, COOH), 7.52 (1H, s, H-6, ArH), 3.89 (6H, s, OCH_3), 2.38 (3H, s, OAc).
^{13}C NMR (CD_3OD) δ_C:	
	20.4 ($OCO\underline{C}H_3$), 56.1 (OCH_3) 61.0 (OCH_3), 110.0, 113.6, 124.7, 142.9, 145.9, 151.6 (6ArC), 168.0 ($\underline{C}O_2H$), 170.1 ($O\underline{C}OCH_3$).
MS *m/z*	346.9756

4.8.2.14 Diethyl 2-(3-acetoxy-2-bromo-4,5-dimethoxybenzoyloxy)-3-(3,4-diacetoxy-2-bromo-5-methoxybenzoyloxy)succinate (4.84)



The acid **4.78** was converted to the acid chloride **4.68** by boiling it in $SOCl_2$ for 5 h. After the reaction the $SOCl_2$ was distilled off under vacuum to give benzoyl chloride **4.68**, which was used in the next step without purification.

The monoacylated alcohol **4.83** (1 g, 1.8 mmol), a catalytic amount of DMAP and benzoyl chloride **4.68** (780 mg, 2.3 mmol) in THF was stirred at room temperature for 2 h. The reaction mixture was poured into water and extracted with EtOAc. The EtOAc extract was washed several times with sodium bicarbonate, brine and flash chromatographed (8:2 hexane:EtOAc) to give diester **4.84** (1.2 g, 78%).

^{1}H NMR ($CDCl_3$) δ_H:

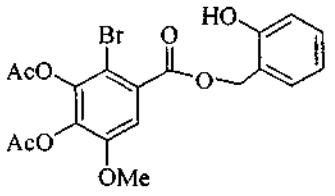
7.52 (1H, s, H-6, ArH), 7.49 (1H, s, H-6', Ar'H), 6.00 (2H, s, COCHCO), 4.24 (4H, m, $OC\underline{H}_2CH_3$) 3.86 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.80 (3H, s, OCH_3) 2.34 (3H, s, OAc) 2.27 (3H, s, OAc), 2.00 (3H, s, OAc), 1.23 (6H, m, $OCH_2C\underline{H}_3$)

^{13}C NMR ($CDCl_3$) δ_C:

13.8 ($OCH_2\underline{C}H_3$), 19.9 ($OCO\underline{C}H_3$) 20.17 ($OCO\underline{C}H_3$), 20.74 ($OCO\underline{C}H_3$) 56.0 (OCH_3), 56.3 (OCH_3), 60.1 (OCH_3), 60.8 ($O\underline{C}H_2CH_3$), 62.4 ($O\underline{C}H_2CH_3$), 71.4 ($OH\underline{C}HCO$), 71.7 ($CO\underline{C}HCO$), 108.9, 109.3, 112.8, 113.3, 124.3, 127.8, 136.5, 142.5, 142.7, 145.7, 151.6 (ArC), 163.1 (CO_2), 165.1 (CO_2), 165.2 (CO_2), 166.6 (CO_2), 166.8 (CO_2), 167.6 (CO_2), 166.8 (CO_2),

MS *m/z* 849.0268

4.8.2.15 2-Hydroxybenzyl 3,4-diacetoxy-2-bromo-5-methoxybenzoate (4.87)

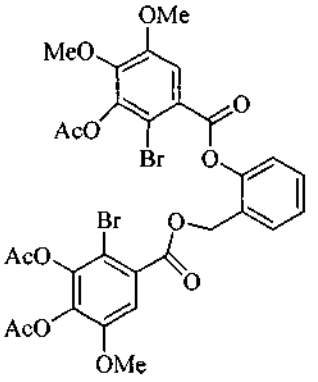


The acid **4.73** was converted to acid chloride **4.67** by boiling in $SOCl_2$ for 5 h. After the reaction the $SOCl_2$ was distilled off under vacuum to give benzoyl chloride **4.67**, which was used in the next step without purification. To a solution of salicyl diol (1 g, 8 mmol) in THF was added Bu_2SnO (2 g, 9.6 mmol), after addition water was separated by azeotropic distillation in a Dean-Stark apparatus for 4 h. Benzoyl chloride **4.67** (800 mg, 2.2 mmol)

in 1 ml THF was added dropwise with a syringe through a septum cap to the stirred solution. The reaction mixture was then stirred at room temperature for 1 h. The solvent was evaporated and the residue dissolved in 8 ml of anhydrous CH_3CN. The solution was then cooled to 0 °C, 0.75 mmol of oxalic acid in 3.5 ml CH_3CN was added, and the mixture was stirred at room temperature for 1 h. The resulting suspension was filtered under vacuum and solid washed several times with CH_3CN. The residue was flash chromatographed (hexane:EtOAc, 7:3) to give monoacylated alcohol **4.87** (0.77 g, 77%).

Mp:	127-130 °C
^{1}H NMR ($CDCl_3$) δ_H:	
	7.38 (1H, s, H-6, ArH), 7.34 (1H, dd, J = 8.3, 1.5 Hz, ArH), 7.29 (2H, m, ArH), 6.93 (1H, dd, J = 7.5 Hz, ArH), 5.39 (2H, s, Ar$\underline{C}H_2$), 3.84 (3H, s, OCH_3), 2.35 (3H, s, OAc), 2.29 (3H, s, OAc).
^{13}C NMR ($CDCl_3$) δ_C:	
	20.1 (OCO$\underline{C}H_3$), 20.2 (OCO$\underline{C}H_3$), 56.5 (OCH_3), 64.3 (Ar$\underline{C}H_2$), 108.8, 112.5, 117.4, 120.6, 121.2, 129.3, 131.0, 131.9, 136.4, 142.6, 151.2, 155.3, (12 ArC), 166.8 (CO_2R), 166.9 (CO_2R), 67.1 (CO_2R)
MS *m/z*	455.3364

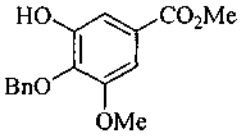
4.8.2.16 2-(3-Acetoxy-2-bromo-4,5-dimethoxybenzoyloxy)benzyl-3,4-diacetoxy-2-bromo-5-methoxybenzoate (4.88)



The monoacylated alcohol **4.87** (1g, 2.2 mmol), a catalytic amount of DMAP and benzoyl chloride **4.68** (800 mg, 2.4 mol) in THF was stirred at room temperature for 2 h. The reaction mixture was poured into water and extracted with EtOAc. The EtOAc extract was washed several times with sodium bicarbonate, brine and flash chromatographed (hexane:EtOAc, 8:2) to give diester **4.88** (1.03 g, 62%).

Mp:	104-106 °C
^{1}H NMR ($CDCl_3$) δ_H:	
	7.60 (1H, dd, *J* = 77.7, 1.2 Hz, ArH), 7.54 (1H, s, ArH), 7.48 (1H, td, *J* = 8.0, 1.3 Hz, ArH), 7.33 (1H, t, *J* = 7.5, ArH), 7.29 (1H, d, J = 8.2, ArH), 7. 32 (1H, s, H-6', ArH), 5.42, (2H, s,Ar$\underline{C}H_2$), 3.92 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 2.40 (3H, s, OAc), 2.33 (3H, s, OAc), 2.28 (3H, s, OAc).
^{13}C NMR ($CDCl_3$) δ_C:	
	20.1 (OCO$\underline{C}H_3$), 20.2 (OCO$\underline{C}H_3$), 20.4 (OCO$\underline{C}H_3$), 56.2 (OCH_3), 56.5 (OCH_3), 61.0 (OCH_3), 62.9 (Ar$\underline{C}H_2$), 108.5, 109.4, 112.3, 112.9, 122.7, 125.3, 126.5, 127.4, 129.4, 130.2, 130.8, 131.1, 142.5, 143.1, 145.8, 149.4, 151.2, 152.1 (18 ArC), 163.7 ($\underline{C}O_2CO_3$), 164.5 ($\underline{C}O_2CH_3$), 166.8 (O$\underline{C}OCH_3$), 167.0 (O$\underline{C}OCH_3$), 167.8 (O$\underline{C}OCH_3$).
MS *m/z*	753.5036

4.8.2.17 Methyl 4-benzyloxy-3-hydroxy-5-methoxybenzoate (4.93)



Methyl 3,4-dihydroxy-5-methoxybenzoate (**4.70**) (5 g, 0.108 mol) and K_2CO_3 (7.5 g, 0216 mol) were added to acetonitrile (100 ml). Benzyl bromide (2.5 ml) was added and the mixture was heated for 1.5 h at 50 °C. After cooling, the mixture was poured into water and (100 ml) and extracted several times with EtOAc. The EtOAc extracts were washed

with water, dried ($MgSO_4$), concentrated and chromatographed (9:1 hexane:EtOAc) to give **4.93** (7.4 g. 83%) as a white solid.

Mp: 74-76 °C

^{1}H NMR ($CDCl_3$) δ_H:

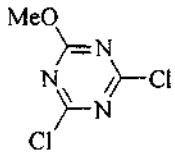
7.38 (5H, m, $ArCH_2$) 7.26 (1H, d, J = 1.5 Hz, H-2 or H-6, ArH), 7.22 (1H, d, J = 1.9 Hz, H-2 or H-6), 5.77 (1H, s, OH), 5.15 (2H, s, $ArC\underline{H}_2$), 3.94 (3H, s, OCH_3), 3.88 (3H, s, CO_2CH_3).

^{13}C NMR (CD_3OD) δ_C:

52.1 ($CO_2\underline{C}H_3$), 55.9 (OCH_3) 75.2 ($Ar\underline{C}H_2$), 105.4, 109.8, 125.7, 128.4(2x), 128.6(2x), 136.7, 138.1, 149.2, 151.9 (12ArC), 166.6 (CO_2).

MS *m/z* 289.1721

4.8.2.18 2,4-Dichloro-6-methoxy-1,3,5-triazine (4.98)



A mixture of 100 ml methanol, 13.5 ml water and (16.5 g, 0.2 mol) sodium bicarbonate was cooled to 10-15 °C. Cyanuric chloride (18.4 g, 0.1 mol) was added and the resulting mixture was warmed to 35 °C then stirred at the same temperature for 30 min. Water was added to the reaction mixture and the precipitated product filtered under vacuum, washed with water and dried under vacuum to yield 2,4-dichloro-6-methoxy-1,3,5-triazine (**4.98**) as a white solid (5.2 g, 28%)

Mp: 81-83 (lit.[53] 80-90)

^{1}H NMR ($CDCl_3$) δ_H:

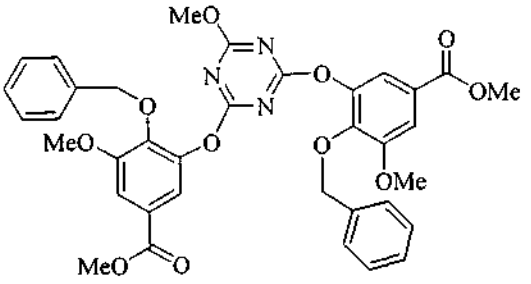
4.12 (3H, s, OMe).

^{13}C NMR (CD_3OD) δ_C:

56.9 (OMe), 172.5 ($N\underline{C}OMe$), 171.4 (NCCl),

MS *m/z* 179.9768

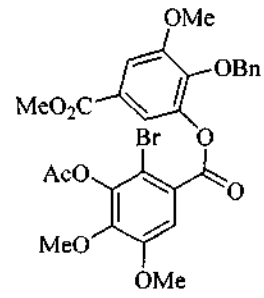
4.8.2.19 Dimethyl 3,3'-[6-methoxy-1,3,5-triazine-2,4-diyl)bis(oxy)]bis(4-benzyloxy-5-methoxybenzoate) (4.99)



A solution of compound **4.98** (72 mg, 0.40 mmol) and compound **4.93** (58 mg, 0.20 mmol) in THF (20 ml) was stirred under nitrogen. N-Methylmorpholine (24 mg, 0.24 mmol) was added and, after stirring for 4 h, the reaction mixture was poured into water and extracted with ethyl acetate. The organic phase was washed successively with saturated $NaHCO_3$ solution, water, 1N HCl, water, brine and dried ($MgSO_4$). The crude product was chromatographed (hexane:EtOAc, 7:3) to afford **4.99** (92 mg, 67%) as a cream white solid. Recrystallisation by slow evaporation of a methanol solution of **4.99** gave the compound as colourless crystals.

Mp:	98-100 °C
^{1}H NMR ($CDCl_3$) δ_H:	
	7.51 (1H, d, J = 1.9 Hz, H2 or H6), 7.43 (1H, d, *J* = 1.9 Hz, H-2or H-6, ArH), 7.23-7.25 (10H, m, $ArCH_2$), 5.06 (4H, s, $ArC\underline{H}_2$), 3.92 (6H, s, $CO_2C\underline{H}_3$), 3.90 (6H, s, OCH_3), 3.77 (3H, s, OMe).
^{13}C NMR (CD_3OD) δ_C:	
	52.2 (2x $CO_2\underline{C}H_3$), 55.4 (OCH_3), 56.2 (OCH_3), 74.8 ($Ar\underline{C}H_2$), 111.2(2x), 116.8(2x), 125.2(2x), 127.6(2x), 127.8(4x), 128.2(4x), 136.9(2x), 144.0(2x), 153.3(2x) (24ArC), 165.9 (CO_2), 173.0 (N$\underline{C}$OMe), 173.6 (CO_2Me).
MS m/z	687.2168

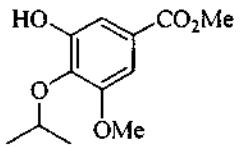
4.8.2.20 2-Benzyloxy-3-methoxy-5-(methoxycarbonyl)phenyl 3-acetoxy-2-bromo-3,4-dimethoxybenzoate (4.94)



To a suspension of 2-bromo-3-acetoxy-4,5-dimethoxybenzoic acid (**4.78**) (200 mg, 0.360 mmol) in dry benzene (3 ml), trifluoroacetic anhydride (0.06 ml, 0.425 mmol) was added. After stirring for 2 h at 60 °C, a solution of methyl 4-benzyloxy-3-hydroxy-5-methoxybenzoate (100 mg, 0.359 mmol) in dry benzene (3 ml) was added to the mixture and stirred for 2 h at 60 °C. The reaction mixture was cooled to room temperature and then diluted with EtOAc. After neutralization with 5% NaOH aqueous solution and extracted with EtOAc, the obtained EtOAc layer was washed with brine, dried ($MgSO_4$), concentrated and flash chromatographed (hexane:EtOAc, 9:1) to give **4.94** (202 mg, 72%) as a light-yellow solid.

Mp:	67-68 °C
^{1}H NMR ($CDCl_3$) δ_H:	7.58 (1H, d, J = 2.0 Hz, H-2' or H-6', ArH), 7.55 (1H, d, J = 2.0 Hz, H-2' or H-6', ArH), 7.36 (1H, s, H-6) 7.22 (5H, m, $ArCH_2$), 5.14 (2H, s, $ArC\underline{H}_2$), 3.96 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 2.40 (3H, s, OAc).
^{13}C NMR ($CDCl_3$) δ_C:	52.2 ($CO_2\underline{C}H_3$), 56.0 (OCH_3), 56.2 (OCH_3), 61.0 (OCH_3), 74.9 ($Ar\underline{C}H_2$), 109.6, 111.5, 113.2, 117.0, 125.3, 127.9, 128.1(2x), 128.2(2x), 136.8, 142.9, 143.6, 144.1, 145.6, 151.7, 153.3 (18 ArC), 163.1 (CO_2), 165.9 (CO_2), 167.8 ($O\underline{C}OCH_3$).
MS *m/z*	589.3421

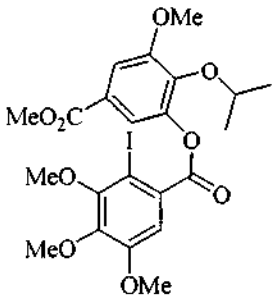
4.8.2.21 Methyl 4-isopropoxy-3-hydroxy-5-methoxybenzoate (4.102)



Methyl 3,4-dihydroxy-5-methoxybenzoate (**4.70**) (2 g, 10 mmol) and K_2CO_3 (7.5 g, 54 mmol) were added to acetonitrile (100 ml). Isopropyl bromide (10 ml) was added and mixture was refluxed for 2 h. After cooling, the mixture was poured into water (100 ml) and extracted several time with EtOAc. The EtOAc extracts were washed with water, dried ($MgSO_4$), concentrated and chromatographed (hexane:EtOAc, 9:1) to give **4.102** (2.2 g, 92%) as a white solid.

Mp:	73-74 °C
^{1}H NMR ($CDCl_3$) δ_H:	7.29 (1H, d, J = 2.0 Hz, H-2 or H-6, ArH), 7.17 (1H, d, J = 1.9 Hz, ArH), 5.96 (1H, s, OH), 4.68 [1H, sept, OC<u>H</u>$(CH_3)_2$], 3.86 (3H, s, CO_2<u>C</u>H_3), 3.86 (3H, s, OCH_3), 1.29 [6H, d, J= 6.2 OCH(C<u>H</u>$_3)_2$].
MS *m/z*	263.0924

4.8.2.22 2-Isopropyloxy-3-methoxy-5-(methoxy carbonyl)phenyl 2-iodo-3,4,5-trimethoxybenzoate (4.103)

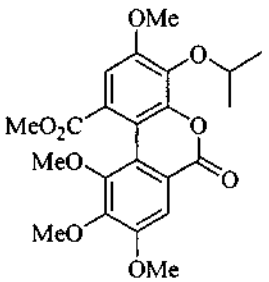


To a suspension of 2-iodo-3,4,5-trimethoxybenzoic acid (**4.101**) (2 g, 5.9 mmol) in dry benzene (9 ml), trifluoroacetic anhydride (0.18 ml, 1.28 mmol) was added. After stirring

for 2h at 60 °C, a solution of Methyl 3-hydroxy-4-isopropoxy-5-methoxybenzoate (**4.102**) (1 g, 3.59 mmol) in dry benzene (9 ml) was added to the mixture and stirred for 2 h at 60 °C. The reaction mixture was cooled to room temperature and then diluted with EtOAc. After neutralization with 5% NaOH aqueous solution and extracted with EtOAc, the obtained EtOAc layer was washed with brine, dried ($MgSO_4$), concentrated and flash chromatographed (hexane:EtOAc, 9:1) to give **4.103** (2.3 g, 70%) as a light yellow solid.

Mp:	82-83 °C
^{1}H NMR ($CDCl_3$) δ_H:	
	7.57 (1H, d, J = 1.8 Hz, H-2' or H-6', ArH), 7.53 (1H, d, J = 1.7 Hz, H-2' or H-6', ArH) 7.48 (1H, s, H-6, ArH), 4.67 [1H, sept, OC<u>H</u>$(CH_3)_2$] 3.95 (3H, s, CO_2CH_3), 3.92 (6H, s, OCH_3), 3.89 (6H, s, OCH_3), 1.24 [6H, d, J = 6.2 Hz, OCH($C\underline{H}_3)_2$].
^{13}C NMR ($CDCl_3$) δ_C:	
	22.7 [OCH($\underline{C}H_3)_2$], 52.2 ($CO_2\underline{C}H_3$), 56.2 (OCH_3), 56.3 (OCH_3) 60.8 (OCH_3), 61.0 (OCH_3), 75.8 [O<u>C</u>H$(CH_3)_2$], 85.0 111.1, 111.2, 117.3, 124.6, 129.2, 143.3, 144.1, 145.5, 153.2, 153.4, 154.1, (12 ArC), 163.7 (CO_2), 166.1 ($\underline{C}O_2CH_3$).
MS *m/z*	596.3256

4.8.2.23 Methyl 4-O-isopropyloxy-3,8,9,10-tetramethoxy-6-oxo-6H-dibenzo[b,d]pyran-1-carboxylate (4.104)

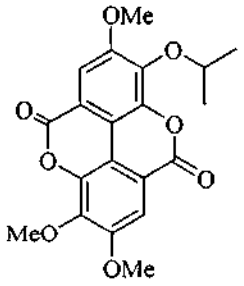


A mixture of ester **4.103** (1 g, 1.8 mmol), $Pd(OAc)_2$ (45 mg, 0.27 mmol), PPh_3 (110 mg, 0.42 mmol) and K_2CO_3 (360 mg, 2.6 mmol) was heated at 10 bar for 1 h at 60 °C. Freshly distilled *N,N*-dimethylacetamide (DMA) was added and the reaction mixture refluxed at

130 °C for 1 h. The mixture was allowed to cool to room temperature and diluted with EtOAc washed sequentially with 2 M HCl, brine, dried ($MgSO_4$), concentrated *in vacuo* and flash chromatographed on silica gel (9.5:0.5 $CHCl_3$:EtOAc) to give **4.104** (400 mg, 52%).

Mp:	112-113 °C
^{1}H NMR ($CDCl_3$) δ_H:	
	7.64 (1H, s, H-6, ArH), 7.24 (1H, s, H-6', ArH), 4.68 [1H, sept, OC<u>H</u>$(CH_3)_2$] 4.03 (3H, s, OCH_3), 3.99 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.59 (3H, s, OCH_3) 1.40 [6H, d, J = 6.2 Hz, OCH(C<u>H</u>$_3)_2$].
^{13}C NMR ($CDCl_3$) δ_C:	
	22.5 [OCH(<u>C</u>$H_3)_2$], 52.0 (OCH_3), 52.2 (CO_2<u>C</u>H_3), 56.2 (OCH_3), 56.3 (OCH_3), 61.1 (OCH_3), 61.2 (OCH_3), 76.7 [O<u>C</u>H$(CH_3)_2$], 107.6, 108.8, 110.1, 117.4, 122.9, 126.1, 136.5, 145.6, 148.3, 150.0, 152.9, 153.7 (12 ArC), 160.2 (CO_2), 169.0 (<u>C</u>O_2CH_3).
MS *m/z*	447.1695

4.8.2.24 4-*O*-Isoproyl-3,3',4'-tri-*O*-methylellagic acid (4.105)

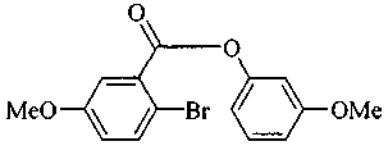


To compound **4.104** (300 mg, 0.68 mmol), KOH solution (2 g in 20 ml) was added. The mixture was heated to reflux for 5h and concentrated hydrochloric acid (Ph = 2) was added to give a white precipitate, which was filtered to give diacid and was used in the next step without purification. A mixture of diacid (30 mg, 0.068 mmol) and CF_3CO_2O (1 ml) in C_6H_6/CH_2Cl_2 1:1 (2 ml) was stirred at room temperature for 6h. Evaporation of solvents

and recrystallisation from AcOEt /hexane gave an ellagic acid derivative **4.105** (26 mg, 99%).

Mp:	137-138 °C
^{1}H NMR ($CDCl_3$) δ_H:	
	7.69 (2H, s, H6 & H-6', ArH), 4.85 [1H, sept, OC<u>H</u>$(CH_3)_2$] 4.22 (3H, s, OCH_3), 4.03 (3H, s, OCH_3), 4.01 (3H, s, OCH_3), 1.43 [6H, d, J = 6.2 Hz, OCH(C<u>H</u>$_3)_2$].
^{13}C NMR ($CDCl_3$) δ_C:	
	22.6 [OCH(<u>C</u>$H_3)_2$], 56.7 (OCH_3), 56.8 (OCH_3), 60.3 (OCH_3), 76.9 [O<u>C</u>H$(CH_3)_2$], 107.7, 107.8, 112.3, 112.4, 113.3, 113.5, 140.3, 141.1, 141.8, 142.1, 154.3, 155.3 (12 ArC), 158.9 (CO_2), 159.0 (CO_2).
MS *m/z*	380.3732

4.8.2.25. 3-Methoxyphenyl 2-bromo-5-methoxybenzoate (4.108)



To a suspension of 2-bromo-5-methoxybenzoic acid (**4.106**) (1 g, 2.9 mmol) in dry benzene (3.5 ml), trifluoroacetic anhydride (0.06 ml, 1.28 mmol) was added. After stirring for 2 h at 60 °C, a solution of 3-methoxyphenol (**4.107**) (0.5 g, 3.59 mmol) in dry benzene (3.5 ml) was added to the mixture and stirred for 2 h at 60 °C. The reaction mixture was cooled to room temperature and then diluted with EtOAc. After neutralization with 5% NaOH aqueous solution and extracted with EtOAc, the obtained EtOAc layer was washed with brine, dried ($MgSO_4$), concentrated and flash chromatographed (9:1 hexane:EtOAc) to give **4.108** (2.3 g, 76%) as a yellow oil.

^{1}H NMR ($CDCl_3$) δ_H:

7.58 (1H, d, J = 8.8 Hz, ArH), 7.51 (1H, d, J = 3.1 Hz, ArH), 7.31 (1H, br t, J = 8.8 Hz, ArH), 6.94 (1H, dd, J = 8.8, 3.2 Hz, ArH), 6.87 (1H, br d, J = 10.5 Hz, ArH), 6.84 (1H, br d,

	J = 8.5 Hz, ArH), 6.82 (1H, br s, ArH), 3.82 (3H, s, OCH_3), 3.81 (3H, s, OCH_3).
^{13}C NMR ($CDCl_3$) δ_C:	
	55.3 ($O\underline{C}H_3$), 55.6 ($O\underline{C}H_3$), 107.4, 111.8, 112.2, 113.6, 116.6, 119.3, 129.7, 131.7, 135.1, 151.4, 158.5, 160.4 (12 ArC), 160.4 (CO_2).
MS m/z	337.0072

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Appendix

List of publications generated by this project:

1. Venusol from *Gunnera perpensa*: structural and activity studies
2. A triazine derivative with a triple-decker sandwich conformation

PLATE 1

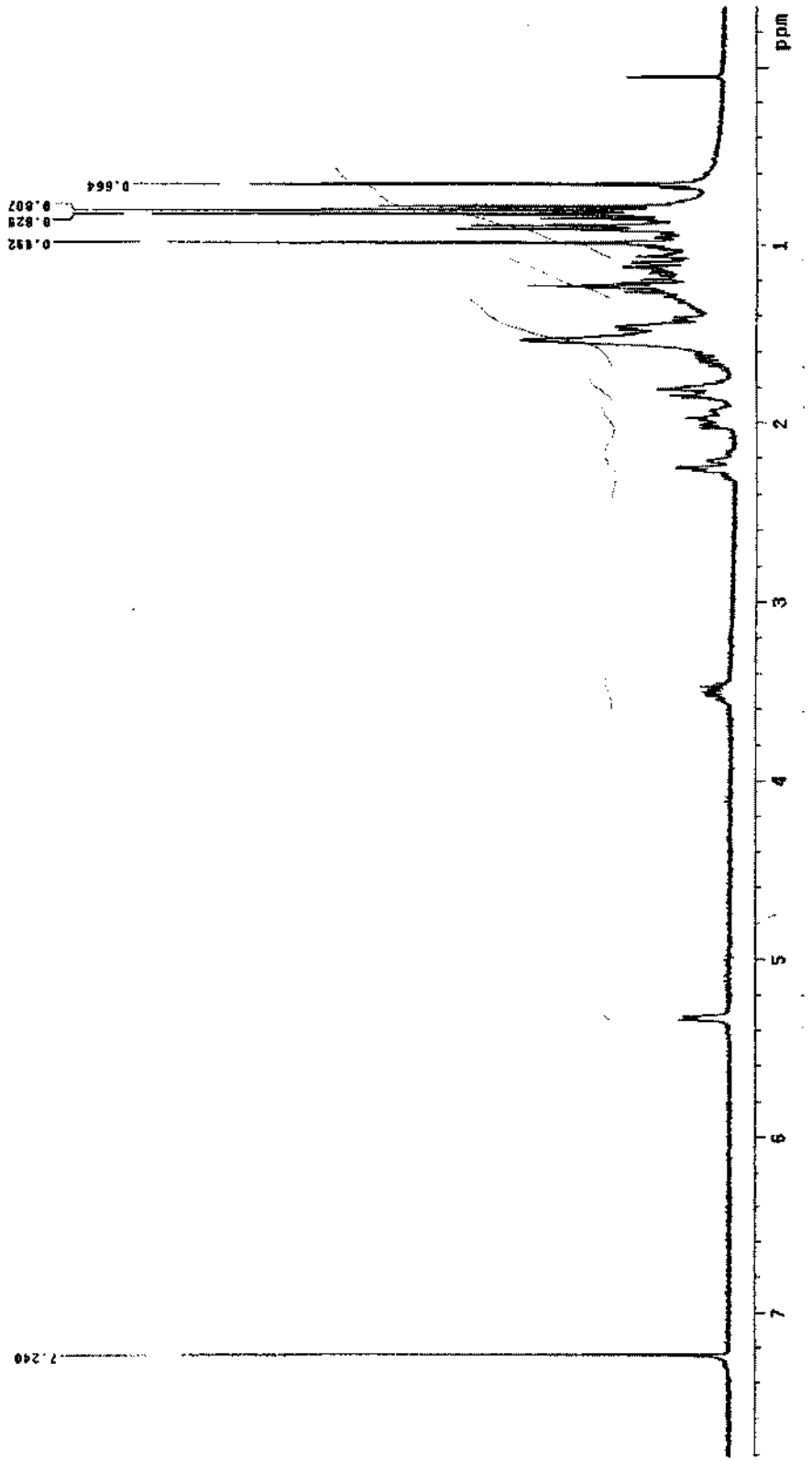
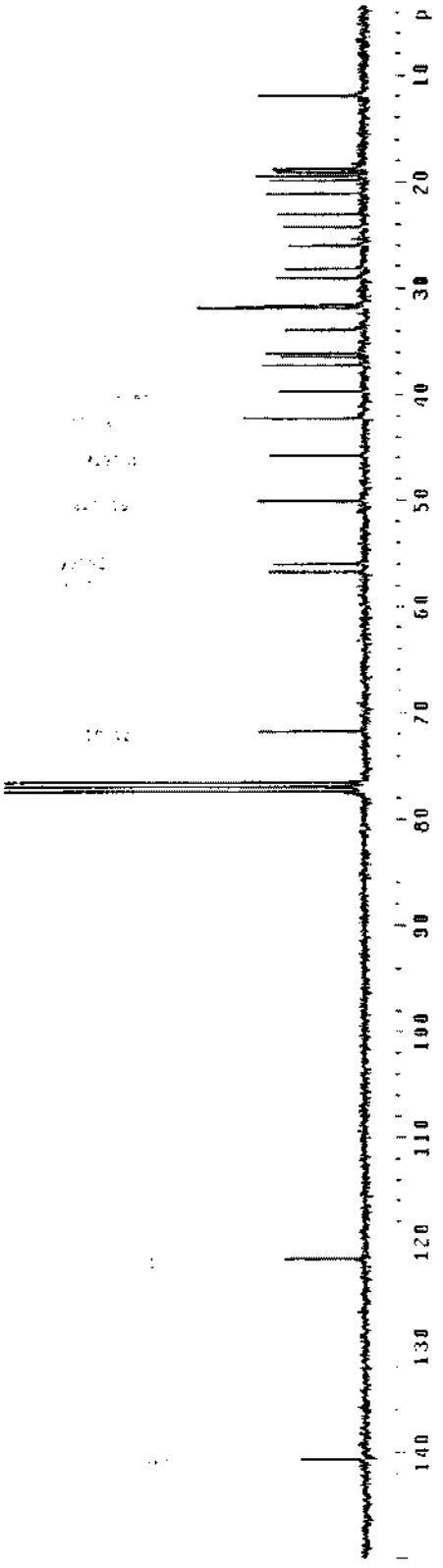


PLATE 2



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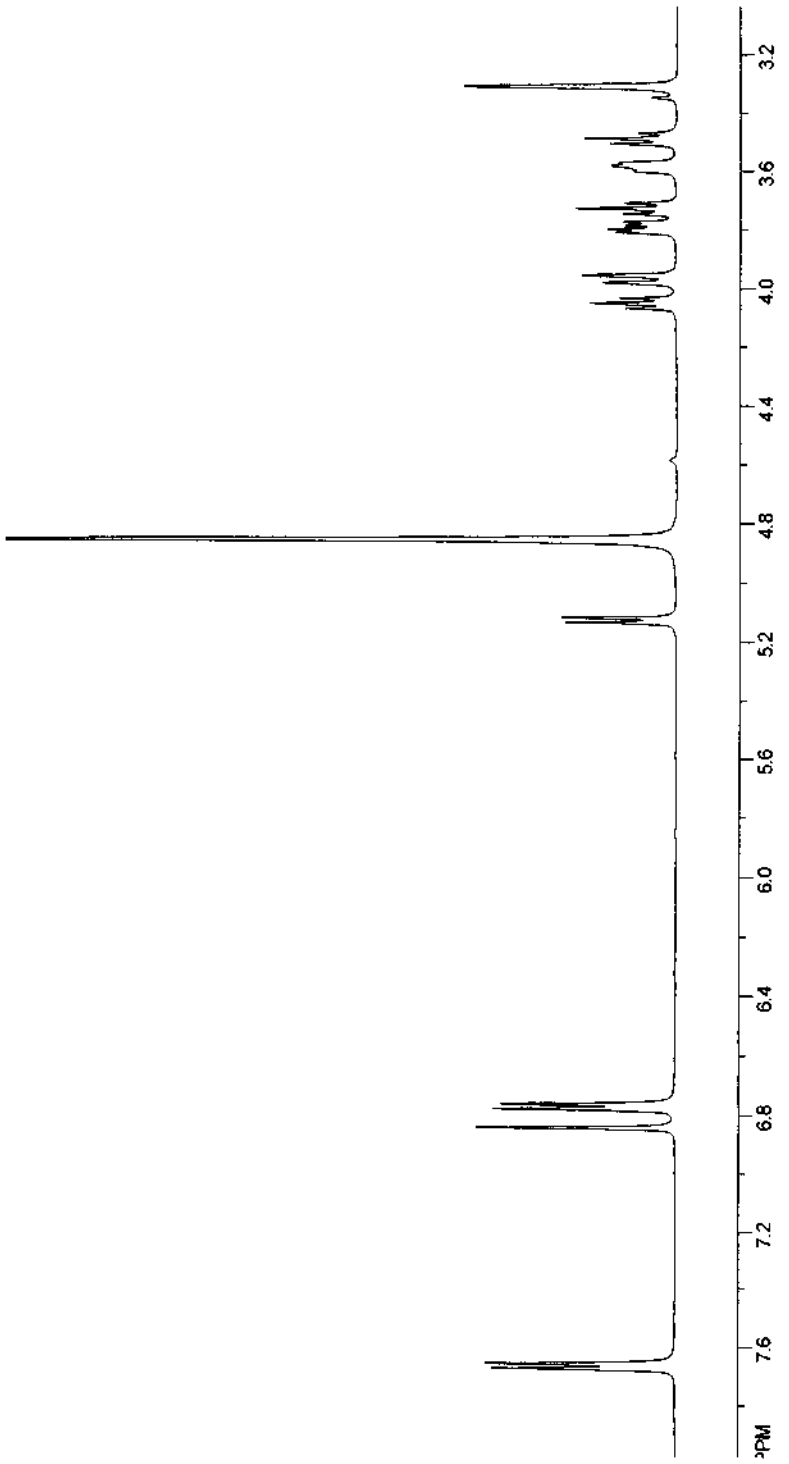

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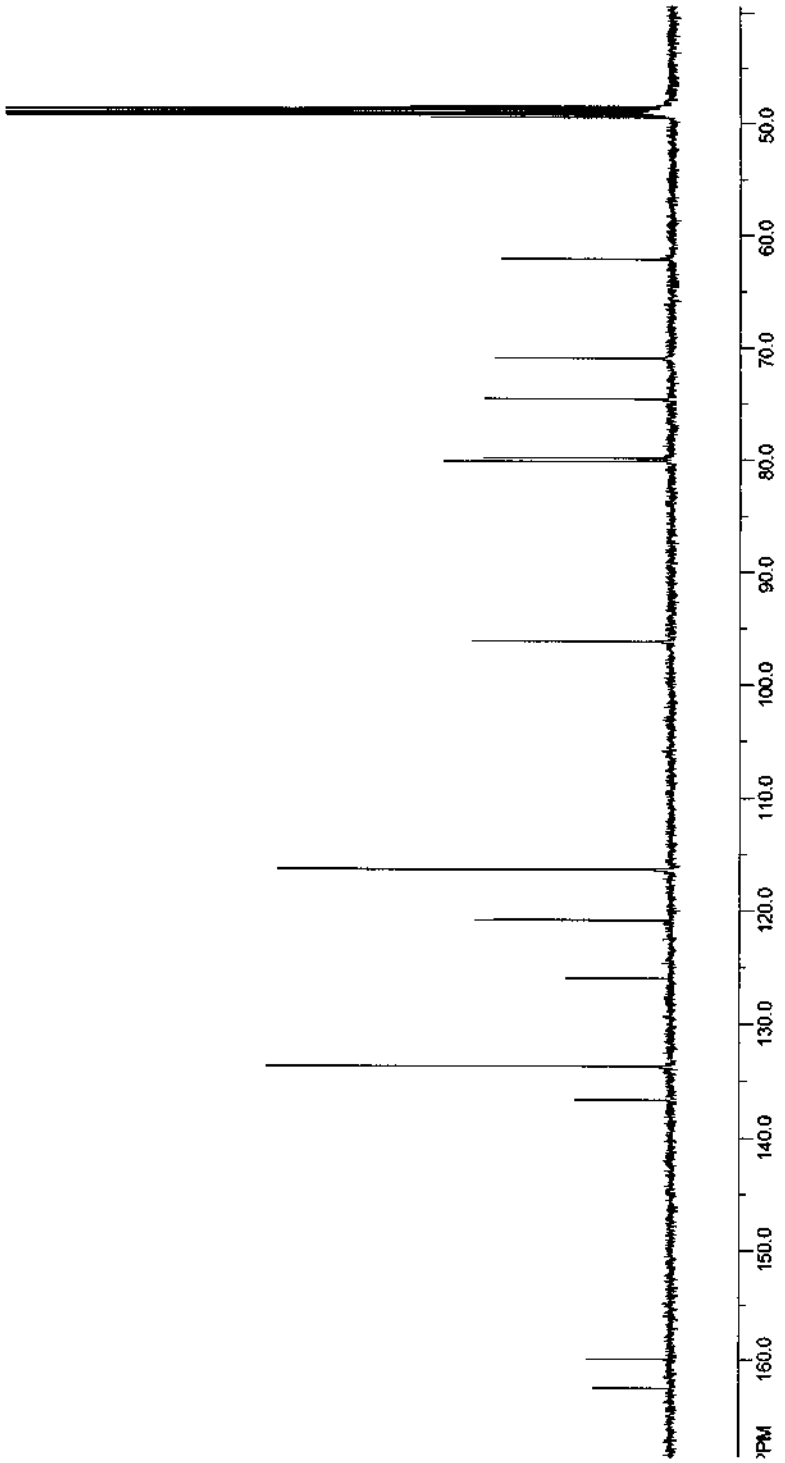

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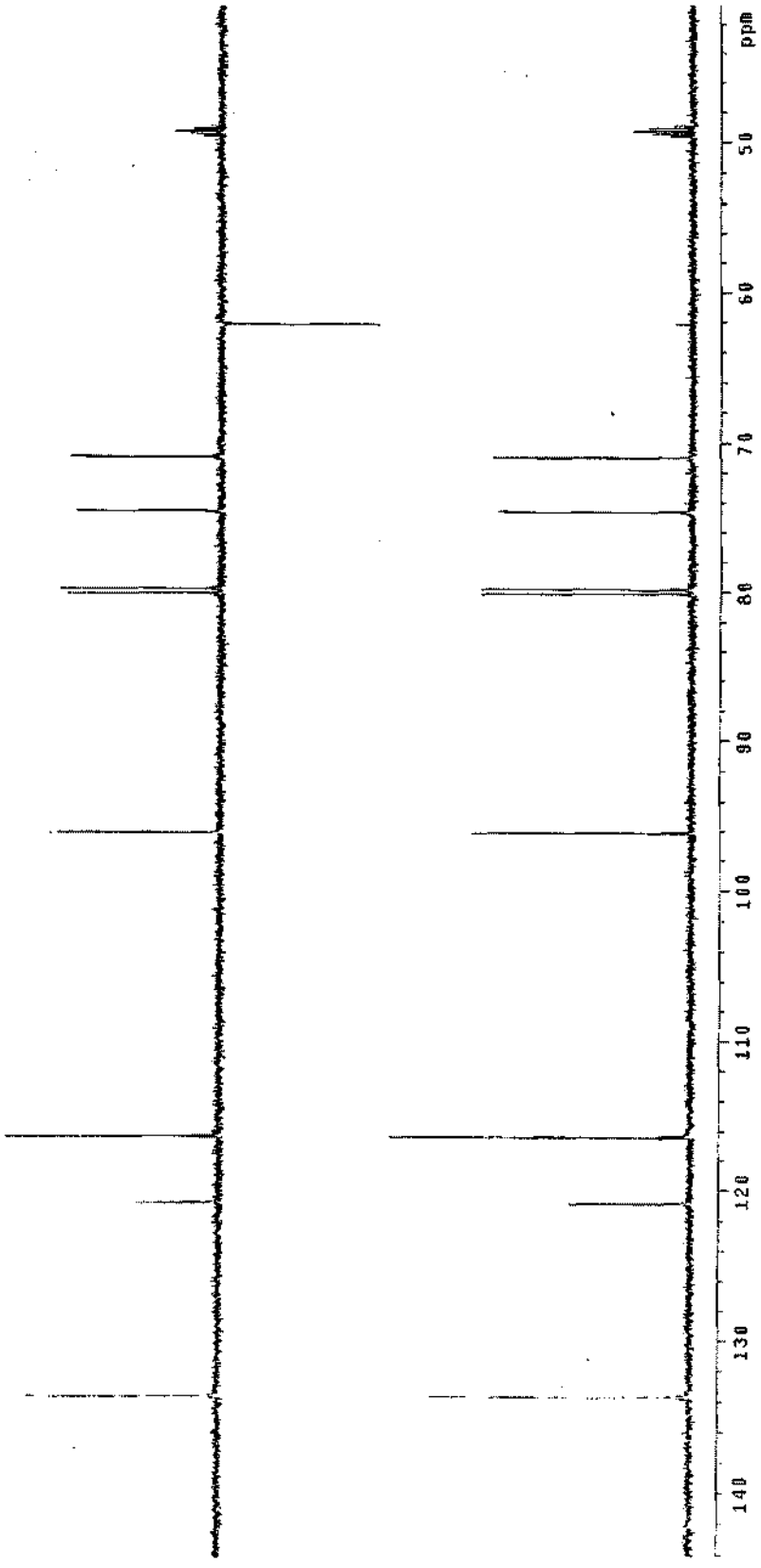

