CHEMISTRY AND BIOLOGICAL ACTIVITY OF HYPERICUM LANCEOLATUM

BY

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THIS THESIS IS MY ORIGINAL WORK AND HAS NOT BEEN PRESENTED FOR A DEGREE IN ANY UNIVERSITY.

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DEDICATED TO MY PARENTS

ATSEDE GIZAW AND DERESE WOLDEAMANUEL

AND MY SISTERS AND BROTHERS

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ABSTRACT

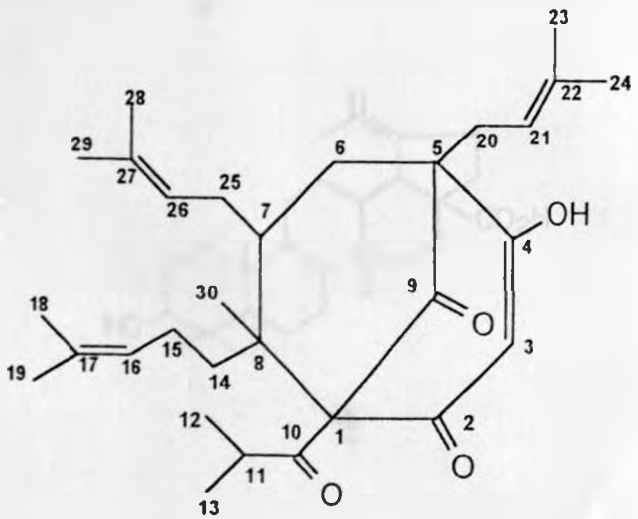
The genus *Hypericum* belongs to the family Guttiferae and is represented by over 400 species. It is found widely distributed in the tropics as well as the temperate regions. This genus is mainly characterized by the presence of xanthones, anthraquinones, fillicinic acid and phloroglucinol acid derivatives. Presently more attention is given to this genus due to the antiviral activity of some of its constituent compounds. It is represented by twelve species in Eastern Africa. One of these is *H. lanceolatum* which is considered to be synonymous with *Hypericum revolutum* by some authors, however, others consider them different taxa.

In this work the phytochemistry and biological activity of *H. lanceolatum* and *H. revolutum* have been investigated. Based on the distribution of their constituent compounds, an attempt has been made to determine the similarities and differences between these two taxa. The bark, flowers and seeds of the plant samples were dried in shade, ground into powder and then separately extracted with various solvents. This was followed by chromatographic (column and preparative thin layer chromatography) separation with normal silica gel, silica gel impregnated with 3% oxalic acid solution in deionized water and Sephadex LH-20 columns. The structure of the pure compounds were determined using NMR (1D and 2D), EIMS, UV and IR spectrometers.

The hexane extract of the bark of *H. revolutum* afforded hyperevolutin A (4-hydroxy-8-exo-

methyl-5,7-exo-bis(3-methylbut-2-enyl)-1-(2-methyl-1-oxopropyl)-8-endo-(4-methylpent-3-enyl)bicyclo[3.3.1] non-3-ene 2,9-dione) (**1**) and the corresponding diketo form (**2**), while the chloroform extract gave stigmasterol (**3**). The hexane and chloroform extract of the stem bark of *H. lanceolatum* afforded β-betulinic acid (**4**). The combined acetone and methanol extract of the flowers and seeds of *H. lanceolatum* afforded I3,II8-biapigenin (5,5",4',4''',7,7''-hexahydroxy-3,8-biflavone) (**5**), quercetin (3,3',4',5,7-pentahydroxyflavone) (**6**) and myricetin (3,3',4',5,5',7-hexahydroxyflavone) (**7**).

The hexane and chloroform extracts of *H. revolutum* had brine shrimp lethality test indicative of potential good bioactivity with an LC_{50} value of 6 and 5 μg/ml, respectively. Hyperevolutin A had a lethality with LC_{50} value of 0.5 μg/ml. Hyperevolutin A exhibited anti-fungal activity against *Saccharomyces cerevisae* and *Trichophyton mentanogrophyte*. The result showed hyperevolutin A had an inhibition zone of 5.5 mm radius at 6 μg per disc against the former organism and an inhibition zone of 7.5 mm radius at 100 μg per disc against the latter organism. Under similar conditions, chloramphenicol had an inhibition zone of 12.5 mm radius at 25 μg per disc on *Sacaromyces cerevisae*.



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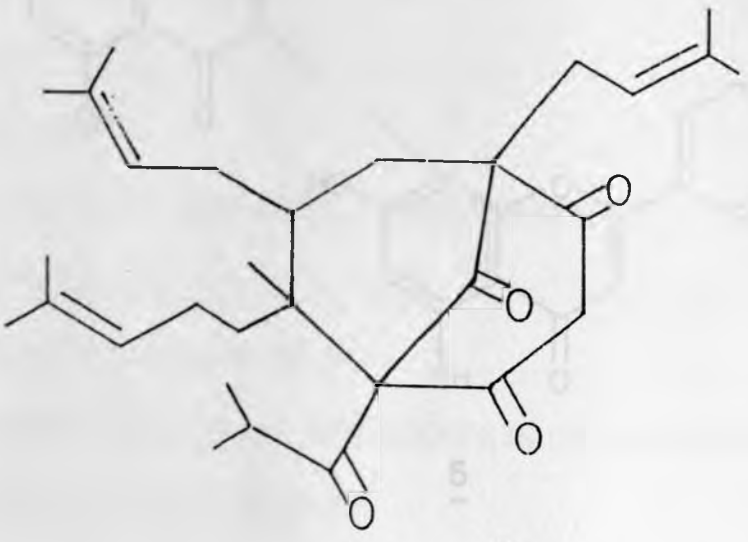

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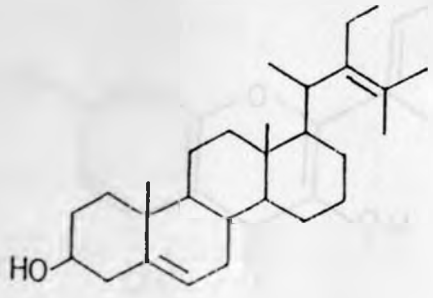

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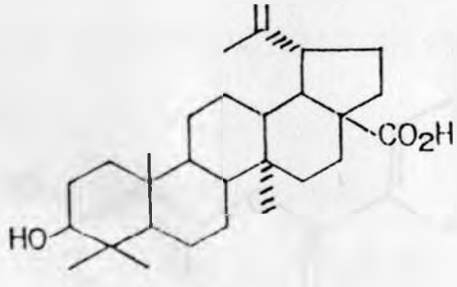

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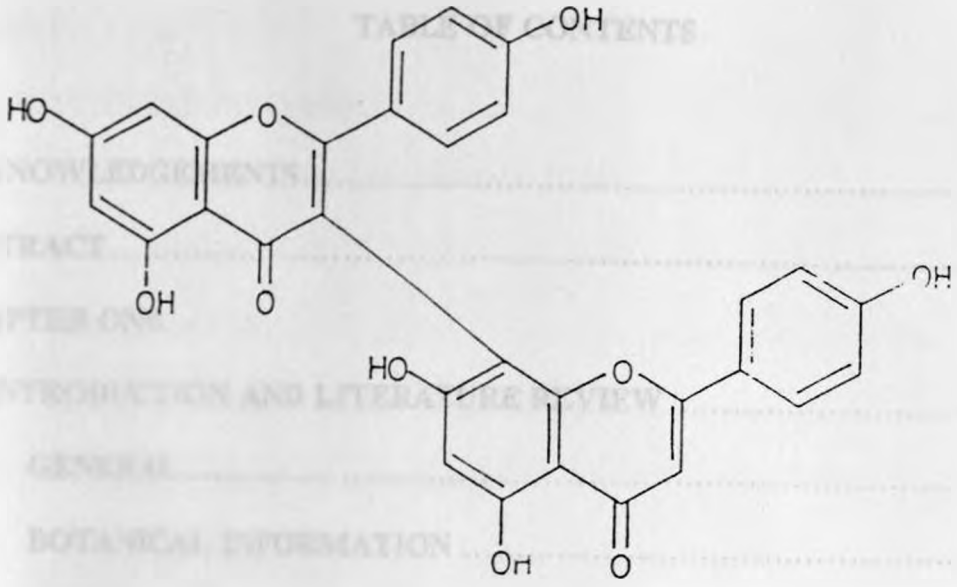

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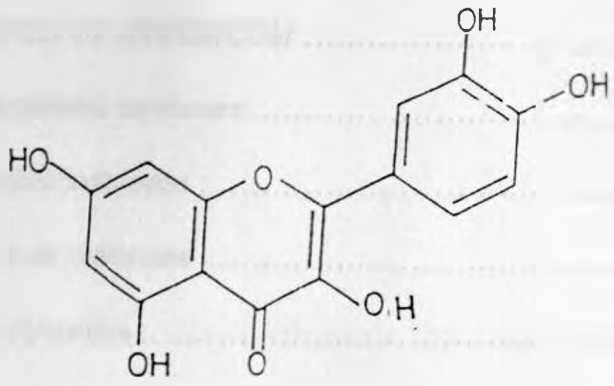

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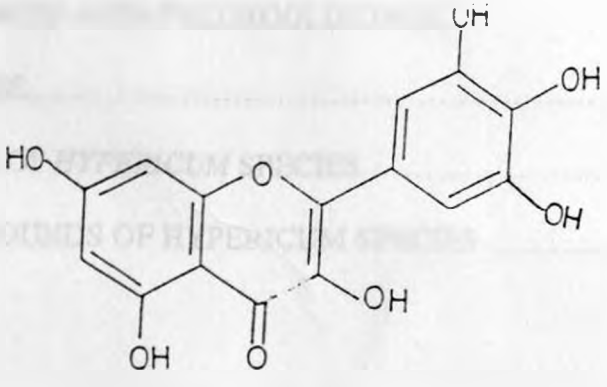

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CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL

The use of medicinal plants as a remedy for illness can be traced back to over five millennia to written documents of the early civilization in China, India, and the Near East. However, it is a tradition without any doubt, as old as mankind. Nevertheless, the potential of plants as sources for new drugs is hardly exploited. Among the estimated 250,000-500,000 [Hamburger and Hostettmann, 1991] plant species, only a small percentage has been investigated phytochemically and the fraction submitted for biological screening is even smaller. Nonetheless, despite the dominance of synthetics as drugs, the plant kingdom serves to provide mankind with more than 25% of the valuable drugs in use [Hamburger and Hostettmann, 1991].

All compounds isolated from plants are not useful, at least as drugs. Therefore, performing random phytochemical research is very much a waste of time and resources. The crude extracts of the plant must be screened initially for biological activity. The active extracts should then be subjected to chromatographic work to isolate the bioactive principles which could then be subjected to thorough biological activity tests. In brief to achieve applied meaning and significance, phytochemical studies must incorporate bioassay. In this line, the National Cancer Institute (NCI), United States, screened some 35,000 plant species for antitumor activities from 1957 to 1981 [Suffness, 1987] and has currently acquired some 20,000 tropical species from

Latin America, Africa and South East Asia [Cassady *et al.*, 1990]. Even these plants should be treated as 'uninvestigated' with respect to any other pharmacological activities. This demonstrates that there are many drugs waiting to be tapped from plants. The problem here could be the fact that phytochemists, in general, have very limited knowledge of biology and pharmacology. This calls for strengthening the already existing collaboration among phytochemists and biologists and pharmacologists. In order to expedite the work, plant extracts and pure compounds must be sent for bioassay to various pharmacological laboratories so that data may be obtained in a very short period of time.

However, obtaining prior information from traditional healers, whenever possible, is helpful in selecting plants compared to random biological activity screening. A correlation between traditional medicinal use of a plant and its biological activity exists [McLaughlin, 1991]. It is therefore to the advantage of natural product chemists to document as much of this information as possible in order to help them select species which need priority attention. Unfortunately, not enough information has been documented on African traditional medicinal plants. Most of the information is only passed orally from generation to generation. Certain African countries have made provisions to protect prescriptions which have been passed from generation to generation and which will continue to be employed. A case in point is Malawi [Hostettmann and Marston, 1990], where an attempt to promote traditional medicine alongside modern medicine has been made. In order to obtain their confidence, traditional healers should be treated as *bona fide* contributors to the modern health system of African states. Therefore, enough effort has to be applied on this line so that Africa's traditional medicinal resources are exploited before the

plants become extinct due to deforestation and other factors. It is in this vein that some plants in the genus *Hypericum* of the Guttiferae family are to be studied in this project.

Some African countries have made efforts to document medicinal plants, for example Kokwaro [1976] has listed some of the medicinal plants used in East Africa. Some of these plants include *Hypericum peplidifolium* and *Hypericum roeperianum* which are used to cure indigestion and enhance women fertility, respectively. In this project effort will be made to determine the biological activity of the crude extract of *H. revolutum* and *H. lanceolatum*. There after the active fractions will be subjected to chromatographic work in order to isolate and identify the various compounds present in the plant. The isolated pure compounds will then be subjected to further bioassay analysis.