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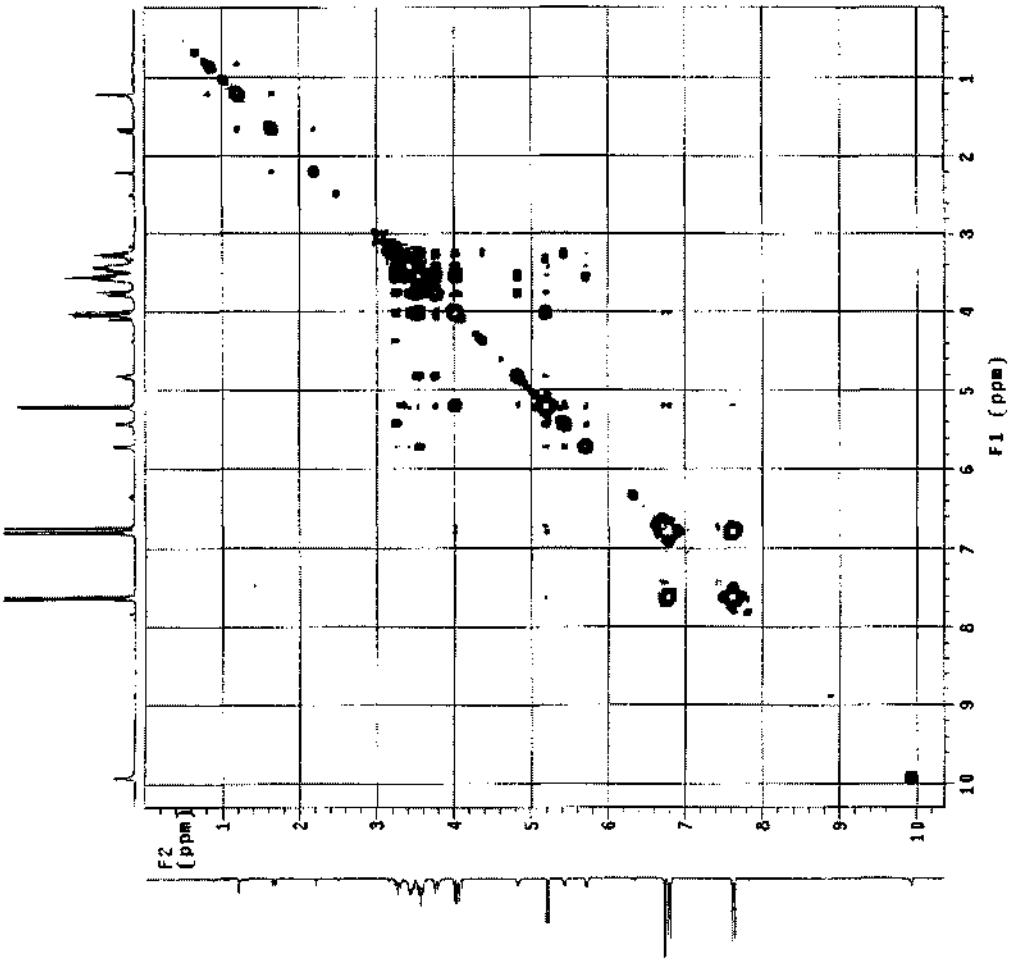

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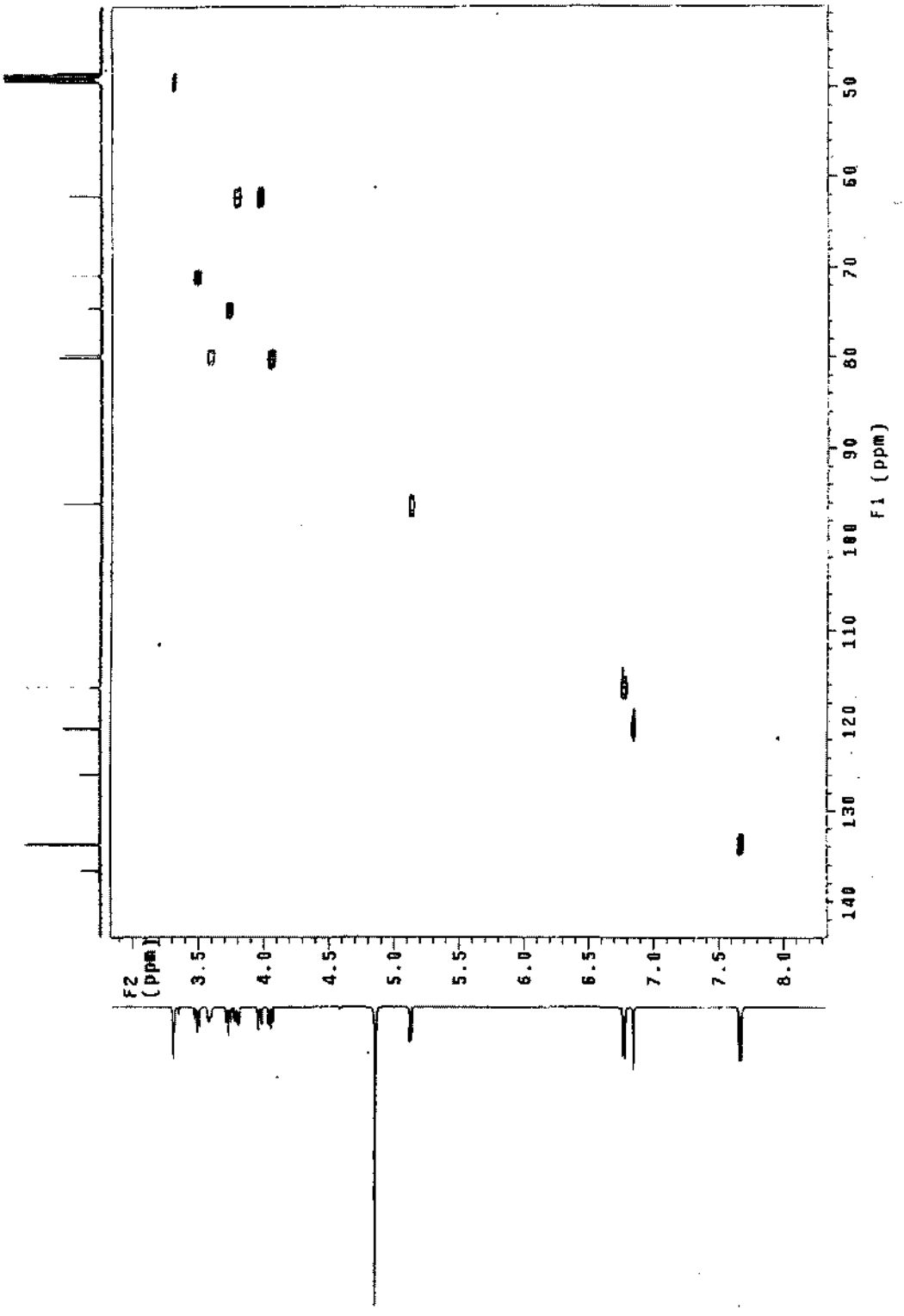
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PLATE 8

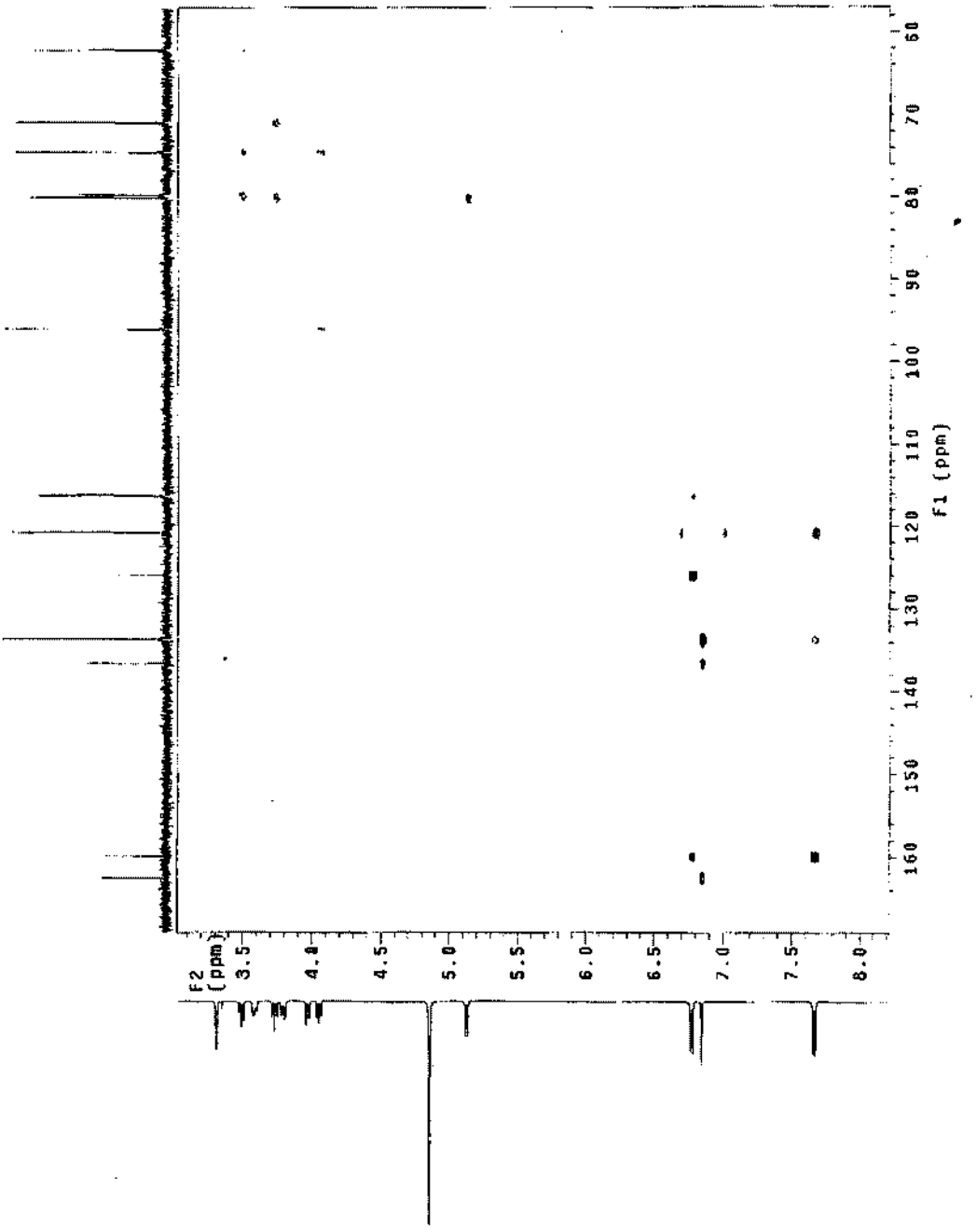
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PLATE 9

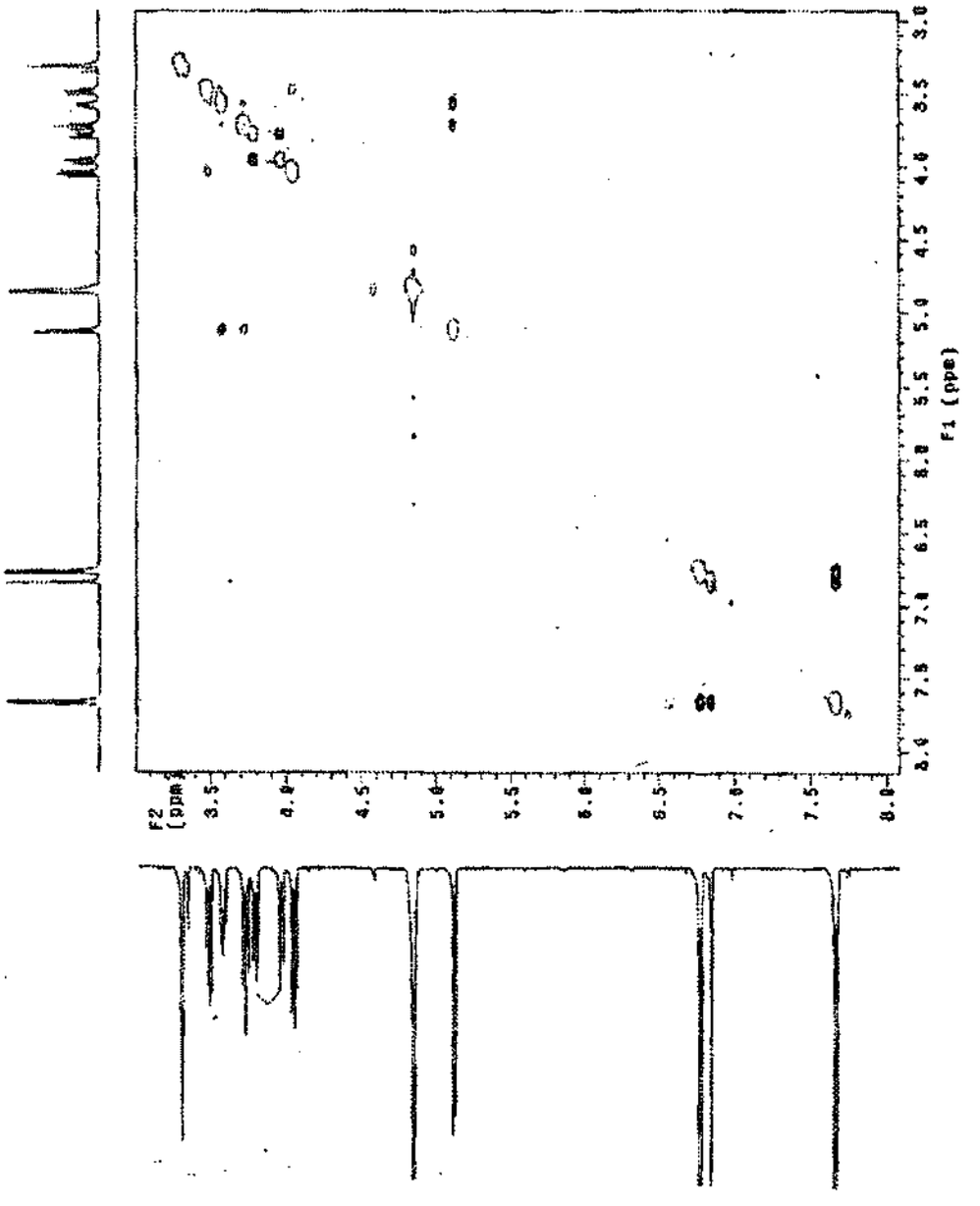


PLATE 10

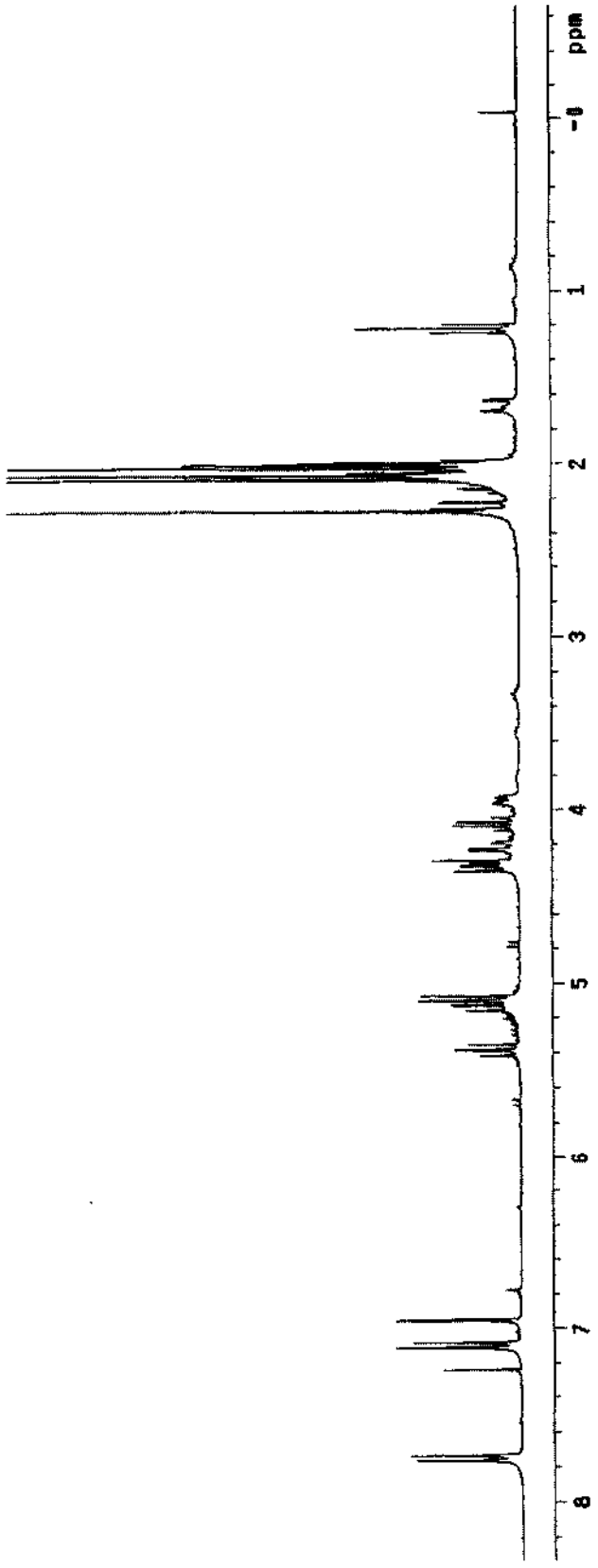


PLATE 11

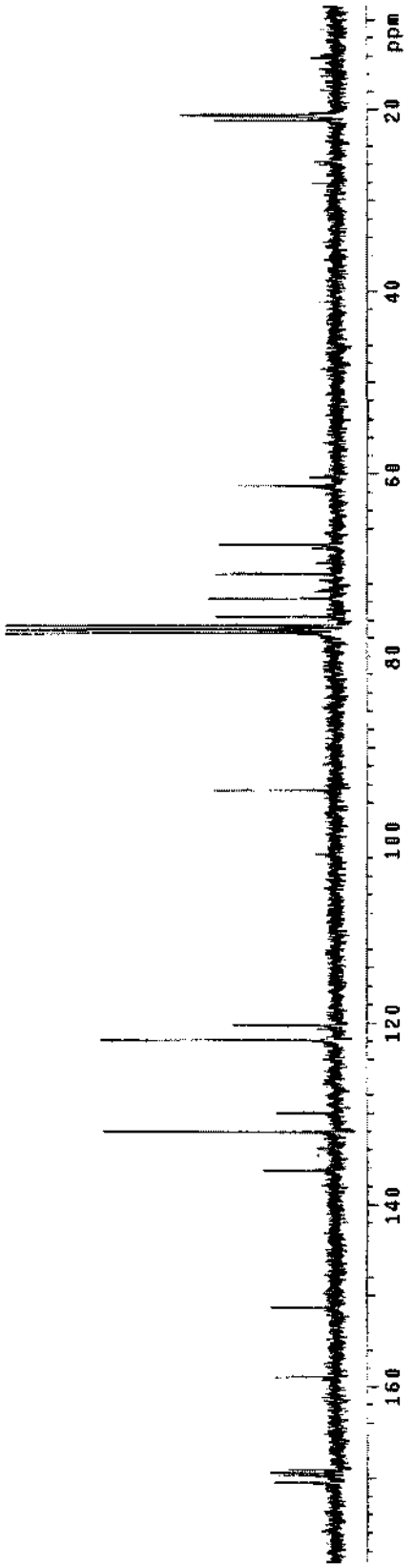


PLATE 12

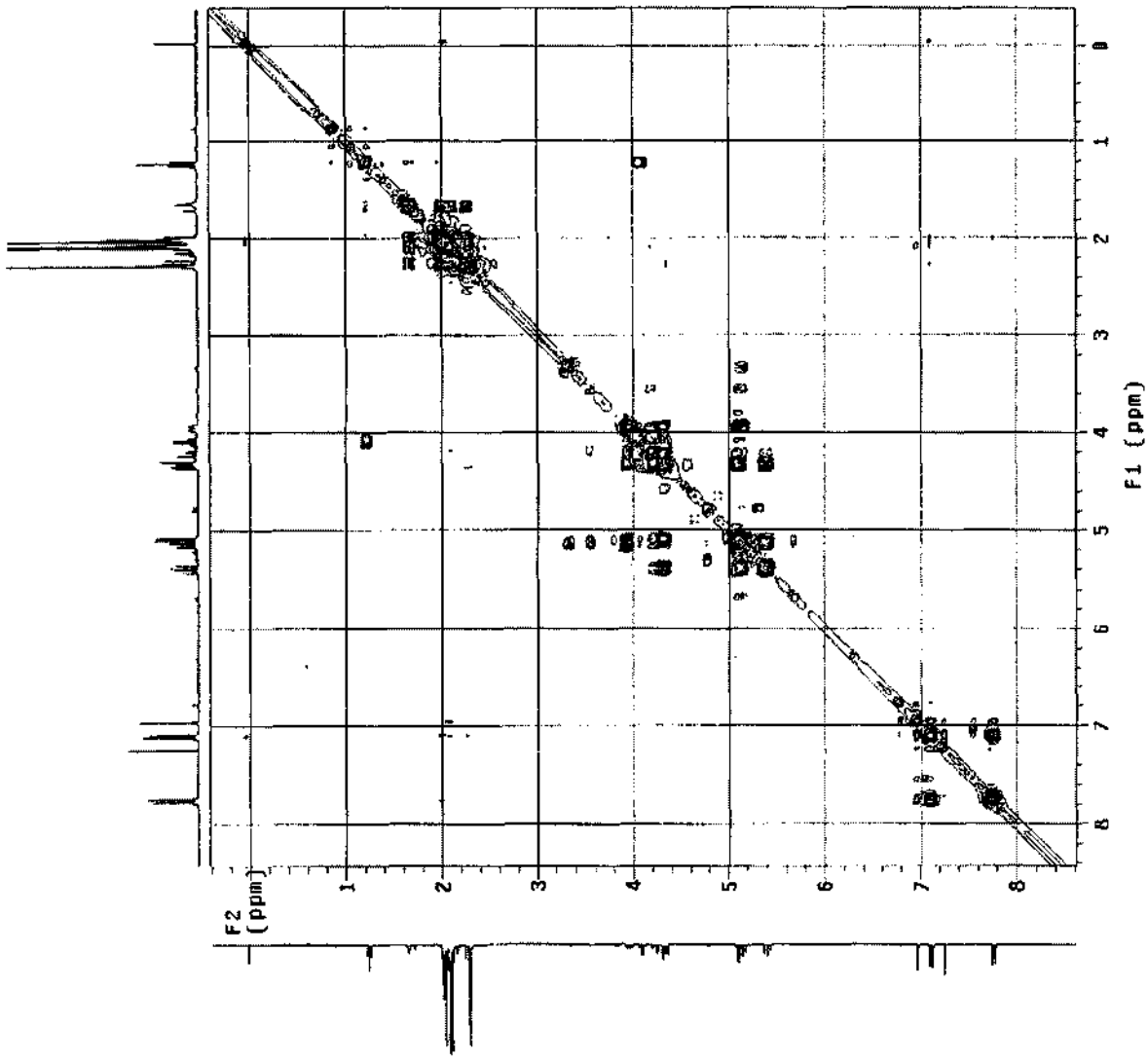


PLATE 13

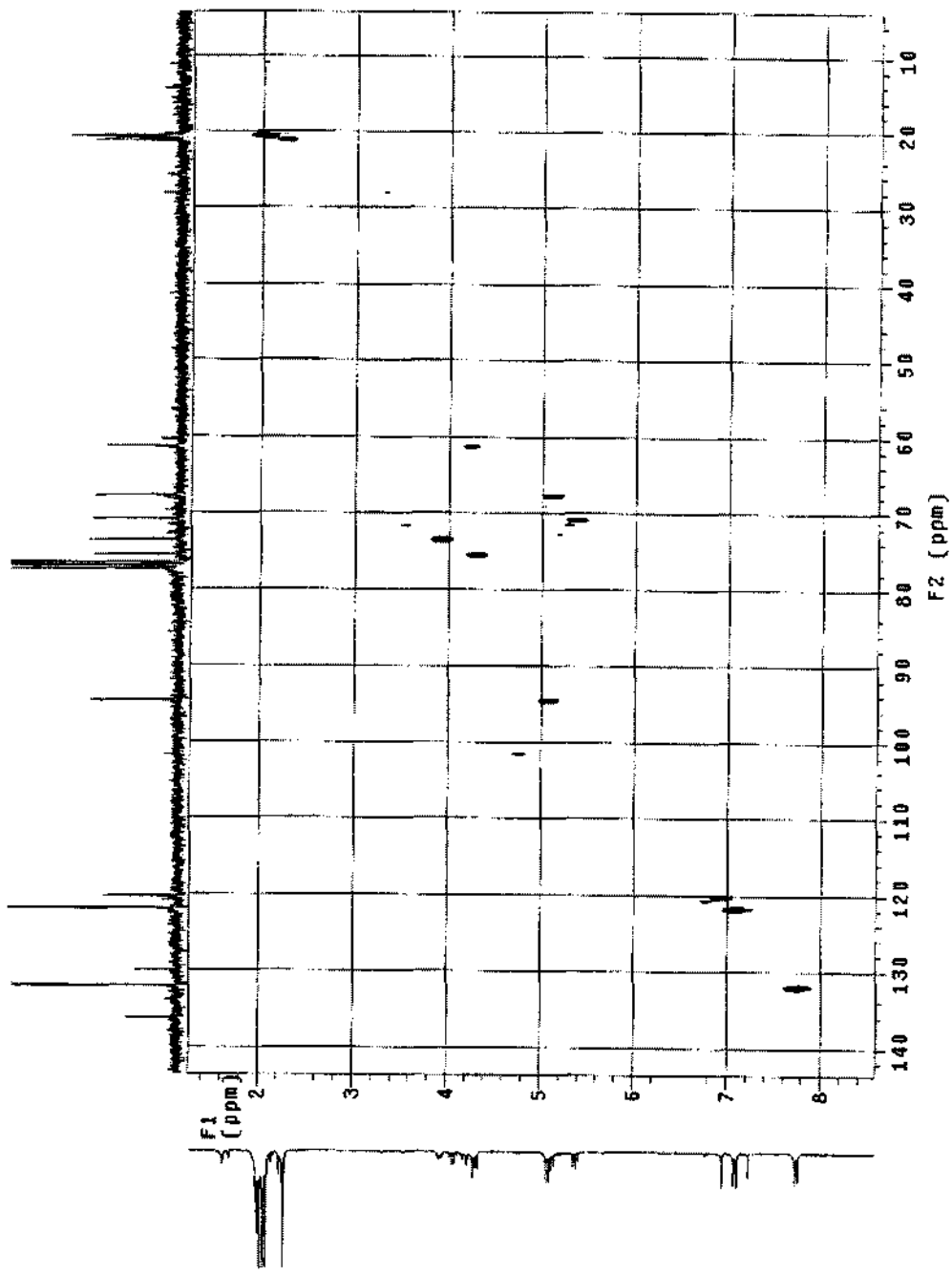


PLATE 14

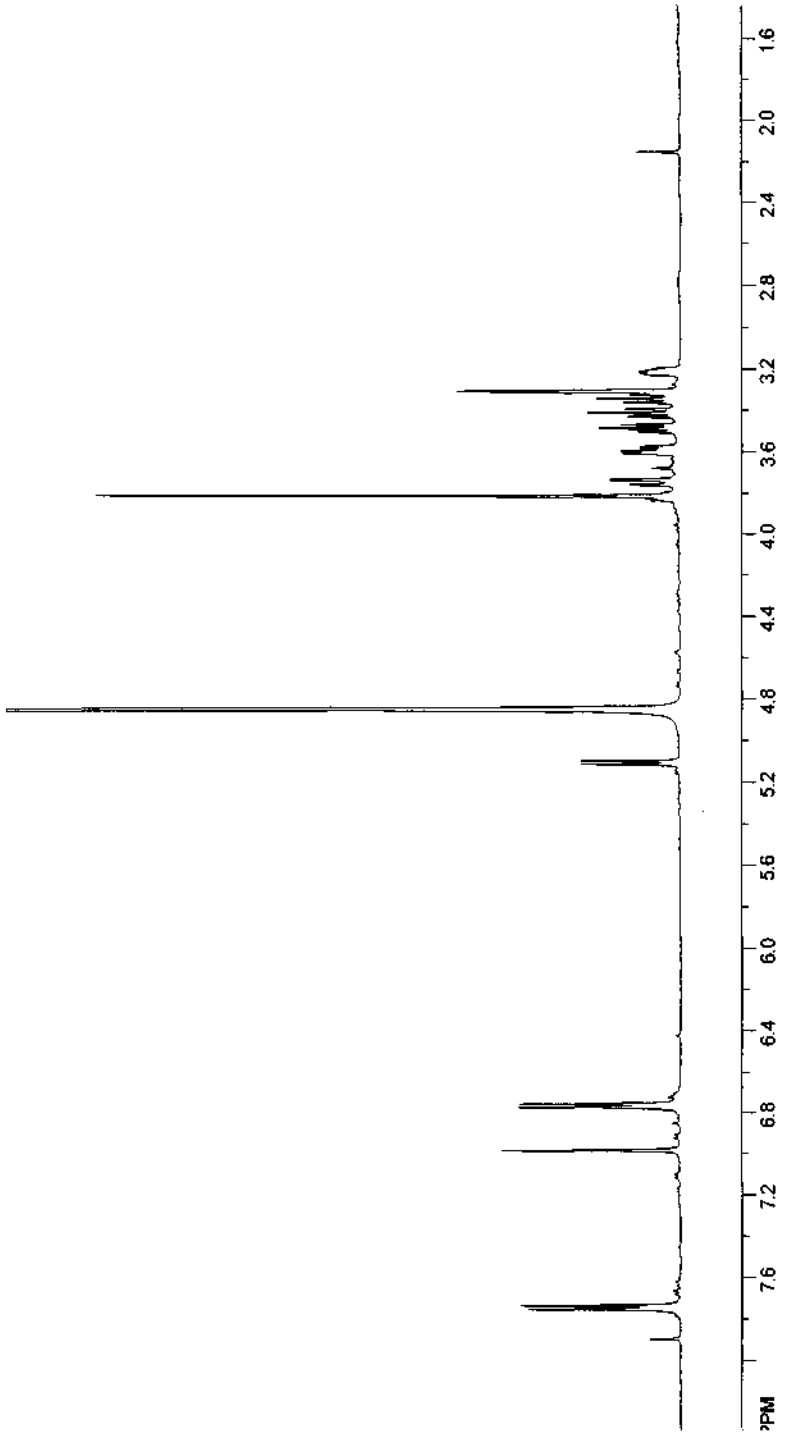


PLATE 15

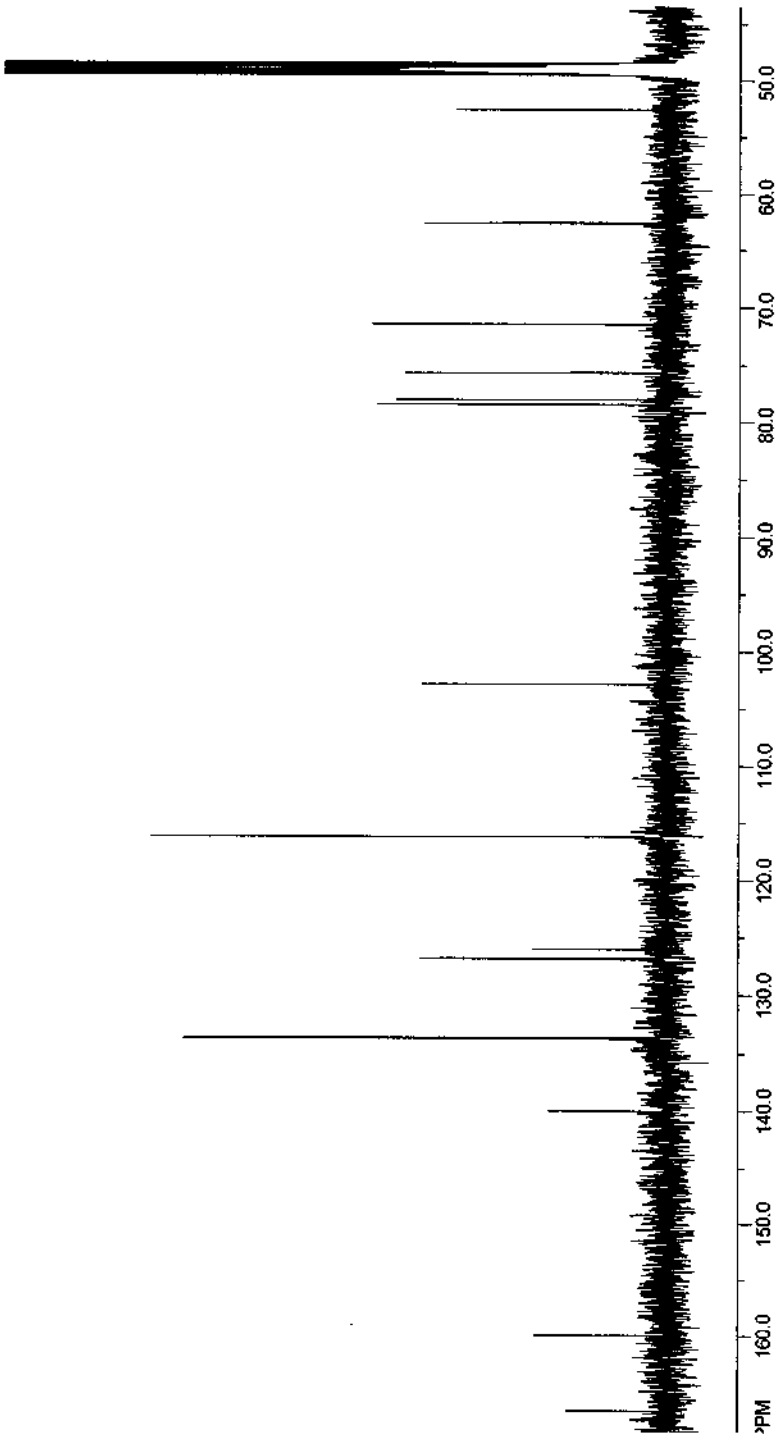


PLATE 16

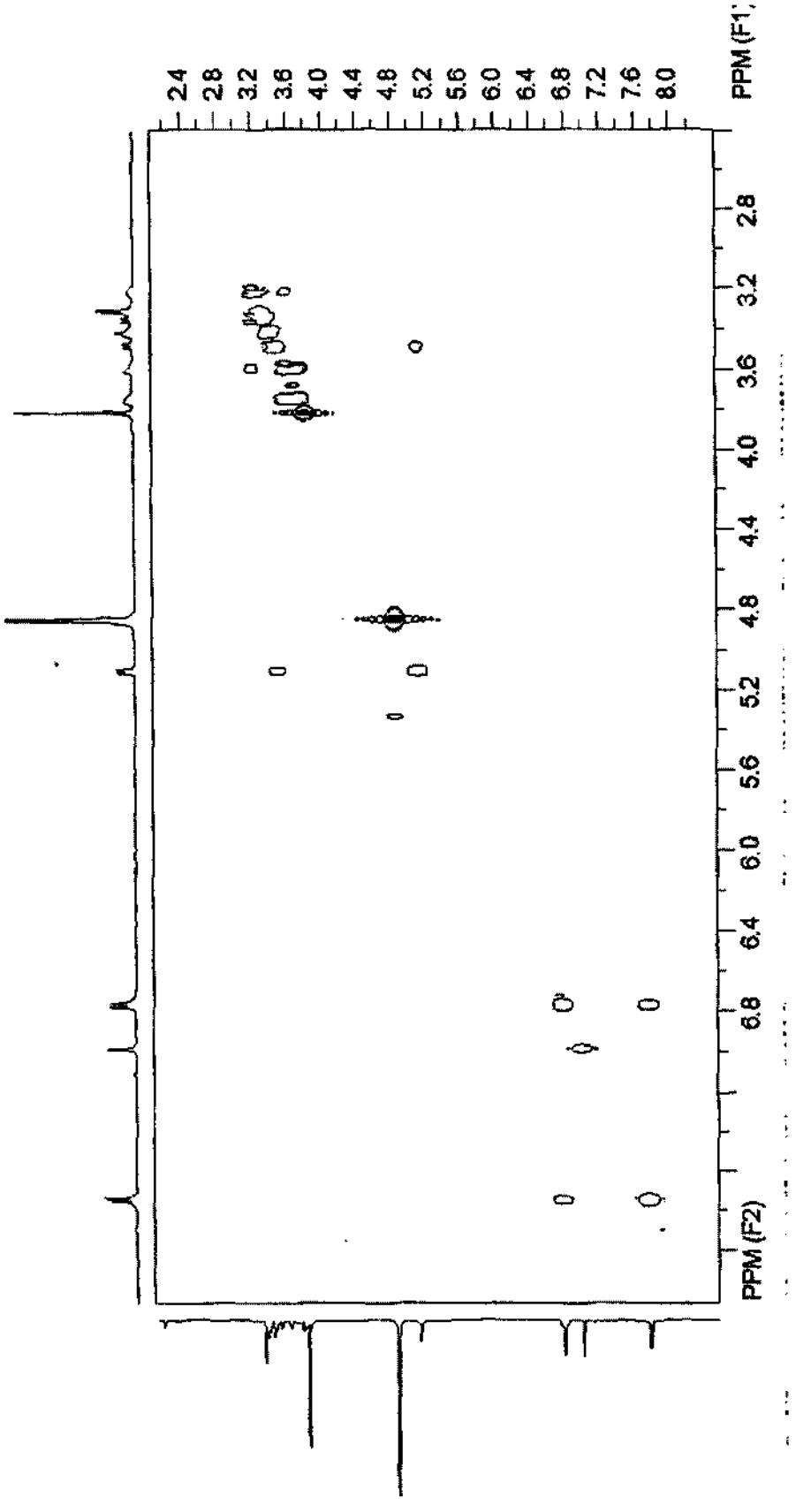

PPM (F1)
2.4
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
6.0
6.4
6.8
7.2
7.6
8.0
PPM (F2)
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
6.0
6.4
6.8

PLATE 17

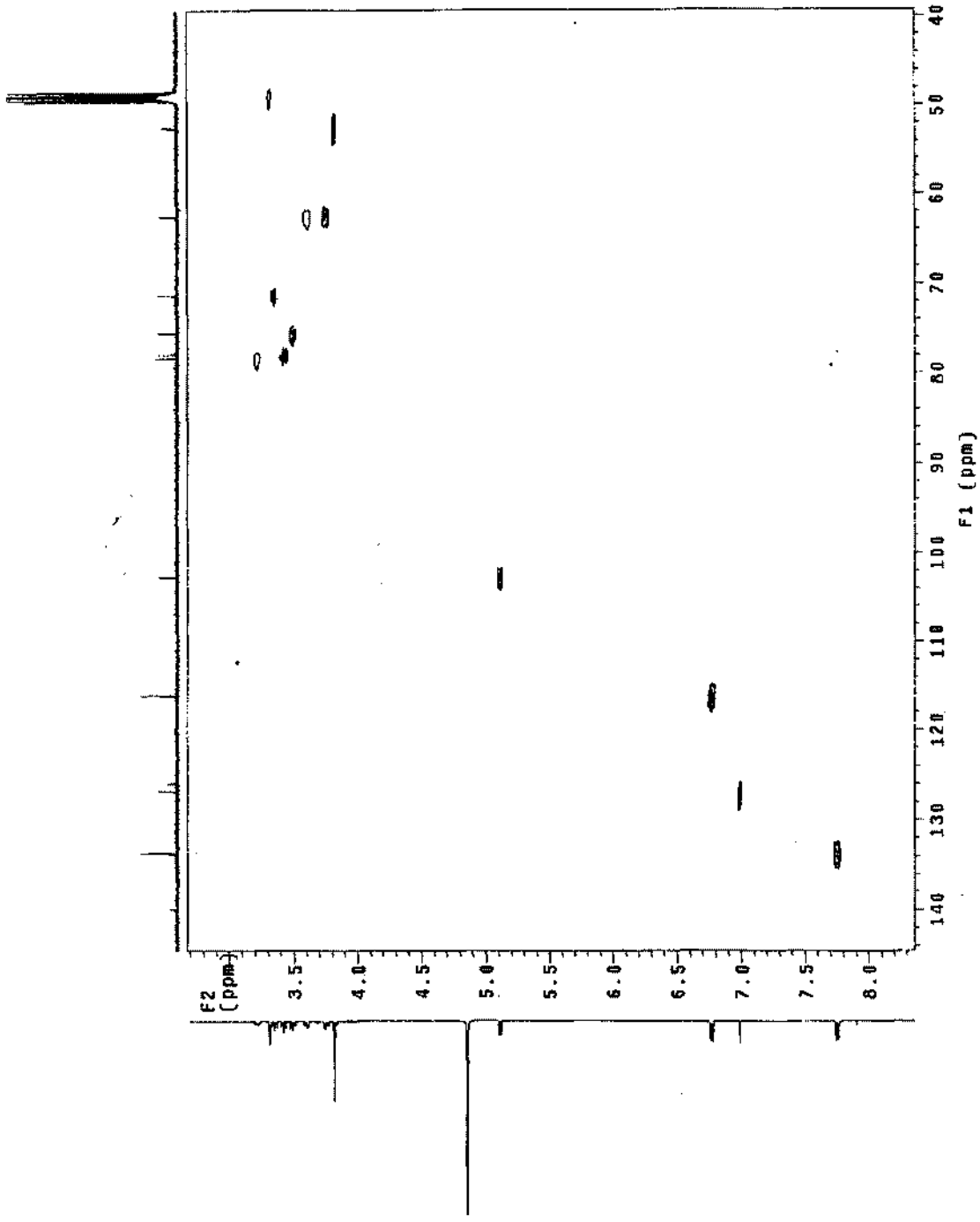

F2 (ppm)
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
F1 (ppm)
40
50
60
70
80
90
100
110
120
130
140

PLATE 18

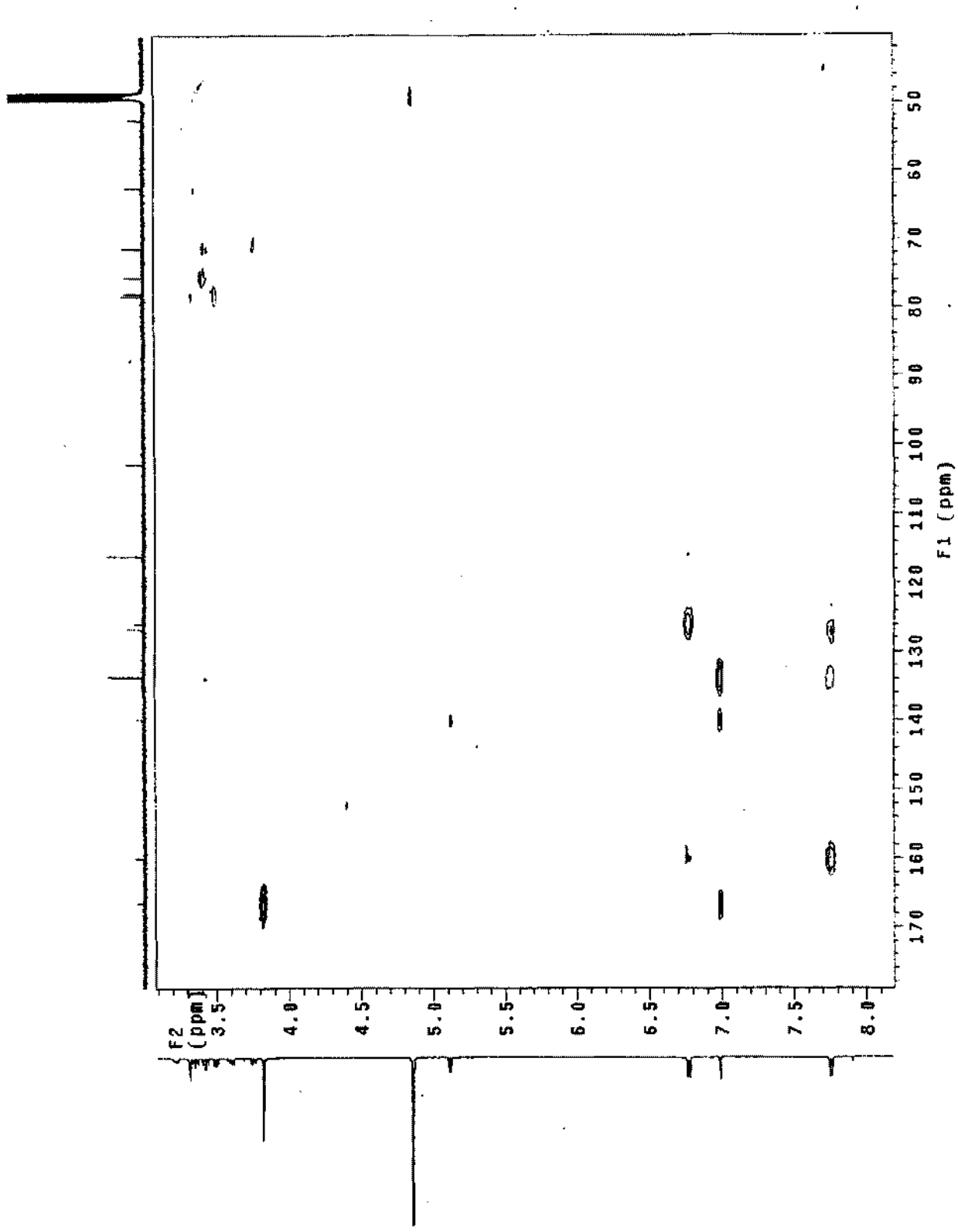

F2 (ppm)
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
F1 (ppm)
50
60
70
80
90
100
110
120
130
140
150
160
170

PLATE 19

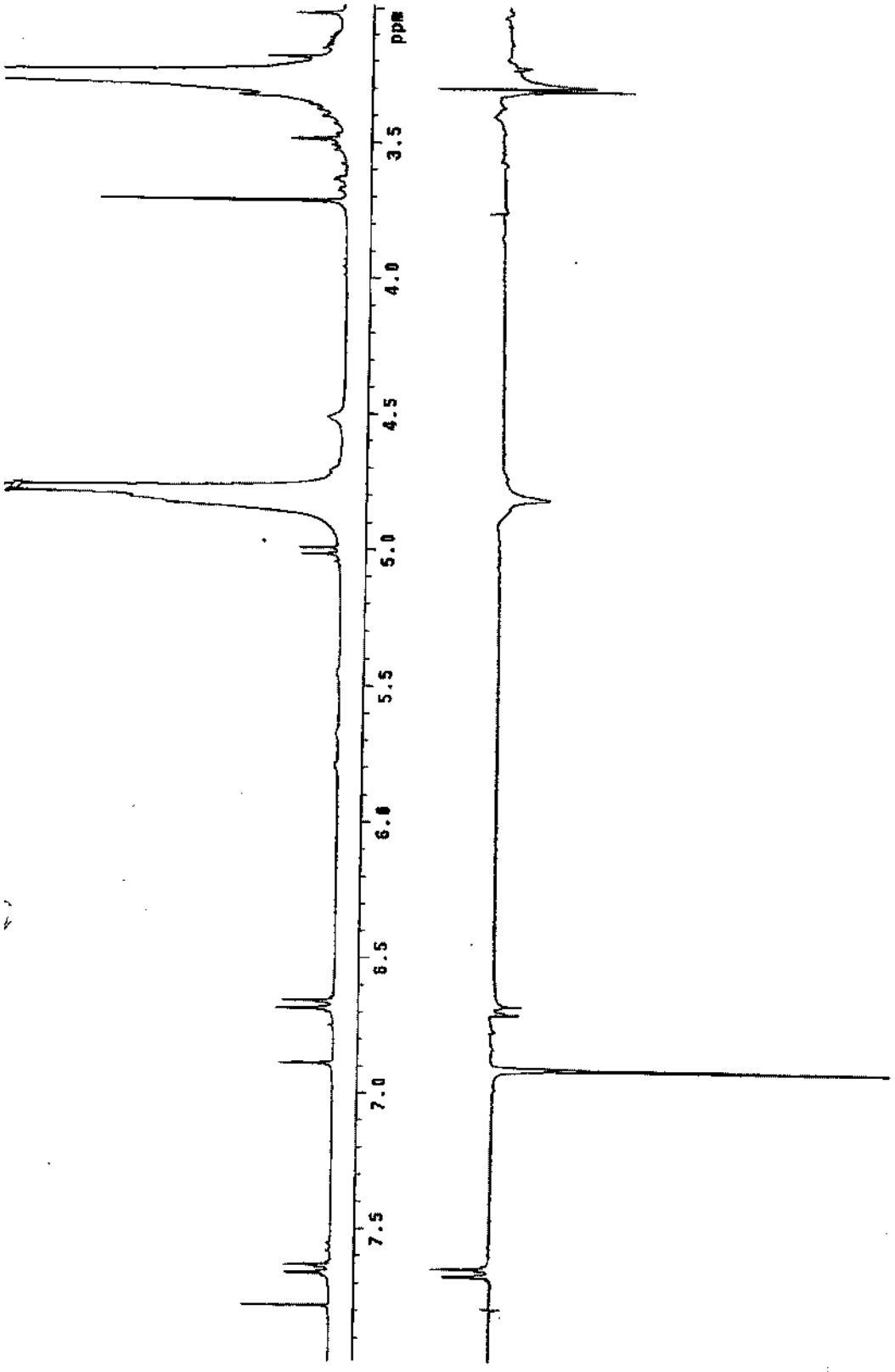


PLATE 20

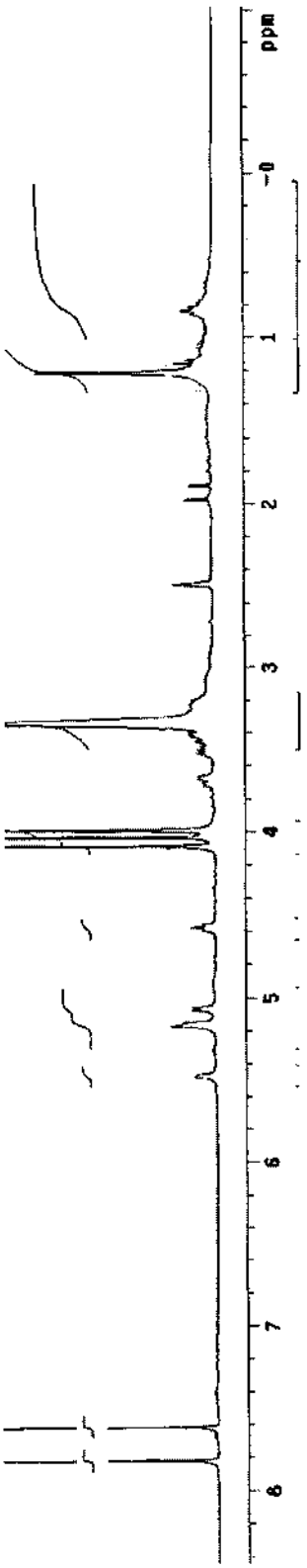


PLATE 21

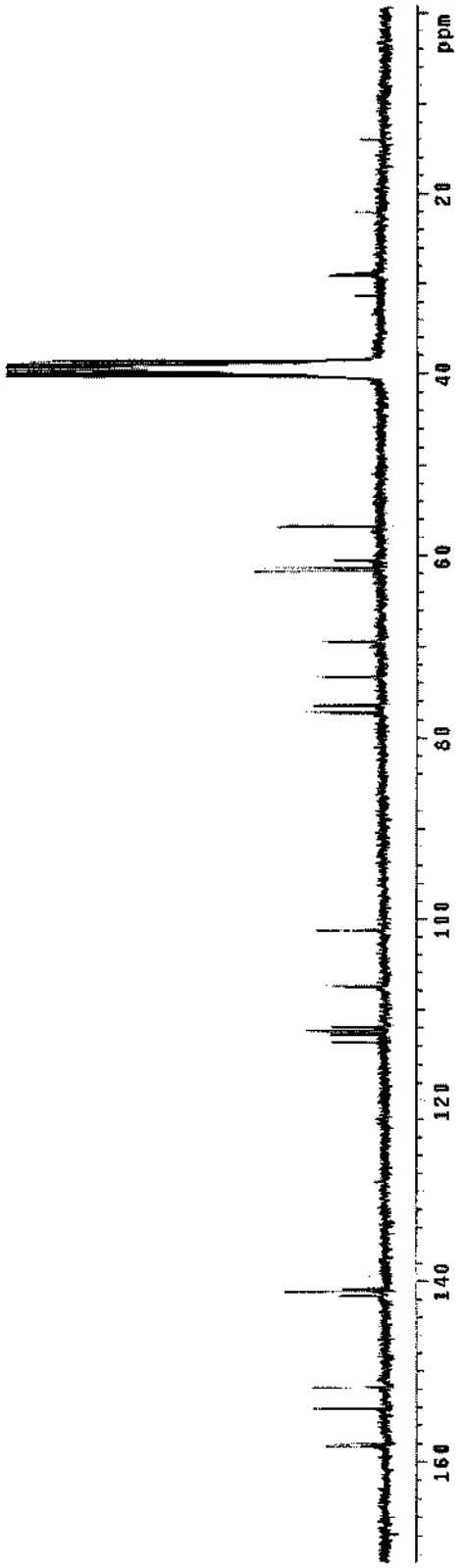


PLATE 22

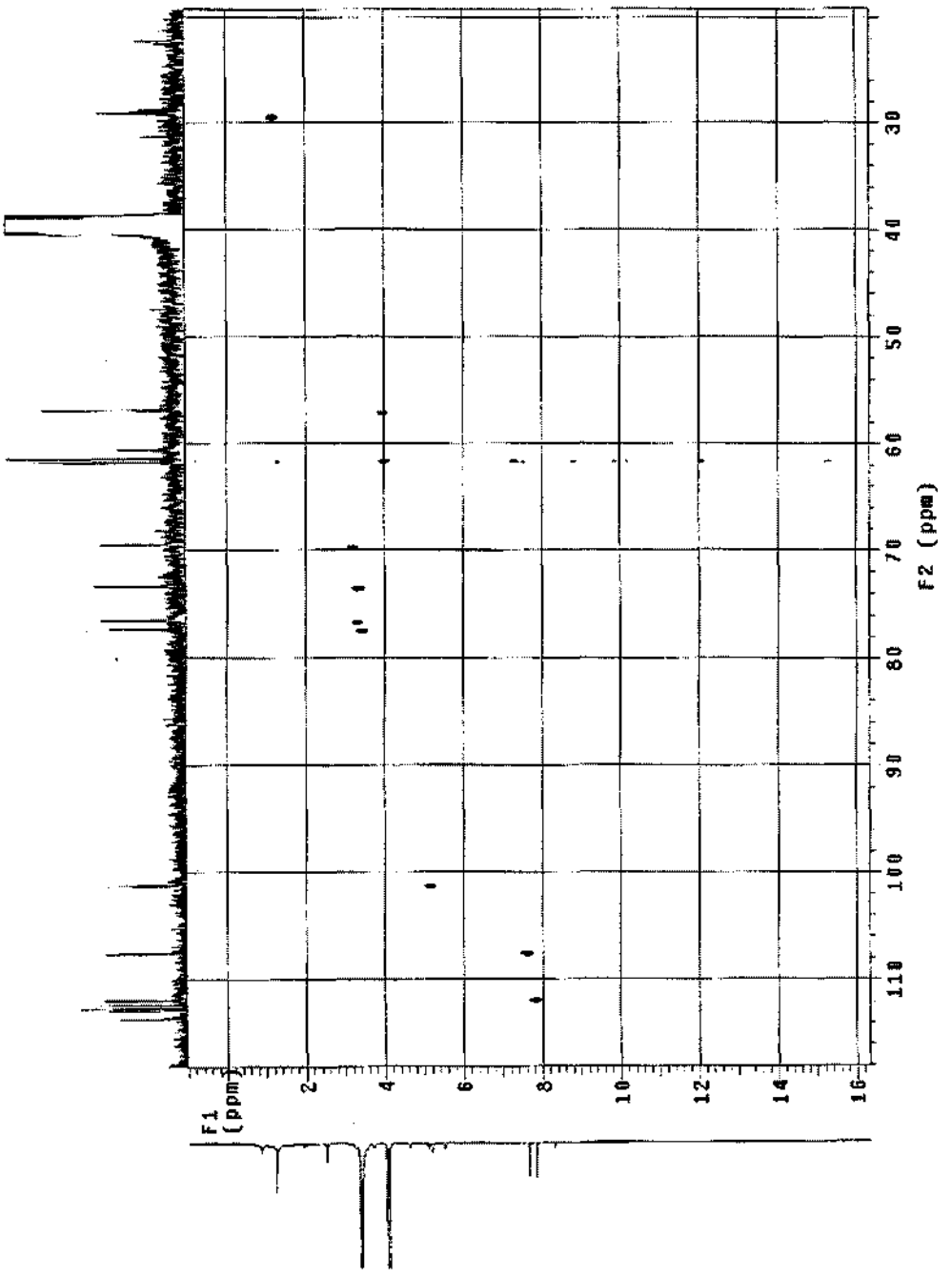


PLATE 23

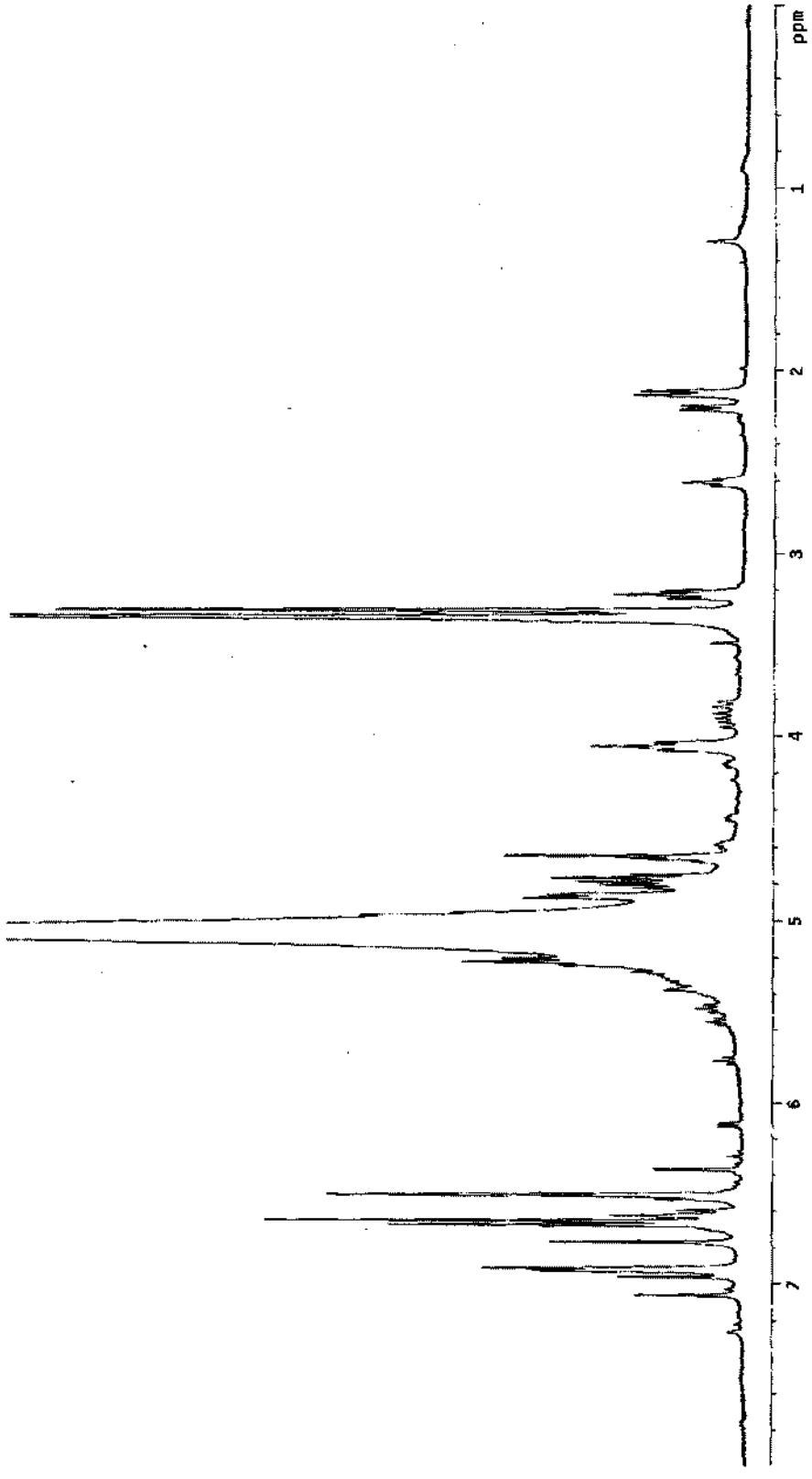


PLATE 24

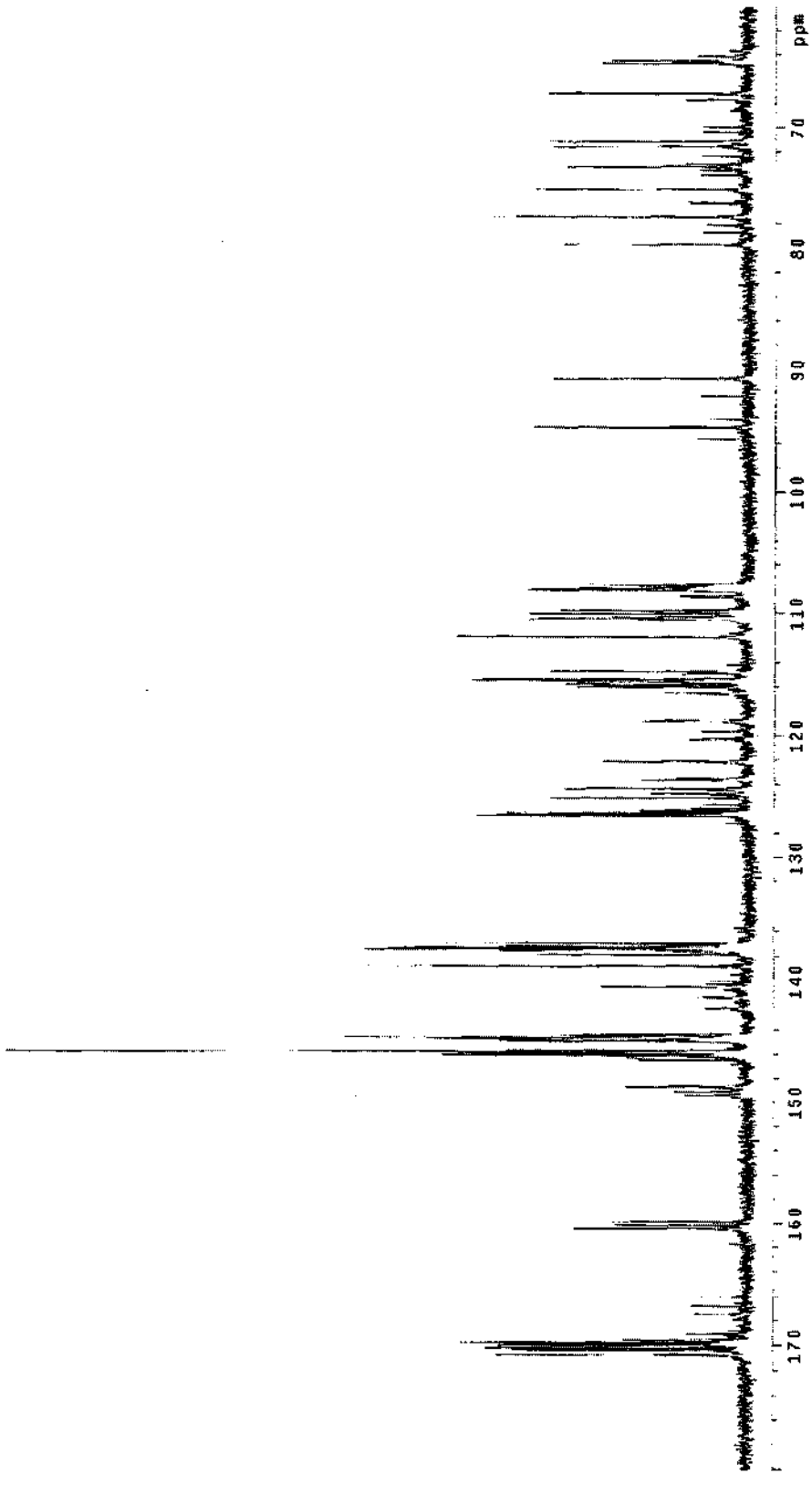


PLATE 25

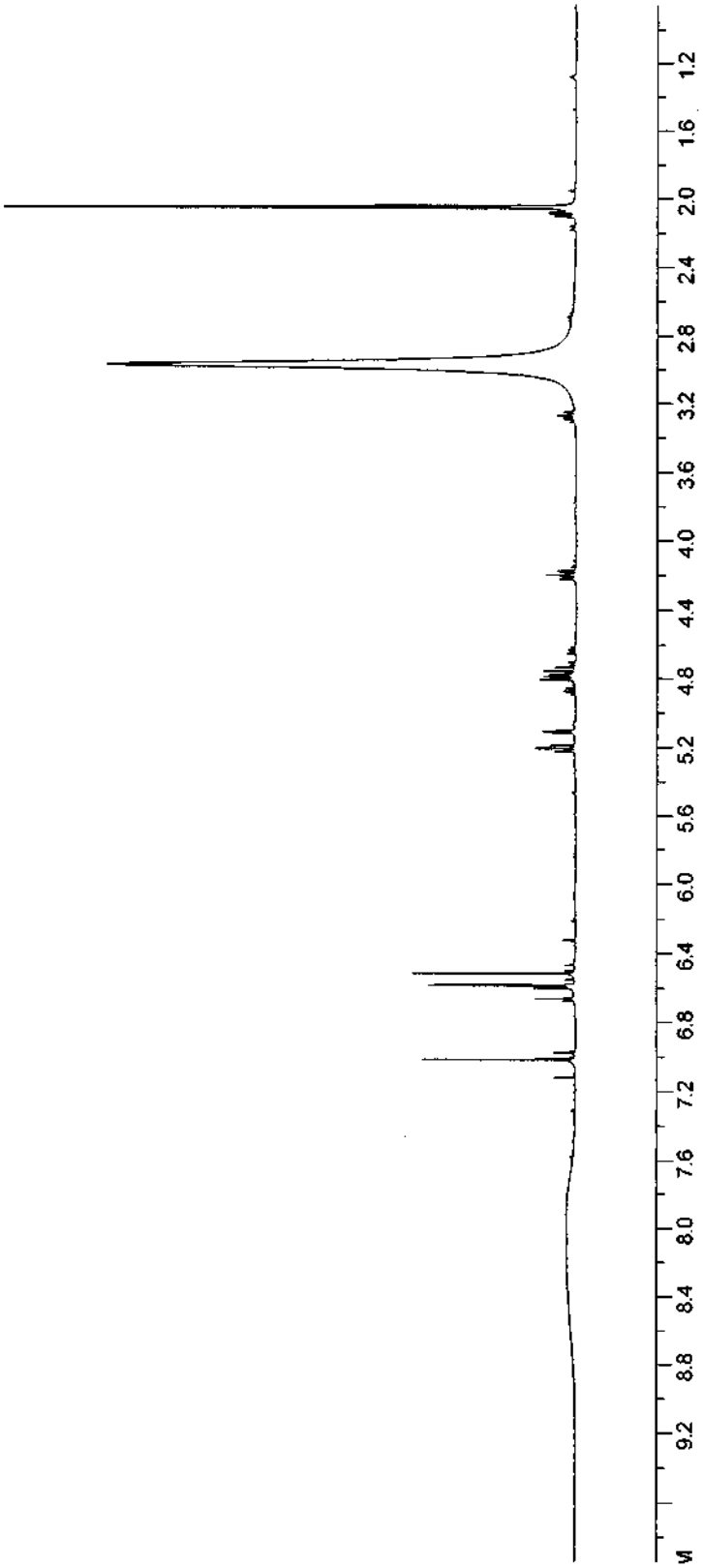


PLATE 26

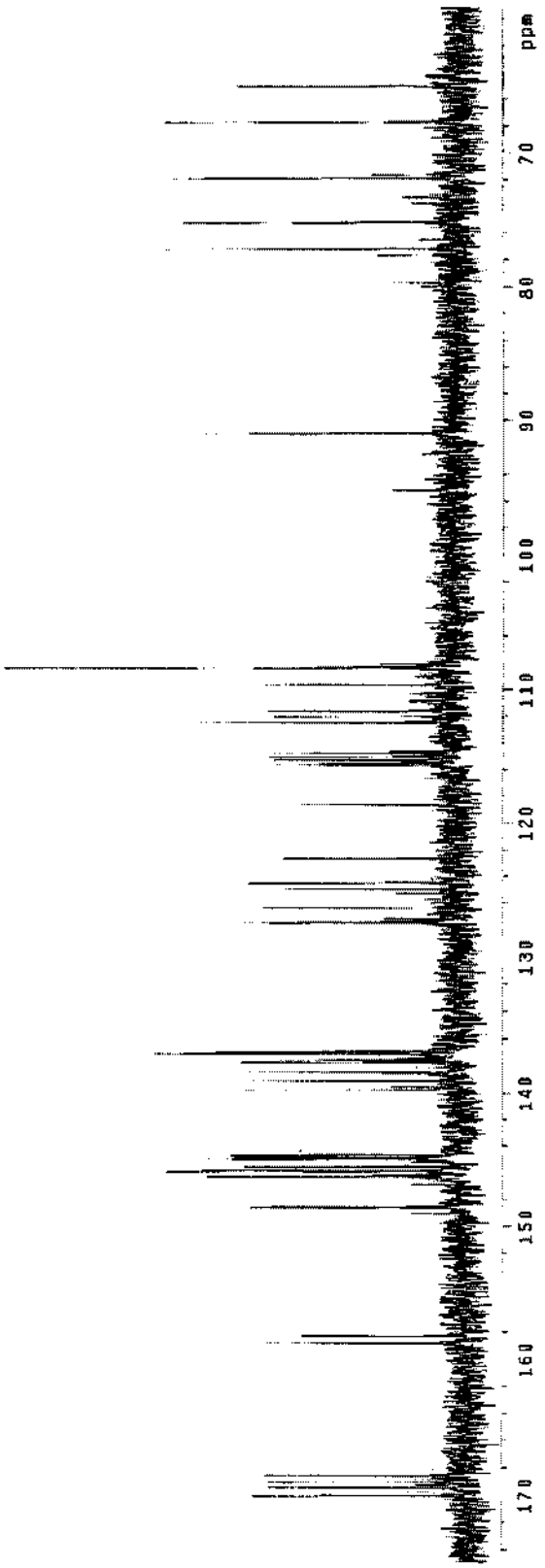


PLATE 27

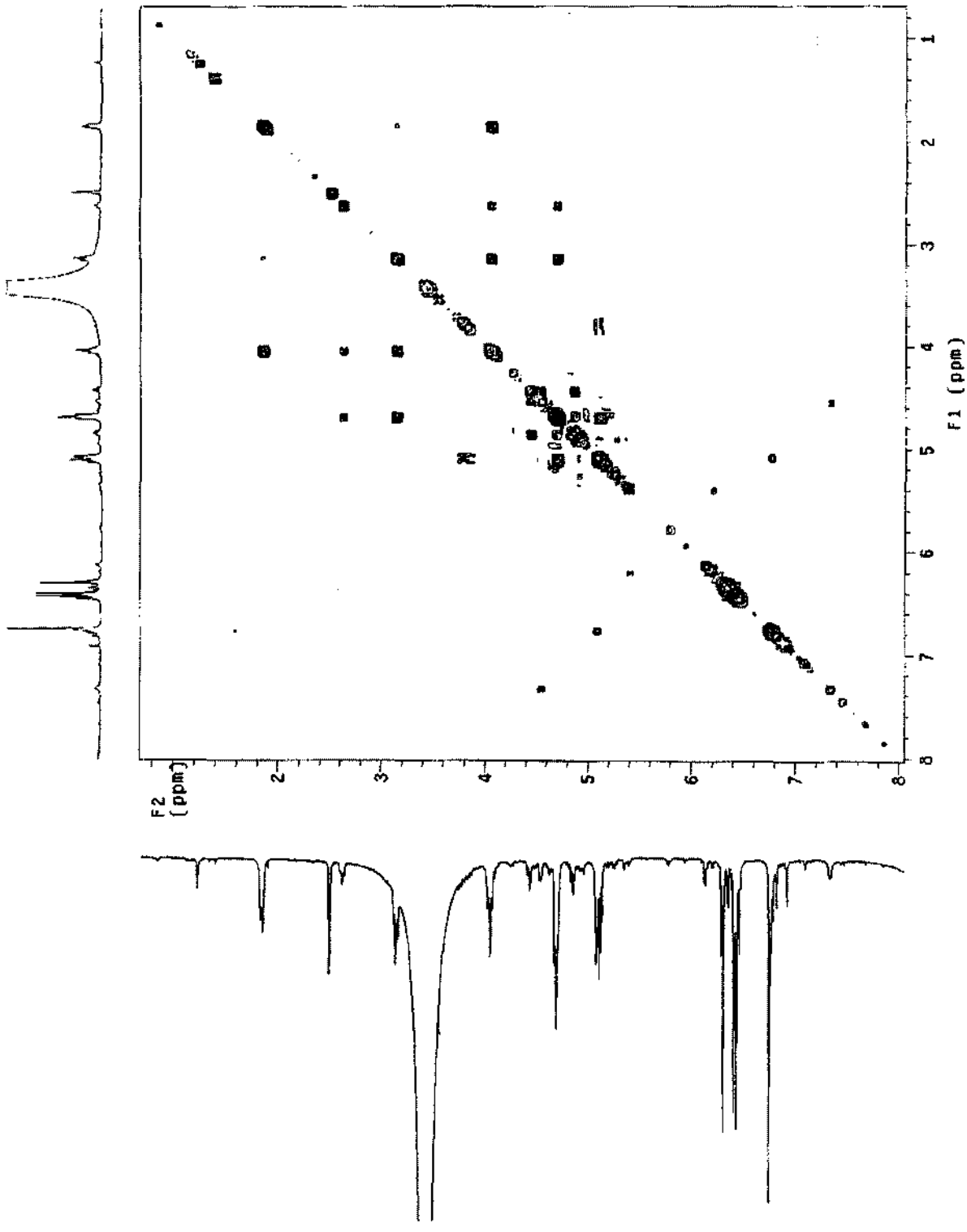


PLATE 28

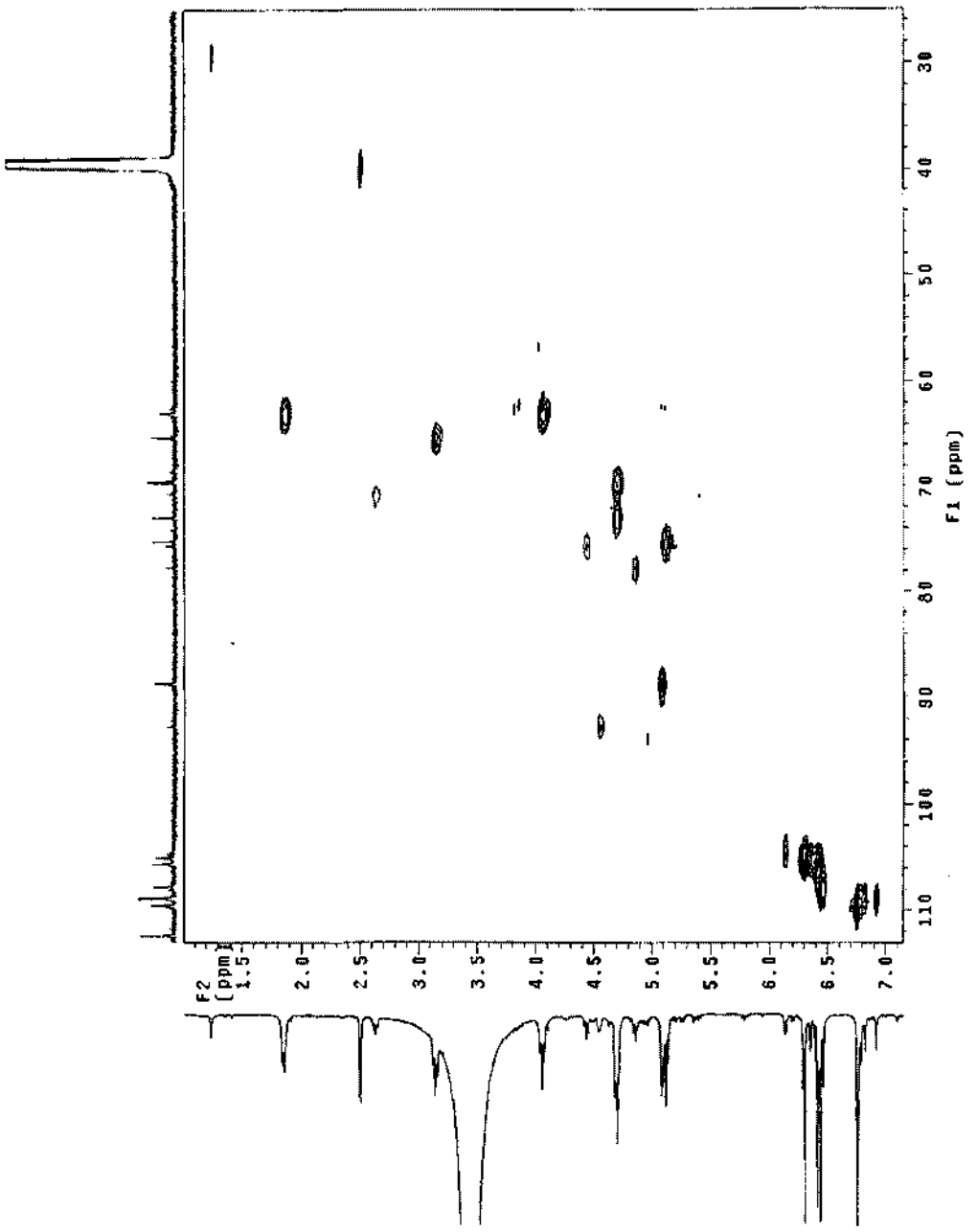

F2 (ppm)
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
F1 (ppm)
30
40
50
60
70
80
90
100
110