1.2 BOTANICAL INFORMATION

The genus *Hypericum* belongs to the family Guttiferae. Guttiferae is a family of flowering plants with dicotyledonous seeds. Plants of this family are mainly restricted to the tropics with the major exception being the genus *Hypericum* which is found widely distributed [Bennett and Lee, 1989]. The family is represented by over 1000 species in 40 genera. It is a family mainly characterized by the occurrence of xanthones [Sultanbawa, 1980]. Xanthones or the related benzophenones have been found in all the major genera and several minor genera of this family. Willis [1973] listed the approximate number of species in each genus, and the number of species

that have been found to contain xanthones for each genus of the family. Following Engler's [Bennett and Lee, 1989] system, there are six sub-families in this family, namely: Kielmeyeroideae, Calophylloideae, Clusiodeae, Moronboideae, Lorostemonoideae, and Hypericoideae. The sub-family Hypericoideae has been classed by some taxonomists [Hutchinson, 1973] as a separate family which is closely related to Guttiferae, the Hypericaceae. The recent surge of interest in studying this group of plants has led to the isolation of several prenylated xanthones [Ishiguro *et al.*, 1995; 1993] supporting its inclusion in the family Guttiferae. This sub-family is divided into three tribes, namely: Cratoxyleae, Vismieae and Hypericeae.

The genus *Hypericum* belongs to the sub-family Hypericoideae and tribe Hypericeae. This genus is represented by over 400 species and is found widely distributed in the tropics as well as the temperate regions. It is represented in Eastern Africa by about 12 species. These are : *H. lanceolatum, H. annulatum, H. lalandii, H. peplidifolium, H. bequaertii, H. roeperianum, H. kiboense, H. scioanum, H. humbretii, H. revolutum, H. quartiianum, and H. conjungens.*

In 1797 Lamarck described *H. lanceolatum* from the island of Reunion which at first glance appear indistinguishable from *H. revolutum* [Robson, 1978a]. Indeed several authors [e.g. Robson, 1958] have treated these two species to be a member of one species, *H. lanceolatum*. However, these are two different species, that is *H. lanceolatum* and *H. revolutum*, which evolved due to parallel evolution from *H. angustifolium* and *H. keniense*, respectively. Table 1.2.1 shows the characters of *H. revolutum* and *H. lanceolatum*.

Contrary to the above view there are still some taxonomists who hold that these two taxa are the same [Beetje, 1994]. Robson [1978b] described *H. revolutum* to be found widely distributed all the way from Yemen down the Great Rift Valley, South Africa and Cameroon whereas *H. lanceolatum* to be found only in Reunion and the Comoro's island. However, despite the limited geographical distribution given by Robson, the *H. lanceolatum* used in this project was collected in Kenya. It is very evident from the above discussion that there is a disagreement among taxonomists concerning the classification of these two taxa. Therefore, chemical investigation of these two plants will be very useful in determining whether these two taxa are the same or not.

TABLE 1.2.1 CHARACTERS OF *H. REVOLUTUM* AND *H. LANCEOLATUM**

	H. revolutum	*H. lanceolatum*
Leaf shape	narrowly elliptic to elliptic, acute; margin plane to recurved; ± discolors	narrowly elliptic to narrowly lanceolate or oblanceolate acute to rounded; margin usually ± revolute, discolors.
leaf cross-veins	3-8	3-8
Leaf parallel venation	disjoint or fragmentary	disjoint or fragmentary
Pedicel (mm)	1-5	5-15
Petals	tinged orange	not tinged
Stamen number per fascile	30-35	20-25
Stamen length relative to petals (longest)	$C \frac{1}{2}$	$C \frac{3}{4}$
Style length (mm)	4-8	9-18
Style length relative to ovary	0.8-1.6	1.8-3
Style union (coherence)	$\frac{3}{4} - \frac{7}{8}$	$\frac{4}{5} - \frac{5}{6}$

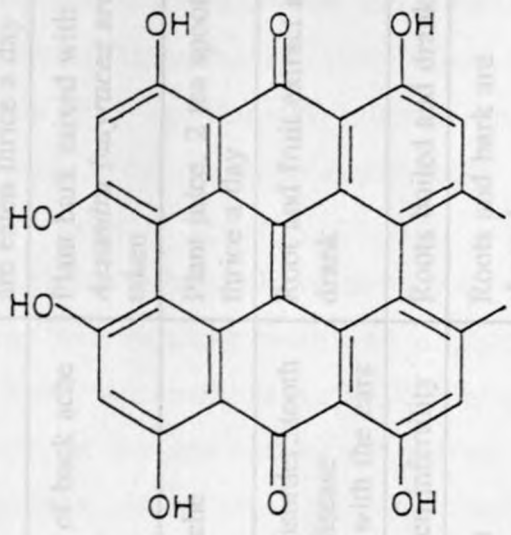
* Adapted from Robson [1978b]

1.3 PHARMACOLOGICAL INFORMATION ON *HYPERICUM*

Plants of the genus *Hypericum* have been used in folk medicine since ancient times [Zdunek and Alfermann, 1992]. Table 1.3.1 shows the traditional medicinal uses of some *Hypericum* species for the treatment of various ailments in some countries. The ingestion of St. John's wort, *H. perforatum*, by grazing animals causes hypericism which is a state of sensitivity to sun light. As a disease of livestock, it affects unpigmented portion of the skin of sheep, cattle, horses, goats and swine, depressing the central nervous system and rendering them hypersensitive to temperature change and handling [Southwell and Campbell, 1991]. The toxicity of the plant is reported by several groups to be due to the chemical compound hypericin (**8**) [Southwell and Campbell, 1991]. Hypericin is also a generic label for hypericin type pigments [Watt and Beryer-Bradwijk, 1962].

The extracts of *H. perforatum* have also been used traditionally since ancient times due to their wound healing and anti-depressive [Zdunek and Alfermann, 1992; Bombardelli and Morazzoni, 1995] properties. Bombardelli and Morazzoni [1995] have reviewed the mechanism of actions, pharmaco-kinetics, clinical trials and side effects of the alcohol extract of this plant for its anti-depressive activity. Clinical trials on 39 patients with psycho-vegetative irritation were done for four weeks on a daily dose of 900 mg of extract. The result demonstrated that about 70% of the treated patients were free of symptoms after four weeks of administration. A dose of 200-900 mg/day is determined to be effective by clinical trials and pharmaco-kinetics study. The anti-

depressive activity of the plant is thought to be due to hypericin (**8**) and/or its derivatives. However, in recent years this idea has been questioned. In fact the pharmacology, the mechanism and the active constituents are not clearly known. Computer modelling [Bombardelli and Morazzoni, 1995] has been used to determine the active constituents by comparing the hypericins, the flavonoids, and the xanthones found in the plant. A best fit was obtained for the flavonoids. Of course, these are theoretical models that need in any case to be tested pharmacologically *in vitro* and *in vivo*. From results obtained thus far one can only conclude that the active ingredients is the alcohol extract *in toto*.



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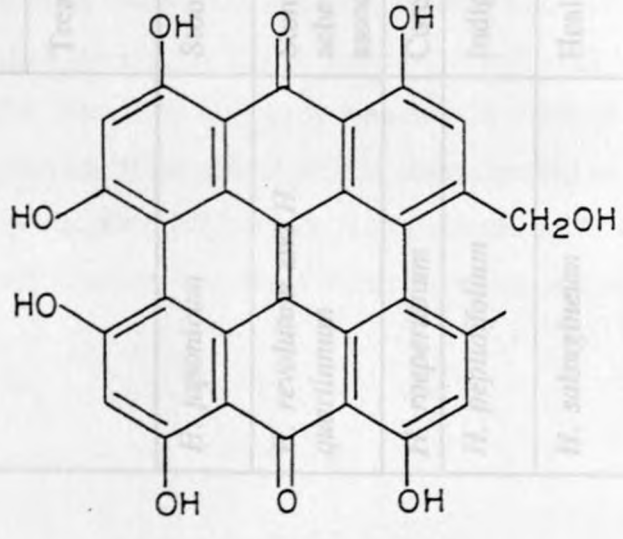

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TABLE 1.3.1: TRADITIONAL MEDICINAL USES OF SOME *HYPERICUM* SPECIES

Botanical name	Ailment treated	Methods of use	Country	Reference
H. cordifolium	Bloody diarrhoea	2-3 g of the flower paste are eaten thrice a day	India	Bhattarai, 1992a
	Treatment of back ache	Plant bark mixed with *Aesandra butyracea* are taken	India	Bhattarai, 1992b
H. japonicum	Stomach ache	Plant juice, 2 tea spoon thrice a day	India	Manandhar, 1992
H. revolutum and *H. quartinnum*	Stomach disorder, tooth ache and disease associated with the ears	Root and fruit extract are drank	Ethiopia	Taddesse and Demissew, 1992
H. roeperianum	Cure women infertility	Roots boiled and drank	Kenya	Kokwaro, 1976
H. peplidifolium	Indigestion	Roots and bark are chewed	Kenya	Kokwaro, 1976
H. salsugineum	Healing of wound	Leaves and fruits extract are applied on wound	Turkey	Sakar, 1990
H. uralum	Chronic dysentery	2-3 g of the fresh dried flowers are made into paste and taken with honey	India	Bhattarai, 1993

Phytochemical and subsequent biological activity tests done on *Hypericum* species have led to the isolation of several anti-microbial [Ishiguro *et al.*, 1990], anti-fungal [Decosted *et al.*, 1987], and anti-tumor [Jayasuriya *et al.*, 1989] compounds.

The anti-viral activities of compounds found in *Hypericum* extracts have been extensively studied since the second half of the eighties. The results demonstrated that hypericin (**8**) and pseudohypericin (**9**) which are found in many *Hypericum* species are effective anti-viral agents against influenza, vescular stomatitis and herpex simplex virus types I and II [Bombardelli and Morazzoni, 1995].

Compounds active against retroviruses and in particular against HIV, the virus which causes AIDS, are urgently required. It was discovered that these two aromatic polycyclic diones are effective against retroviruses [Meruelo *et al.*, 1988]. These compounds inhibit the propagation of Friend leukemia virus (FV) and Radiation leukemia virus (RadLV) [Merulo *et al.*, 1988] both *in vivo* and *in vitro*. These retrovirus animal model systems can be used to evaluate compounds as possible therapeutic agents against HIV. These compounds directly inactivate mouse and human retroviruses including HIV [Meruelo *et al.*, 1988]. As a result of these and other studies, hypericin (**8**) is currently being used in phase 1 clinical trials in patients with AIDS [Wood *et al.*, 1990]. A remarkable property of these compounds is the fact that their administration to mice at the low dose sufficient to prevent retroviral-induced disease appears to be devoid of undesirable side effects. This observation in experimental animals can be extrapolated to humans since *Hypericum* extracts have been used for a long time for the treatment of various diseases without any undesired effects. Takahashi *et. al* [1989] found that hypericin (**8**) and pseudohypericin (**9**) inhibit protein kinase C with IC_{50} values of 1.7 and 15 μg/ml respectively. The substantial difference between the potency of these two compounds might seem surprising in view of the near identity of their chemical structure. This suggests that it might be profitable to evaluate other complex plant quinones, as well as synthetic equivalents, in order to identify active structural features of these compounds and possibly obtain more potent ones.

Many studies done on the anti-viral activities of hypericin (**8**) have not considered the role of light. Hypericin (**8**) is known to be a photosensitizer [Duran and Song, 1986] which in the presence of light in the 650-700 nm visible range, generates singlet oxygen ($^1O^*_2$) and possibly other reactive species. This is thought to be the property responsible for the phototoxic symptoms observed in grazing animals that ingest some *Hypericum* plants. Singlet oxygen is highly reactive in biological systems [MacRobert *et al.*, 1989]. When virus hypericin (**8**) mixtures are exposed to normal room light, or typical fluorescent lamps found in biosafety cabinets, then hypericin (**8**) will be photoactivated with consequent production of singlet oxygen (Type II Photosensitization) and possibly radicals (Type I Photosensitization). Hudson *et. al* [1991] have shown that hypericin (**8**) has impressive light-mediated anti-viral activities against MCMV, SV, and HIV-1 viruses. In the absence of light the activities were diminished, though still significant.

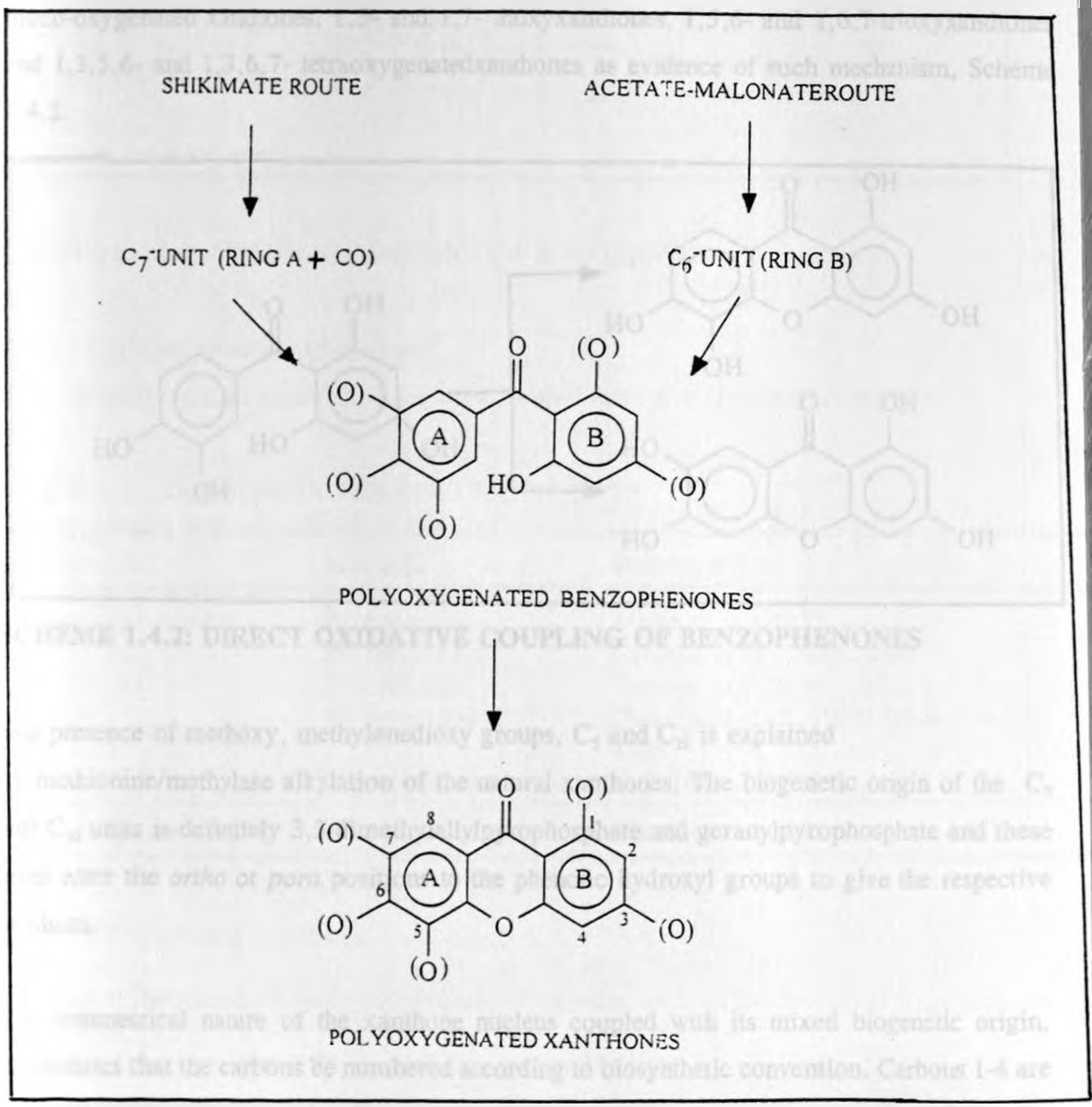
1.4 CHEMICAL BACKGROUND

Literature survey on the phytochemical study of the genus *Hypericum* revealed that as a member of the Guttiferae the most common secondary metabolites in this genus are xanthones. However, other secondary metabolites, some of which have considerable biological activity are also present. These mainly include anthraquinones, flavonoids, and derivatives of fillicinic acid and phloroglucinol, and acylphloroglucinol.

1.4.1 XANTHONES OF *HYPERICUM*

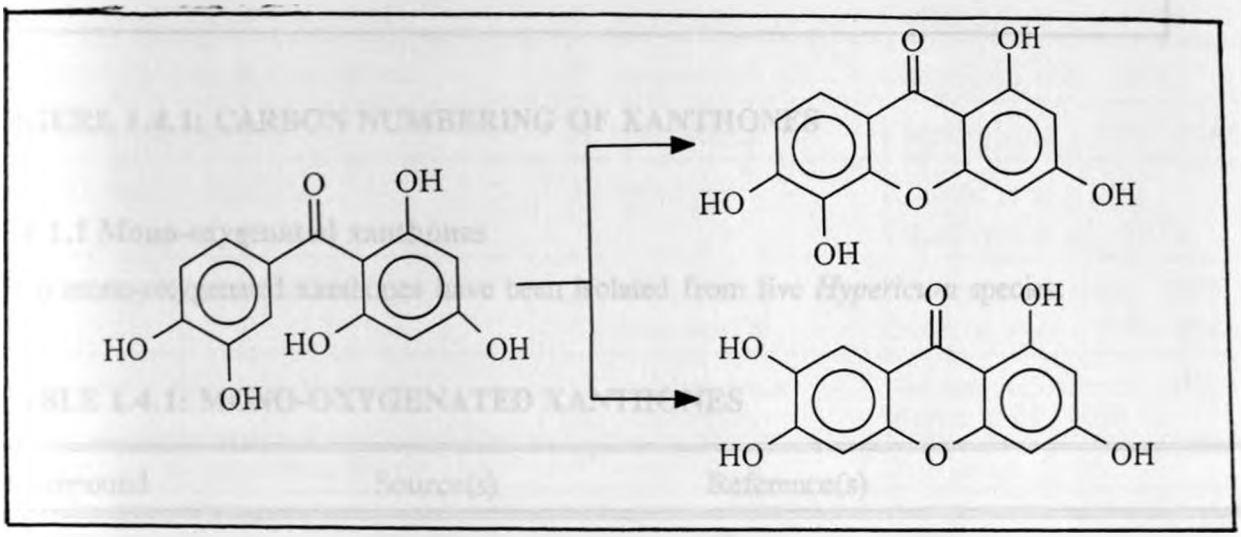
Xanthones or the related benzophenones have been found in most genera of the Guttiferae. They are found in at least 130 species of the Guttiferae and 22 species of the genus *Hypericum* [Bennett and Lee, 1989; Cardona *et al.*, 1990; Ishiguro *et al.*, 1993, 1995, Rath *et al.*, 1996]. It appears, from the number of chemically investigated species, that a large proportion of the species contain xanthones.

When the xanthones from Guttiferae are examined it is seen that ring A and the attached CO group (C_7-unit) are provided by the shikimic acid pathway where as ring B (C_6-unit) arises via the acetate-malonate polyketide route [Sultanbawa, 1980; Benett and Lee, 1988]. Therefore, polyhydroxy benzophenones or their biogenetic equivalents could be intermediates in the formation of xanthones. These ideas are summarized in Scheme 1.4.1 below.



SCHEME 1.4.1: BIOSYNTHESIS OF XANTHONES IN HIGHER PLANTS

A number of mechanisms [Gottlieb, 1968; Markham, 1965] have been suggested for the transformation of polyoxygenated benzophenones to polyoxygenated xanthones. However, Carpenter *et. al* [1969] concluded that direct oxidative coupling of the benzophenones leads to a simpler explanation of the wide variety of oxidative coupling in natural xanthones. They have also emphasized the frequent co-occurrence of pairs of corresponding xanthones like 5- and 7-mono-oxygenated xanthones, 1,5- and 1,7- dioxyxanthones, 1,5,6- and 1,6,7-trioxyxanthones and 1,3,5,6- and 1,3,6,7- tetraoxygenatedxanthones as evidence of such mechanism, Scheme 1.4.2.



SCHEME 1.4.2: DIRECT OXIDATIVE COUPLING OF BENZOPHENONES

The presence of methoxy, methylenedioxy groups, C_5 and C_{10} is explained by methionine/methylase alkylation of the natural xanthones. The biogenetic origin of the C_5 and C_{10} units is definitely 3,3-dimethylallylpyrophosphate and geranylpyrophosphate and these units enter the *ortho* or *para* positions to the phenolic hydroxyl groups to give the respective products.

The symmetrical nature of the xanthone nucleus coupled with its mixed biogenetic origin, necessitates that the carbons be numbered according to biosynthetic convention. Carbons 1-4 are assigned to the acetate ring A (often characterized by 1,3-dioxygenation) and carbons 5-8 to the shikimate derived ring B. This is demonstrated in Figure 1.4.1 below.

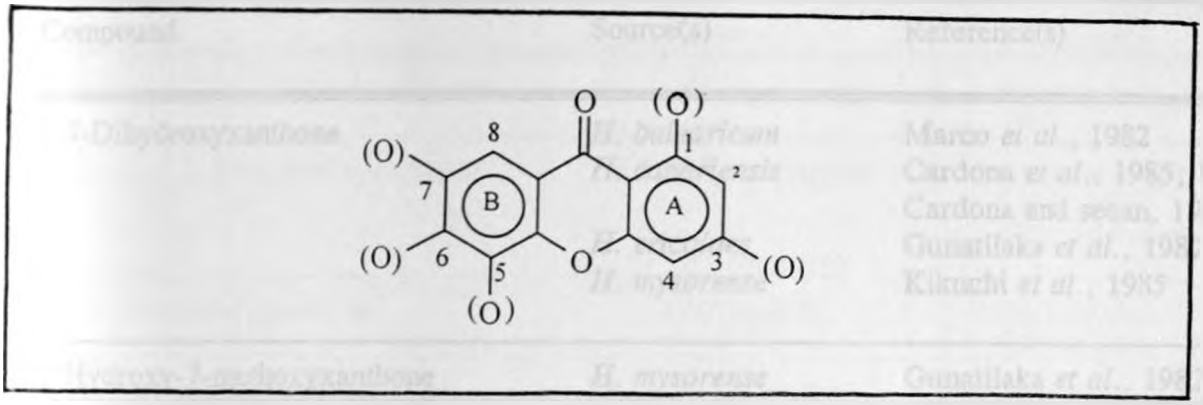


FIGURE 1.4.1: CARBON NUMBERING OF XANTHONES

1.4.1.1 Mono-oxygenated xanthones

Two mono-oxygenated xanthones have been isolated from five *Hypericum* species.

TABLE 1.4.1: MONO-OXYGENATED XANTHONES

Compound	Source(s)	Reference(s)
2-Hydroxyxanthone	*H. balearicum*	Marco *et al.*, 1982
	H. canariensis	Cardona *et al.*, 1985, 1986
	H. ericoides	Cardona and Seoan, 1982a
	H. mysorense	Gunatilaka *et al.*,1982; Kikuchi *et al.*, 1985
	H. roeperanum	Rath *et al.*, 1996
2-Methoxyxanthone	*H. mysorense*	Kikuchi *et al.*, 1985

1.4.1.2 Dioxygenated xanthones

Majority of the recently isolated dioxygenated xanthones are from the species of Hypericoidea, reflecting the recent interest in studying this groups of plants.

TABLE 1.4.2: DIOXYGENATED XANTHONES

Compound	Source(s)	Reference(s)
1,7-Dihydroxyxanthone	*H. balearicum*	Marco *et al.*, 1982
	H. canariensis	Cardona *et al.*, 1985; 1986
		Cardona and seoan, 1982a
	H. ericoides	Gunatilaka *et al.*, 1982
	H. mysorense	Kikuchi *et al.*, 1985
1-Hydroxy-7-methoxyxanthone	*H. mysorense*	Gunatilaka *et al.*, 1982
2-Hydroxy-3-methoxyxanthone	*H. mysorense*	Gunatilaka *et al.*, 1982
2,5-Hydroxyxanthone	*H. canariensis*	Cardona *et al.*, 1985; 1986
2,3-Dimethoxyxanthone	*H. mysorense*	Kikuchi *et al.*, 1985
		Gunatilaka *et al.*, 1979
2-Hydroxy-5-methoxyxanthone	*H. androsaemum*	Nielsen and Arends, 1979
	H. canariensis	Cardona *et al.*, 1985; 1986
3-Hydroxy-2-methoxyxanthone	*H. androsaemum*	Nielsen and Arends, 1979
	H. balearicum	Marco *et al.*, 1982
5-Hydroxy-2-methoxyxanthone	*H. roeperanum*	Rath *et al.*, 1996

1.4.1.3 Trioxygenated xanthones

About ten trioxygenated xanthones have been isolated from the genus *Hypericum*.