PLATE 29

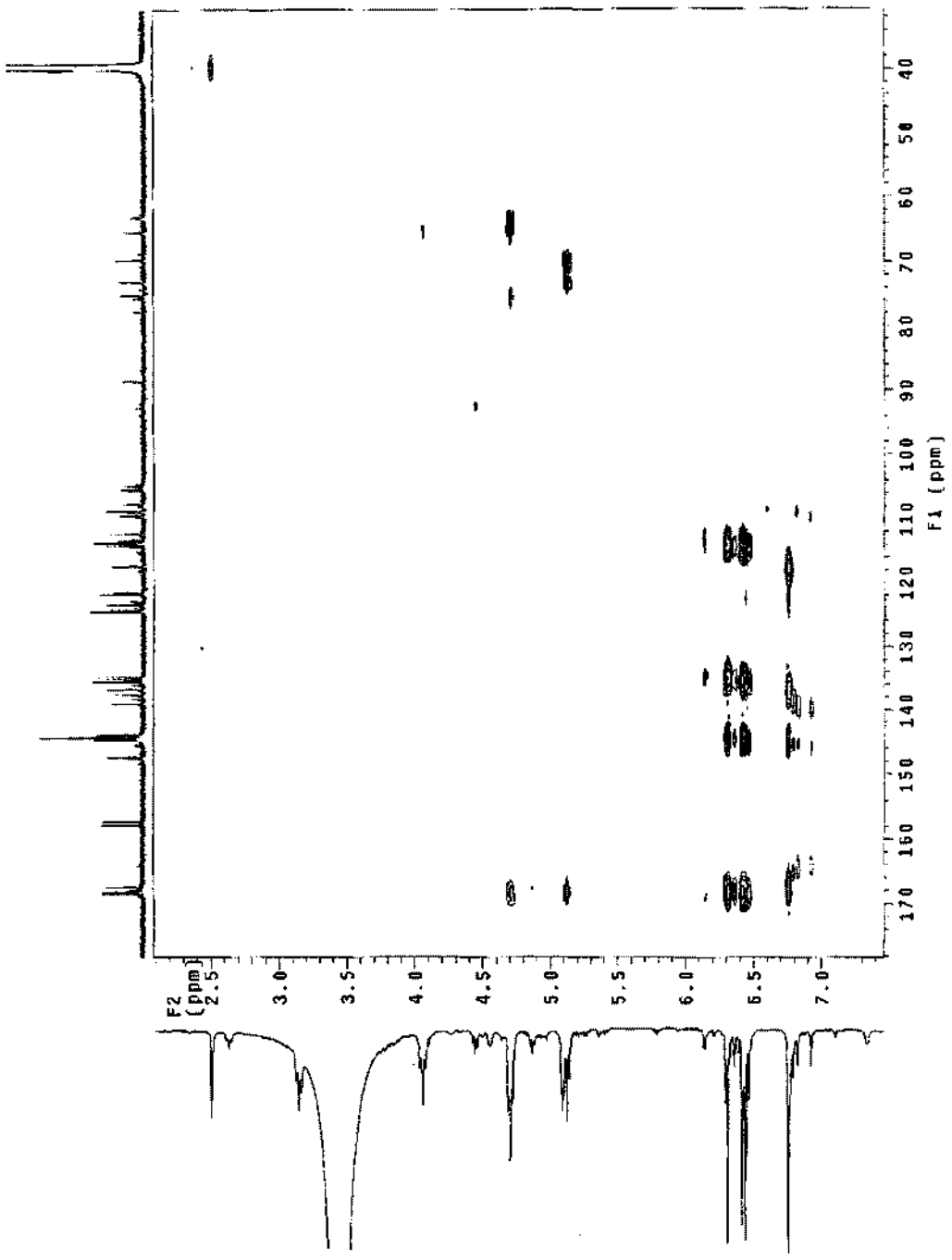

F2 (ppm)
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
F1 (ppm)
40
50
60
70
80
90
100
110
120
130
140
150
160
170

PLATE 30

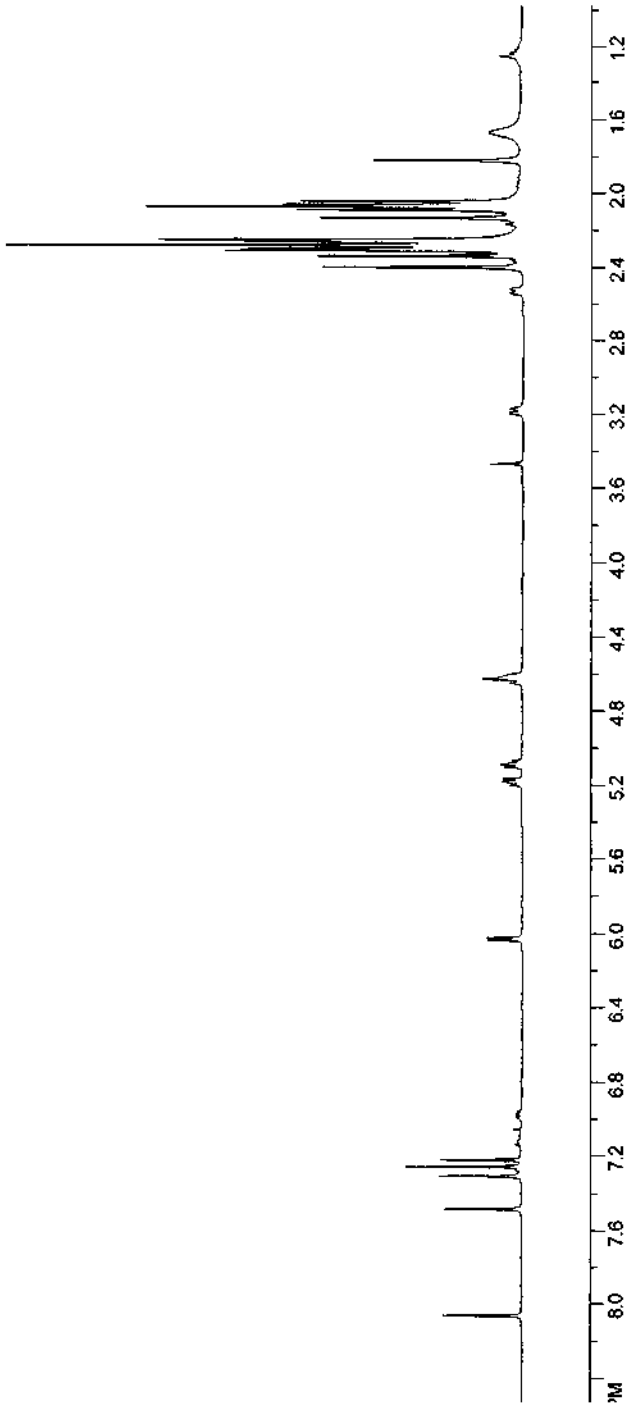


PLATE 31

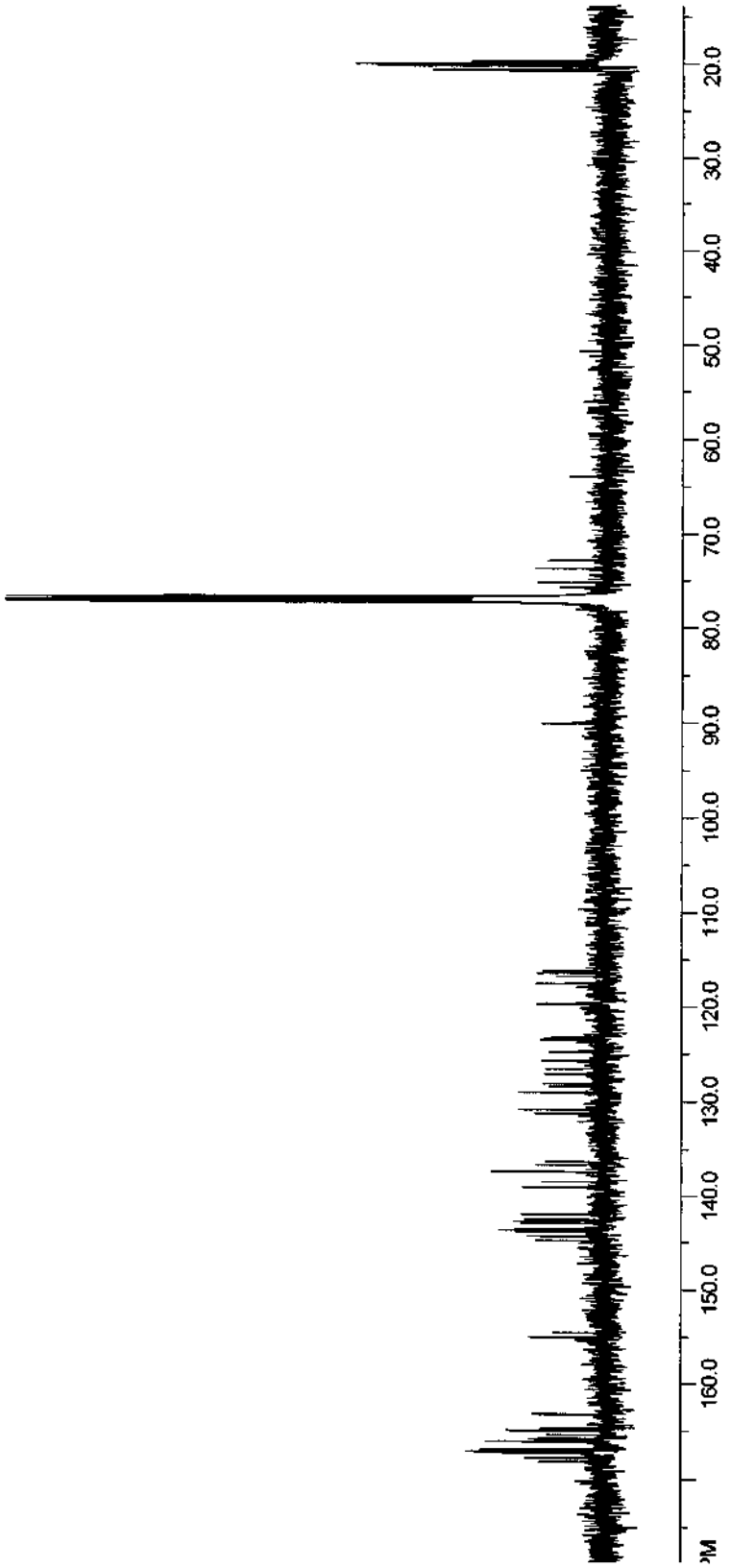

20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0
110.0
120.0
130.0
140.0
150.0
160.0
PM