TABLE 1.4.3: TRIOXYGENATED XANTHONES

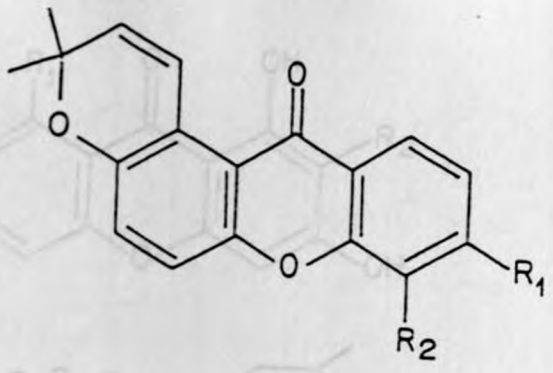
Compound	Source(s)	Reference(s)
1-Hydroxy-6,7-dimethoxyxanthone	*H. mysorense*	Gunatilaka *et al.*, 1982
2-Hydroxy-3,4-dimethoxyxanthone	*H. canariensis*	Cardona *et al.*, 1985; 1986
	H. sampsonii	Chen and chen, 1985
1,3,7-Trihydroxyxanthone	*H. degenii*	Kitanov and Akhtardjiev, 1979
2,3,4-Trimethoxyxanthone	*H. ericoides*	Cardona and Seoan, 1982a
3-Hydroxy-2,5-dihydroxyxanthone	*H. androsaemum*	Nielsen and Arends, 1979
4-Hydroxy-2,3-dimethoxyxanthone 3,4-Dihydroxy-2-methoxyxanthone 3-Hydroxy-2,4-dimethoxyxanthone 3,6-Dihydroxy-2-methoxyxanthone	*H. reflexum*	Cardona *et al.*, 1990
1,5-Dihydroxy-2-methoxyxanthone	*H. roeperanum*	Rath *et al.*, 1996

TABLE 1.4.4: TETRAOXYGENATED XANTHONES

Compound	Source(s)	Reference(s)
1,6-Dihydroxy-5,7-dimethoxyxanthone	*H. canariensis*	Cardona *et al.*, 1985; 1986
2-Hydroxy-5,6,7-trimethoxyxanthone	*H. ericoides*	Cardona and Seoan, 1982a
1,3,5,6-Tetrahydroxyxanthone	*H. androsaemum*	Nielsen and Arends, 1979
4-Hydroxy-2,3,6-trimethoxyxanthone 2,4-Dihydroxy-3,6-dimethoxyxanthone	*H. reflexum*	Cardona *et al.*, 1990
1,3,6,7-Tetrahydroxyxanthone	*H. androsaemum*	Nielsen and Arends, 1979
	H. aucheiri	Kitanov and Blinova, 1980
1,5,6-Trihydroxy-3-methoxyxanthone	*H. androsaemum*	Nielsen and Arends, 1979

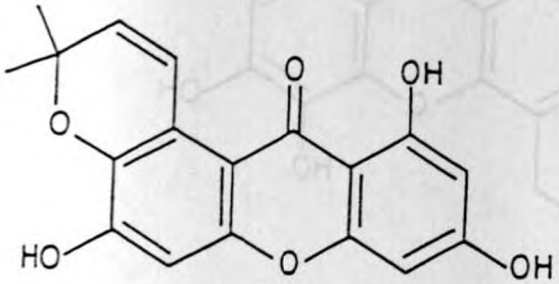
TABLE 1.4.5: ALKYLATED XANTHONES

Compound	Source(s)	Reference(s)
Hypericarin (**10**)	*H. canariensis*	Cardona *et al.*, 1985
Hyperxanthone (**11**)	*H. sampsonii*	Chen and Chen, 1985
Toxyloxanthone B (**12**)	*H. androsaemum*	Nielsen and Arends, 1979
	H. sampsonii	Chen and Chen, 1986
	H. patulum	Ishiguro *et al.*, 1995
13 **14**	*H. reflexum*	Cardona *et al.*, 1990
Paxanthone (**15**) Paxanthone B (**16**) Garcinone B (**17**) γ-Mangostin (**18**) **19**	*H. patulum*	Ishiguro *et al.*, 1995
Maculatoxanthone (**20**)	*H. maculatum*	Arends, 1969
Sarothralin (**21**)	*H. japonicum*	Ishiguro *et al.*, 1985
2-Deprenylrheediaxanthone B (**22**) 5-O-methyl-2-deprenylrheediaxanthone B (**23**) Isojacareubin (**24**) 5-O-methylisojacareubin (**25**) Calycinoxanthon (**26**) Roeperanone (**27**) 5-O-demethylpaxaxanthonin (**28**)	*H. roeperanum*	Rath *et al.*, 1996



10 $R_1 = H$, $R_2 = OH$

11 $R_1 = OH$, $R_2 = H$



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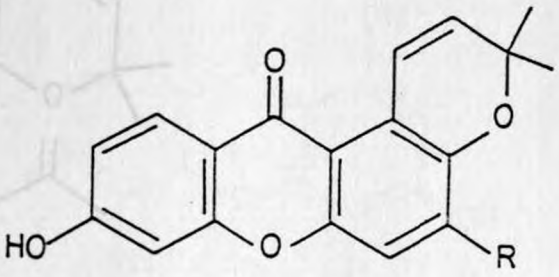
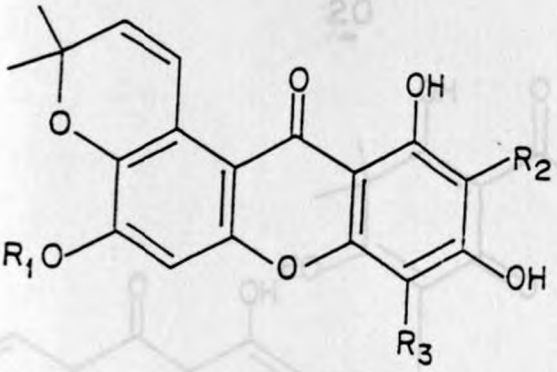
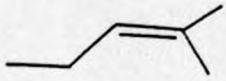


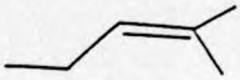
13 R = H

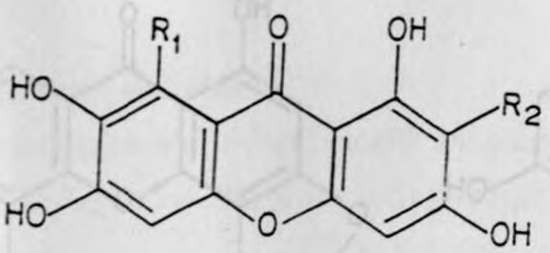
14 R = OH

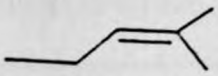


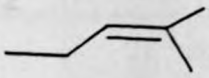
15 $R_1 = CH_3$, $R_2 = R_3 = H$

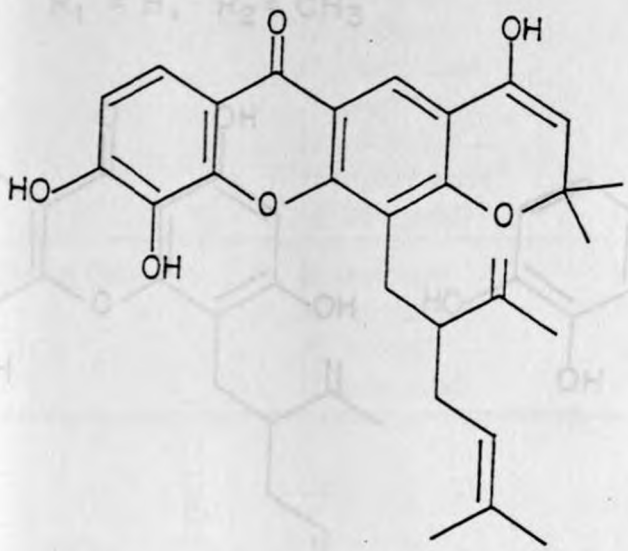
16 $R_1 = R_2 = H$, $R_3 =$ 

17 $R_1 = R_3 = H$, $R_2 =$ 



18 $R_1 = R_2 =$ 

19 $R_1 =$  , $R_2 = H$



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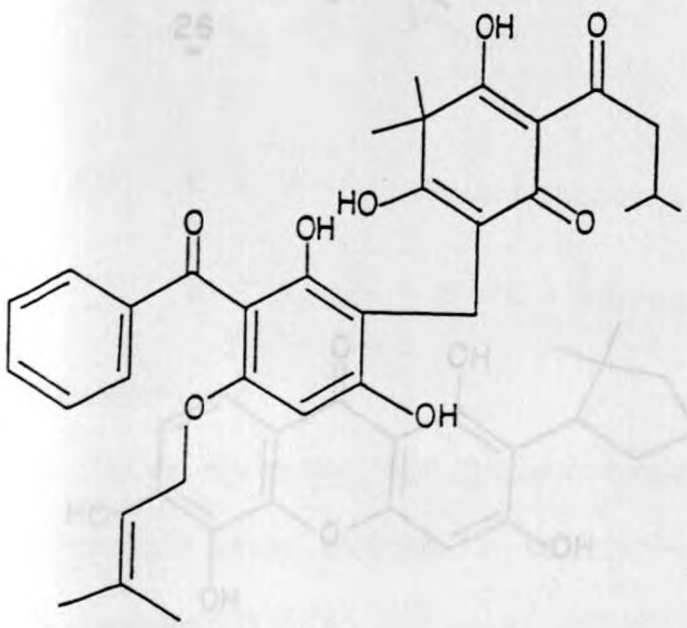

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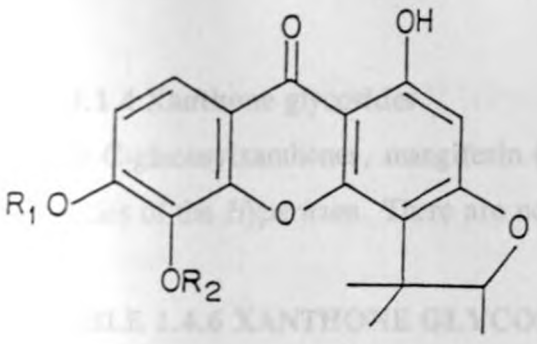
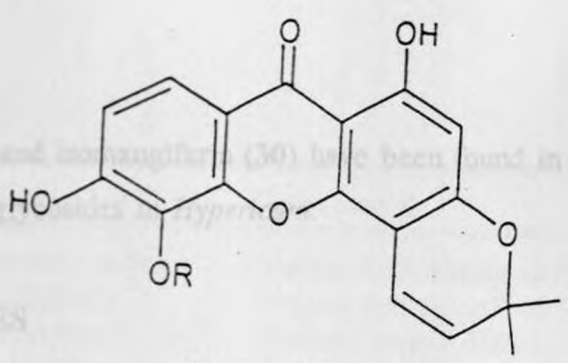


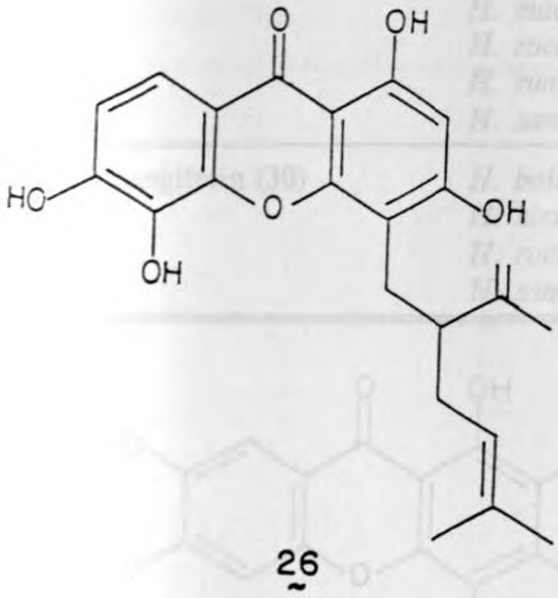
22 $R_1 = R_2 = H$

23 $R_1 = H,\ R_2 = CH_3$



24 $R = H$

25 $R = CH_3$



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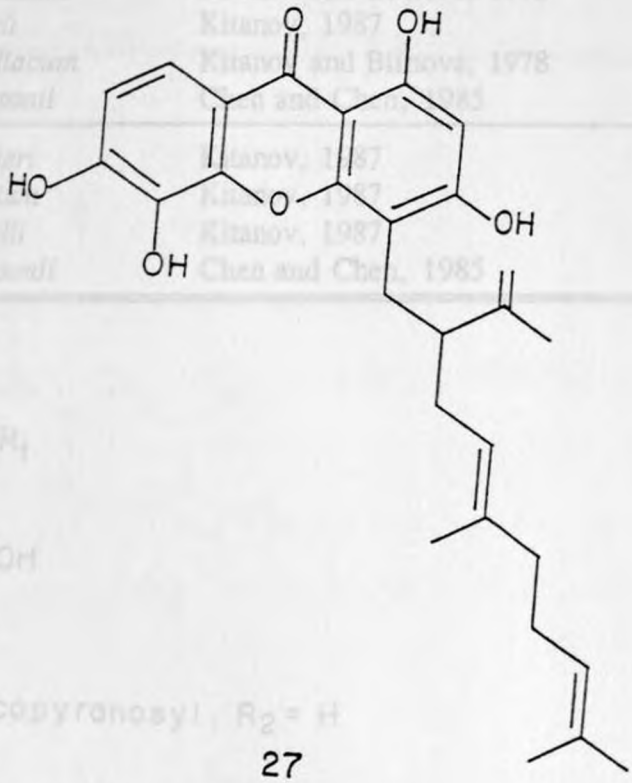

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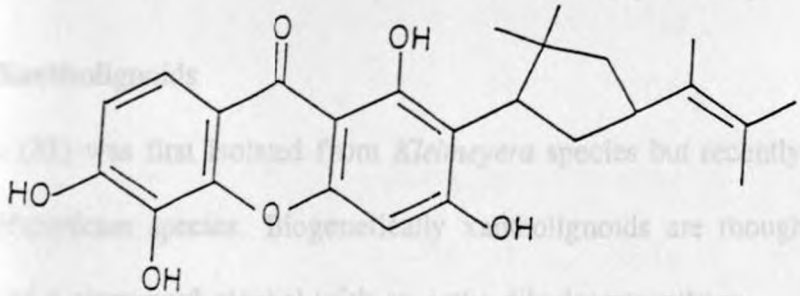

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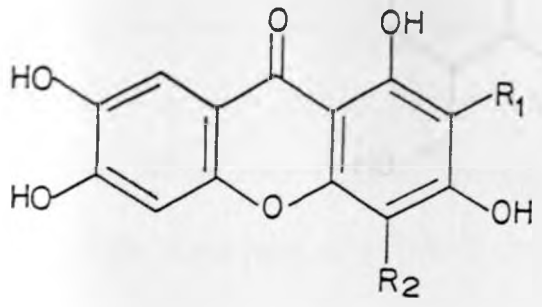
1.4.1.4 Xanthone glycosides

The C-glucosylxanthones, mangiferin (**29**) and isomangiferin (**30**) have been found in several species of the *Hypericum*. There are no O-glycosides in *Hypericum*.

TABLE 1.4.6 XANTHONE GLYCOSIDES

Compound	Source(s)	Reference(s)
Mangiferin (**29**)	*H. aucheiri*	Kitanov and Blinova, 1980
	H. barbatum	Kitanov and Blinova, 1978
	H. boissieri	Kitanov, 1987
	H. humifusum	Lebreton and Bouchez, 1967
	H. maculatum	Kitanov and Blinova, 1978
	H. rocheli	Kitanov, 1987
	H. rumeliacum	Kitanov and Blinova, 1978
	H. sampsonii	Chen and Chen, 1985
Isomangiferin (**30**)	*H. boissieri*	Kitanov, 1987
	H. hirsutum	Kitanov, 1987
	H. rochelli	Kitanov, 1987
	H. sampsonii	Chen and Chen, 1985



29 $R_1 = \beta - D -$ Glucopyranosyl, $R_2 = H$

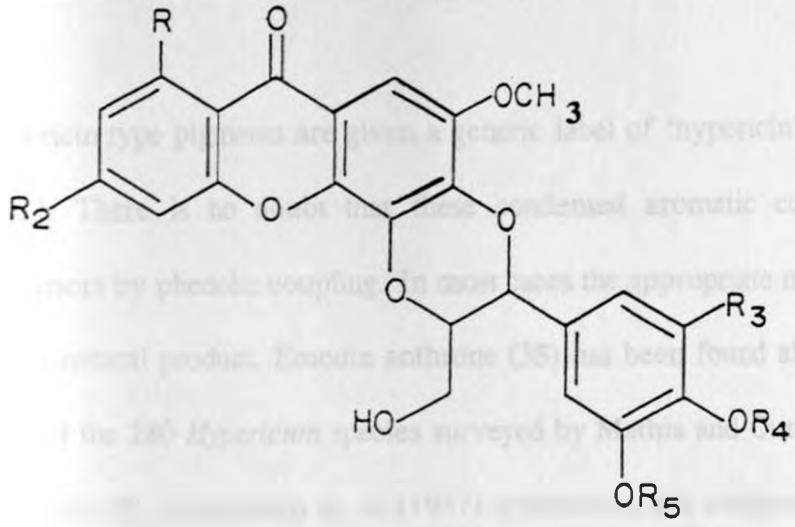
30 $R_1 = H$, $R = \beta - D -$ Glucopyranosyl

1.4.1.5 Xantholignoids

Kielcorin (**31**) was first isolated from *Klelmeyera* species but recently has been isolated from several *Hypericum* species. Biogenetically xantholignoids are thought to be formed by the coupling of a cinnamoyl alcohol with an *ortho*-dihydroxyxanthone.

TABLE 1.4.7: XANTHONOLIGNOIDS

Compound	Source(s)	Reference(s)
Kielcorin (**31**)	*H. androsaemum*	Nielsen and Arends, 1979; 1978
	H. calycinum	Nielsen and Arends,1978
	H. canariensis	Cardona *et al.*, 1985
	H. ericoides	Cardona and Seoan, 1982a
	H. maculatum	Nielsen and Arends, 1978
	H. perforatum	Nielsen and Arends, 1978
	H. reflexum	Cardona *et al.*, 1990
6-Methoxykielcorin (**32**)	*H. reflexum*	Cardona *et al.*, 1990
Candesin C (**33**)	*H. canariensis*	Cardona *et al.*, 1985
Hypericorin (**34**)	*H. canariensis*	Cardona *et al.*, 1985
	H. mysorense	Vishwakarma, 1986

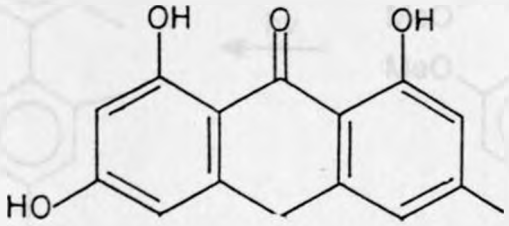


	R_1	R_2	R_3	R_4	R_5
31	H	H	H	H	CH_3
32	H	OCH_3	H	H	OCH_3
33	OH	H	OCH_3	H	CH_3
34	H	H	OCH_3	H	CH_3

1.4.2 HYPERICIN

The aromatic polycyclic dione hypericin (**8**) was isolated for the first time isolated from the flowers of *H. perforatum* in 1830 by Buchner [Bombardelli and Morazzoni, 1995; Thompson, 1971], who then named it as hypericum red. Cerny [Bombardelli and Morazzoni, 1995] isolated this compound and renamed it as hypericin (**8**), its present name, in 1911. The dark red needle like crystals of hypericin (**8**) were not obtained until 1942 [Thompson, 1971]. The correct structure of this compound was established in 1953 [Bombardelli and Morazzoni, 1995] by chemical transformation and some spectroscopic methods.

Hypericin type pigments are given a generic label of 'hypericin' [Watt and Beryer-Brandwijk, 1962]. There is no doubt that these condensed aromatic compounds arise from simpler precursors by phenolic coupling. In most cases the appropriate monomer, or a close relative, is also a natural product. Emodin anthrone (**35**) has been found along with hypericin (**8**). About 60% of the 280 *Hypericum* species surveyed by Mathis and Ourisson [1963] tested positive for hypericin (**8**). Brockmann *et. al* [1957] synthesized this compound for the first time in 1957.



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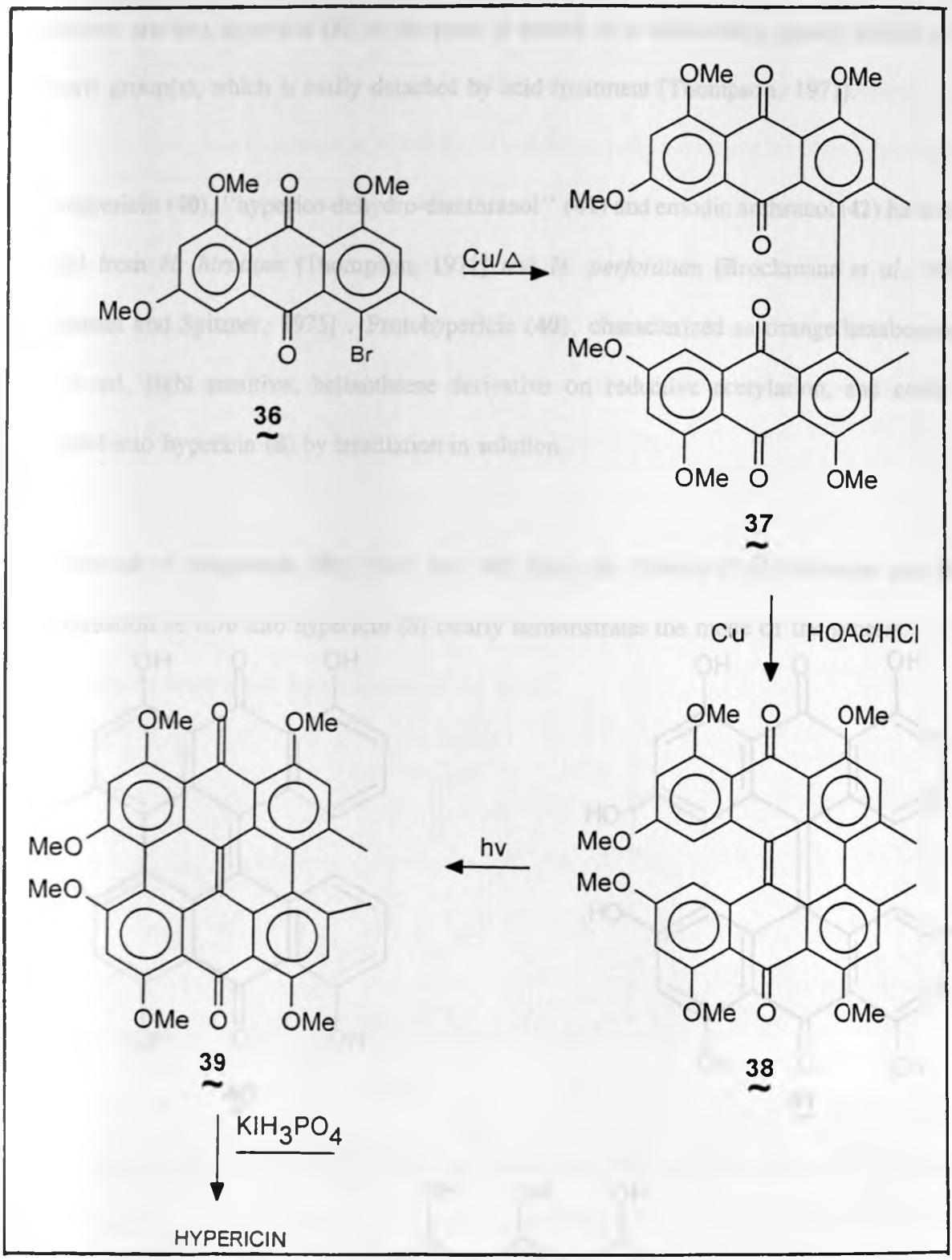
When the bianthraquinone (**37**), obtained from 4-bromo-emodin trimethyl ether (**36**) by an Ulmann reaction, was reduced with copper in acetic acid/hydrochloric acid in the absence of light, it gave the helianthrone (**38**) which was converted into the naphthodianthrone (**39**) by

irradiation. Demethylation of this compound by heating with potassium iodide in phosphoric acid gave a product in all respect identical with hypericin. The synthesis is outlined below:

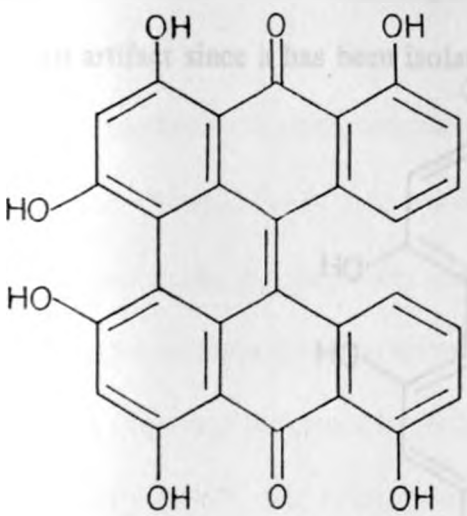


SCHEME 1.4.2.1: SYNTHESIS OF HYPERICIN

The exact form in which hypericin occurs *in vivo* is not known. The natural pigment is much more soluble than the purified crystalline material but has the same electronic spectrum. The indications are that hypericin (**8**) in the plant is bound to a solubilizing moiety linked to β-hydroxyl group(s), which is easily detached by acid treatment [Thompson, 1971].

Protohypericin (**40**), "hyperico-dehydro-dianthranol" (**41**) and emodin anthranol (**42**) have been isolated from *H. hirsutum* [Thompson, 1971] and *H. perforatum* [Brockmann *et al.*, 1974; Brockmann and Spitzner, 1975] . Protohypericin (**40**), characterized as orange hexabenzoate, gave a red, light sensitive, helianthrene derivative on reductive acetylation, and could be converted into hypericin (**8**) by irradiation in solution.

The isolation of compounds (**40**), (**41**), and (**42**) from the flowers of *H. hirsutum*, and their transformation *in vitro* into hypericin (**8**) clearly demonstrates the mode of the genesis.