PLATE 32

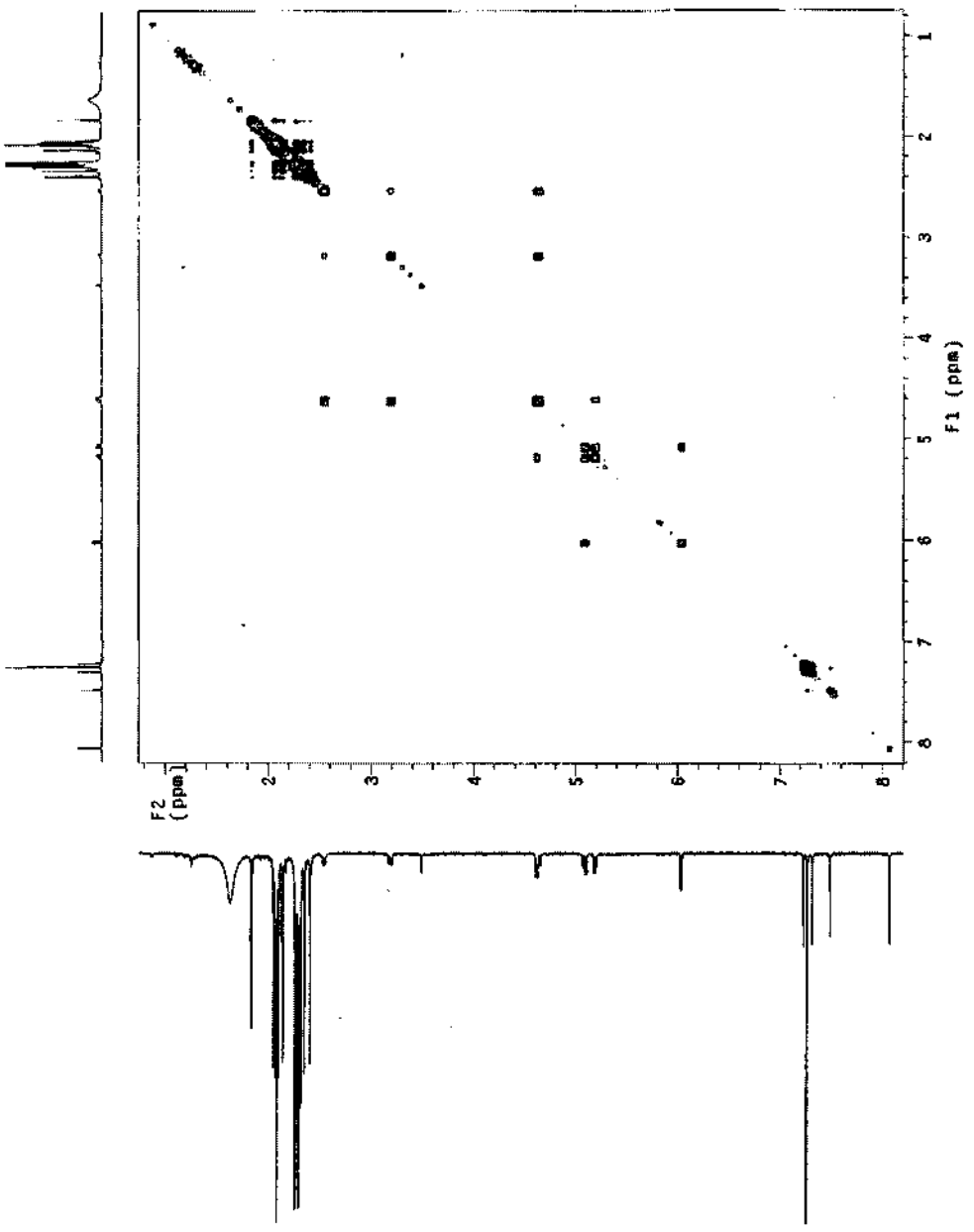


PLATE 33

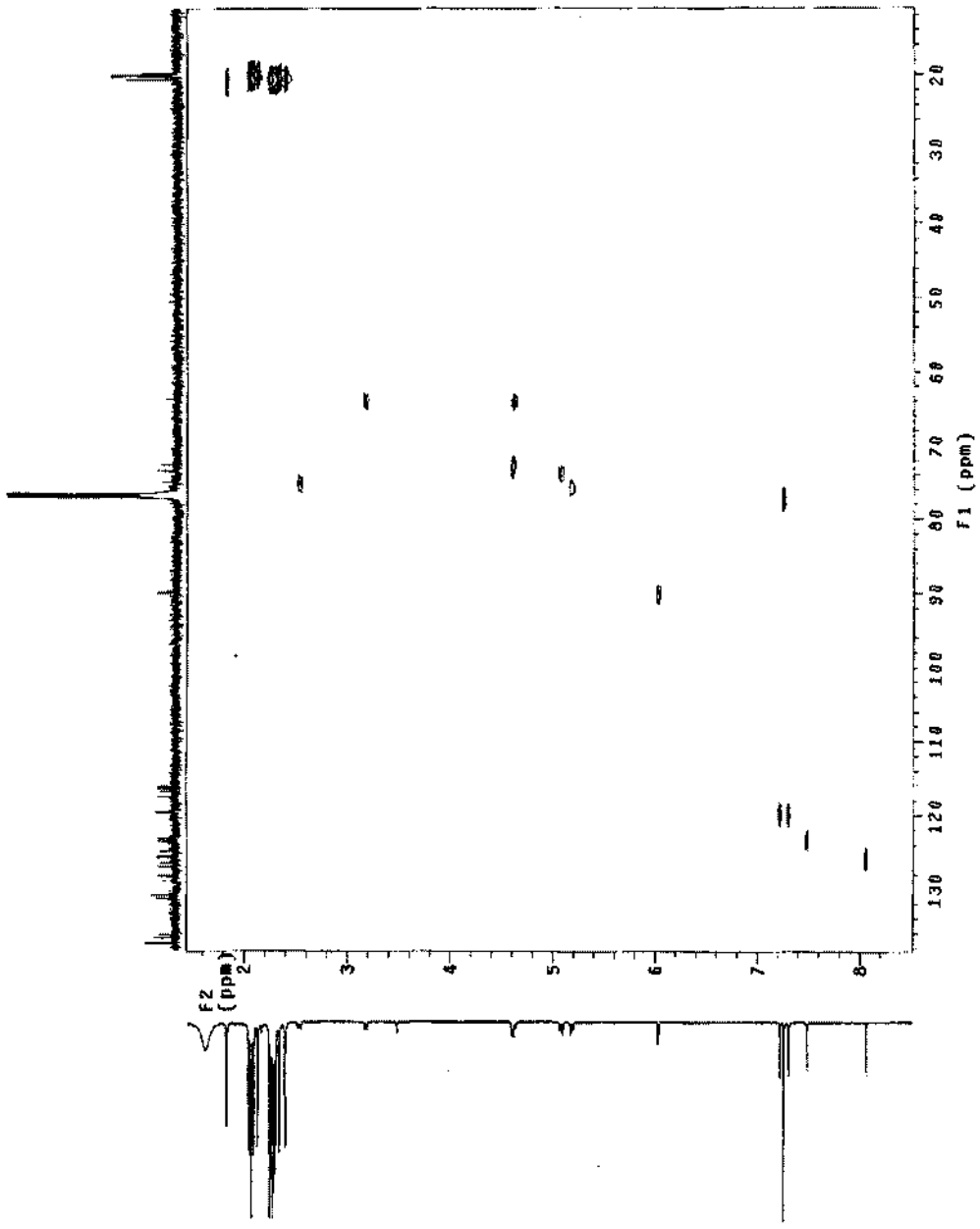


PLATE 34

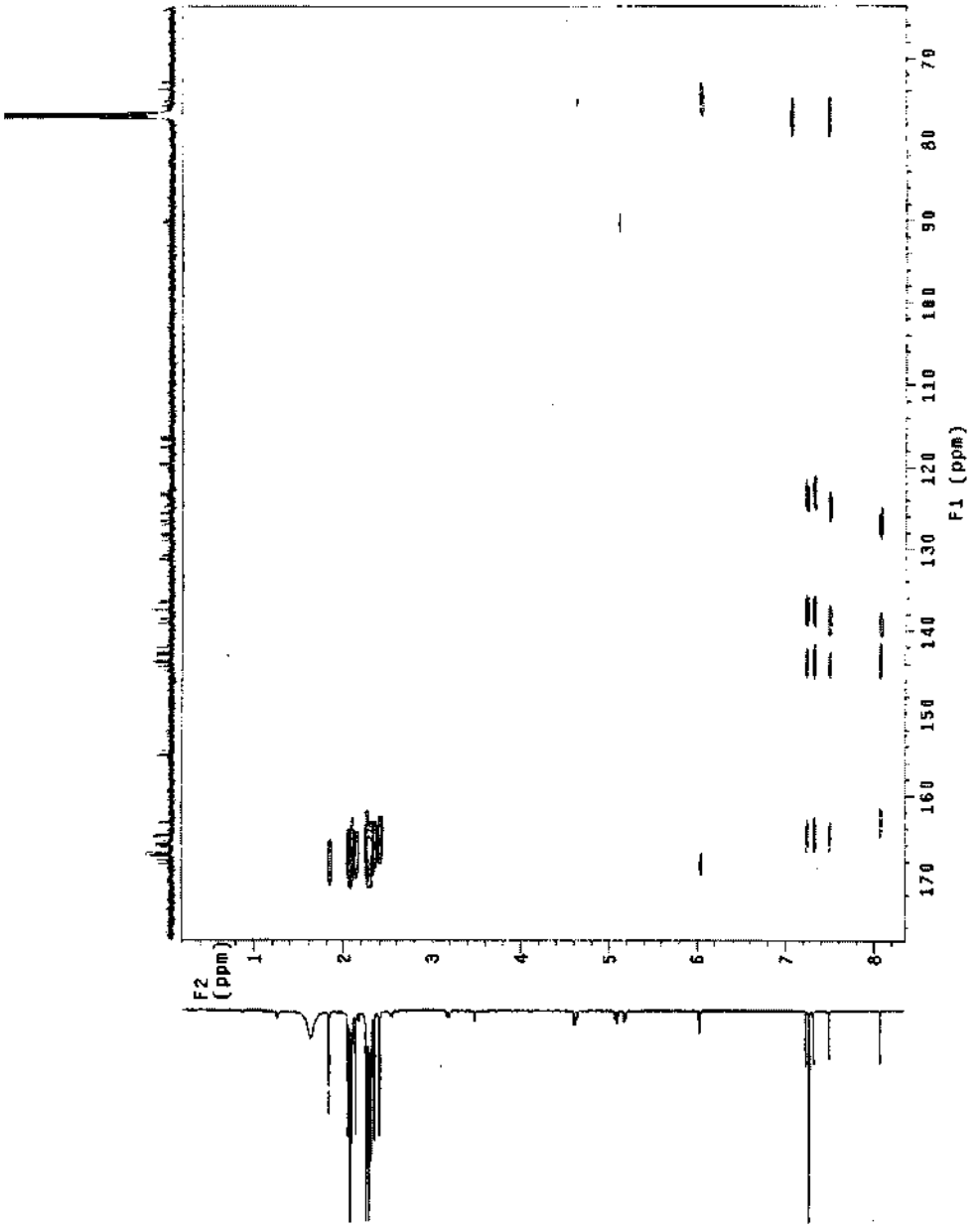


Plate 35

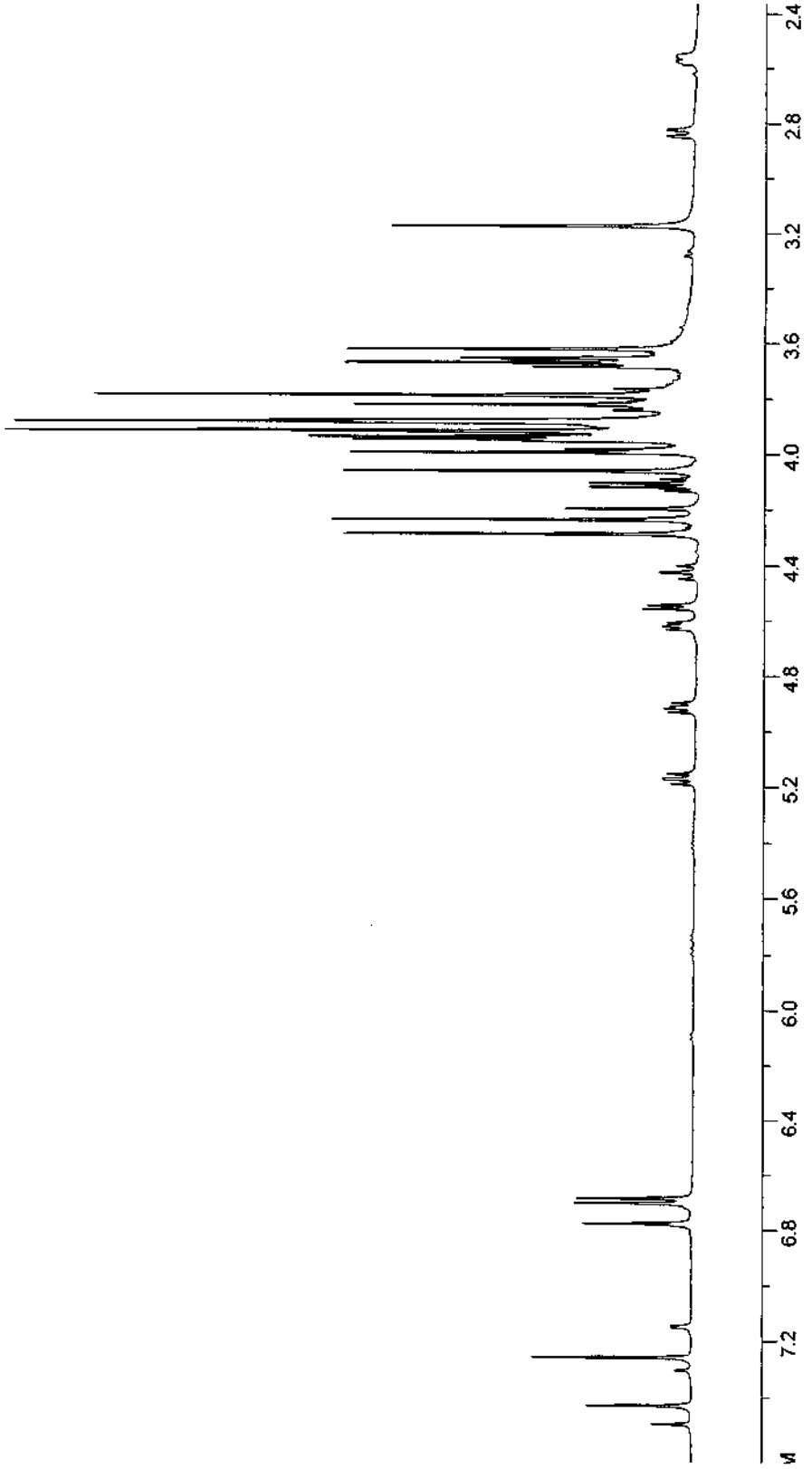


Plate 36

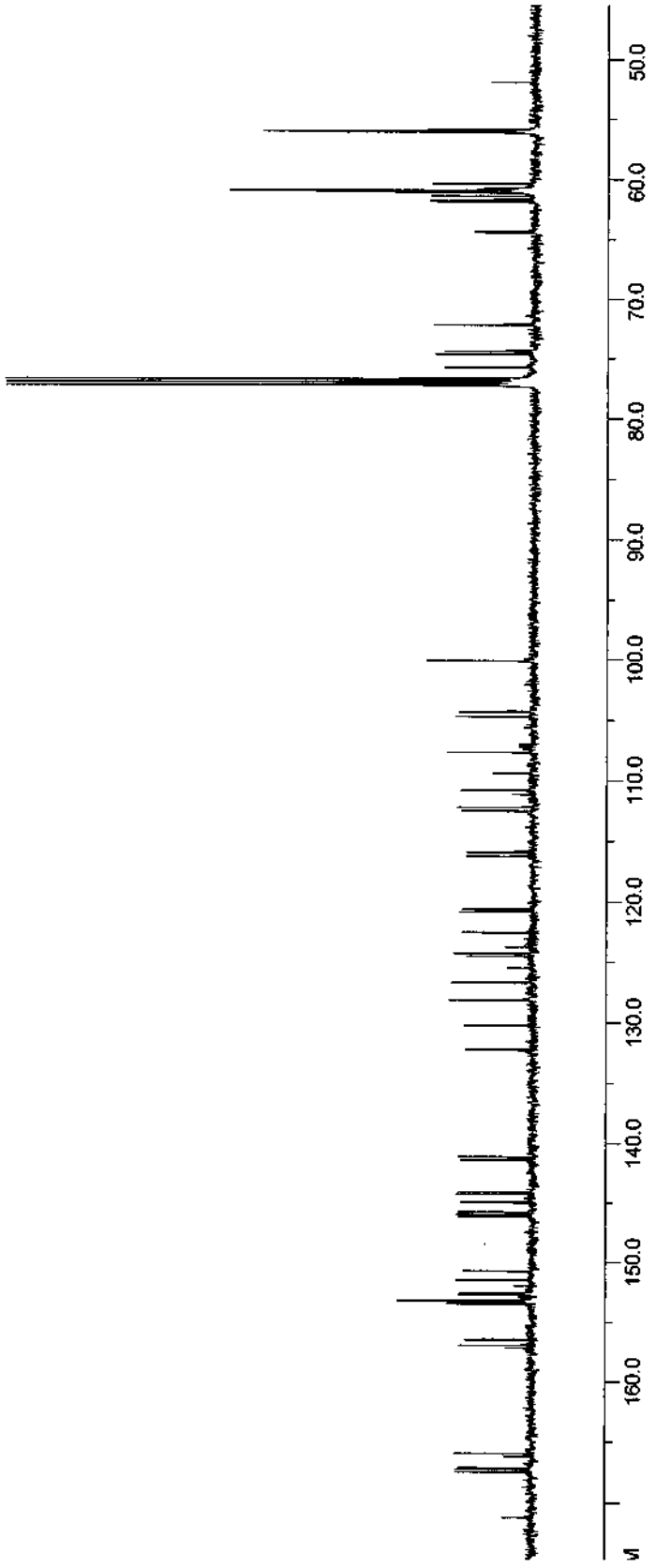


Plate 37

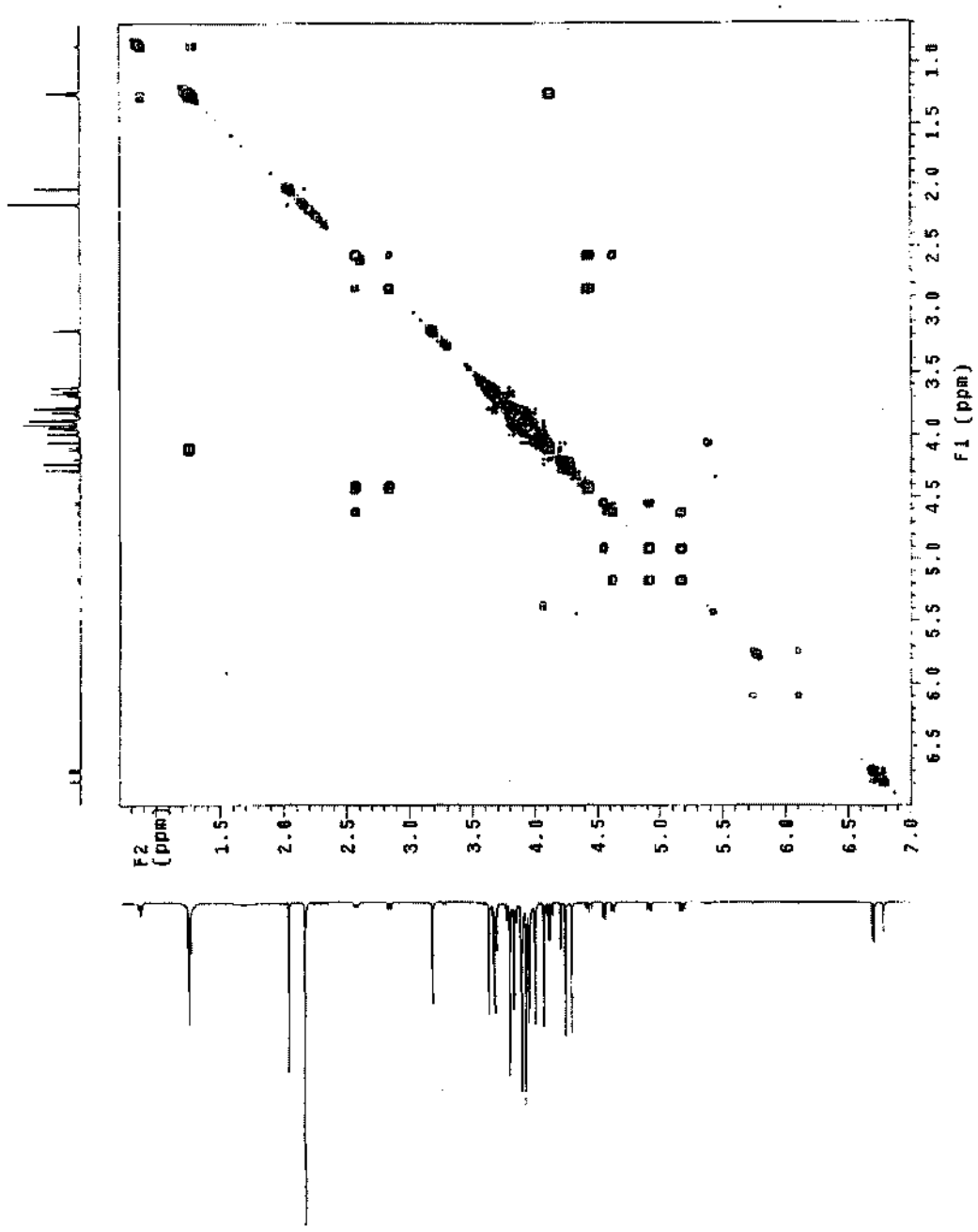


Plate 38

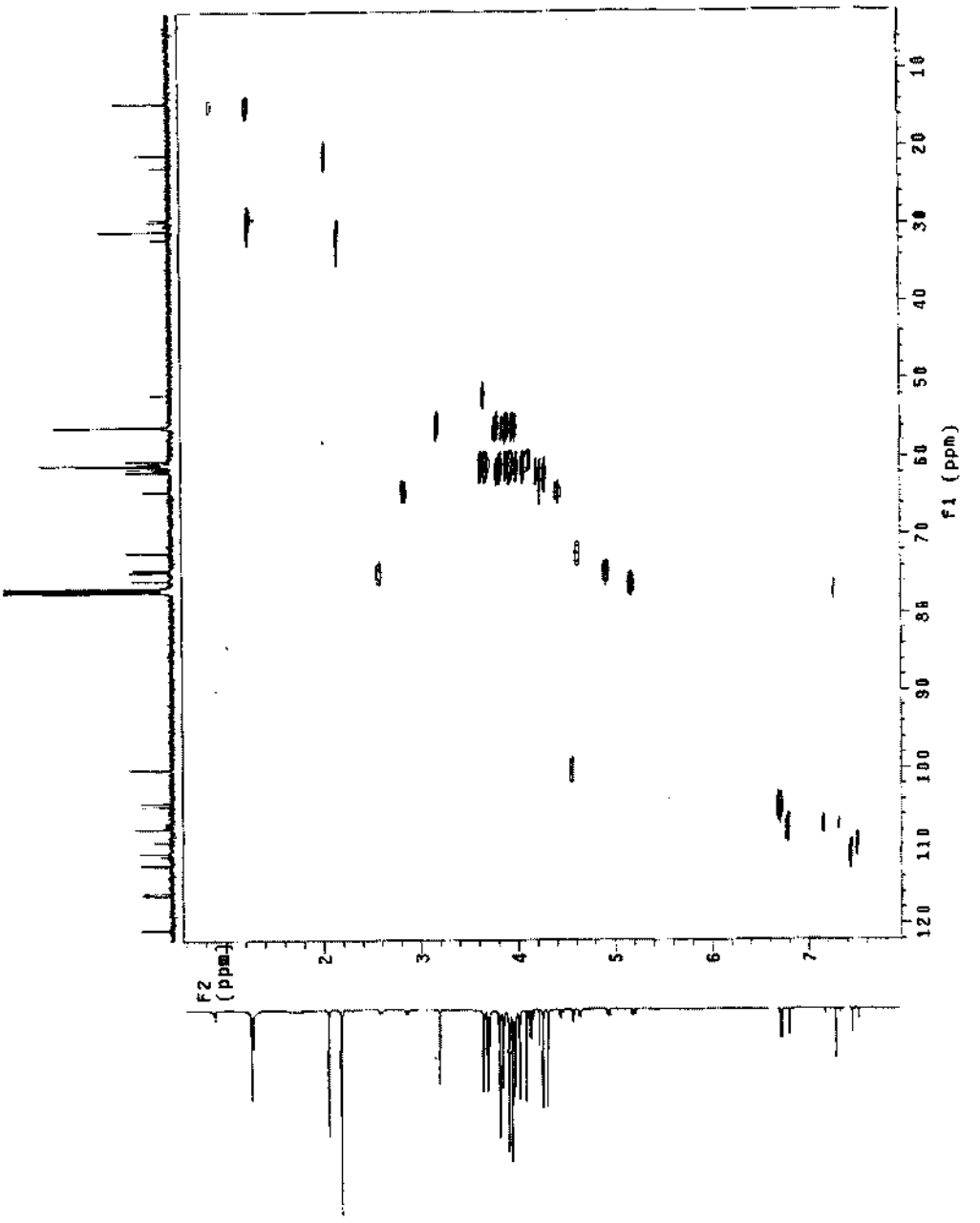


Plate 39

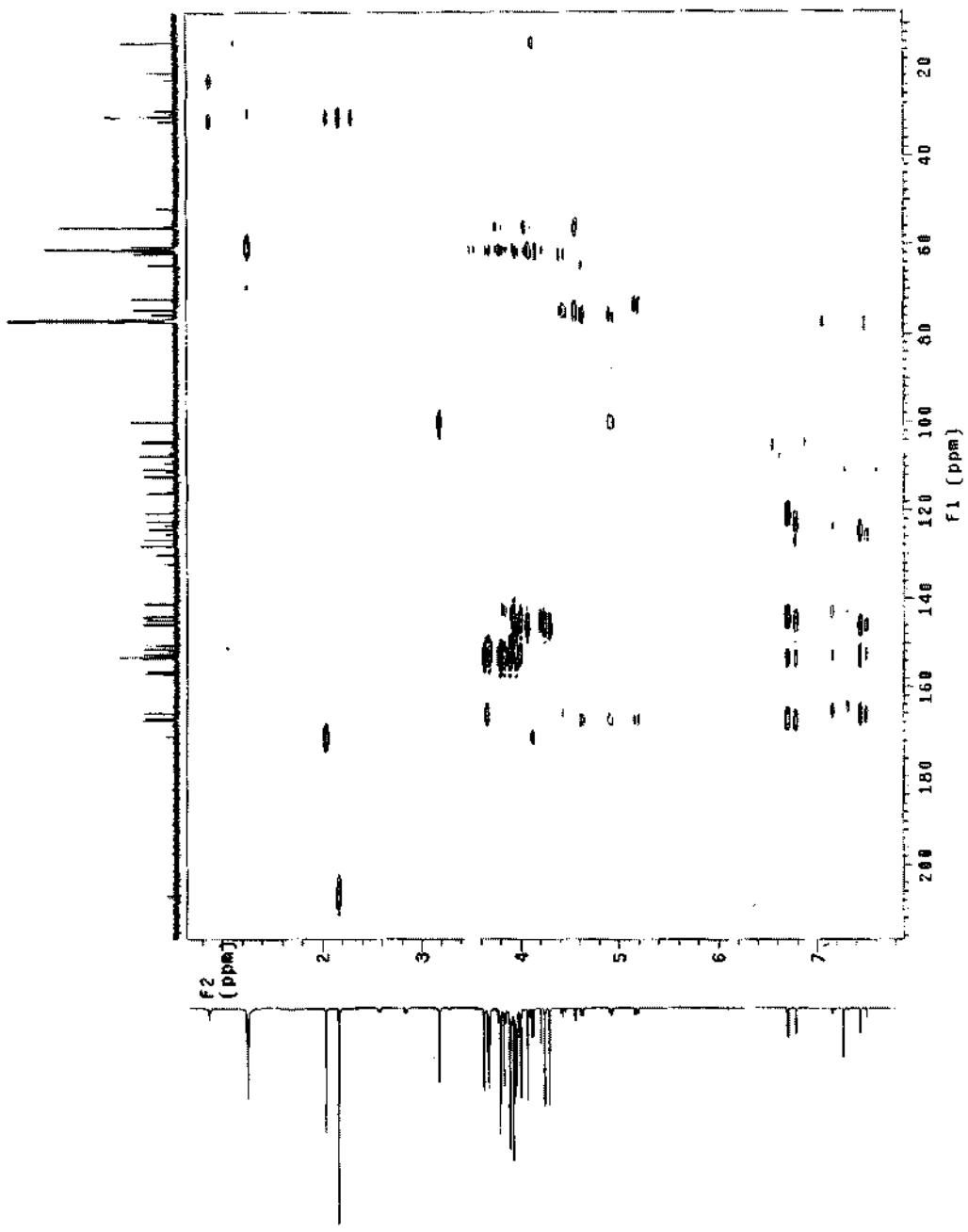


Plate 40

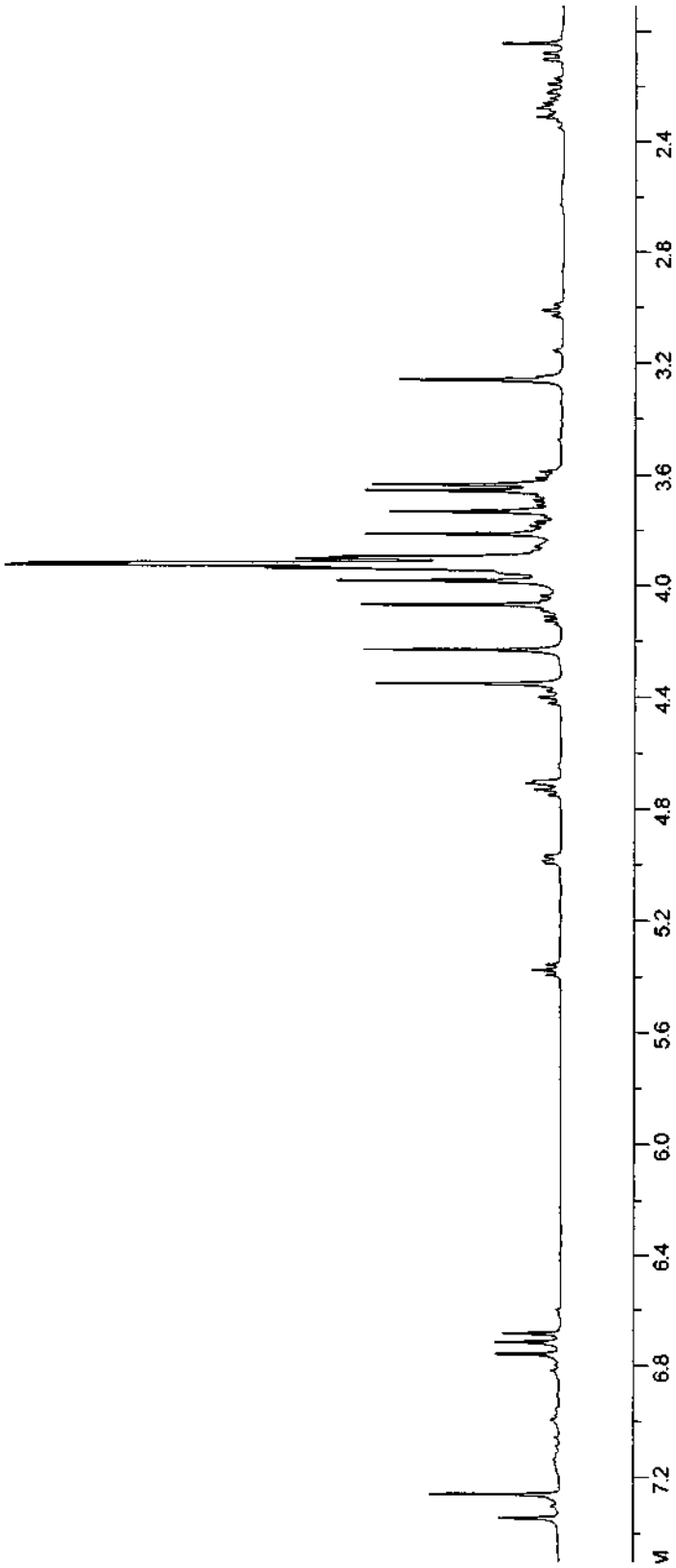


Plate 41

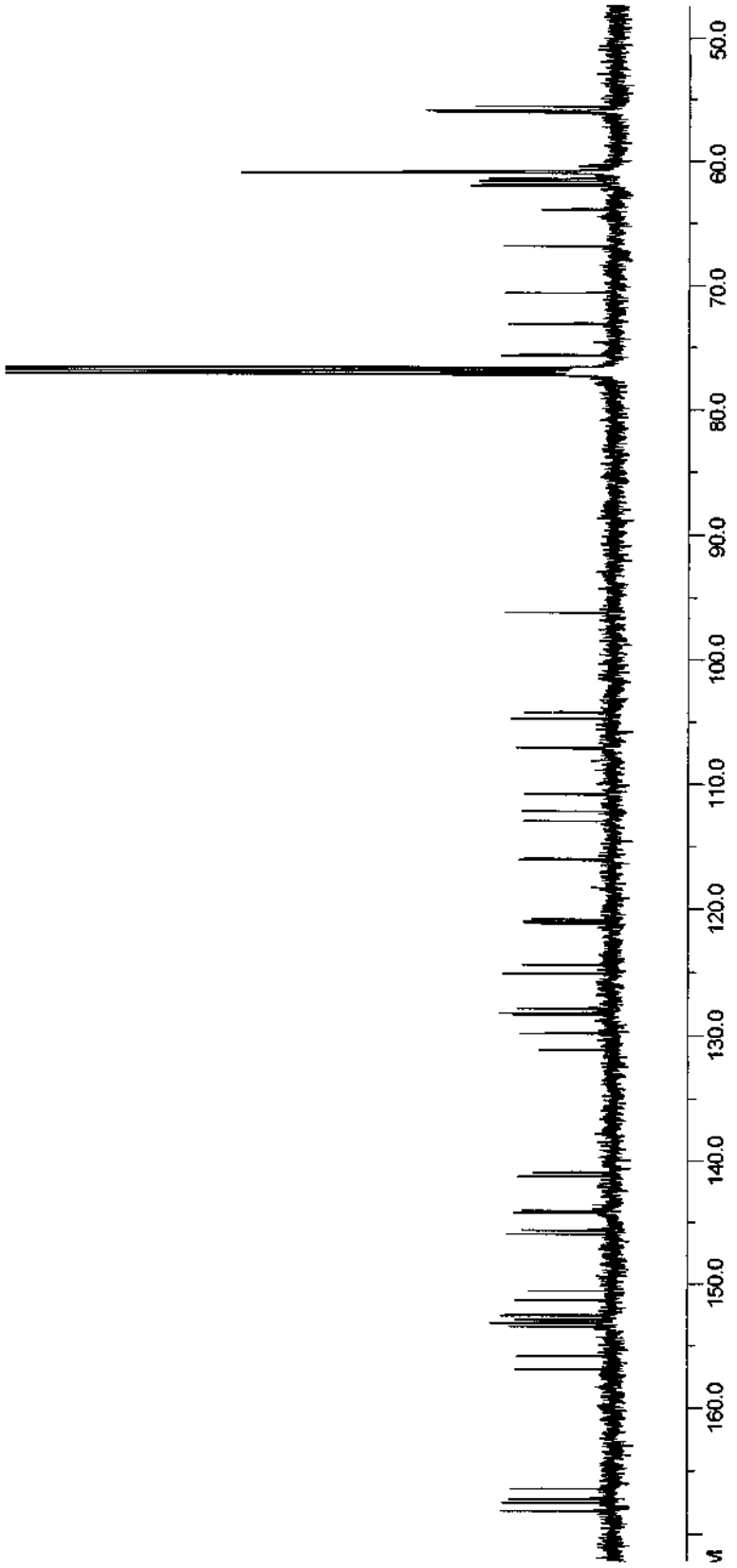


Plate 42

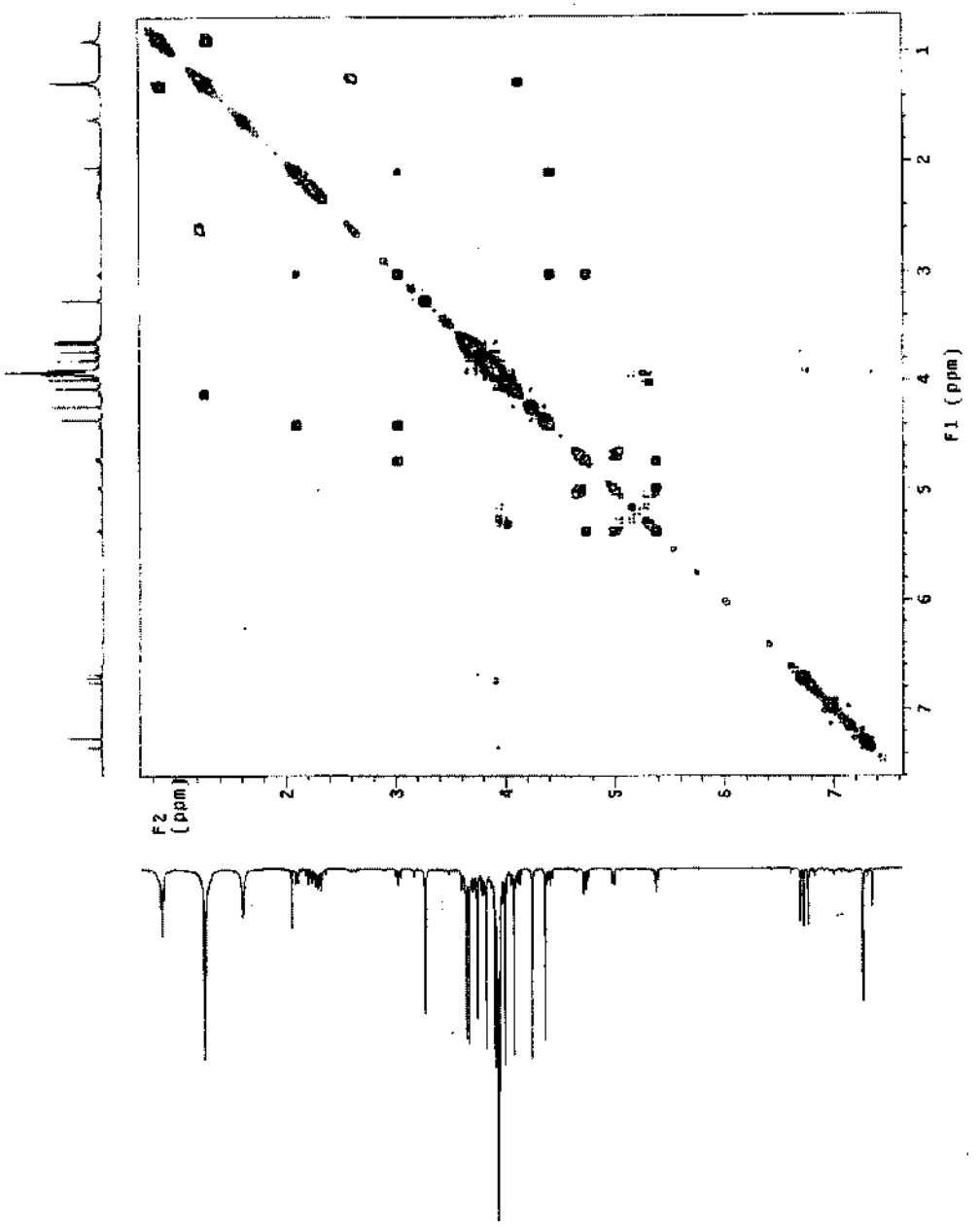


Plate 43

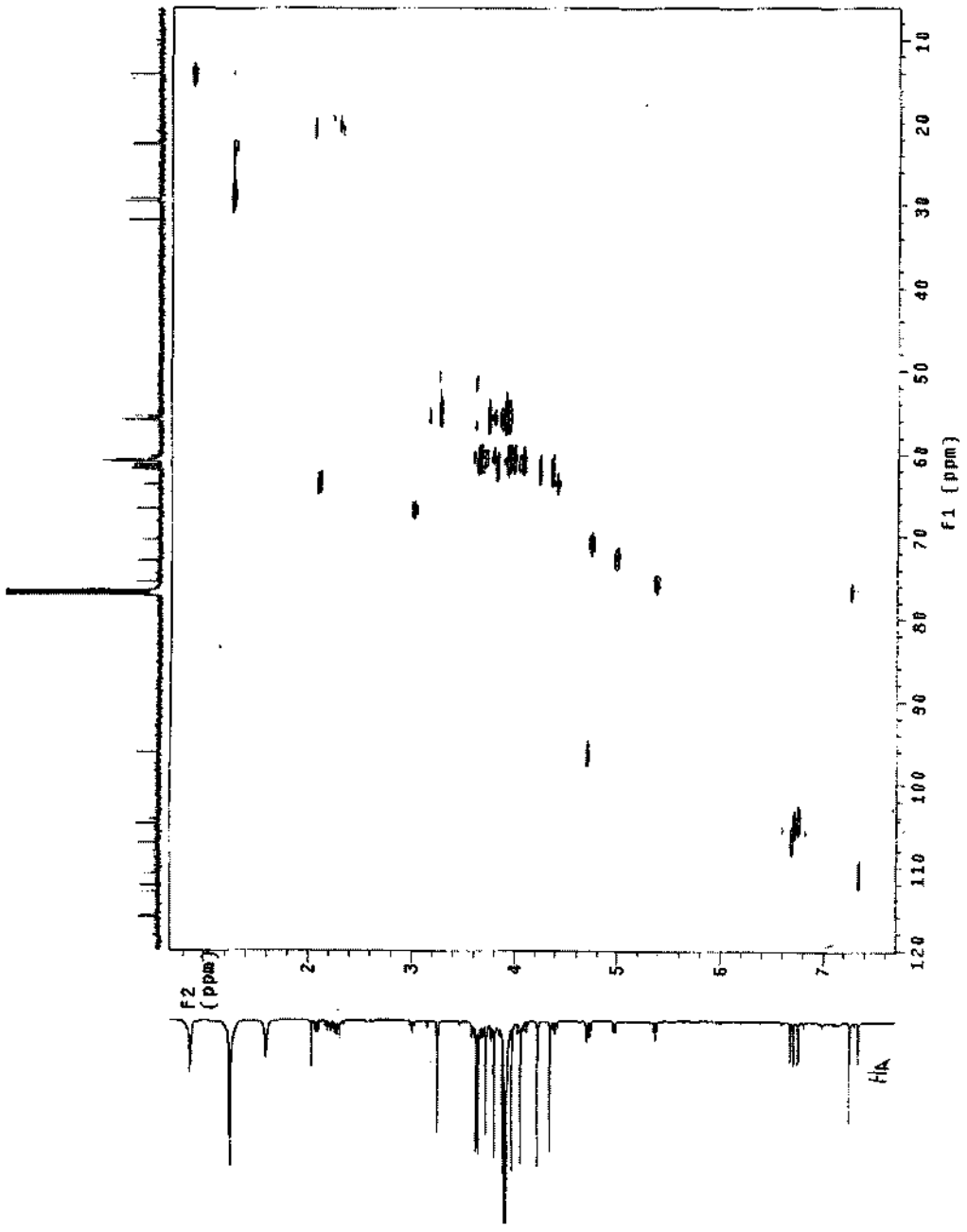


Plate 44

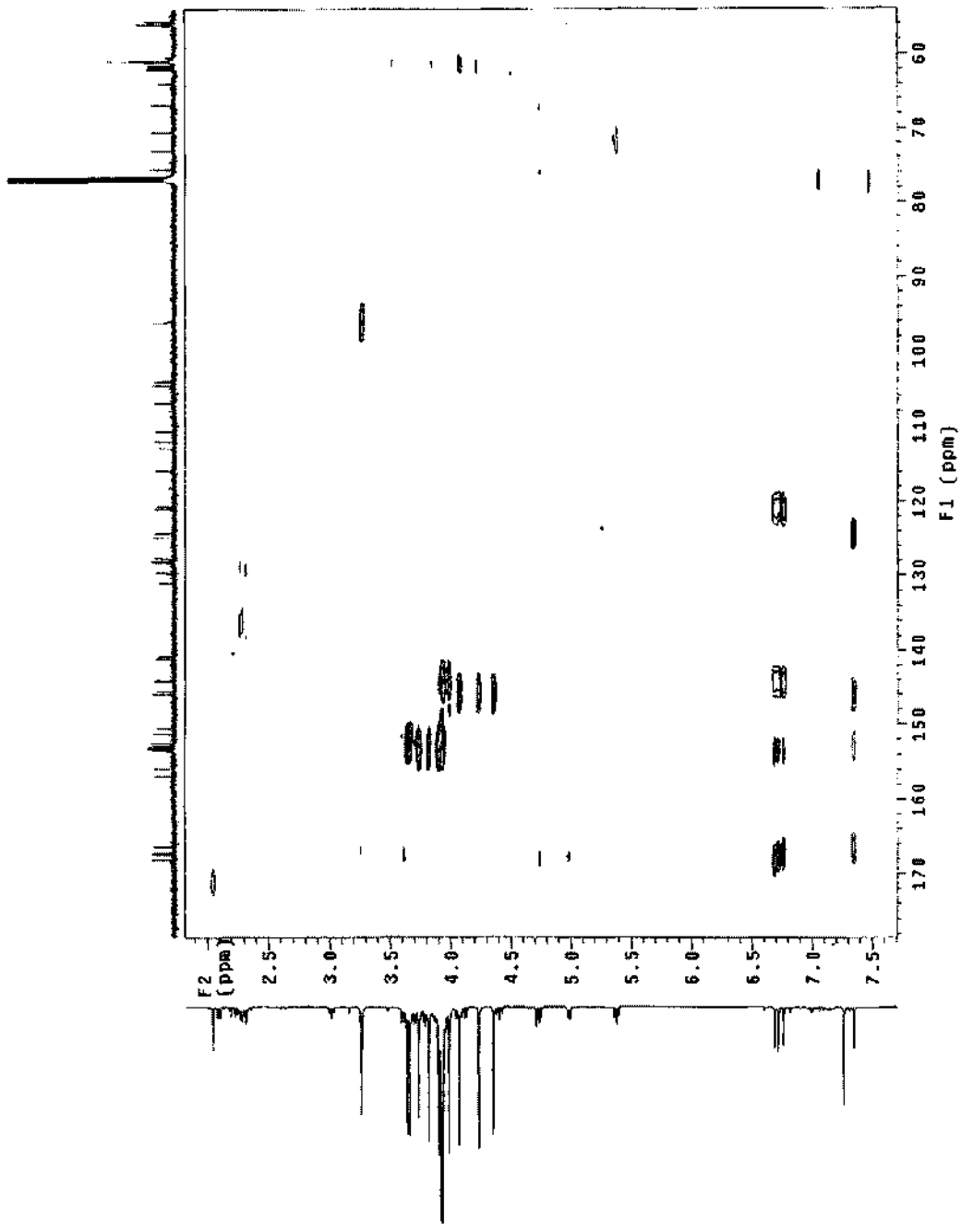

F2 (ppm)
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
F1 (ppm)
60
70
80
90
100
110
120
130
140
150
160
170

PLATE 11

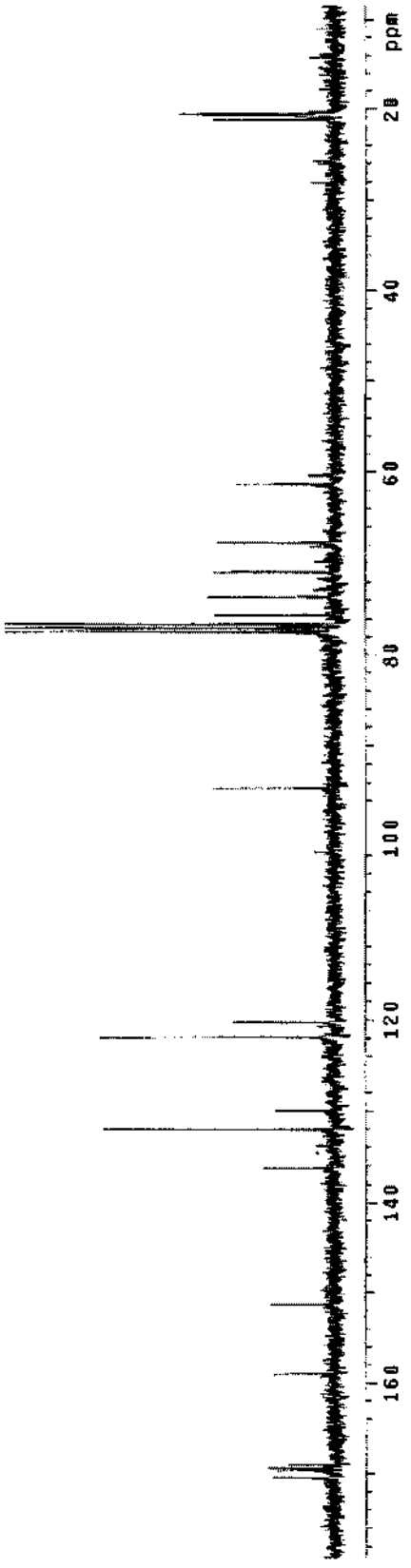


PLATE 12

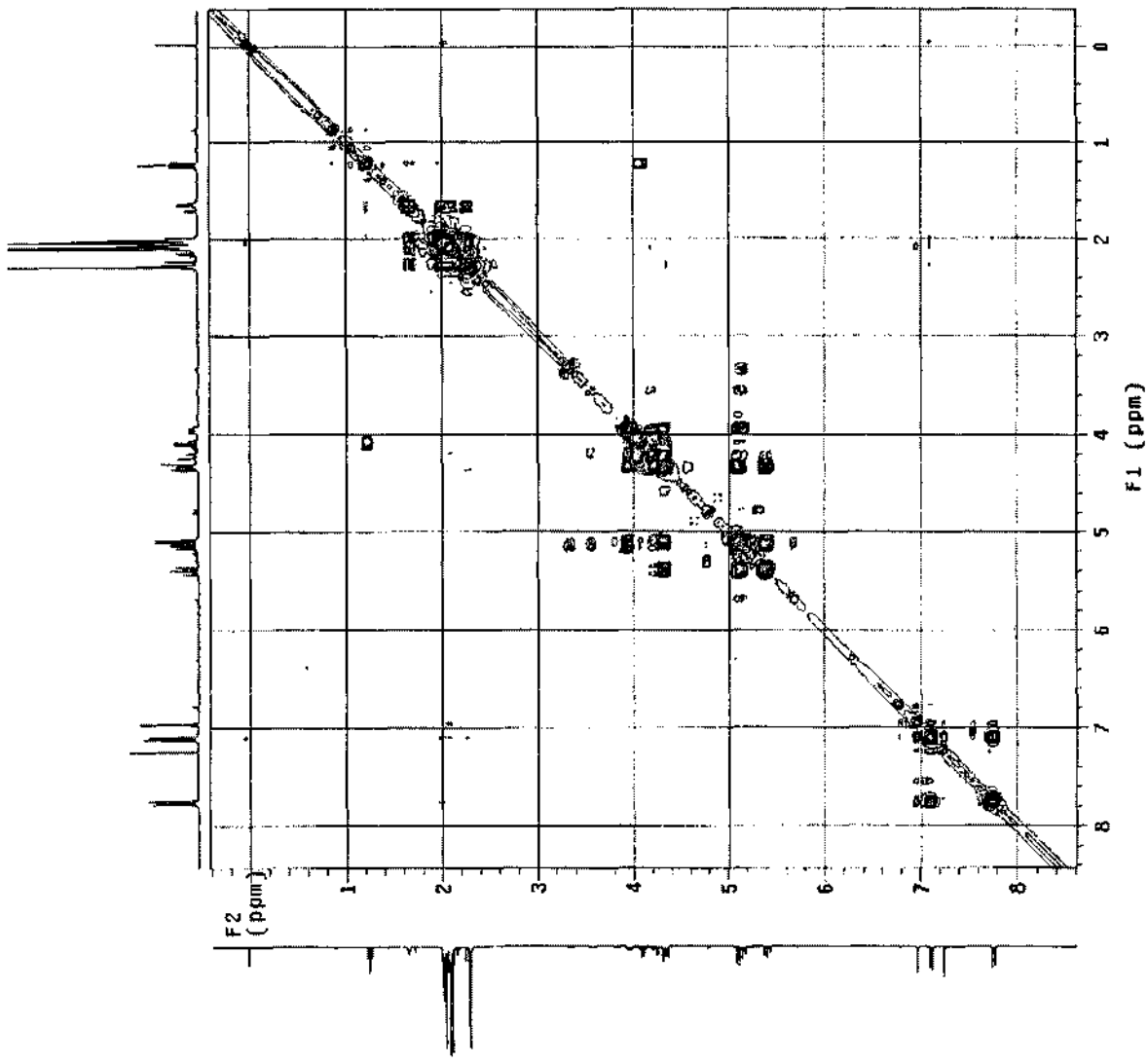


PLATE 13

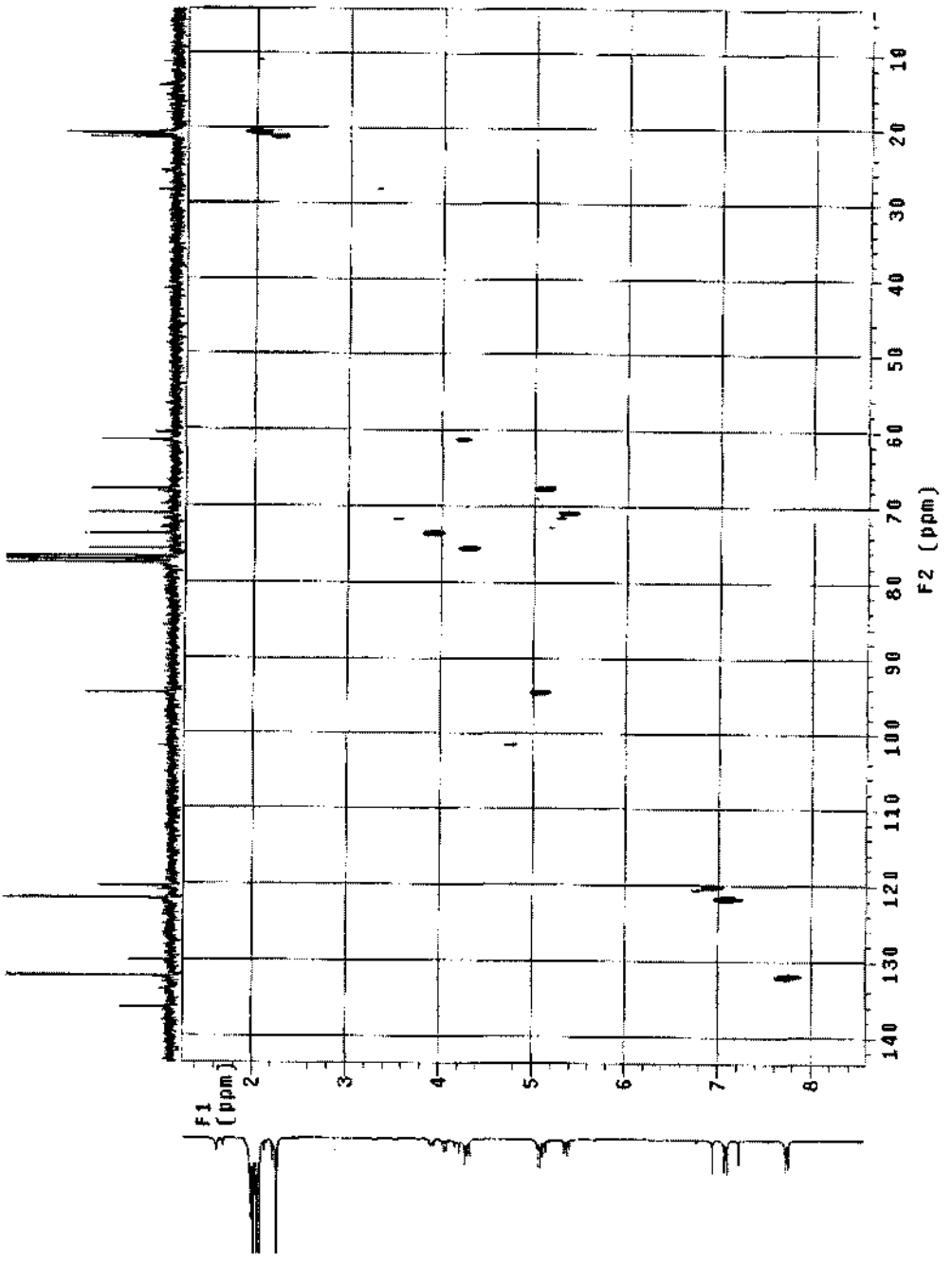


PLATE 14

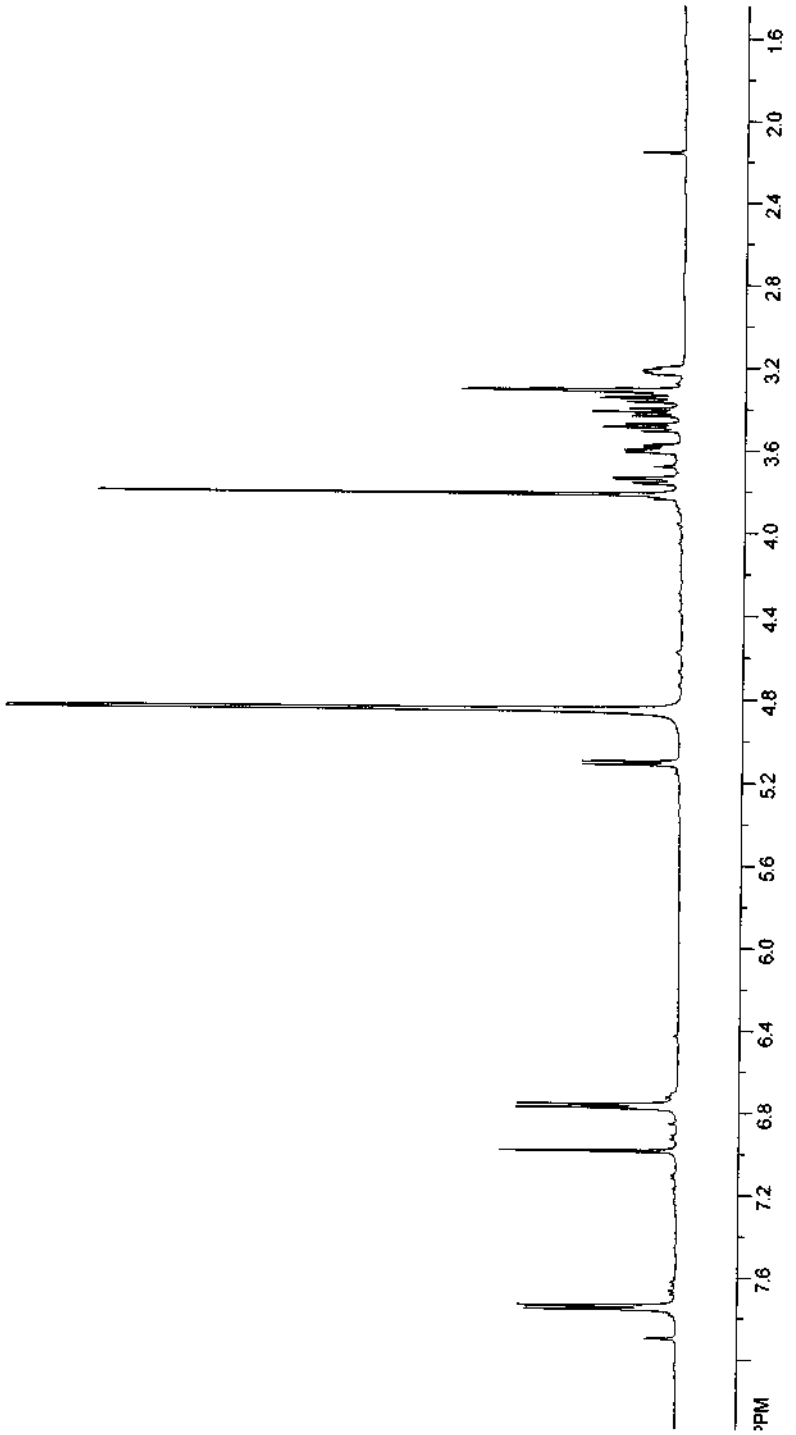


PLATE 15

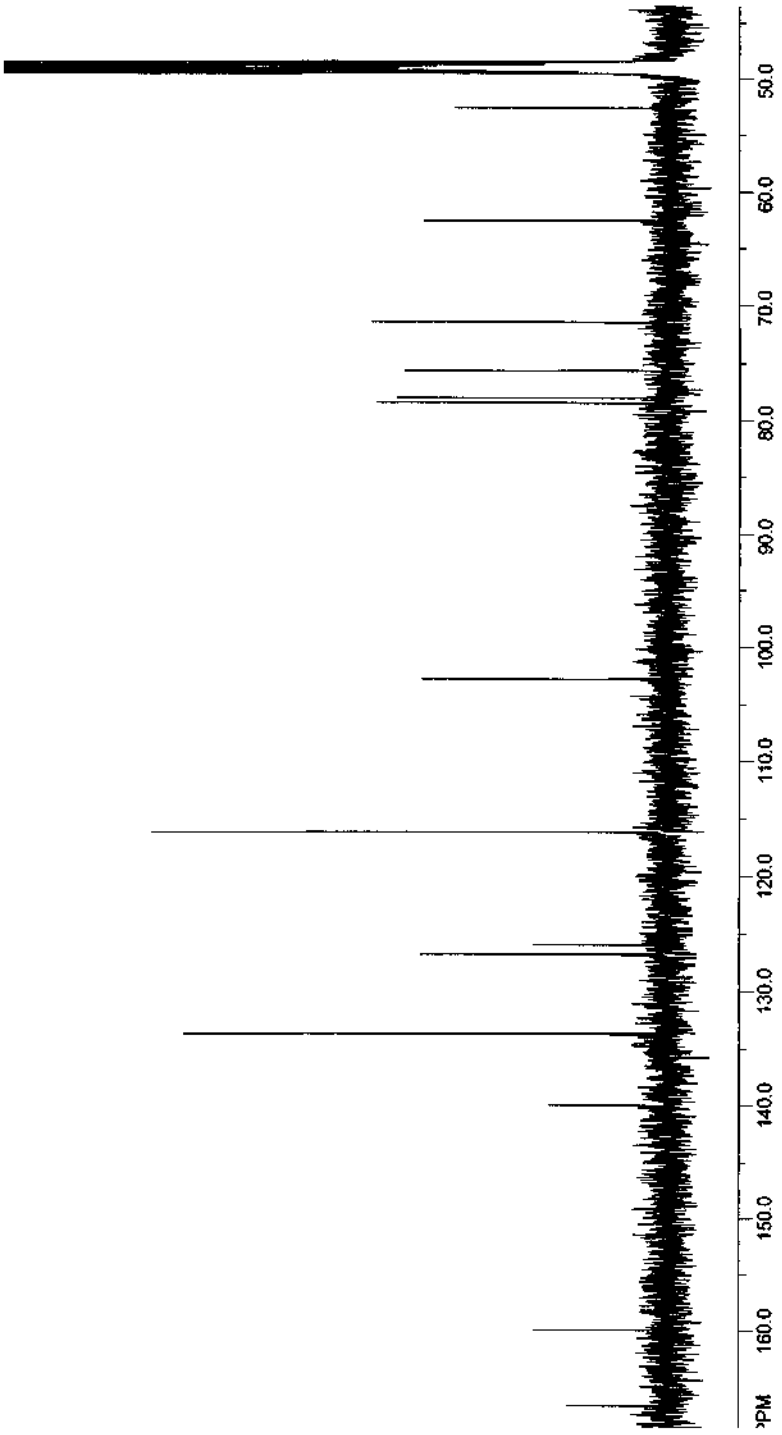


PLATE 16

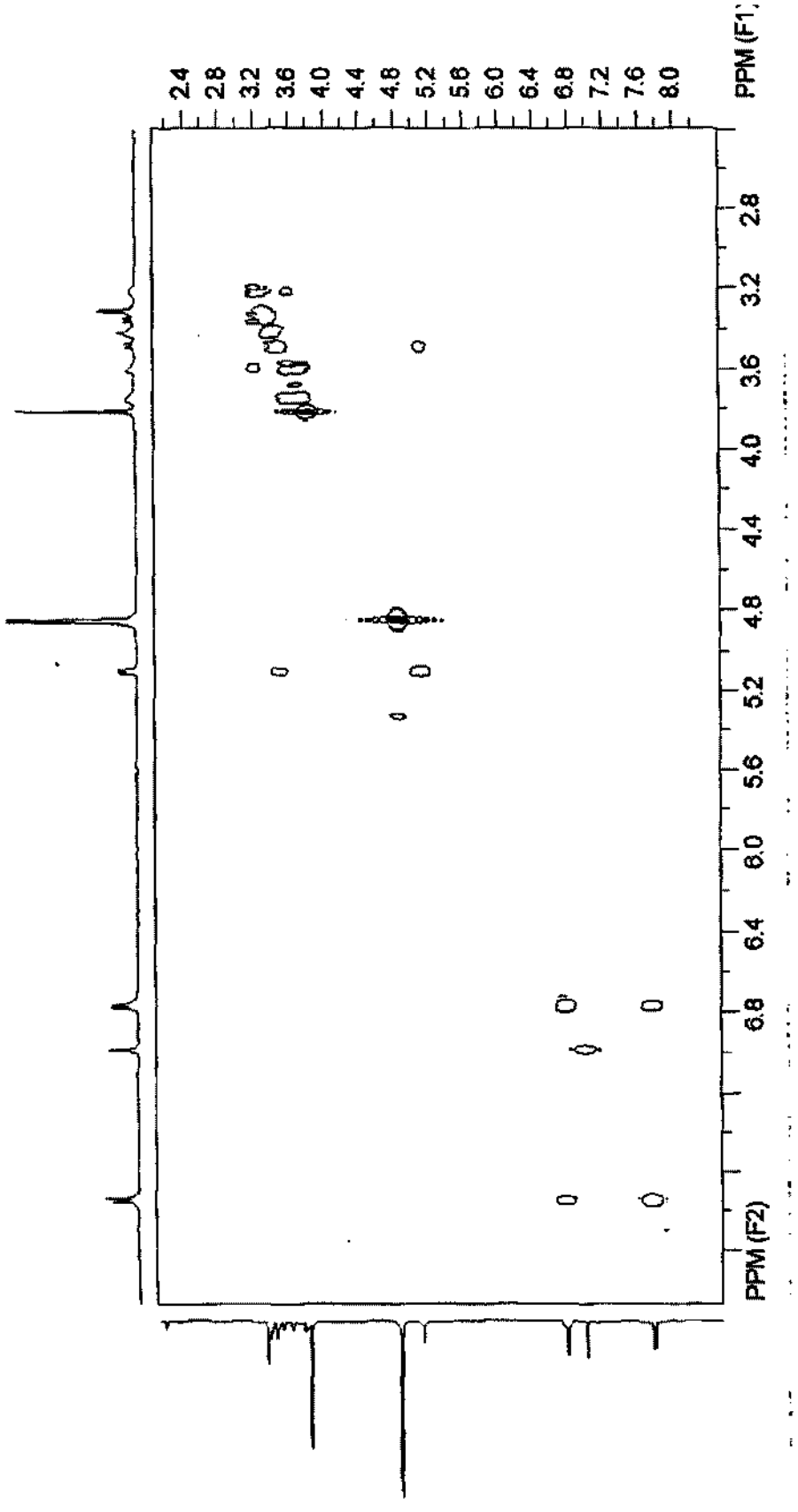

PPM (F1)
2.4
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
6.0
6.4
6.8
7.2
7.6
8.0
PPM (F2)
2.8
3.2
3.6
4.0
4.4
4.8
5.2
5.6
6.0
6.4
6.8

PLATE 17

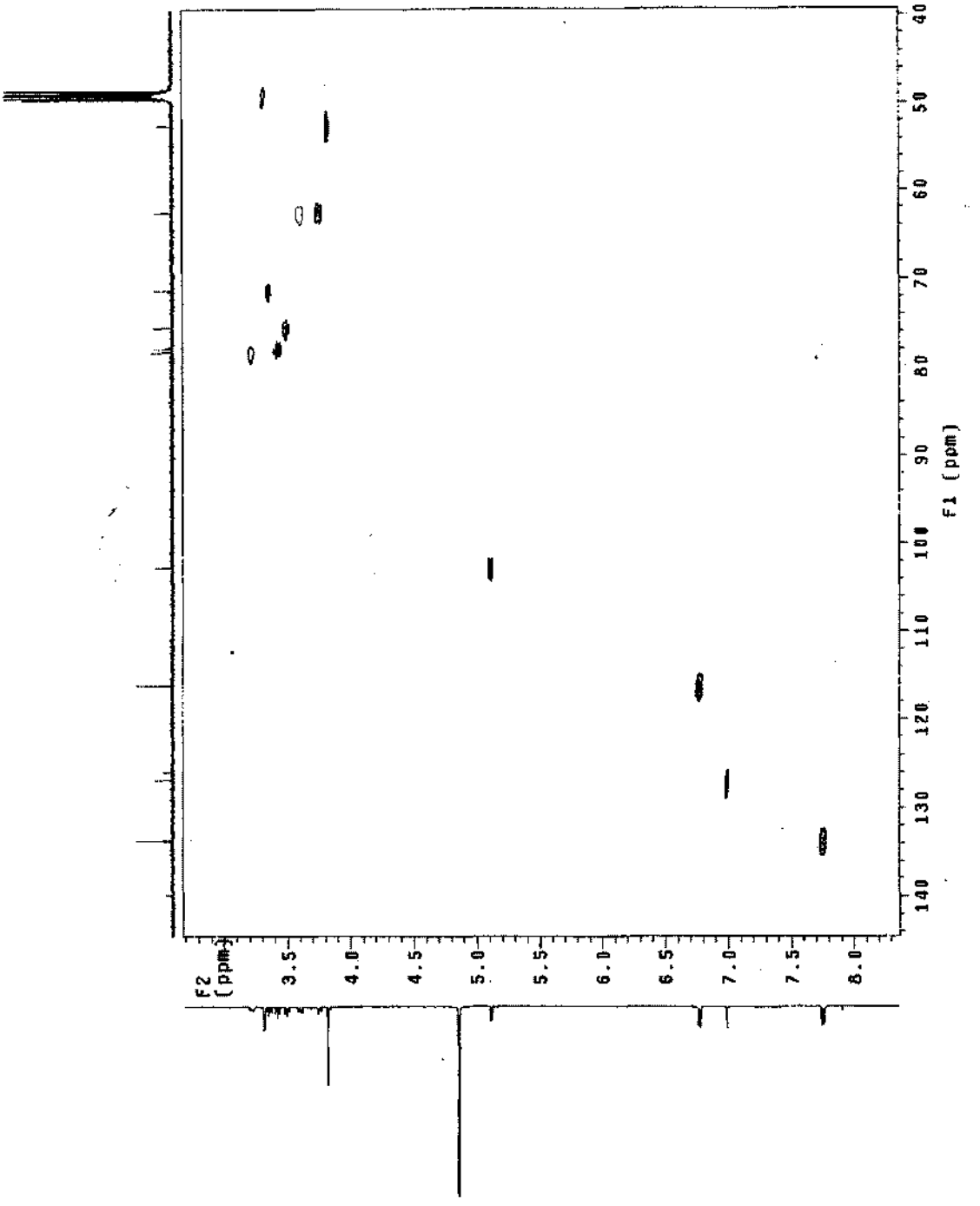
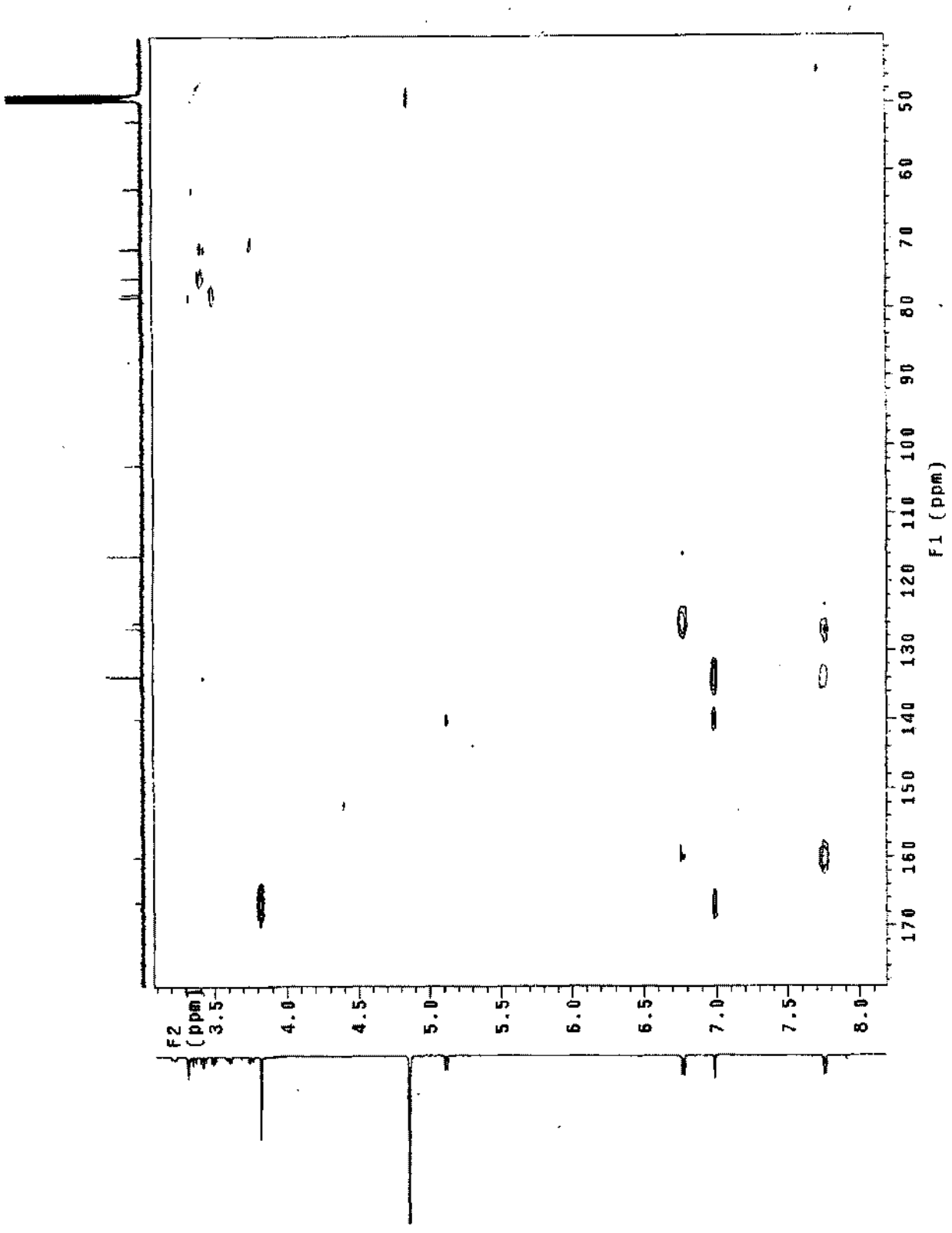