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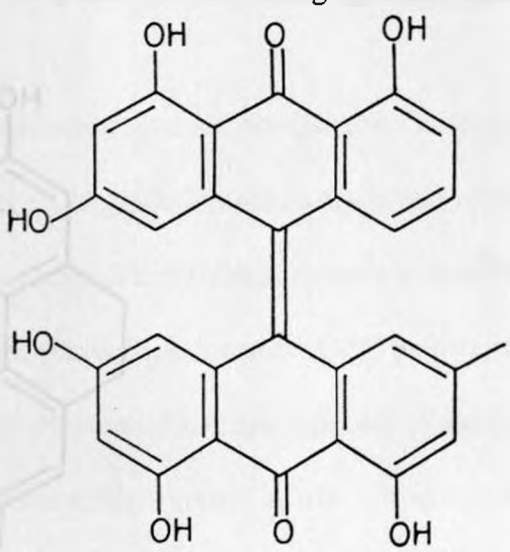

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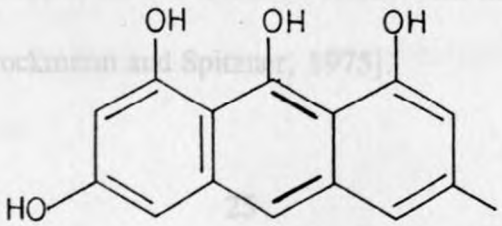
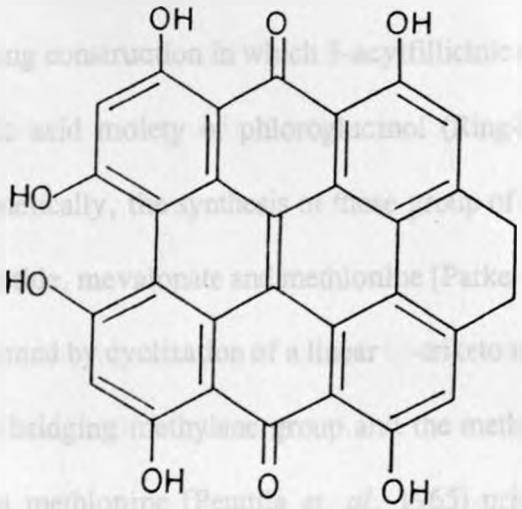


42

As mentioned earlier protohypericin (**40**) is readily converted into hypericin on exposure to light. Melvyn *et. al* [1988] for the first time monitored the progress of this transformation using ^{1}H-NMR spectroscopy. They isolated these two compounds from the fruiting bodies of *Dermocybe austroveneta* . Accordingly when a fresh solution of *D. austroveneta* pigment prepared in the dark in Me_2CO-d_6 was examined at 400 MHz, a spectrum fully consistent with the structure (**40**) was observed. Periodic exposure of this solution to light resulted in the gradual appearance of proton resonances consistent with the presence of hypericin (**8**) together with the concomitant diminution in intensity due to protohypericin (**40**) itself. After 8 minutes of cumulative exposure to sunlight the solution contained no protohypericin (**40**). Evaporation of the solution and chromatographic purification of the residue gave hypericin (**8**).

Pseudohypericin (**9**) and cyclopseudohypericin (**43**) were isolated from the flowers of *H. perforatum*. Photooxidation of (**9**) gave (**43**). However, compound (**43**) is a natural product and not an artifact since it has been isolated in the dark.



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All the compounds described above have also been isolated from the flowers of *H. perforatum* [Brockmann *et al.*, 1974; Brockmann and Spitzner, 1975].

1.4.3 FILLICINIC ACID AND PHLOROGLUCINOL DERIVATIVES

Plants of the genus *Hypericum* elaborate several fillicinic acid and phloroglucinol derivatives. The basic skeleton of these group of compounds is as shown below:

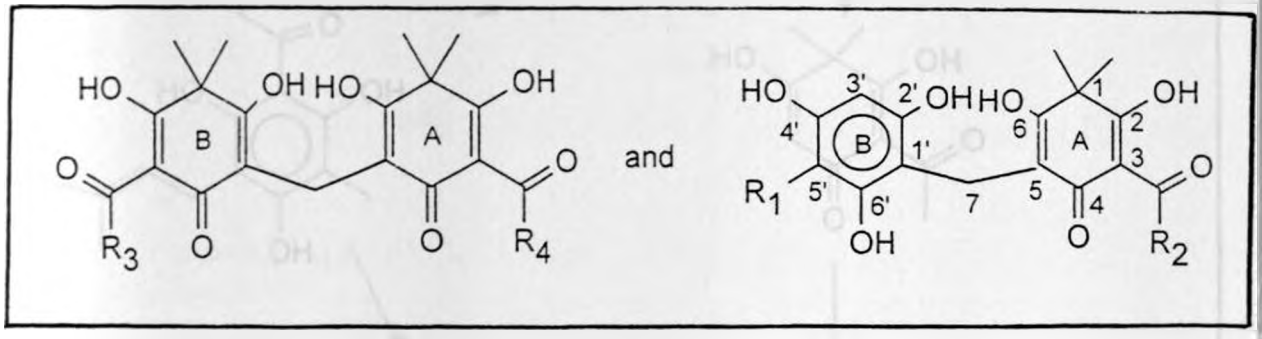
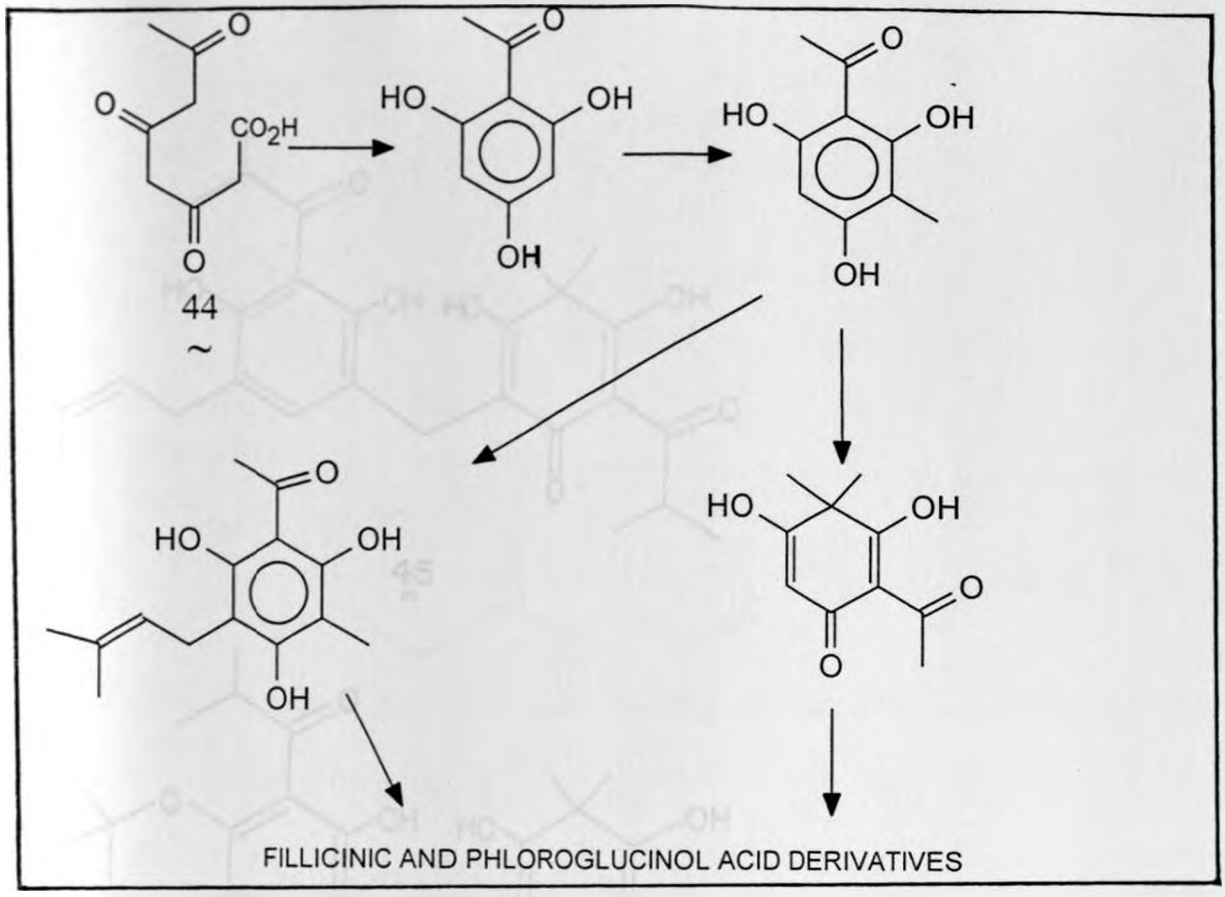


FIGURE 1.4.3.1: BASIC SKELETON OF FILLICINIC ACID AND PHLOROGLUCINOL DERIVATIVES

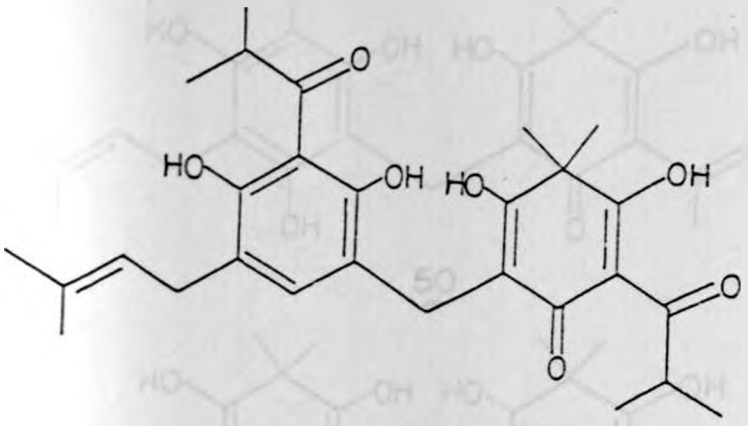
It is represented as two ring construction in which 3-acylfillicinic acid moiety (Ring-A) is linked to another 3-acylfillicinic acid moiety or phloroglucinol (Ring-B) derivative, by means of a methylene bridge. Biogenetically, the synthesis of these group of compounds appears to involve combination of the polyketide, mevalonate and methionine [Parker and Johnson, 1968] pathways. The two rings may be formed by cyclization of a linear -triketo acid intermediate (**44**) [Douglas and Money, 1967]. The bridging methylene group and the methyl groups of the fillicinic acid moiety are derived from methionine [Penttila *et. al*, 1965] prior to condensation of the two rings. The isopentenyl side chains and the corresponding five carbon atoms of the chromene systems are probably derived from mevalonate [Richards and Hendrckson, 1964]. The biosynthesis is outlined below:



SCHEME 1.4.3.2: BIOSYNTHESIS OF PHLOROGLUCINOL AND FILLICINIC ACID DERIVATIVES

The extracts of *H. uliginosum*, which is widely distributed in Mexico and Central America, upon chromatographic separation gave uliginosin A (**45**) and uliginosin B (**46**) [Parker and Johnson, 1968].

Bioassay-directed fractionation of the hexane extract of *H. drummondii* afforded drummondin A (**47**), B (**48**), C (**49**), and F (**50**), albaspidin A-A (**51**) and albaspidin P-P (**52**) [Jayasuriya *et al.*, 1989]. On further study of the hexane extract of the stems and leaves of this plant drummondin D (**53**), isodrummondin D (**54**), and drummondin E (**55**) [Jayasuriya *et al.*, 1991] were isolated.



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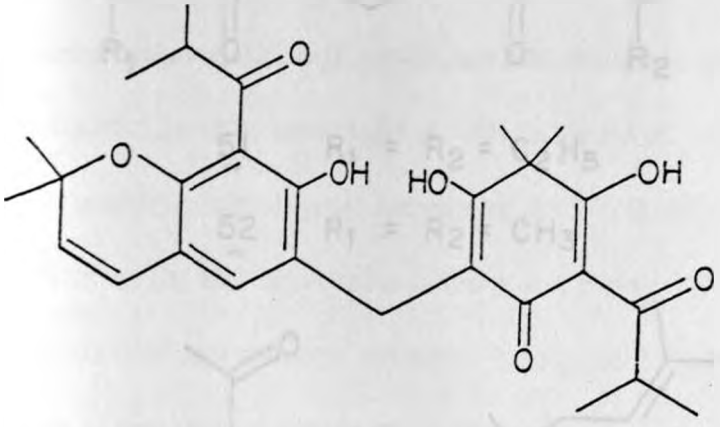

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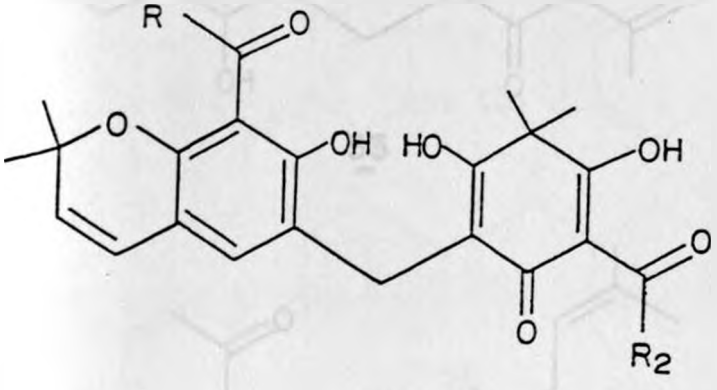
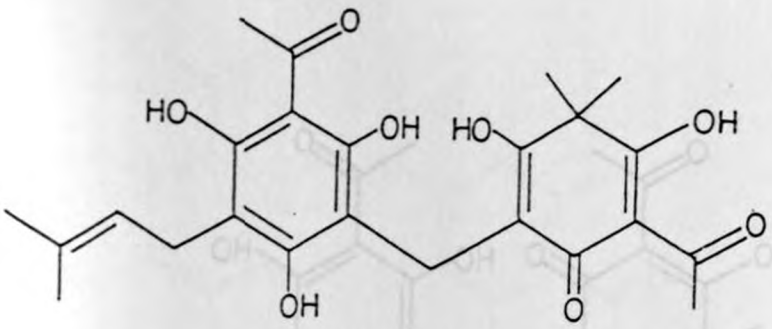


47 $R_1 = R_2 = C_2H_5$

48 $R_1 = CH_3$, $R_2 = C_2H_5$

49 $R_1 = R_2 = CH_3$



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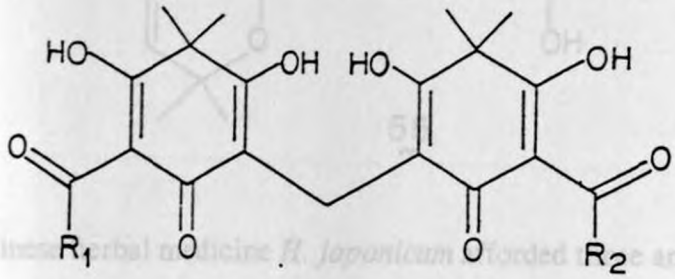
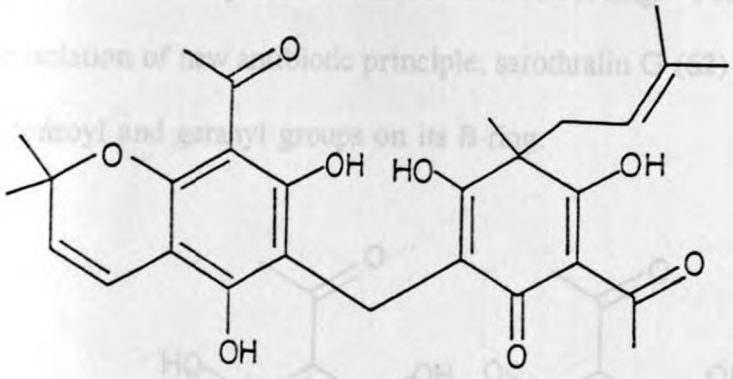


51 $R_1 = R_2 = C_2H_5$

52 $R_1 = R_2 = CH_3$



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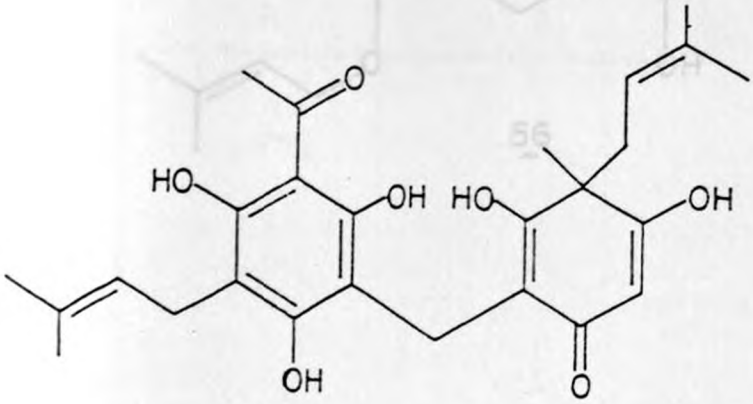

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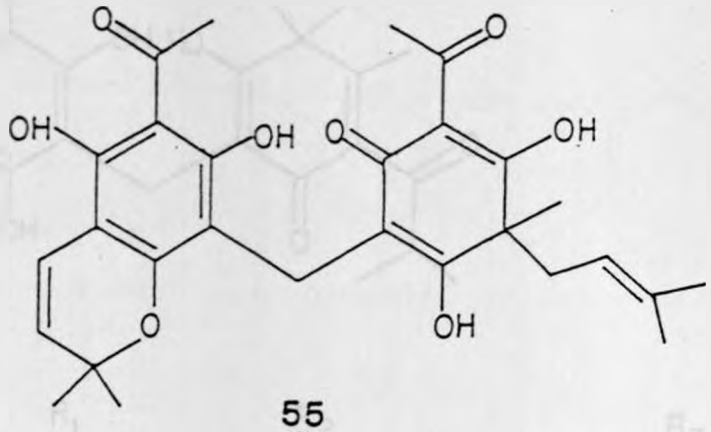
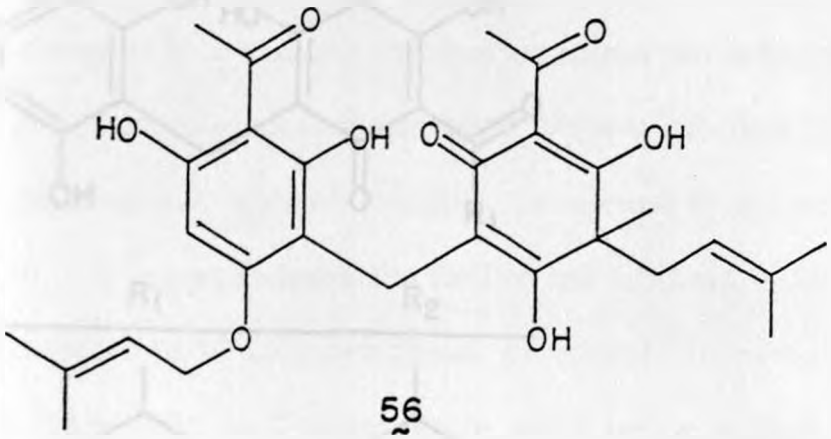
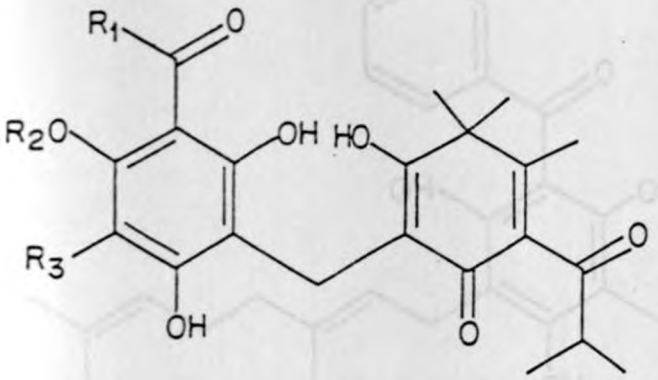


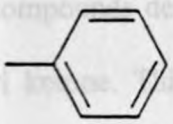
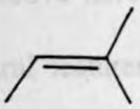
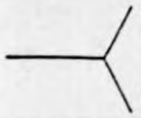
55

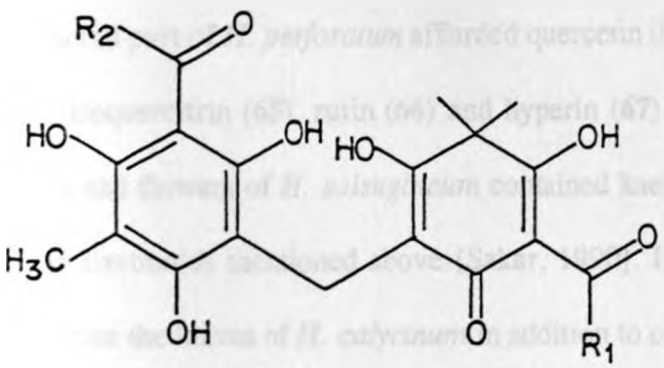
The Chinese herbal medicine *H. japonicum* afforded three antimicrobial compounds; sarothralin B (**56**), sarothralin (**57**), sarothralin A (**58**) [Ishiguro *et. al*,1985; Ishiguro *et. al*, 1986a] and three new antibiotic homologous; saroaspidin A (**59**), B (**60**), and C (**61**) [Ishiguro *et. al*, 1986b] which differ on the acyl side-chains of the A and B rings. Further investigation of this plant led to the isolation of new antibiotic principle, sarothralin G (**62**) [Ishiguro et. al, 1990], which has both benzoyl and geranyl groups on its B-ring.

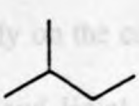
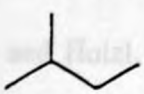
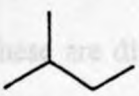


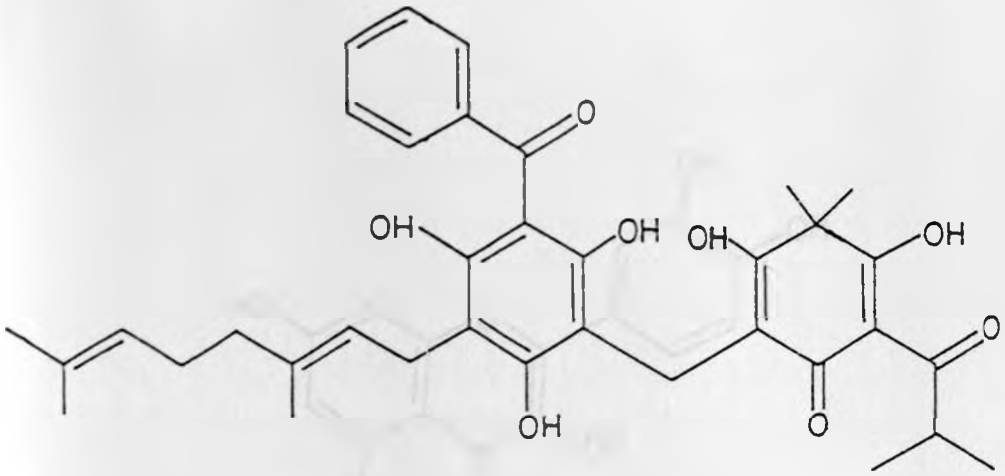
56



	R_1	R_2	R_3
57			H
58		H	



	R_1	R_2
59		
60		
61		

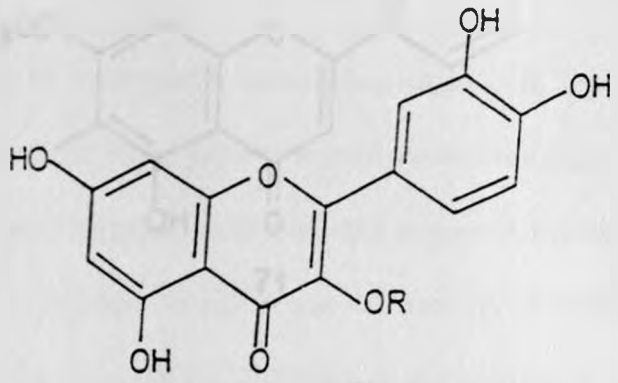


62

All these compounds described above have in common an enolic 1,3-diketo system or a 2-hydroxyaryl ketone. This structural features are evident from their infrared spectra, a broad absorption in the 3000 cm^{-1} together with intense peaks at 1600-1650 cm^{-1} region.

1.4.4 FLAVONOIDS OF *HYPERICUM* SPECIES

The aerial part of *H. perforatum* afforded quercetin (**63**) and the glycosidic quercetins; quercitrin (**64**), isoquercitrin (**65**), rutin (**66**) and hyperin (**67**) [Bombardelli and Morrazonni, 1995]. The leaves and flowers of *H. salsugineum* contained kaempferol (**68**) and myricetin (**69**) in addition to the flavonoids mentioned above [Sakar, 1990]. Doganca and Oksuz [1989] isolated luteolin (**70**) from the leaves of *H. calycinum* in addition to compounds **63, 66,** and **67** mentioned above. *H. ericoides* which grows exclusively in the East and South-east of Spain and North Africa afforded compounds **63, 67** and a new flavone, 6-C-methyl-7-O-methylapigenin (**71**) [Cardona and Seoan, 1982b]. This new C-methyl flavone, which are rare in nature, is the first one to be isolated from Guttiferae. Further study on the constituent of *H. perforatum* led to the isolation of I3,II8-biapigenin (**72**) [Berghofer and Holzl, 1987] and I3',II8-biapigenin (amentoflavone) (**73**) [Berghofer and Holzl, 1989]. These are dimers of apigenin which differ only in the way the apigenin groups are connected.