F2 (ppm)
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
F1 (ppm)
40
50
60
70
80
90
100
110
120
130
140

PLATE 18


F2 (ppm)
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
F1 (ppm)
50
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70
80
90
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110
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PLATE 19

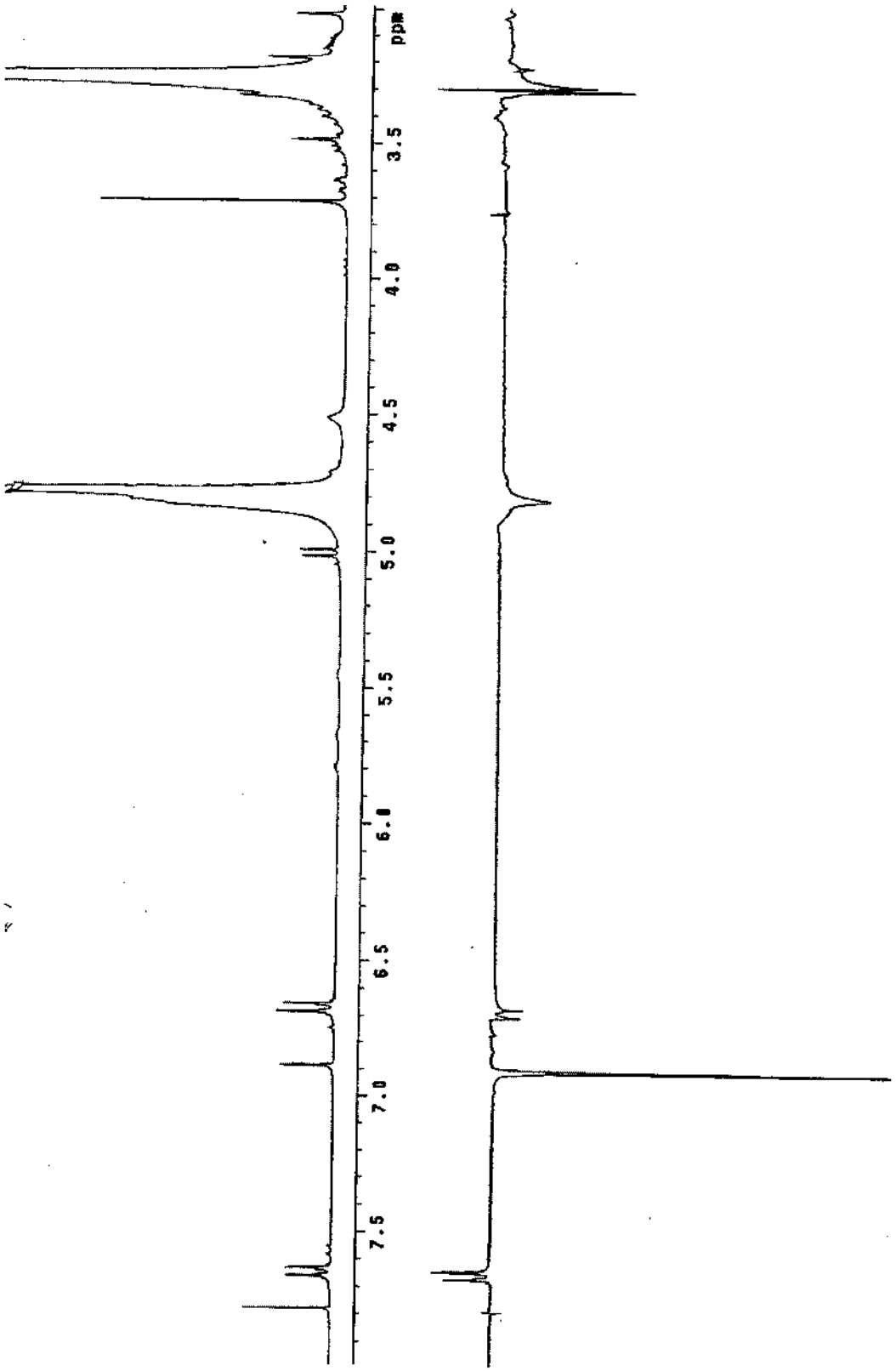

PLATE 20

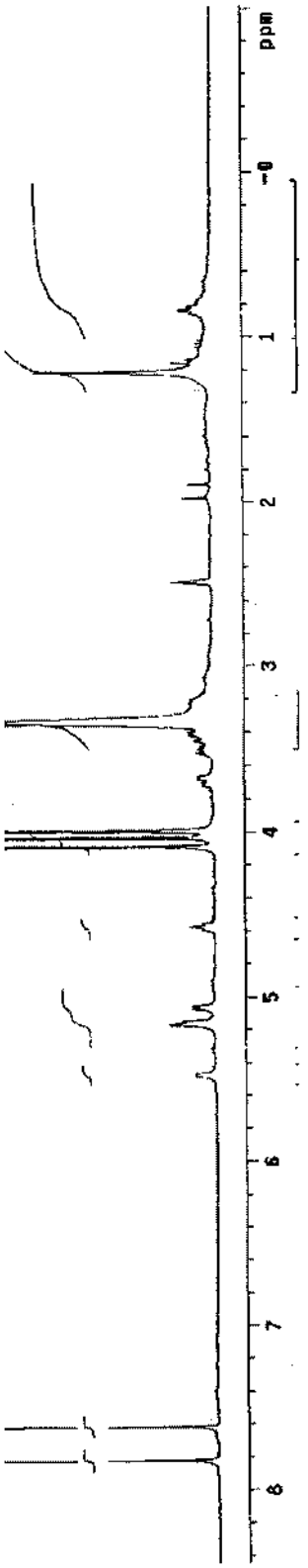

PLATE 21

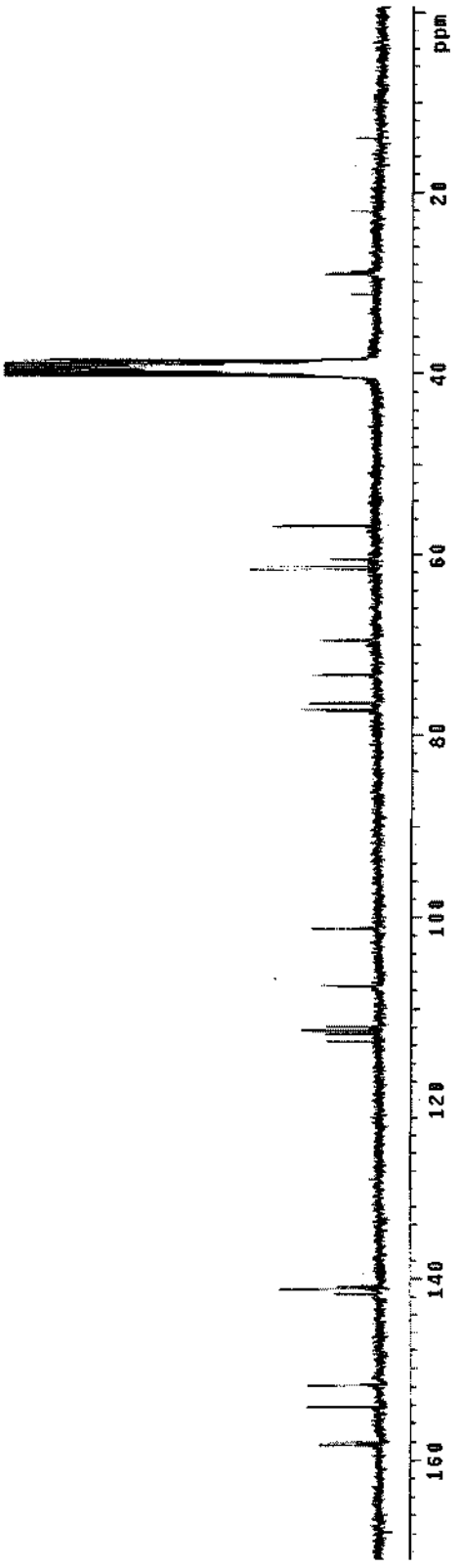

PLATE 22

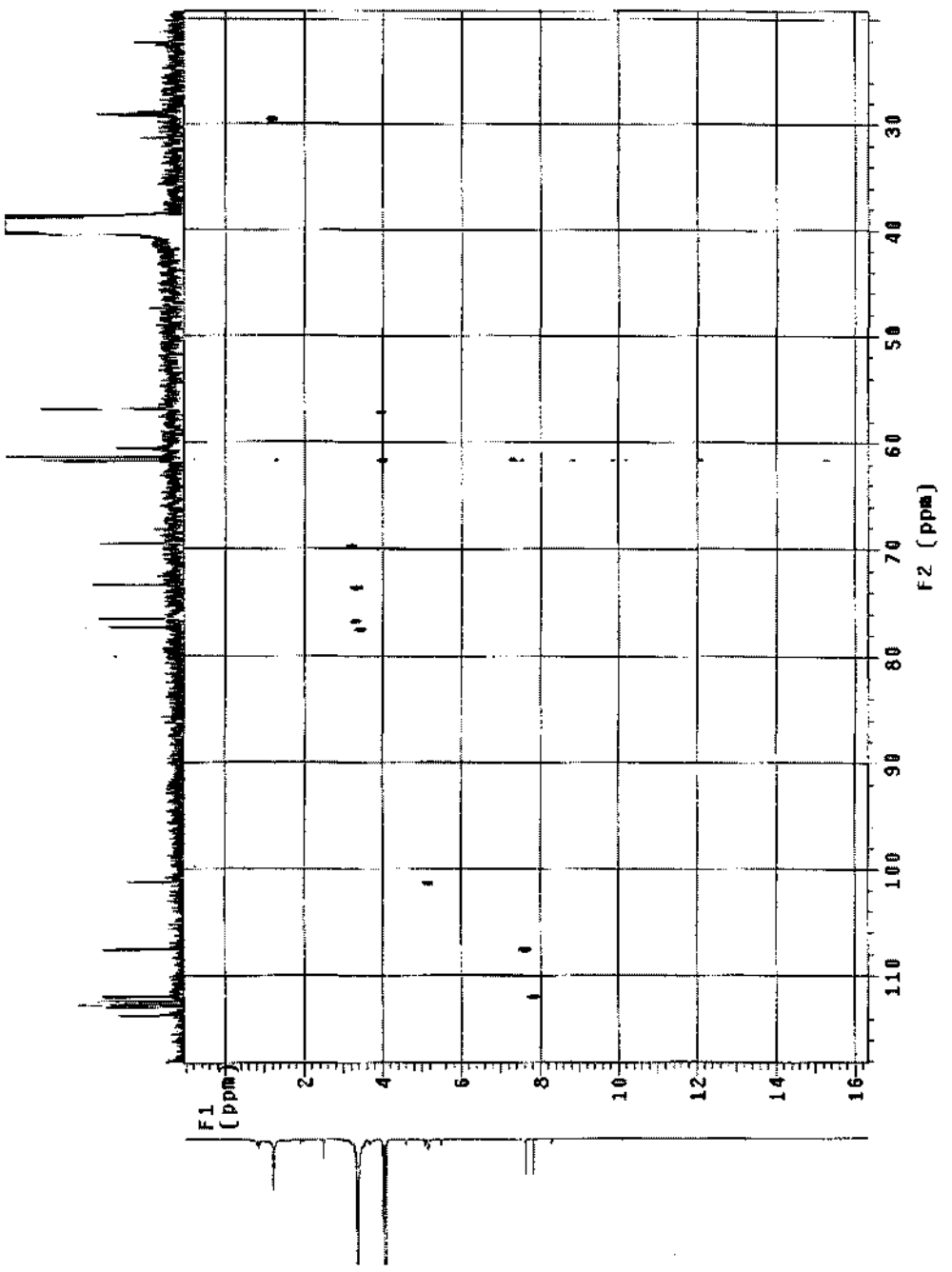

PLATE 23

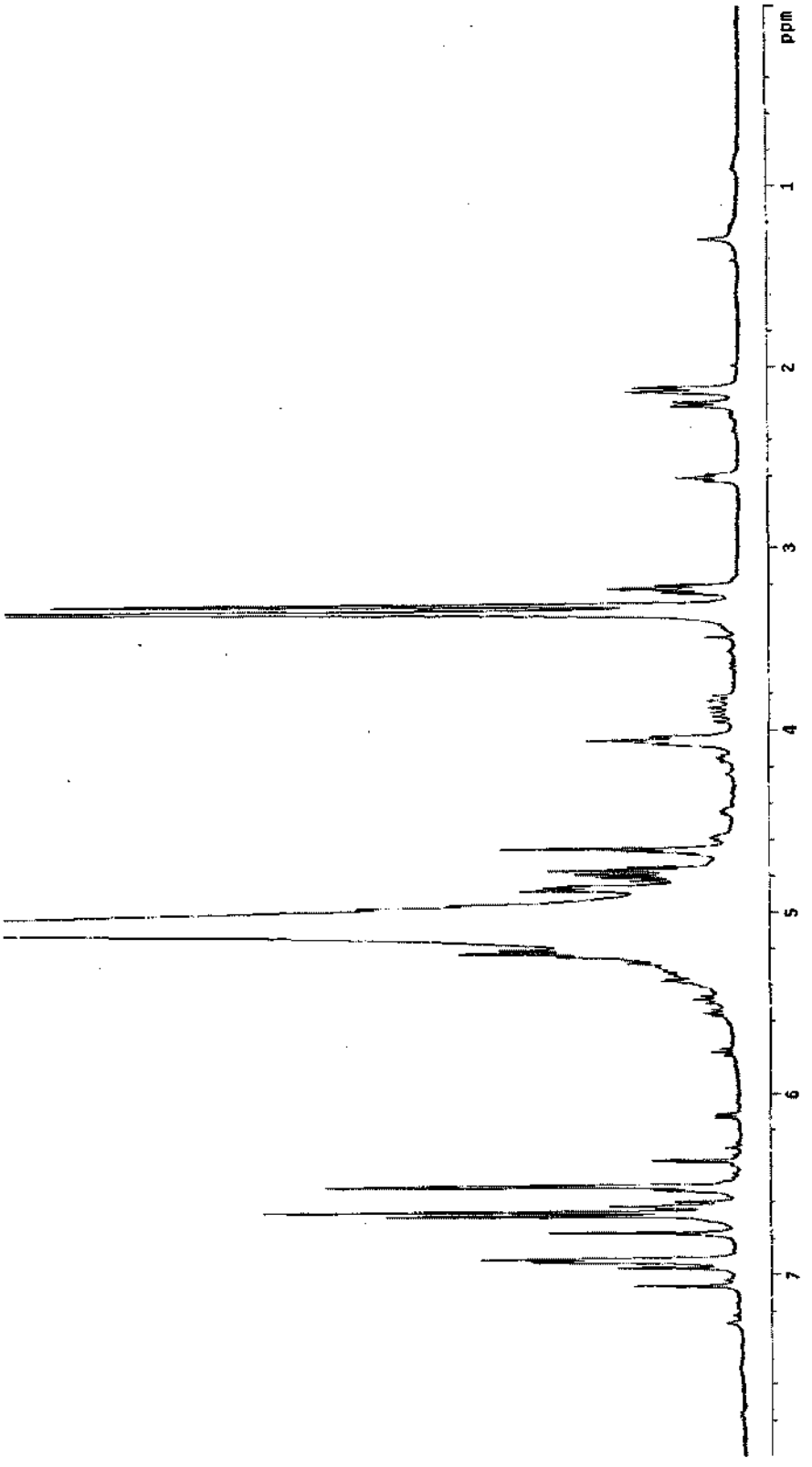

PLATE 24

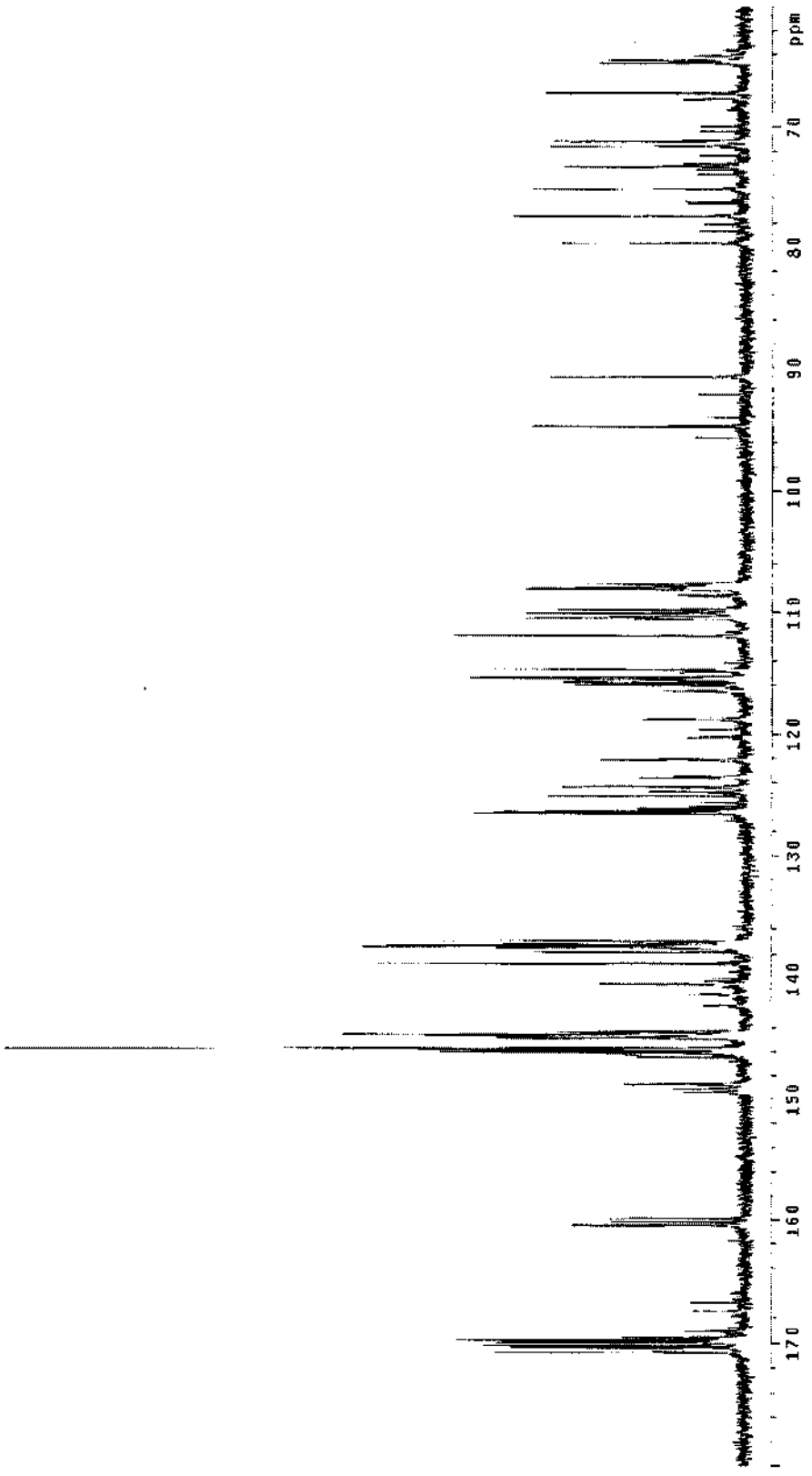

PLATE 25

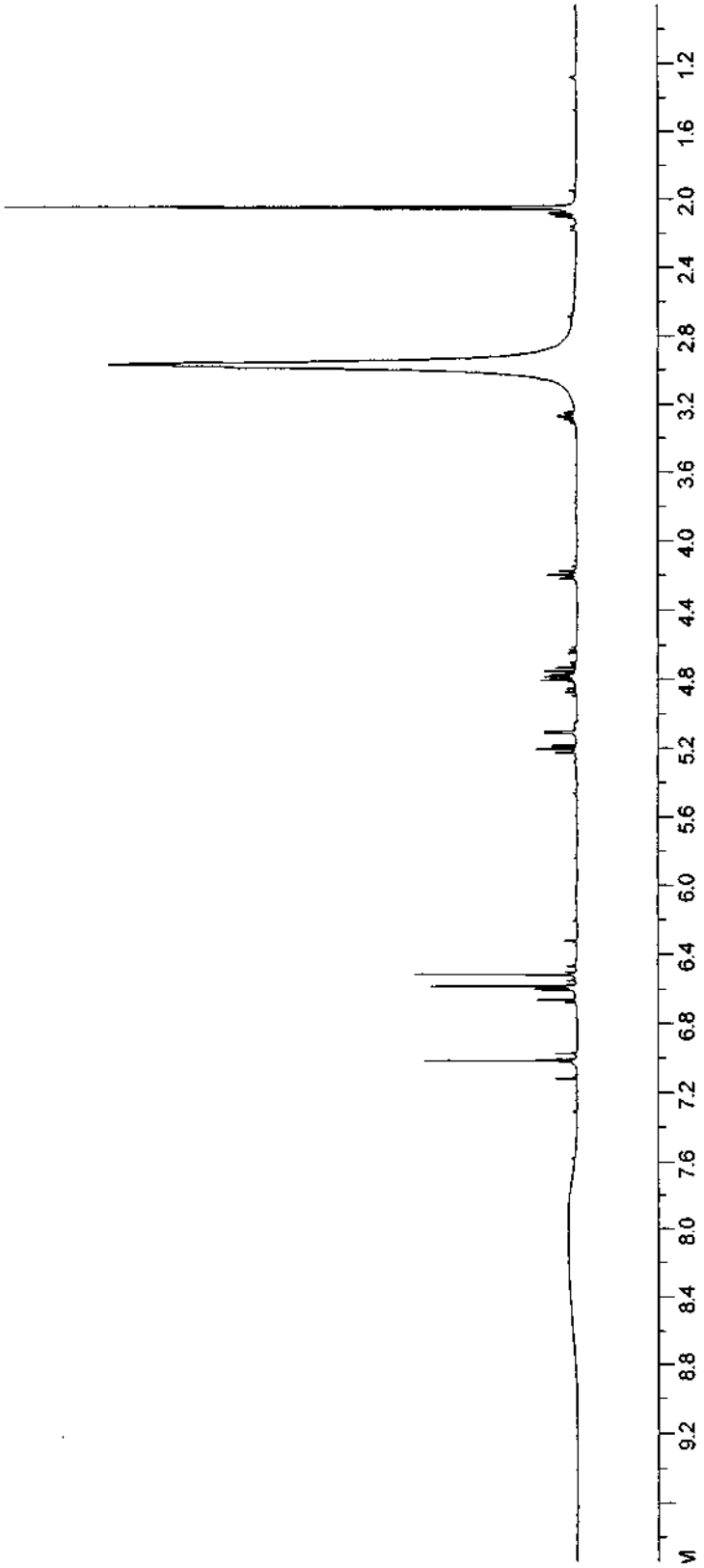

PLATE 26

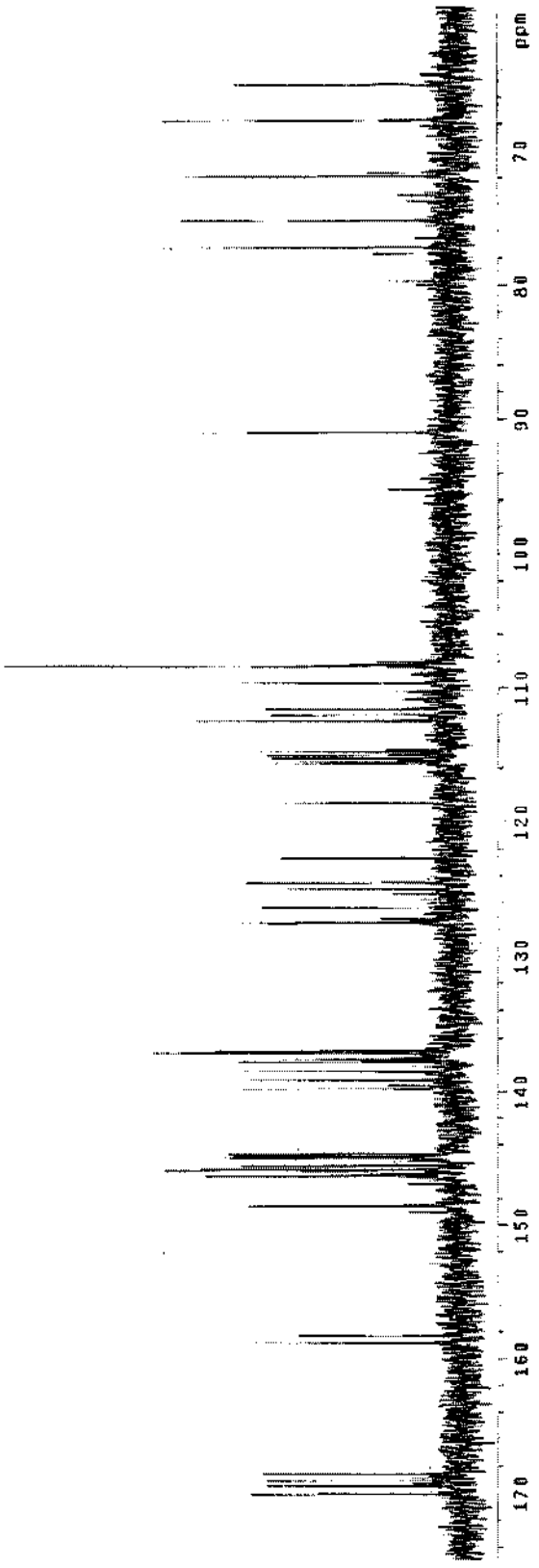

PLATE 27

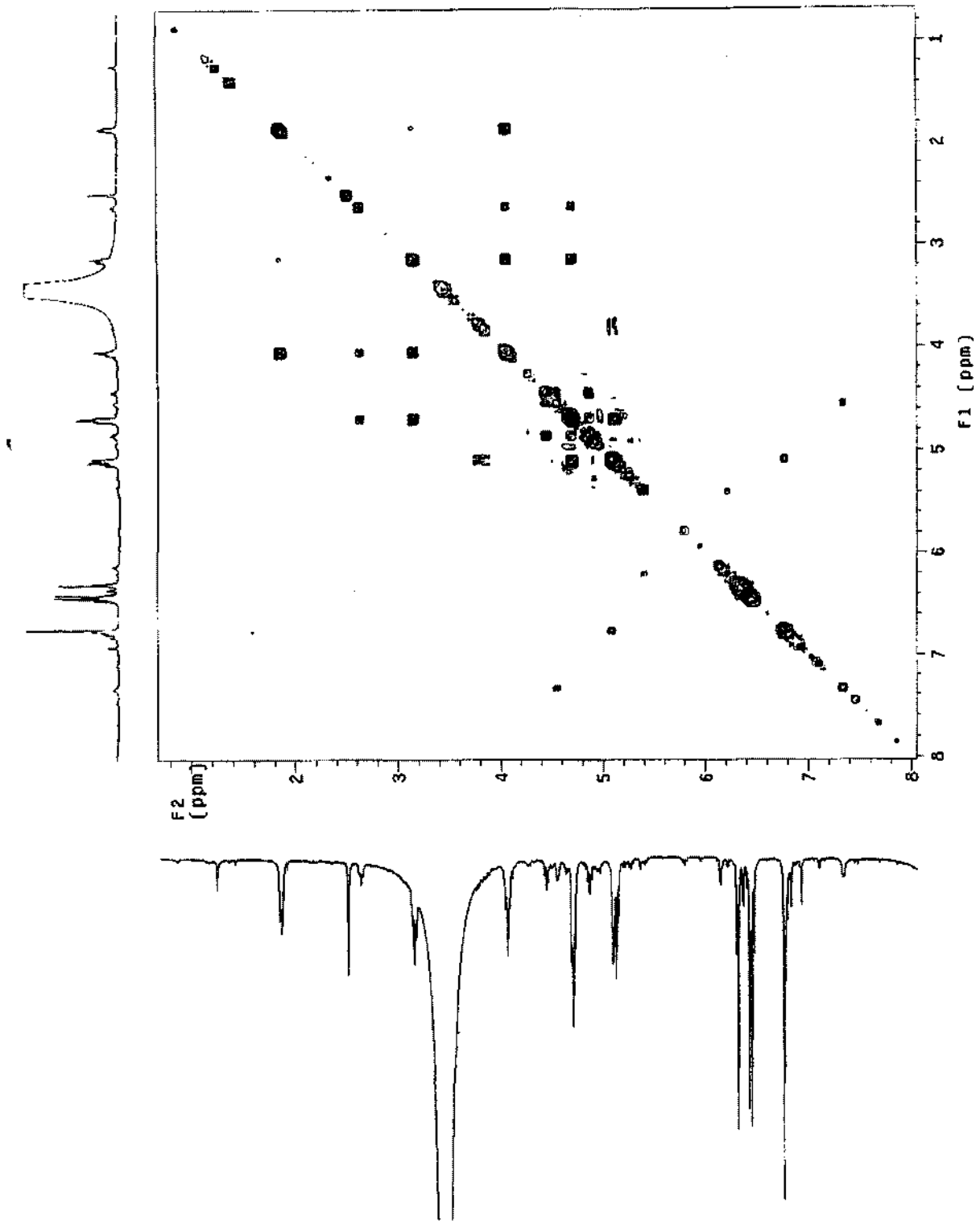

PLATE 28

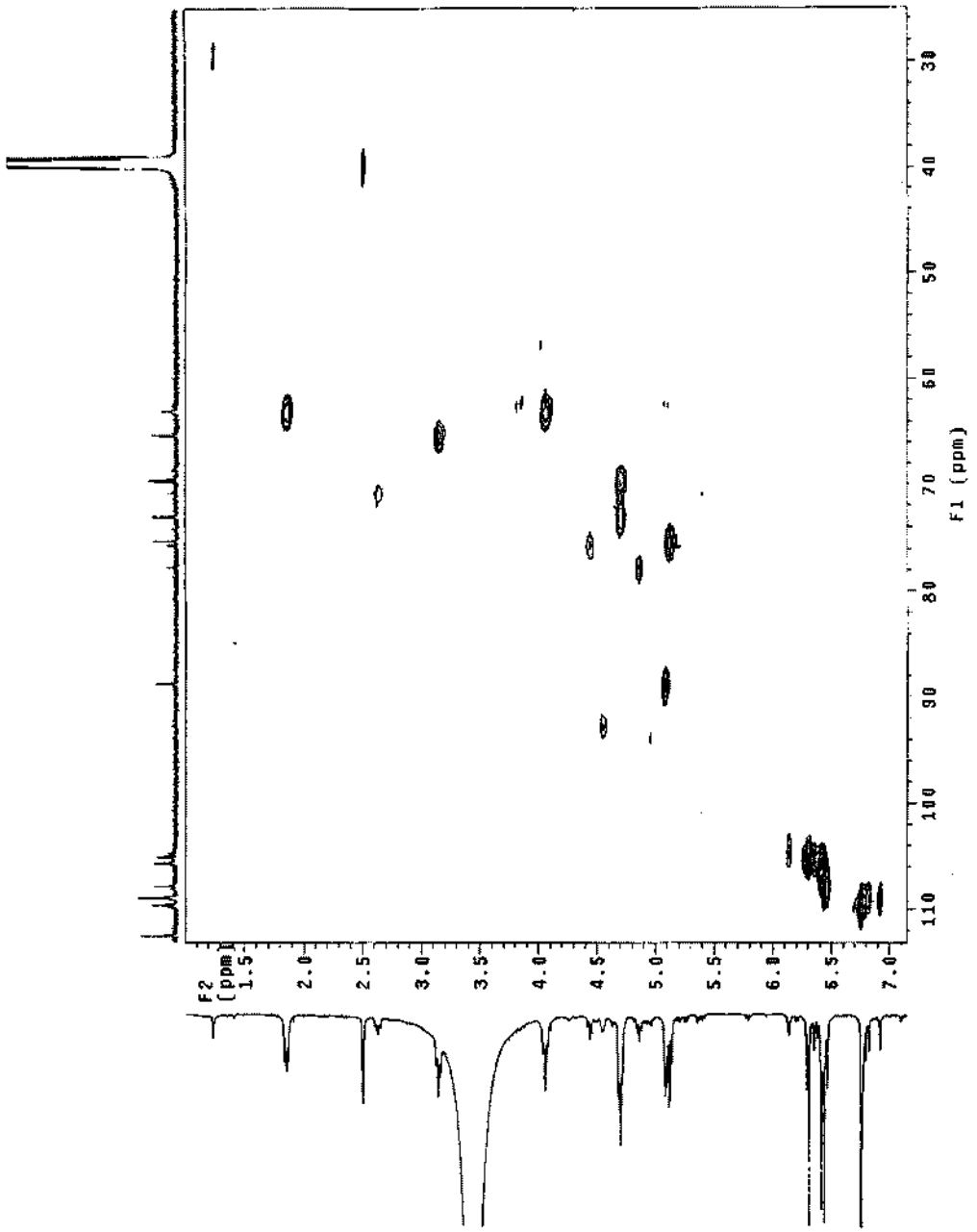
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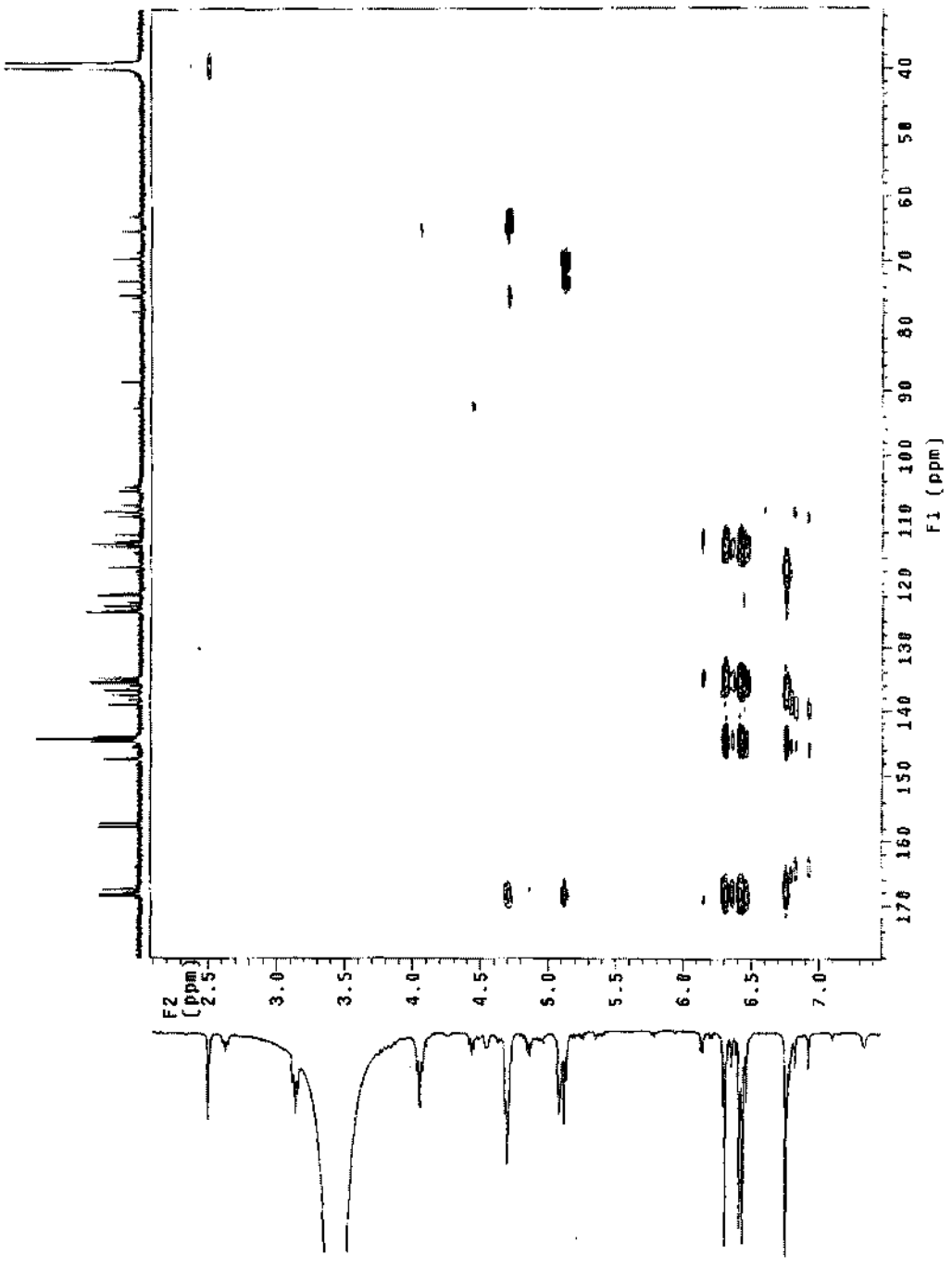
PLATE 29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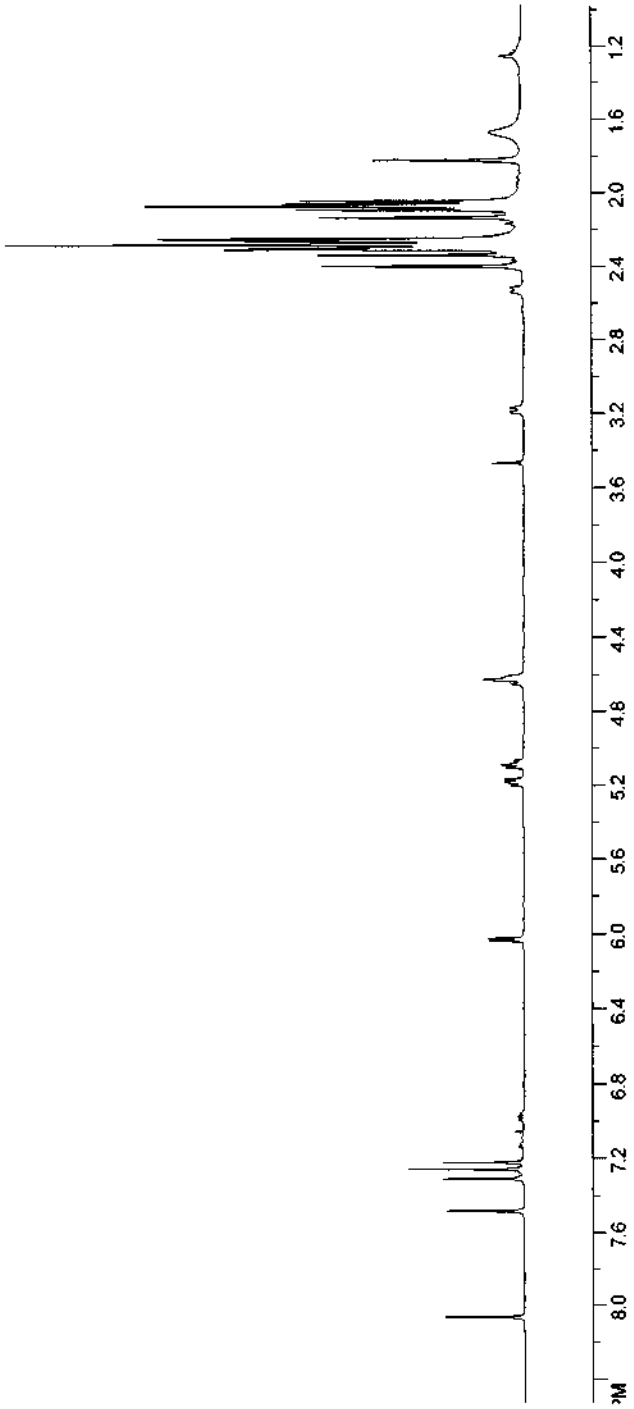

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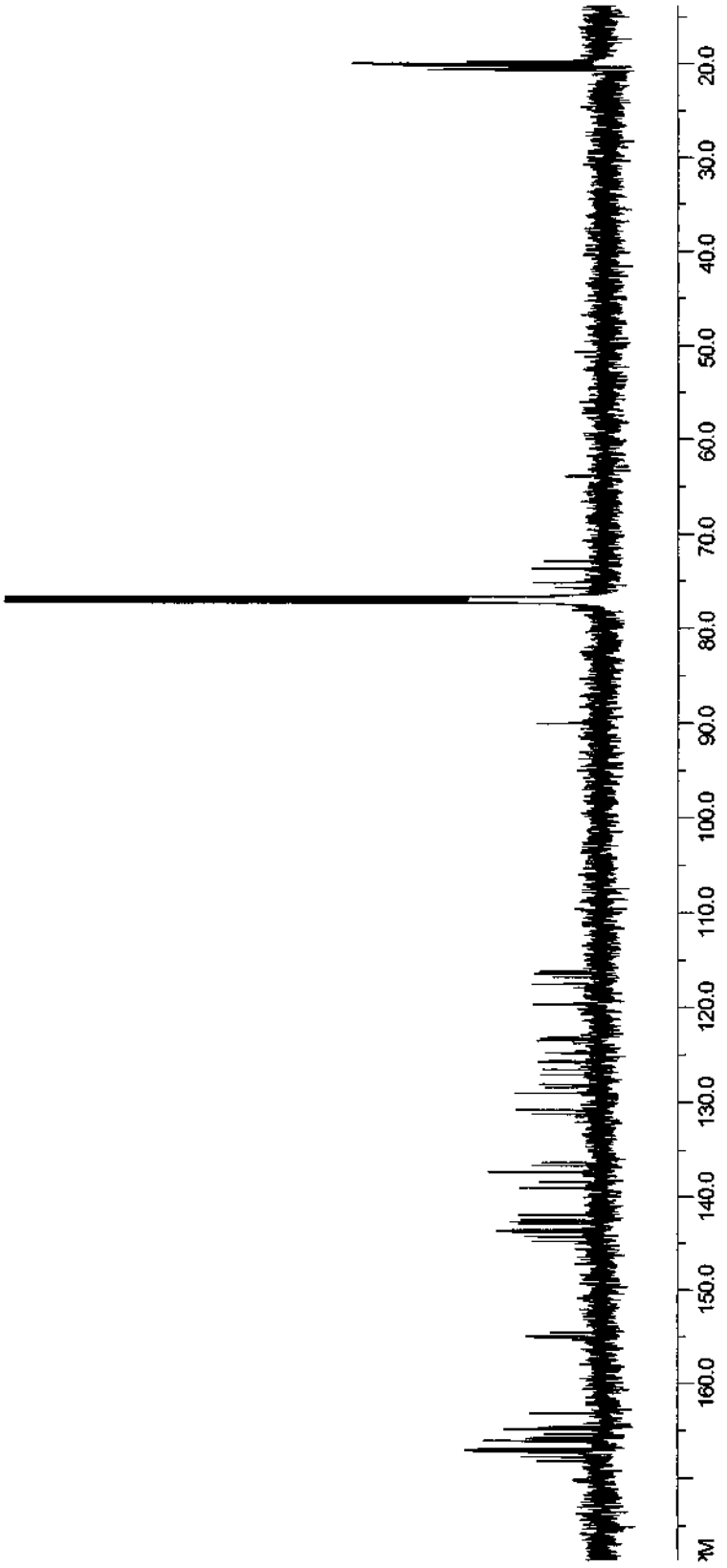
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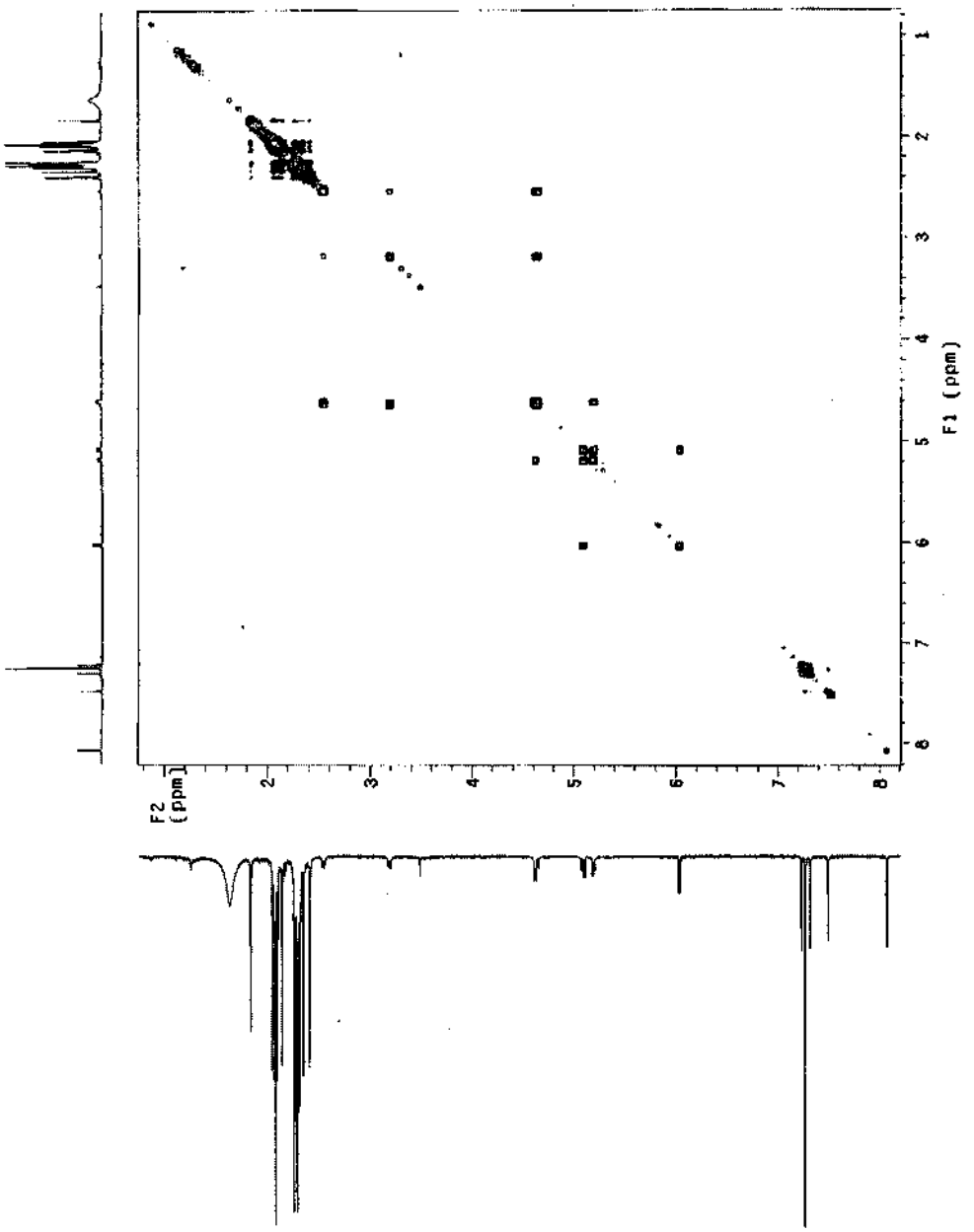

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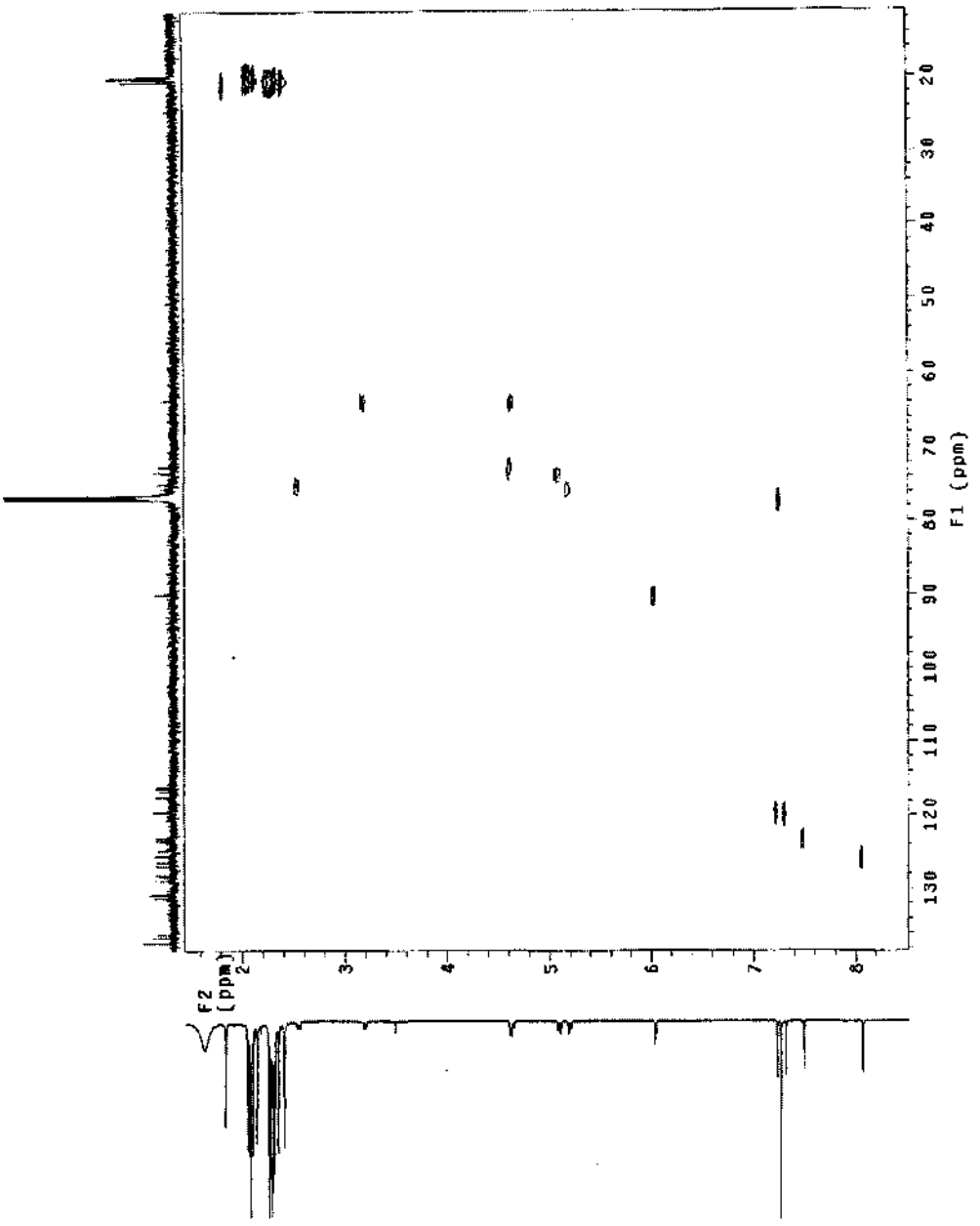

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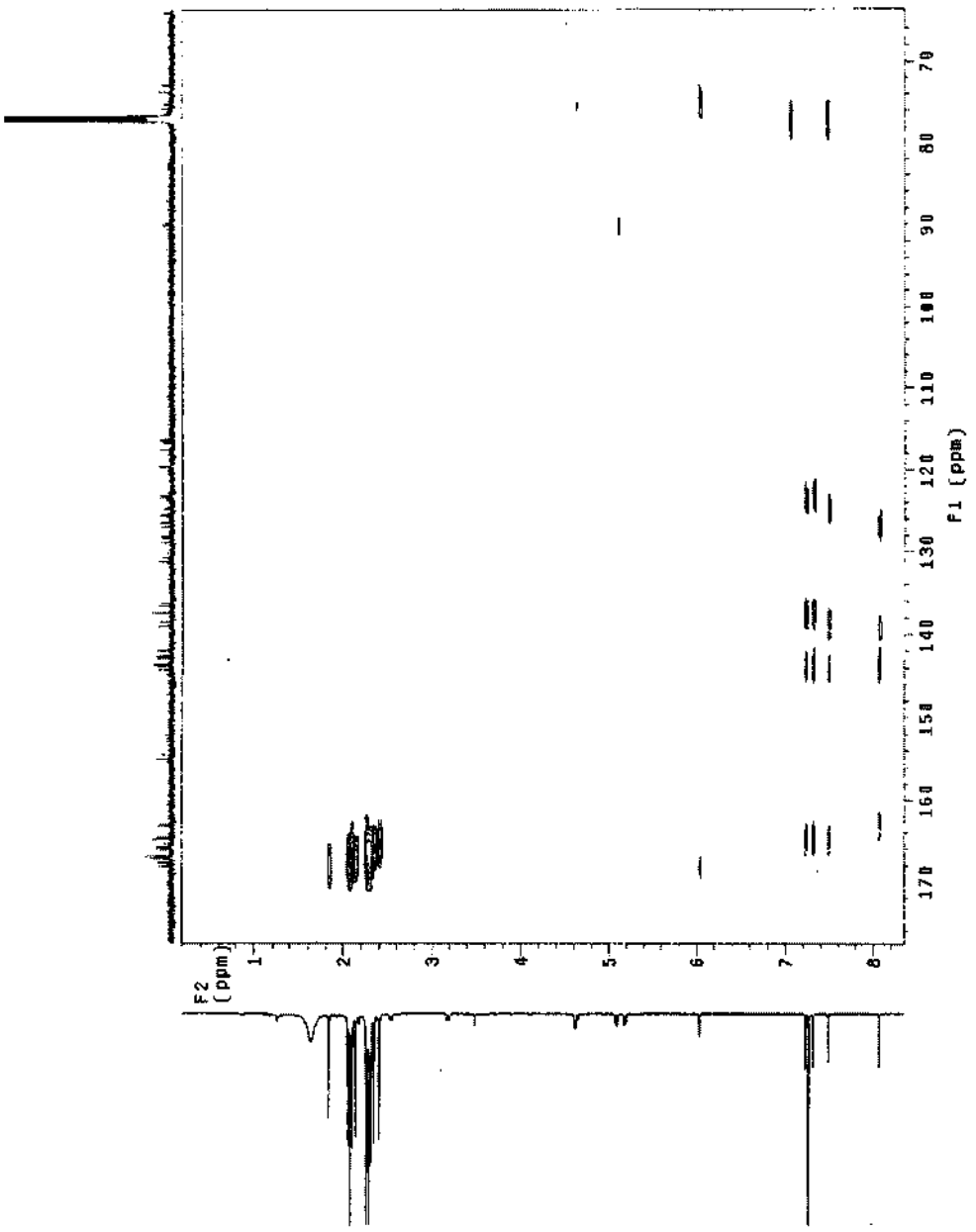

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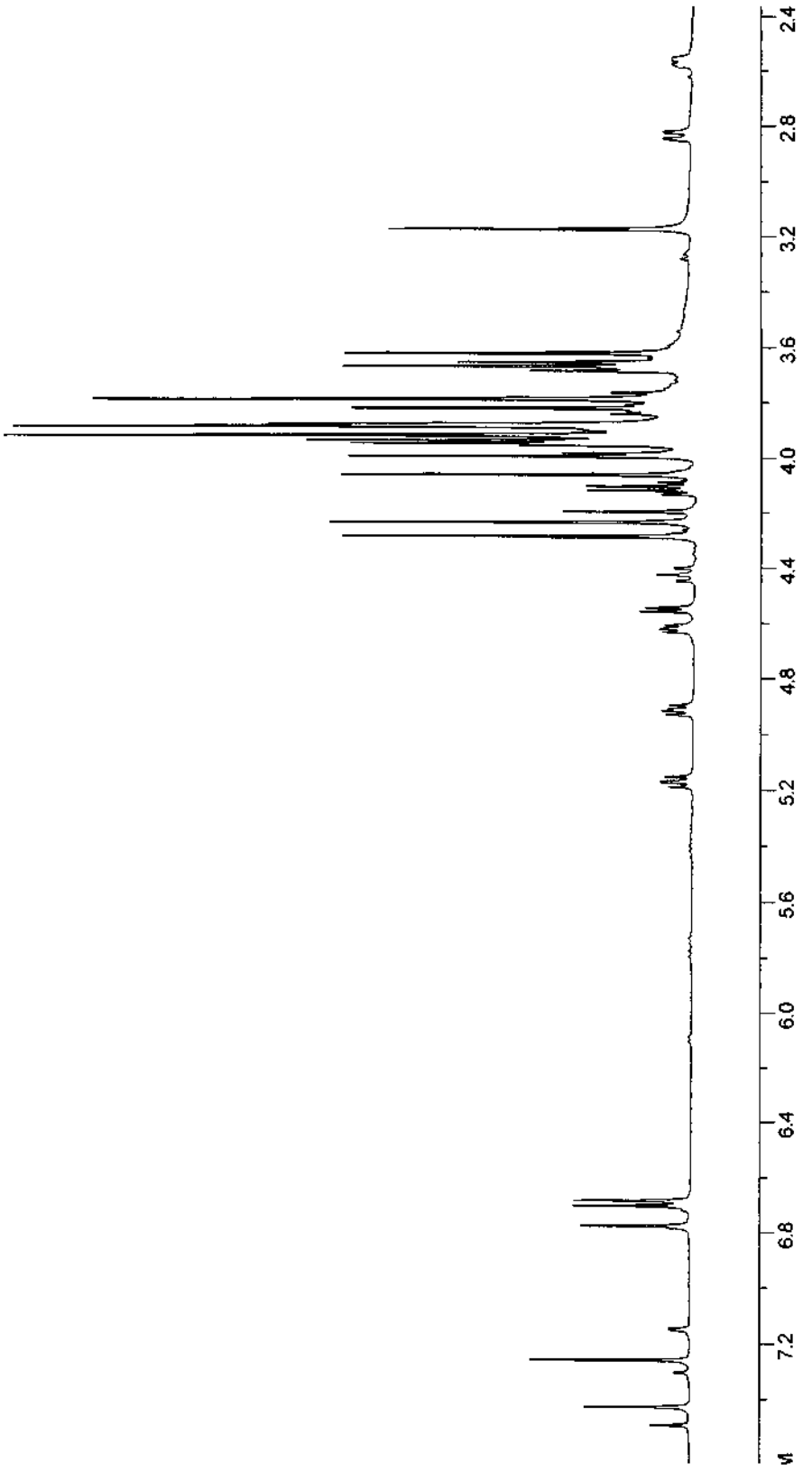

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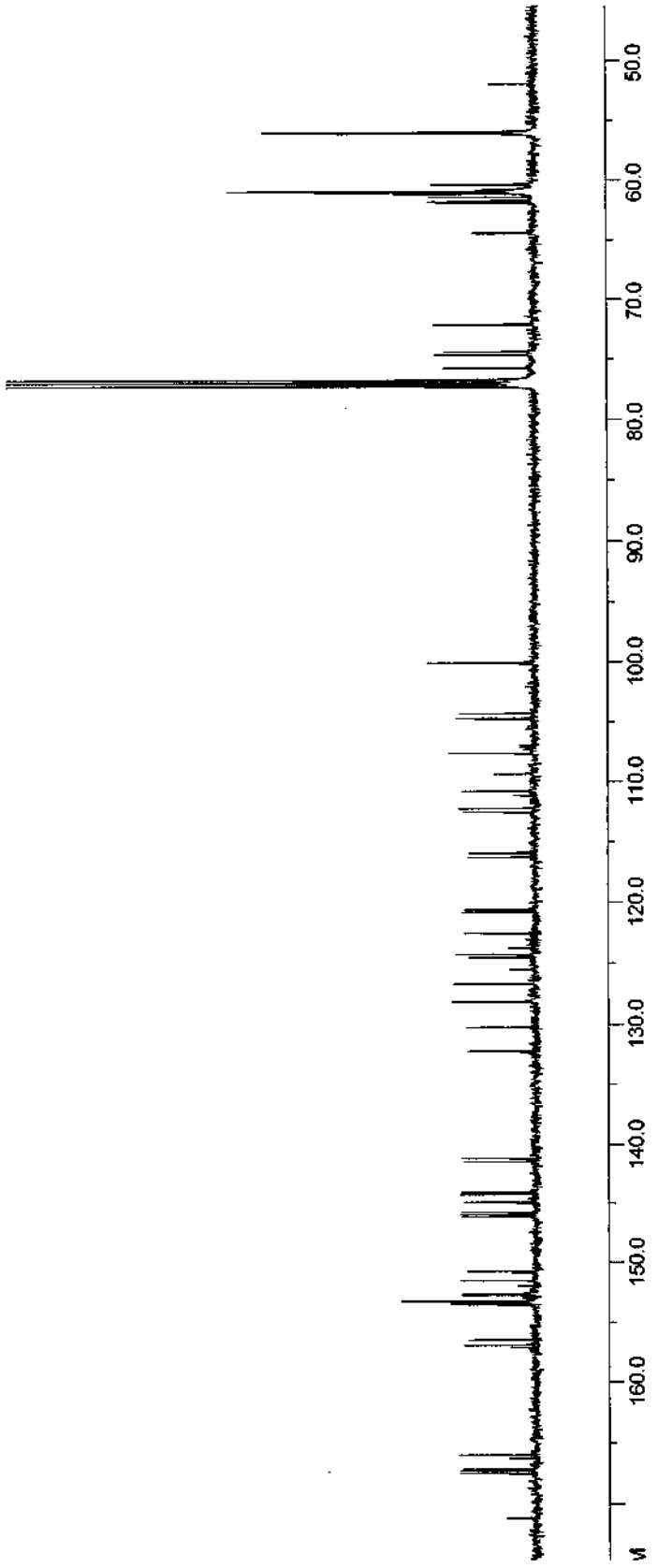

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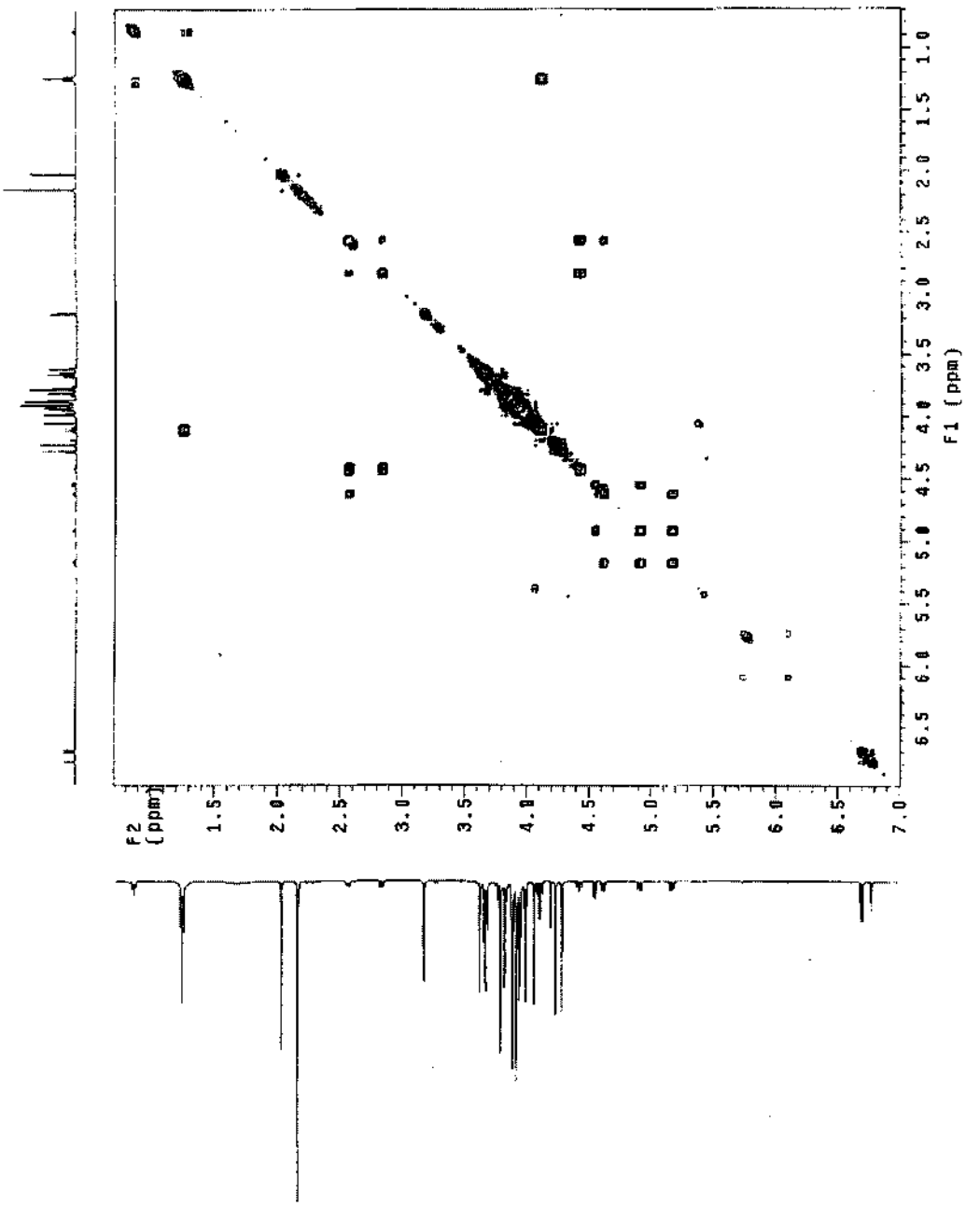

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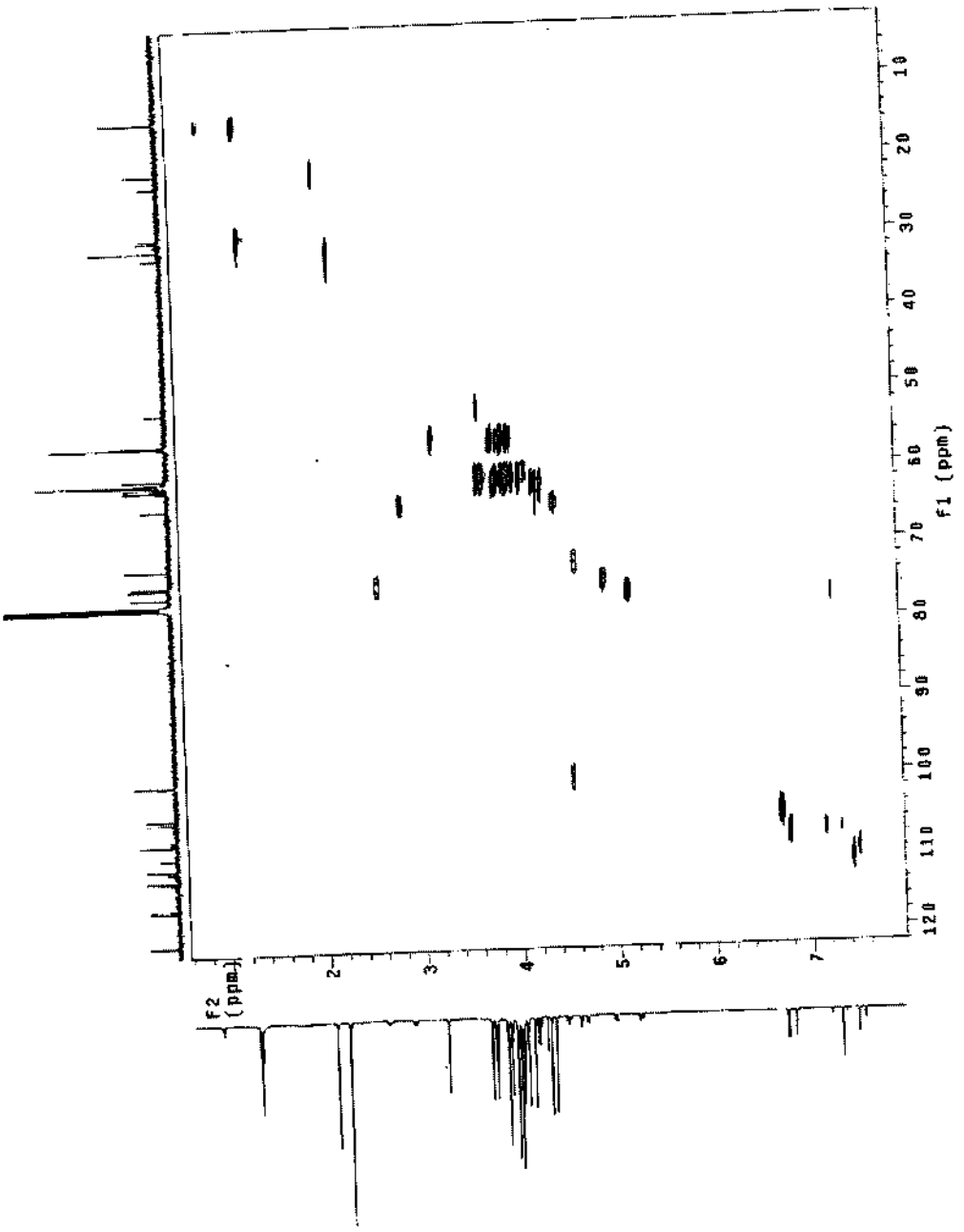

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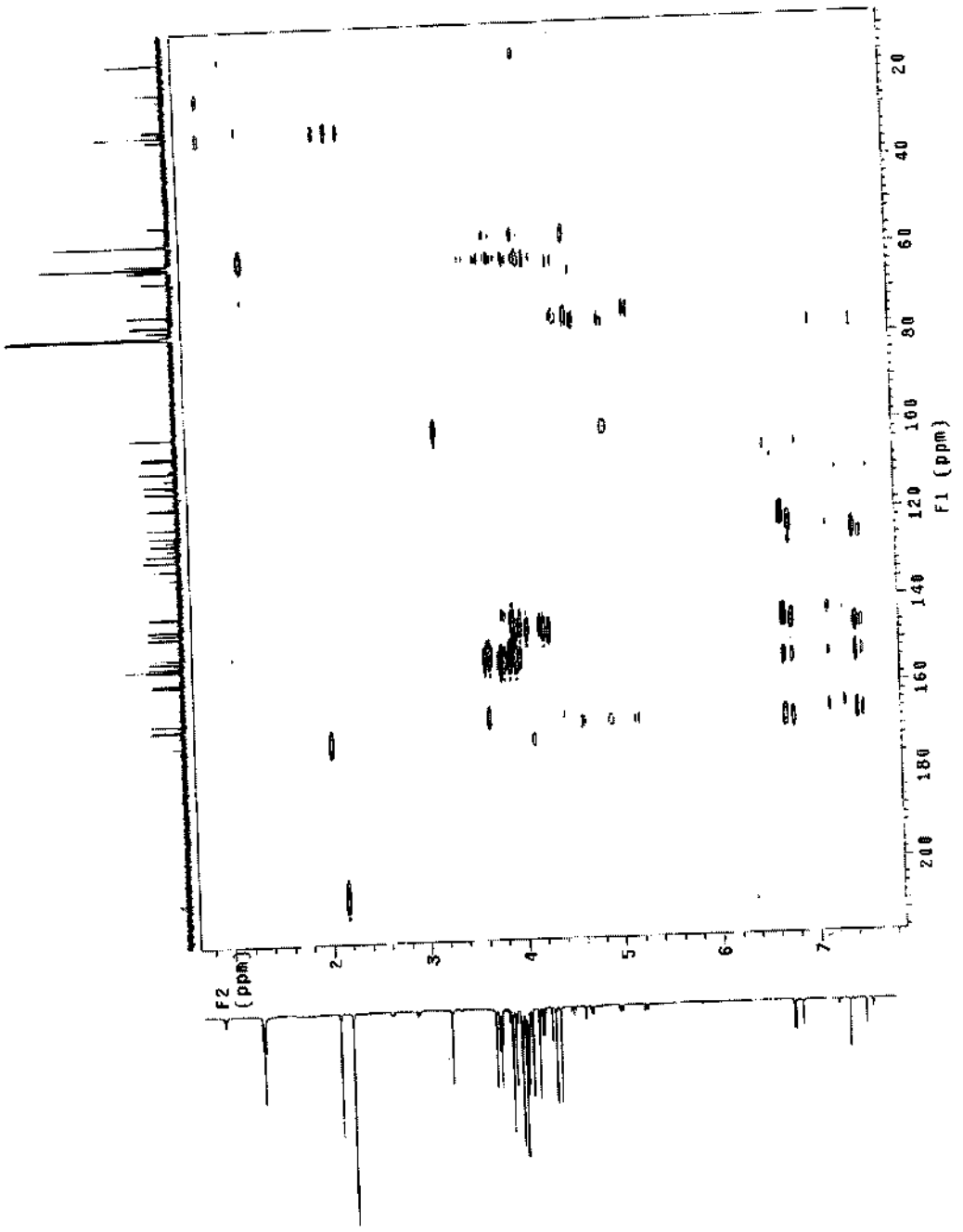

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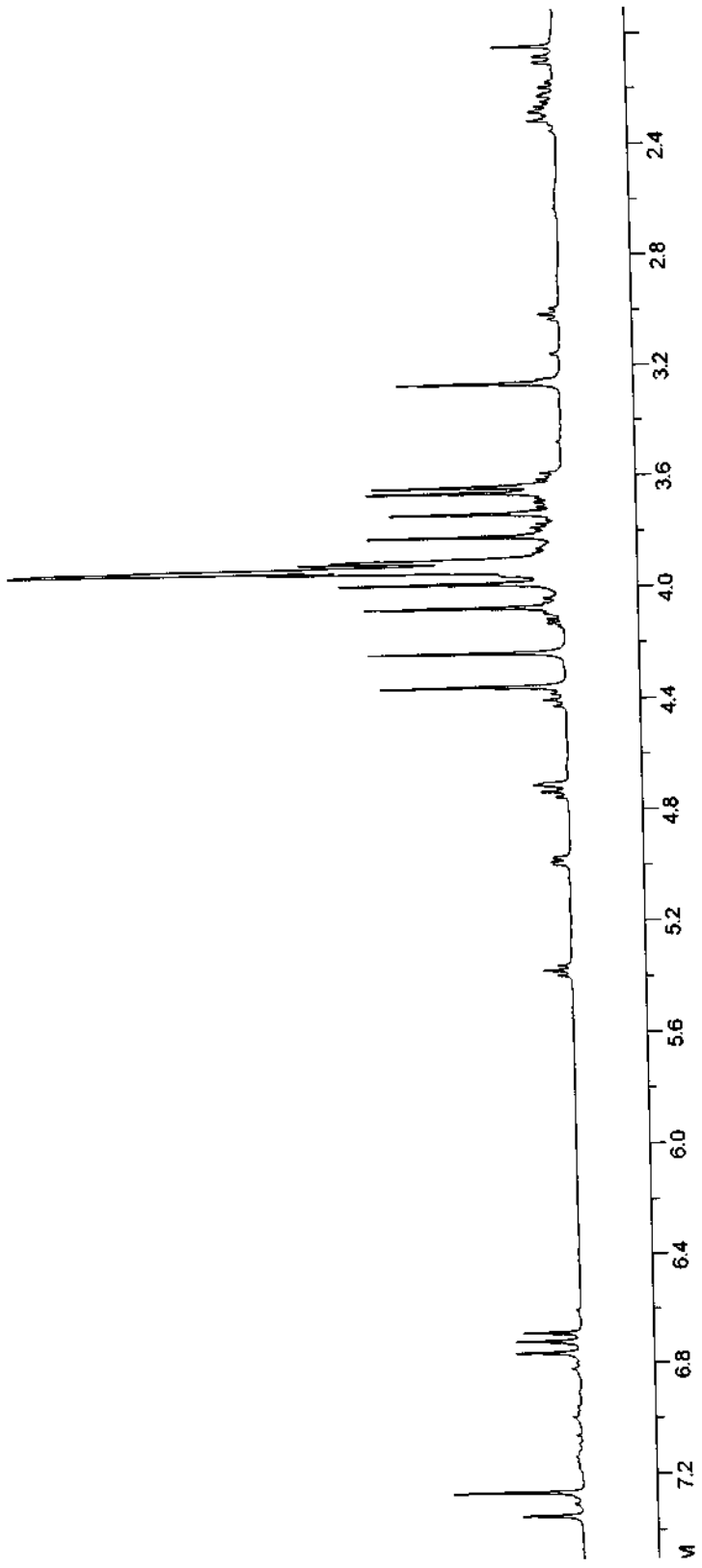

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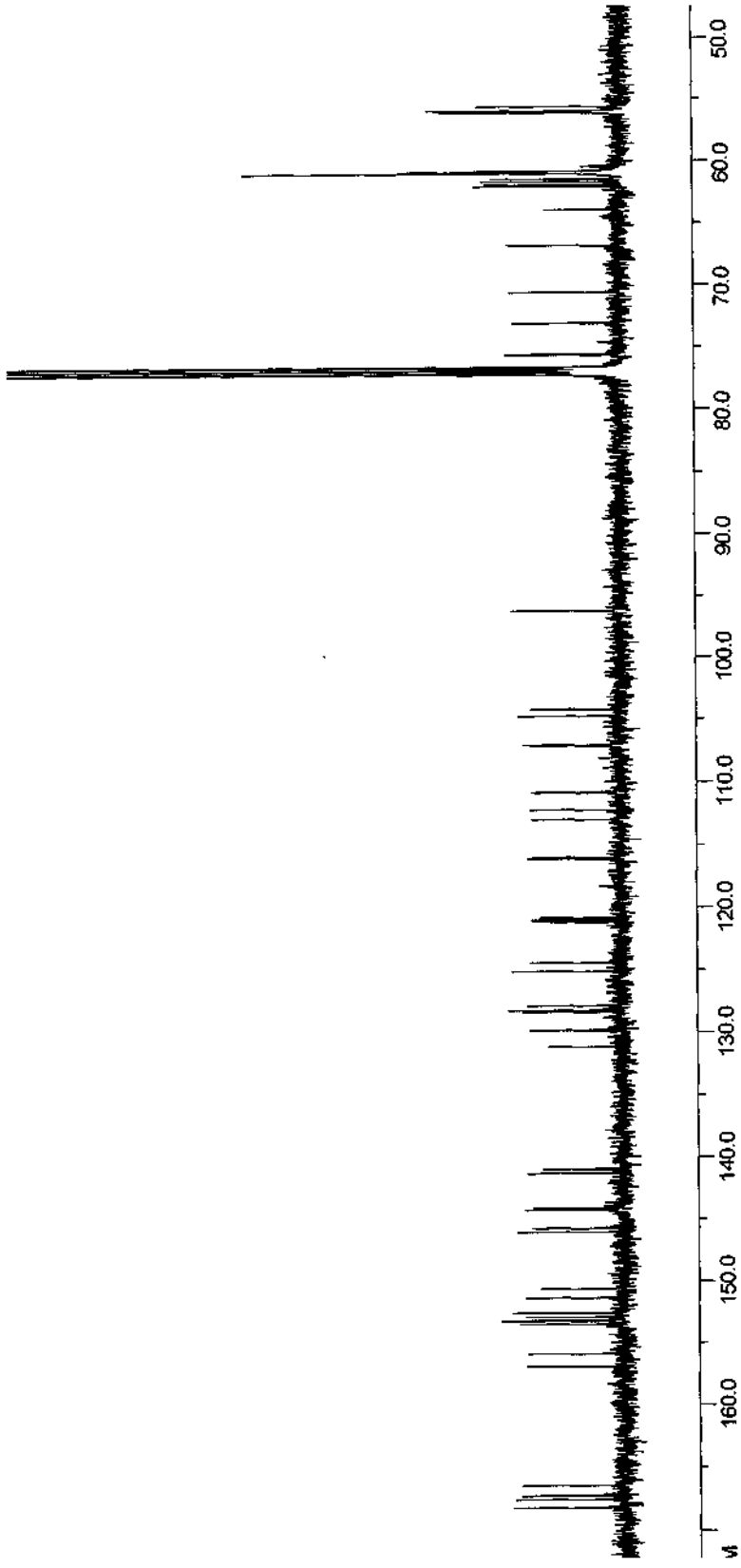

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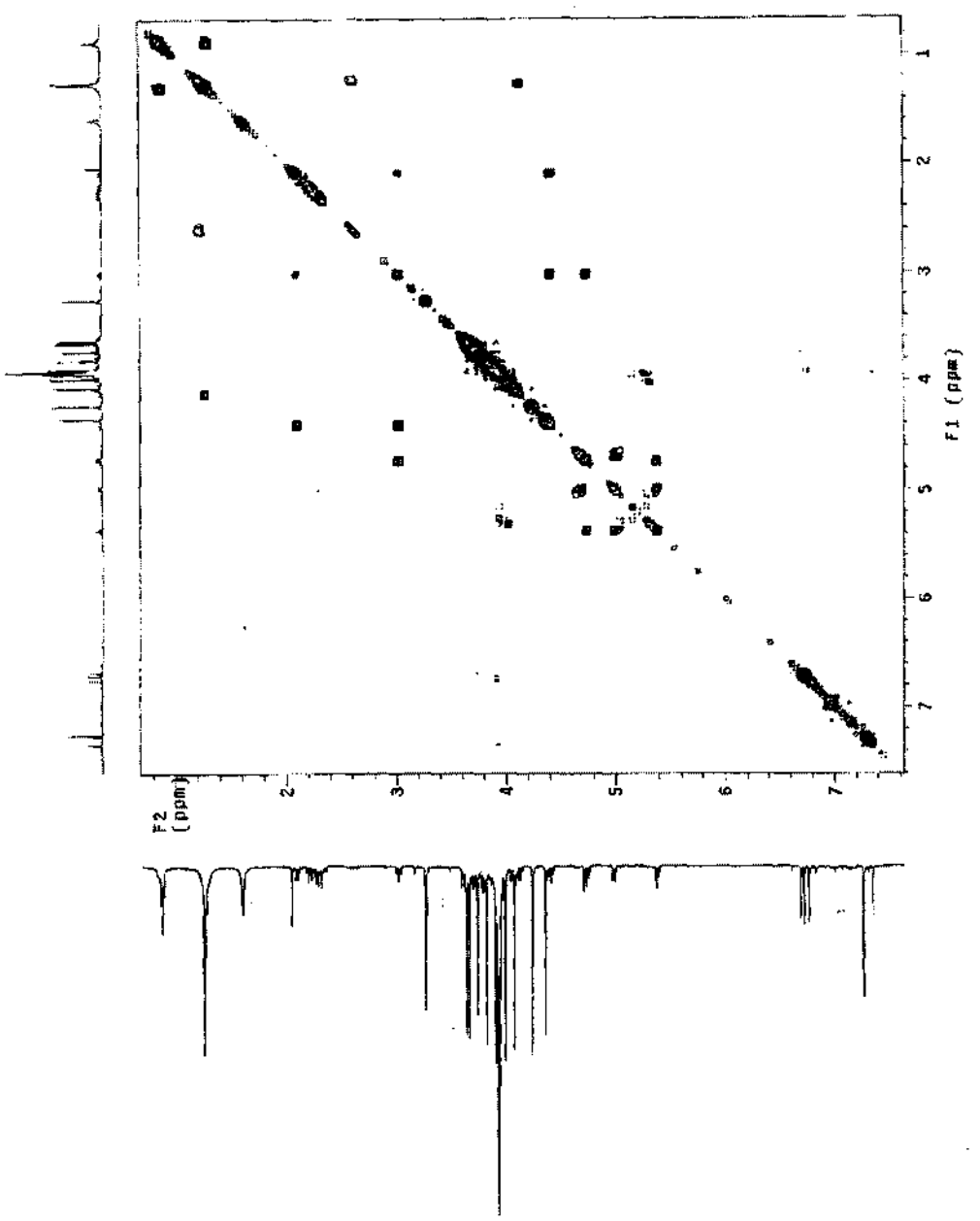

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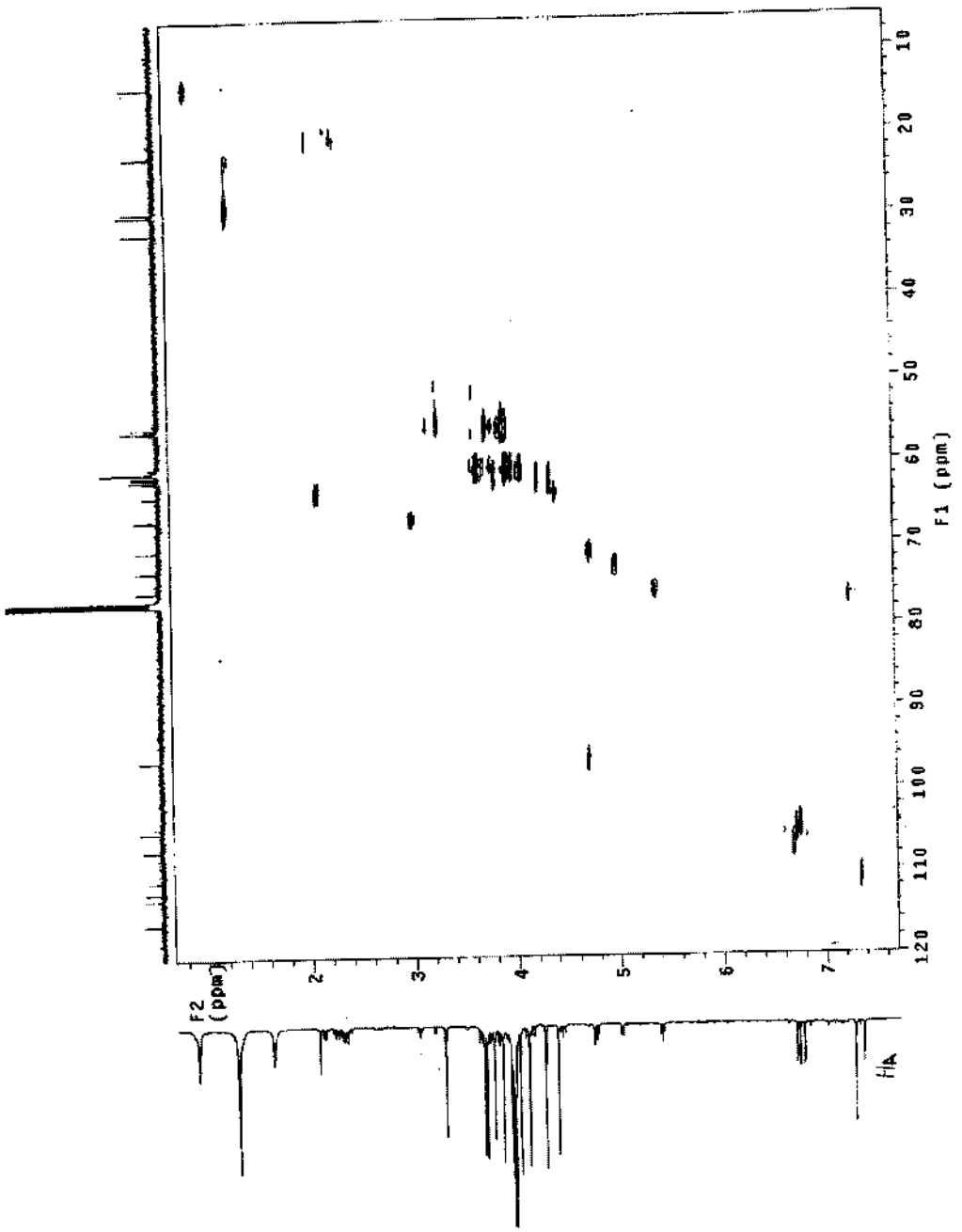

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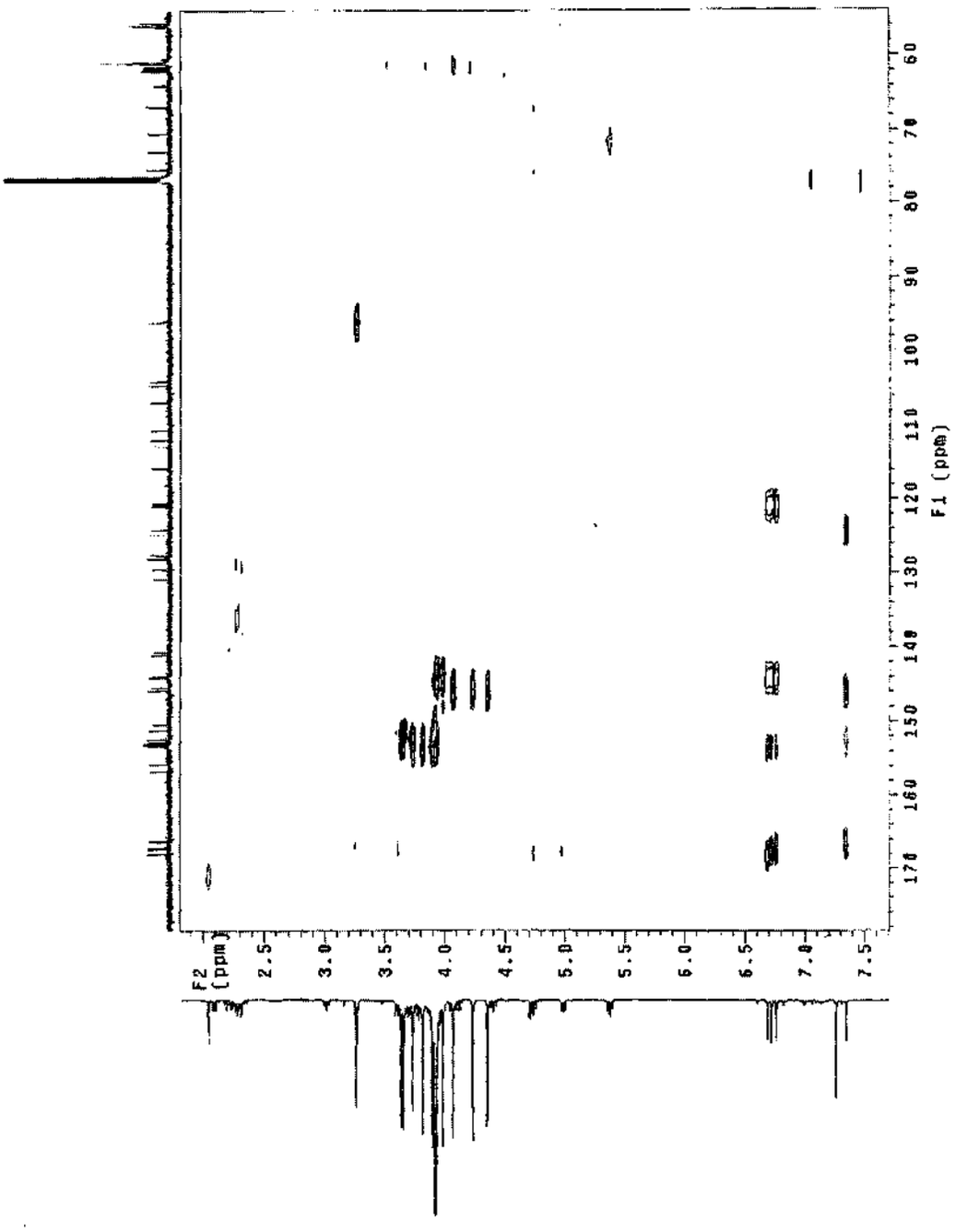
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Venusol from *Gunnera perpensa*: structural and activity studies

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Abstract

From the aqueous extract of the dry rhizomes of *Gunnera perpensa* the minor components pyrogallol, succinic acid, lactic acid, and the trimethyl ether of ellagic acid glucoside were isolated. The major constituent was identified as *Z*-venusol, a phenylpropanoid glucoside. Its structure was verified by X-ray diffraction. Tests on isolated uterine smooth muscle from rats showed that the whole extract stimulated a direct contractile response and induced a state of continuous contractility of the uterus once all additives had been removed from the organ bath. By contrast, venusol did not trigger the direct contractile response but induced the state of continuous contractility once the organ bath was flushed.

Keywords: *Z*-venusol; X-ray structure; Lespedezic acid; Uterine contraction; Expulsion of placenta; Spontaneous contractility

1. Introduction

Gunnera perpensa (Gunneraceae), known as river pumpkin, or "uGhobo" in isiZulu, is a perennial shrub which grows along stream banks in many parts of South Africa. It is best known in the province of Kwa-Zulu Natal where it has been used traditionally as a "muthi" (medicinal) plant by the indigenous Zulu people for a long time. One of the first references to it in the Western literature is by the missionary Father J. Gerstner (1939).

Traditionally the finely ground roots of the plant are boiled in water and administered orally to an animal (cow) after calving in order to expel the retained placenta. The extract operates by inducing vigorous uterine contractions. Human uses of the aqueous decoction are relief of pain in rheumatic fever, to ensure an easy childbirth, and to treat infertility in women (Hutchings, 1996). It is of interest to note that Dr. Tod Collins (1996) a western trained veterinarian from the Underberg district of KwaZulu-Natal, speaks highly of his practical experiences with "uGhobo" and endorses the use of the plant by stock farmers.

Some preliminary screening has been done on South African traditional remedies used during labour (Kaido et al. 1997). Masika and Afolayan (2002) have tested other indigenous plants as ethnoveterinary medicines. Despite its widespread use the chemical composition of *Gunnera perpensa* is not known.

2. Results and discussion

2.1. Isolation of components

Gunnera perpensa roots were extracted with boiling water (as is done traditionally) and the dark red solution partitioned against ethyl acetate containing approximately 10% ethanol. Subsequent separation (flash column chromatography with methanol and chloroform) afforded small quantities of succinic acid, lactic acid, pyrogallol and the glucoside of ellagic acid trimethyl ether (**1**). It is known that ellagic acid is a potent antagonist, in particular on histamine liberators (Bhargava and Westfall, 1969). The activity of venusol

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may well be modulated by the presence of the other compounds.

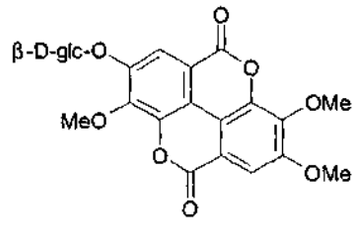
The ethyl acetate extract of aq. *Gunnera* solution yielded buff needles of the compound venusol (0.18% yield based on dry plant material). Recrystallisation afforded orthorhombic needles, suitable for X-ray analysis. Information from this, coupled with the usual ^{1}H- and ^{13}C-NMR and high resolution mass spectral data, enabled us to establish the structure unambiguously as *Z*-venusol, (7,8-dihydroxy-6-(hydroxymethyl)-3-[(*Z*)-(4-hydroxyphenyl)methylidene]tetrahydro-4aH-pyrano[2,3-b][1,4]dioxin-2-one (IUPAC nomenclature). The structure is shown in **2**, but the usual numbering (Proliac et al. 1981) has been retained.

Z-Venusol has been isolated previously by Proliac et al. (1981) and Pagani (1990). Both the *Z* and *E* isomers were obtained recently by Viornery et al. (2000). In all these instances the plant source was *Umbilicus pendulinus* or *Umbilicus ruperstris* (Crassulaceae).

From molecular modeling experiments and NOE data Viornery et al. (2000) was able to distinguish between the *Z*- and *E*-isomers of venusol. This designation of stereochemistry also confirmed the earlier work of Shigemori et al (1990) on lespedezic acid methyl ester (**3**). The chemical relationship between venusol (**2**) and methyl lespedezate (**3**) is a close one. In the aq. *Gunnera* extract we found traces of (**3**) accompanying the major component venusol (**2**). This is likely to be an artefact resulting from the action of methanol (used in the extraction process) on the venusol. Shigemori et al. (1990) were able to show that potassium lespedezate and its geometrical isomer, potassium isolespedezate operate as an "internal clock" in the nyctinastic plant *Cassia mimosoides* and are involved in the leaf-opening and leaf-closing mechanisms.

Recently Ohnuki et al. (1998) have considered the leaf-opening and leaf-closing mechanisms at the molecular level as a result of their findings in nyctinastic plants. These authors were able to isolate both isomers of potassium lespedezate from *Lespedeza cuneata* (leaf-openers). In addition they also isolated the leaf-closing substance potassium D-idarate (**6**). Their detailed studies indicate that leaf movement of nyctinastic plants is controlled by the competitive interaction between leaf-opening [K-lespedezate, (*E* or *Z* isomer)] substances and leaf-closing [K-D-idarate (**6**)] substances. In the evening the enzyme β-glucosidase simply converts K-lespedezate to its deactivated form, potassium 4-hydroxyphenylpyruvate (**7**), i.e. the leaf-opening mechanism is "switched off" by enzymic deactivation of lespedezic acid.

We believe that the above studies are relevant to our work on *Gunnera* as they illustrate the point that all "players" in the activation of the uterine muscles need



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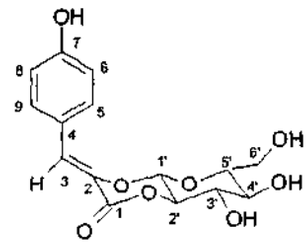
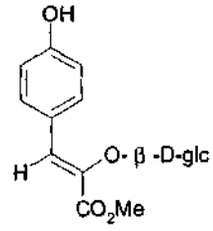
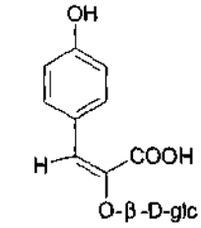


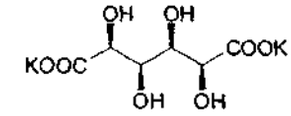
(Z)-Venusol (2)



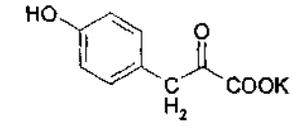
(Z)-Methyl lespedezate (3)
Me = H (Z)-Lespedezic acid (4)



(E)-Lespedezic Acid (5)



(6)



(7)

to be present for the system to function. In the nyctinastic plants all the interacting compounds are present in the plant sap, just as they appear to be present in the crude *Gunnera* extracts. At present our investigations have only yielded one of the compounds involved and this may explain the type of activity observed (see Section 2.3).

While venusol is not a new compound, it has now been isolated from a new plant source (family *Gunneraceae*). From our X-ray data of the compound the Z-stereochemistry around the C-2/C-3 double bond is established unambiguously. The final mechanism remains unclear, but there is good evidence that Z-venusol brings about contraction of uterine muscle (see below).

2.2. Biological activity

The effect of aq. *Gunnera* extract, ethyl acetate extract, ethyl acetate–methanol extract, and pure *Z*-venusol, were tested on uterine and ileal muscle obtained from Sprague–Dawley rats. The procedure has been described previously (Matonhodze et al., 2002).

The aq. *Gunnera* extract directly stimulates a contractile response for both the ileum and uterus muscle. Once the plant extract and the added acetylcholine are rinsed from the organ bath, the tissue enters into a state of continuous spontaneous contractility. Pre-incubation with atropine reduced the contractile response significantly. We speculate from the above that the muscarinic receptor system is involved in the direct contractile response to the whole plant extract.

Neither the organic extracts nor the pure Z-venusol (2) elicited a direct contractile response. However, the venusol did induce a state of spontaneous contractility once the test substance was flushed from the organ bath. It is possible that the venusol exerts its action in conjunction with substances present in the whole extract. This aspect of the work will be explored in a forthcoming publication.

3. Experimental

3.1. General

^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 MHz NMR spectrometer. High resolution mass spectra were measured on a Kratos MS 80 RF double-focussing magnetic sector instrument at 70 eV. The X-ray structure was obtained with an Oxford Xcalibur 2 CCD diffractometer, (M_oK_α) radiation.

3.2. Plant material

Gunnera perpensa roots were collected in May 2002 on a farm in the Underberg district of Kwa-Zulu Natal and allowed to dry out in a shady place. A voucher specimen (leaves and root) was deposited in the Bews Herbarium, University of Natal, Pietermaritzburg (lodged under S.E. Drewes, N.U. 8). Verification of identity was done by Dr. Trevor Edwards, curator of the herbarium.

3.3. Extraction and isolation

The dry root material (910 g) was milled finely and extracted with boiling water (7 l) for 3 h. Preliminary tests had shown that even polar organic solvents, such as ethanol, dissolved almost none of the material. After filtration and autoclaving the red-brown solution was partitioned against ethyl acetate. This was a slow process since an emulsion formed readily which necessitated long periods of waiting between shaking. Emulsion-formation was largely inhibited by resorting to ethyl acetate–methanol (5:1) mixtures and this also resulted in more material being extracted. Concentration of the ethyl acetate–methanol extract to about 2 l, followed by drying (anhyd $MgSO_4$) and subsequent further concentration afforded a brown oil which crystallized on standing (1.67 g, representing 0.18% yield

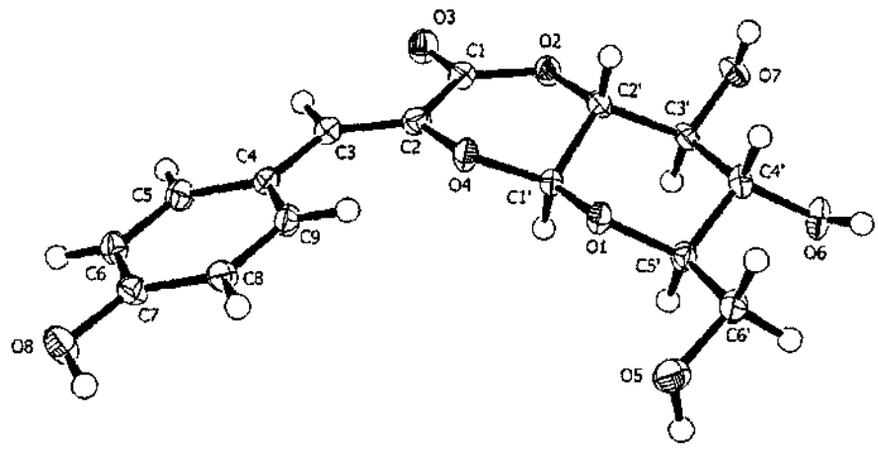


Fig. 1. X-ray structure of (Z) venusol (2).

based on dry plant material). The crude crystalline material subsequently proved to be *Z*-venusol which was difficult to get absolutely pure. Final purification required separation by flash chromatography and elution with methanol–chloroform (3:17). The white needles had mp 259 °C (Proliac et al., 1981, quote 259–260 °C) and proved to be suitable for X-ray analysis.

Examination of a tlc plate of the mother liquor of the *Z*-venusol [run in MeOH–$CHCl_3$ (3:17)] and visualized with vanillin–H_2SO_4 dip reagent revealed the presence of at least another seven compounds. Several of these were subsequently identified and proved to be very minor components of known structure. These compounds were: R_F 0.90, 4-*O*-β-D-glucopyranosyl-3,3′,4′-tri-O-methyl-ellagic acid (**1**); R_F 0.81, Blue fluorescent compound (phytosterol); R_F 0.76, Blue fluorescent compound; R_F 0.60, No fluorescence, purple on heating with dip reagent—lactic acid, 15 mg; R_F 0.54, Faint fluorescence, red with dip reagent—pyrogallol, characterized as the triacetate, 37 mg; R_F 0.41, Venusol—strongly fluorescent, buff coloured with dip reagent, 1.67 g; R_F 0.36, Weak fluorescence, green blue with dip reagent—(*Z*) methyl lespedezate, 10 mg; R_F 0.16, Fluorescent—pale purple with dip reagent.

Succinic acid mp 182 °C, in very low concentration was isolated from the mother liquor of venusol. It is not readily detected by tlc.

The ellagic acid derivative, 4-*O*-β-D-glucopyranosyl-3,3′,4′-tri-*O*-methylellagic acid (**1**) was present in low concentration but was readily identified by ^1H and ^{13}C NMR (Khac et al., 1990; Li et al. 1999) as well as by high-resolution mass spectrometry of its aglycone. H-R EI-MS *m/z* 344.05434 M^+ calc. for $C_{17}H_{12}O_8 = 344.05322$.

The methyl *Z*-lespedezate (**3**) was readily identified by comparison with the spectral data provided by Viornery et al. (2000). The NMR features which distiguish it from Z-venusol are the sharp methoxy peak at δ_H 3.81 and at δ_C 52.6 (deuteriomethanol). In addition, the anomeric cabon resonates at δ_C 102.8 whereas in venusol it is at δ_C 96.2.

3.4. *Z*-venusol

Fine white needles, mp 259 °C, $[\alpha]_D^{20} = 84.5$ (MeOH, 0.245). ^1H and ^{13}C spectra run in D_2O and CD_3OD were compared with those recorded by Proliac et al. (1981) in DMSO and by Viornery et al. (2000) in C_2D_6CO, and leave no doubt about the identity of the compound. Its stereochemistry about the C-2/C-3 double bond was unambiguously proved to be *Z* by X-ray diffraction (see below) (Fig. 1).

Acetylation of *Z*-venusol afforded an oil, $[\alpha]_D^{20} = 25$ (MeOH, 0.028). ^1H NMR (500 MHz, $CDCl_3$): δ 2.05, 2.09, 2.12 (9H, *s*, 3′, 4′, 6′-OAc), 2.30 (3H, *s*, 7-OAc), 3.95 (1H, *ddd*, *J* 10.1, 4.4, 2.2, H-5′), 4.21 (1H, dd, *J* = 12.7, 2.2, H-6′a), 4.32 (1H, dd, J 12.8, 4.4, H-6′b), 4.32 (1H, dd, *J* 9.9, 7.9, H-2′), 5.09 (1H, *d*, *J* = 7.9, H-1′), 5.13 (1H, *dd*, *J* 10.1, 9.2, H-4′), 5.39 (1H, *dd*, *J* 9.9, 9.2, H-3′), 6.98 (1H, s, H-3), 7.12 (2H, *d*, *J* = 8.7, H-5/9), 7.77 (2H, d, *J* = 8.7, H-6/8); ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.5, 20.6, 20.7 (C-3′, 4′, 6′-OAc), 21.1 (C-7-OAc), 61.3 (C-6′), 67.8 (C-4′), 70.9 (C-3′), 73.6 (C-5′), 75.6 (C-2′), 94.6 (C-1′), 120.3 (C-3), 121.9 (C-6/8), 130.0 (C-4), 132.1 (C-5/9), 136.2 (C-2), 151.3 (C-7),159.0 (C-1), 169.1, 169.4, 169.7 (C-3′, 4′, 6′-OAc), 170.5 (C-7-OAc); H-R EIMS m/z 492.126648. M^+ calc for $C_{23}H_{24}O_{12} = 492.126777$. EI-MS *m/z* (rel. int.): 492 (M^+) (77), 450 (10), 408 (3), 289 (22), 194 (48), 176 (88), 169 (100), 163 (20), 134 (60), 127 (35), 109 (95), 43 (58).

Table 1
X-ray data for Z-venusol (2)

Molecular formula	$C_{15}H_{16}O_8$
Formula mass	324.28
Temperature	120 (2) K
Wavelength	0.71073 ?
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions $b = 9.2990(16)$ Å, $\beta = 90°$ $c = 28.397(5)$ Å, $\gamma = 90°$	$a = 5.113(2)$ Å, $\alpha = 90°$
Volume	1350.1(7) Å
Z	4
F(000)	680
Crystal size	0.40×0.20×0.10 mm^3
Theta range for data collection	4.24–31.87°
Reflections collected	13,403
Independent reflections	4251 [*R*(int) = 0.0335]
Completeness to theta = 25.00°	99.1%
Absorption correction	None
Refinement method	Full-matrix least squares on F^2
Goodness of fit on F^2	1.009
Final R indices [$I > 2$ sigma (I)]	$R1 = 0.0364$, $wR2 = 0.0814$
R indices (all data)	$R1 = 0.0455$, $wR2 = 0.0850$
Absolute structure parameter	−0.7 (7)

The complete set of data (atomic coordinates, equivalent isotropic displacement parameters, bond lengths and bond angles, anisotropic displacement parameters, hydrogen coordinates and torsion angles) will be lodged with the Cambridge Crystallographic center.

3.5. *X-ray crystrallography*

The X-ray data is collected in Table 1.

Acknowledgements

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A triazine derivative with a triple-decker sandwich conformation

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Key indicators

Single-crystal X-ray study
T = 295 K
Mean σ(C–C) = 0.005 Å
R factor = 0.066
wR factor = 0.224
Data-to-parameter ratio = 16.1

For details of how these key indicators were automatically derived from the article, see http://journals.iucr.org/e.

In dimethyl 6,6′-bis(benzyloxy)-5,5′-dimethoxy)-3,3′-(6-methoxy-1,3,5-triazine-2,4-diyldioxy)dibenzoate, $C_{36}H_{33}N_3O_{11}$, a new triazine derivative, the benzyloxy groups adopt an interesting conformation in which they are positioned above and below the triazine ring 'core' of the molecule to form an intramolecular triple-decker sandwich. The ester carbonyl O atoms of adjacent molecules in the crystal structure participate in non-conventional (alkyl)C—H···O hydrogen bonds [averaging 2.45 (1)Å] about an inversion centre to give a weakly hydrogen-bonded dimer.

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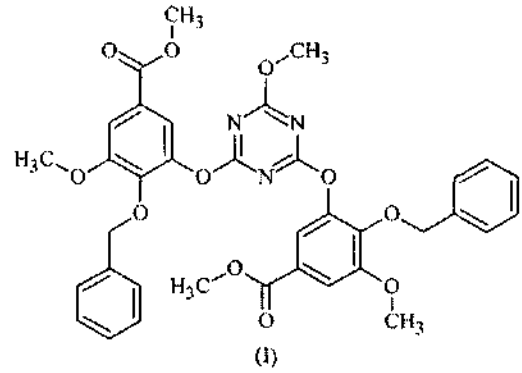
Comment

A new triazine derivative (I) was isolated in crystalline form after work-up of the reaction of 2,4-dichloro-6-methoxy-1,3,5-triazine and methyl 4-benzyloxy-3-hydroxy-5-methoxybenzoate in the presence of *N*-methylmorpholine (NMM) using conditions described in the literature (Kunishima *et al.*, 1999). The structure of (I) is of general interest since phenoxytriazine derivatives have recently been shown to form intriguing supramolecular structures when a suitable hydrogen-bonding substituent is present on the phenoxy ring (Aakeroy *et al.*, 2005; Lebel *et al.*, 2006; Suss *et al.*, 2005).



(I)

The triazine ring core of (I) is sandwiched between the two phenyl rings of the benzyloxy groups (Figs. 1 and 2). The dihedral angles between the top (C11–C15) and bottom (C36–C33) phenyl rings and the triazine ring are 10.9 (2) and 12.9 (2)°, respectively. The distance between the centroid of the C11–C15 ring and the triazine ring is 3.58 (1) Å, while that between the C33–C36 ring is somewhat longer at 3.85 (1) Å. Although the intramolecular ring···ring separations in (I) exceed the graphite spacing (3.35 Å) and the rings are canted, the possibility that the triple-decker sandwich conformation of (I) is brought about by $\pi\cdots\pi$ interactions cannot be excluded,

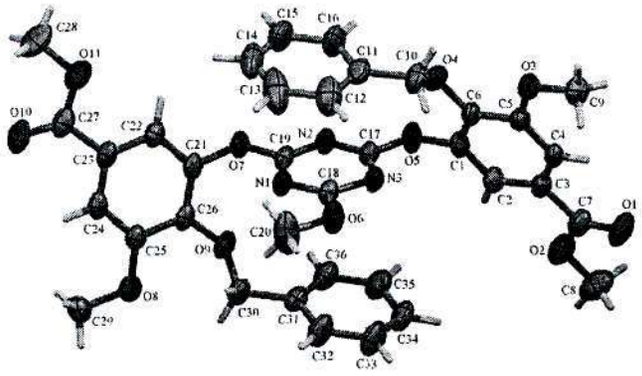


Figure 1
The molecular structure of (I) (45% probability displacement ellipsoids for all non-H atoms).

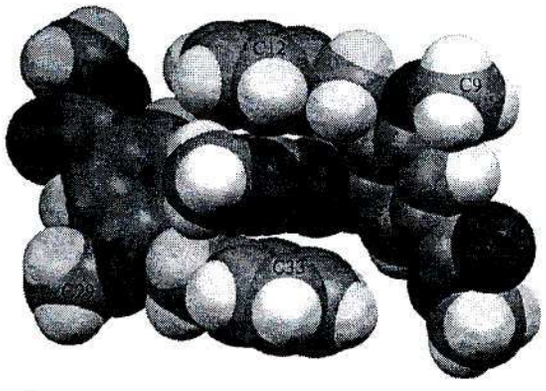


Figure 2
Space-filling plot (van der Waals radii) for (I), viewed approximately down the O6—C18 bond, illustrating the triple-decker sandwich arrangement of the phenyl and central triazine ring systems.

particularly since intramolecular interactions of this type and magnitude have been described previously (Hunter *et al.*, 2001; Munro *et al.*, 2003). Bond distances and angles are normal and warrant no further discussion.

The anisotropic displacement parameters for the C atoms of both benzylic phenyl rings reflect libration of these groups in the room-temperature X-ray structure. Collection of the X-ray data at 100 (2) K to remedy this situation, however, afforded a data set of poorer quality (R_{int} = 0.055) due to partial fracturing of the crystal below 200 K.

The unit-cell contents of (I) comprise the expected pair of molecules situated about the inversion centre and located at general positions in the triclinic unit cell. More interesting is the observation that molecules of (I) associate by weak non-conventional hydrogen bonds (Table 1) to form a centrosymmetric hydrogen-bonded dimer (Fig. 3). The C–H donors in these nominally weak (Steiner, 2002) C—H···O(carbonyl) interactions are not particularly acidic, yet the contact distances are shorter than the sum of the van der Waals radii of oxygen and hydrogen (2.72 Å) by more than 0.25 Å. This fact suggests that the interactions listed in Table 1 are more than simple non-bonded contacts in the case of (I).

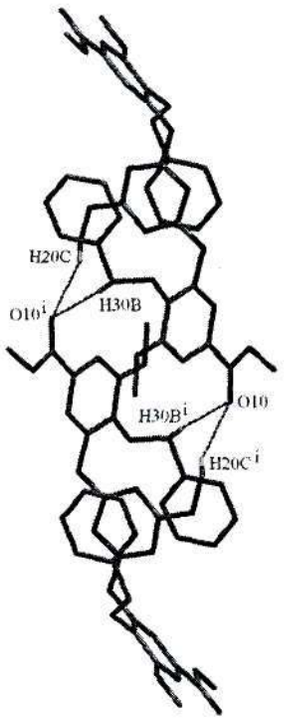


Figure 3
Hydrogen-bonded dimer with inversion symmetry. Atoms involved in the interaction have been labelled. Hydrogen bonds are shown as dashed lines. All other H atoms have been omitted for clarity. [Symmetry code: (i) $-x, 2-y, -z$.]

Experimental

A mixture of 100 ml of methanol, 13.5 ml of water and 16.5 g (0.2 mol) of sodium bicarbonate was cooled to 283–288 K. Cyanuric chloride (18.4 g, 0.1 mol) was added and the resulting mixture was warmed to 308 K then stirred at 308 K for 20 min and at room temperature for 30 min. Water was added to the reaction mixture and the precipitated product filtered under vacuum, washed with water and dried under vacuum to yield 2,4-dichloro-6-methoxy-1,3,5-triazine as a white solid (5.2 g, 28% yield). A solution of 2,4-dichloro-6-methoxy-1,3,5-triazine (72 mg, 0.40 mmol) and methyl 4-benzyloxy-3-hydroxy-5-methoxybenzoate (58 mg, 0.20 mmol) in THF (20 ml) was stirred under nitrogen. *N*-Methylmorpholine (24 mg, 0.24 mmol) was added and, after stirring for 4 h, the reaction mixture was poured into water and extracted with ethyl acetate. The organic phase was washed successively with a saturated $NaHCO_3$ solution, water, 1 *N* HCl, water and brine and dried over $MgSO_4$. The crude product was purified by column chromatography on SiO_2 (hexane–ethyl acetate = 7:3) to afford compound (I) as a cream–white solid (92 mg, 67%). Recrystallisation by slow evaporation of a methanol solution of (I) gave the compound as colourless crystals (m.p. 371–373 K). Atom numbering consistent with the crystallographic labelling scheme of Fig. 1 applies to the following characterization data where necessary. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.51 (2H, *d*, *J* = 1.9 Hz, H4/H24), 7.43 (2H, *d*, *J* = 1.9 Hz, H2/H22), 7.23–7.25 (10H, *m*, Ar-H), 5.06 (4H, *s*, $-CH_2-$), 3.92 (6H, *s*, COOMe), 3.90 (6H, *s*, C9-OMe/C29-OMe), 3.77 (3H, *s*, C206-OMe). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 173.6 (C17/C19), 173.0 (C18), 165.9 (C=O), 153.3 (C5/C25), 144.7 (C6/C26), 144.0 (C3/

C23) 136.9 (C11/C31), 128.2 (*m*-C, phenyl), 127.8 (*p*-C, phenyl), 127.6 (*o*-C, phenyl), 125.2 (C1/C21), 116.8 (C2/C22), 111.2 (C4/C24), 74.8 (C10/C30), 56.2 (C9/C29), 55.4 (C20), 52.2 (C8/C28).

Crystal data

$C_{36}H_{33}N_3O_{11}$	V = 1696.0 (2) Å^3
M_r = 683.65	Z = 2
Triclinic, $P\overline{1}$	D_x = 1.339 Mg m^{-3}
a = 8.5540 (7) Å	Mo $K\alpha$ radiation
b = 11.1363 (8) Å	μ = 0.1 mm^{-1}
c = 17.9894 (13) Å	T = 295 (2) K
α = 92.746 (6)°	Rhomb, colourless
β = 94.278 (6)°	0.6 × 0.4 × 0.25 mm
γ = 96.246 (6)°	

Data collection

Oxford Diffraction Xcalibur 2 CCD diffractometer	7354 independent reflections
	4422 reflections with $I > 2\sigma(I)$
ω scans	R_{int} = 0.024
Absorption correction: none	θ_{max} = 27.1°
13313 measured reflections	

Refinement

Refinement on F^2	$w = 1/[\sigma^2(F_o^2) + (0.1153P)^2 + 0.3795P]$ where $P = (F_o^2 + 2F_c^2)/3$
$R[F^2 > 2\sigma(F^2)]$ = 0.066	
$wR(F^2)$ = 0.224	
S = 1.06	$(\Delta/\sigma)_{max}$ = 0.001
7354 reflections	$\Delta\rho_{max}$ = 0.29 e Å^{-3}
456 parameters	$\Delta\rho_{min}$ = −0.19 e Å^{-3}
H-atom parameters constrained	

Table 1
Hydrogen-bond geometry (Å, °).

D—H···A	D—H	H···A	D···A	D—H···A
C20—H20C···O10^{i}	0.96	2.45	3.272 (6)	144
C30—H30B···O10^{i}	0.97	2.46	3.381 (4)	157

Symmetry code: (i) $-x, -y+2, -z$.

Methyl H atoms were placed geometrically and refined as rigid rotating groups (torsion angles from the electron density), with C—H = 0.96Å and U_{iso}(H) = 1.5U_{eq}(C). Methylene H atoms were placed geometrically and refined using a riding model, with C—H = 0.97Å and U_{iso}(H) = 1.2U_{eq}(C). Aromatic H atoms were placed geometrically and refined using a riding model, with C—H = 0.93Å and U_{iso}(H) = 1.2U_{eq}(C).

Data collection: *CrysAlis CCD* (Oxford Diffraction, 2004); cell refinement: *CrysAlis RED* (Oxford Diffraction, 2004); data reduction: *CrysAlis RED*; program(s) used to solve structure: *SHELXS97* (Sheldrick, 1997); program(s) used to refine structure: *SHELXL97* (Sheldrick, 1997); molecular graphics: *ORTEP-3* (Farrugia, 1997); software used to prepare material for publication: *WinGX* (Farrugia, 1999).

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