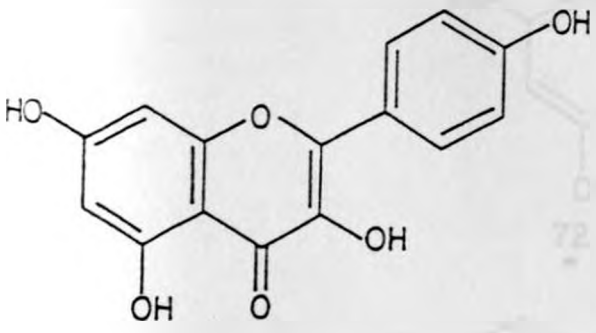
63 R = H

64 R = Rha

65 R = Glu

66 R = Rha – Glu

67 R = Gal



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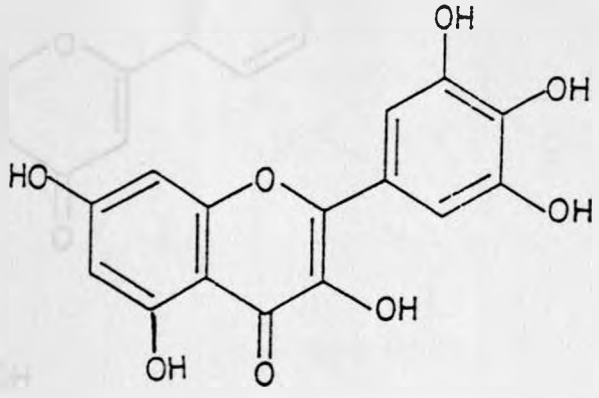
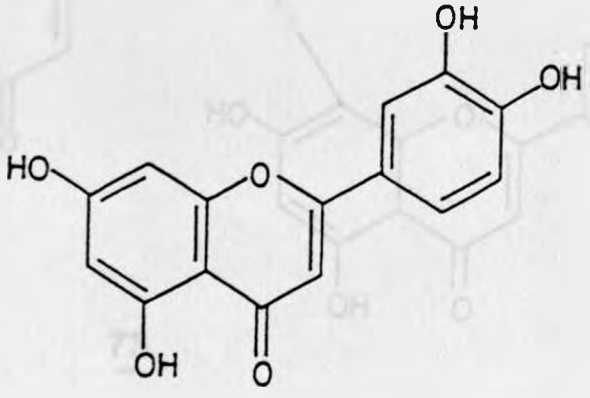


69



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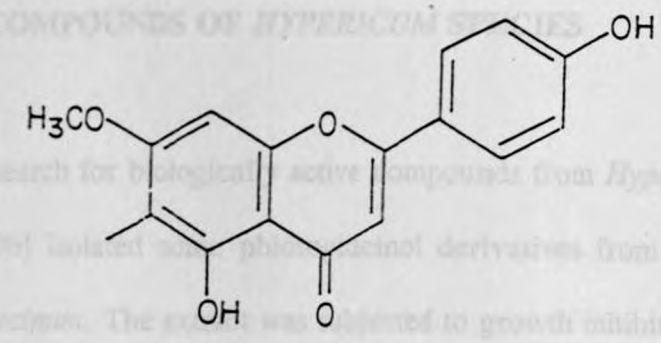
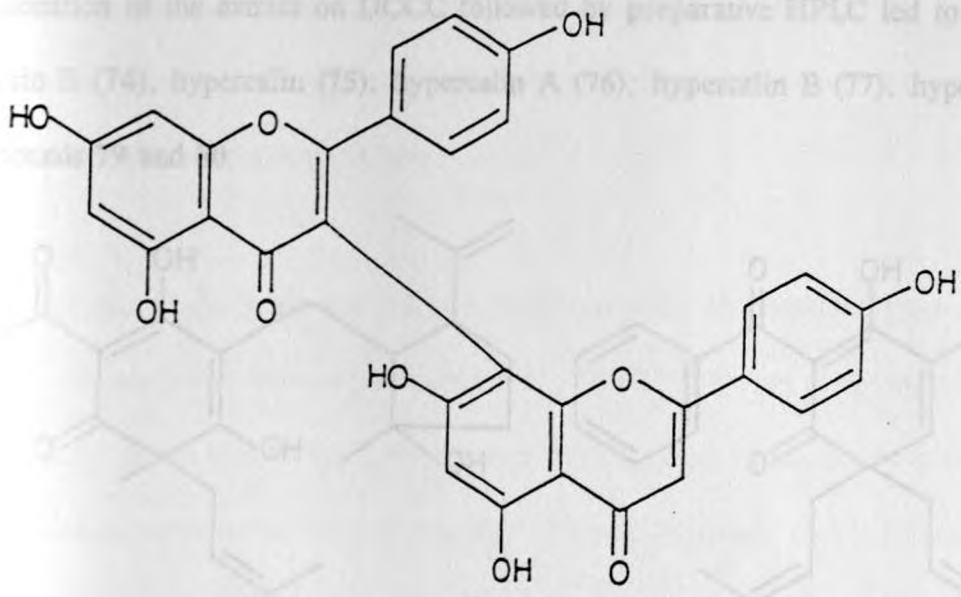
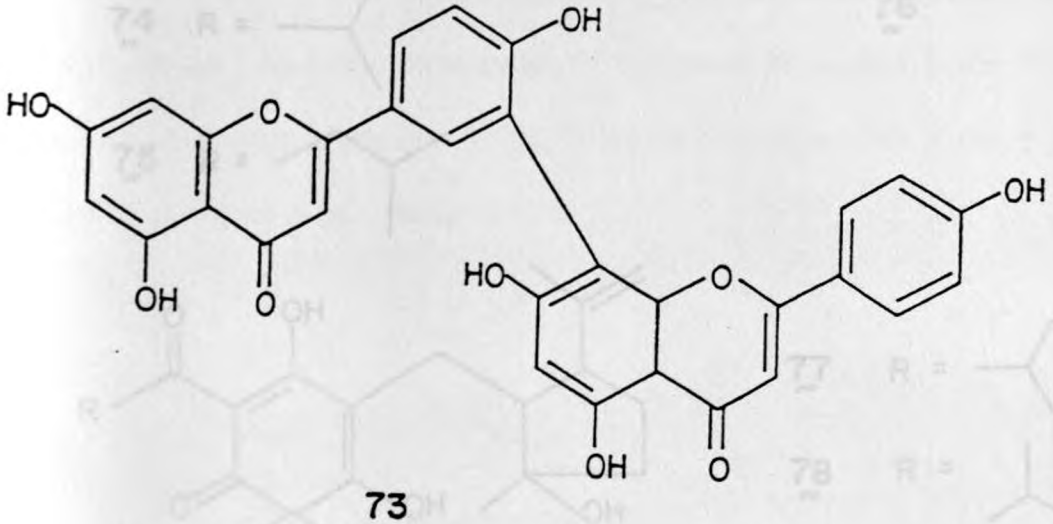


71



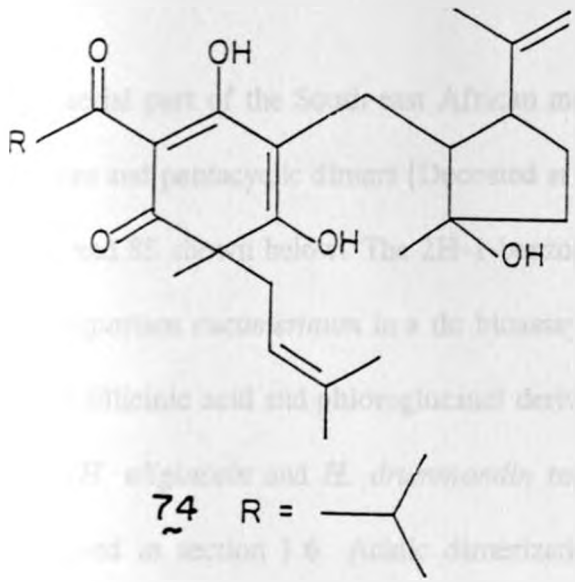
72



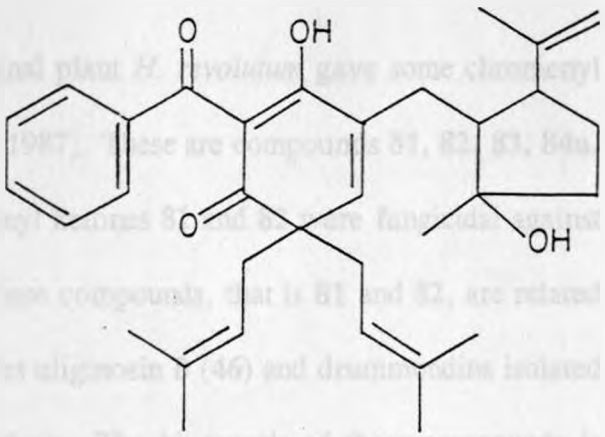
73

1.4.5 OTHER COMPOUNDS OF *HYPERICUM* SPECIES

Extending their search for biologically active compounds from *Hypericum* species Decosted *et al.* [1989a; 1989b] isolated some phloroglucinol derivatives from the light petroleum ether extract of *H. calycinum*. The extract was subjected to growth inhibitory testing against Co-115 human colon carcinoma cell line. It was found to be active with an ED_{50} value of 2.7 μg/ml. Fractionation of the extract on DCCC followed by preparative HPLC led to the isolation of chinesin II (**74**), hypercalin (**75**), hypercalin A (**76**), hypercalin B (**77**), hypercalin (**78**) and compounds **79** and **80**.

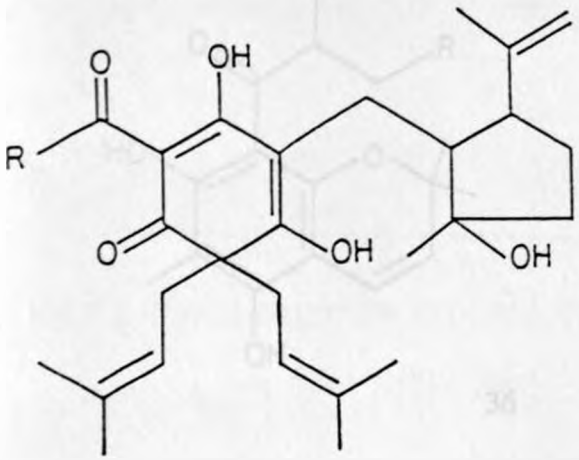


74 R =



76

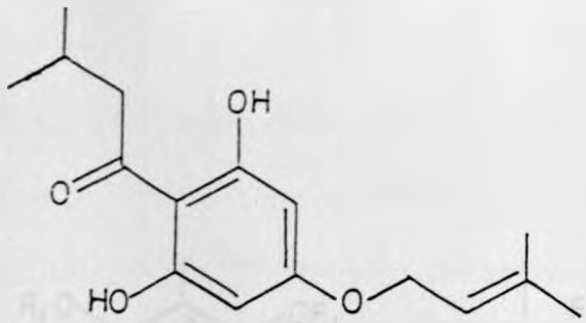
75 R =



77 R =

78 R =

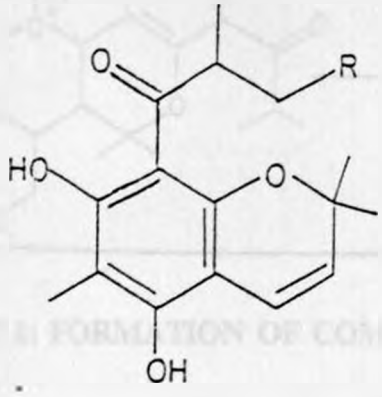
79 R =



80

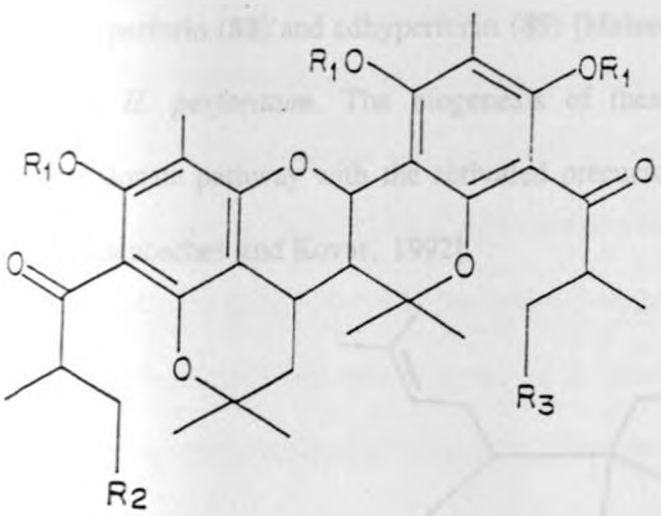
All the above compounds with the exception of compound **80** had growth inhibitory activity against Co-115 human carcinoma cell line. However, compound **80** showed anti-fungal activity against *Cladosporium cucumerinum* in a tlc bioassay. Biogenetically, the synthesis of these compounds involve acetogenesis to phloroglucinol followed by alkylation with isopentyl/dimethylallyl pyrophosphate.

The aerial part of the South-east African medicinal plant *H. revolutum* gave some chromenyl ketones and pentacyclic dimers [Decosted *et al.*, 1987]. These are compounds **81, 82, 83, 84a, 84b,** and **85** shown below. The 2H-1-benzopyranyl ketones **81** and **82** were fungicidal against *Cladosporium cucumerinum* in a tlc bioassay. These compounds, that is **81** and **82**, are related to the fillicinic acid and phloroglucinol derivatives uliginosin B (**46**) and drummondins isolated from *H. uliginosin* and *H. drummondin* respectively. The biogenesis of these compounds is discussed in section 1.6. Acidic dimerization of compound **81** resulted in the formation of compound **83.** A possible mechanism for the formation of compound **83** is shown in Scheme 1.4.5.1 below [Decosted *et al.*, 1987].

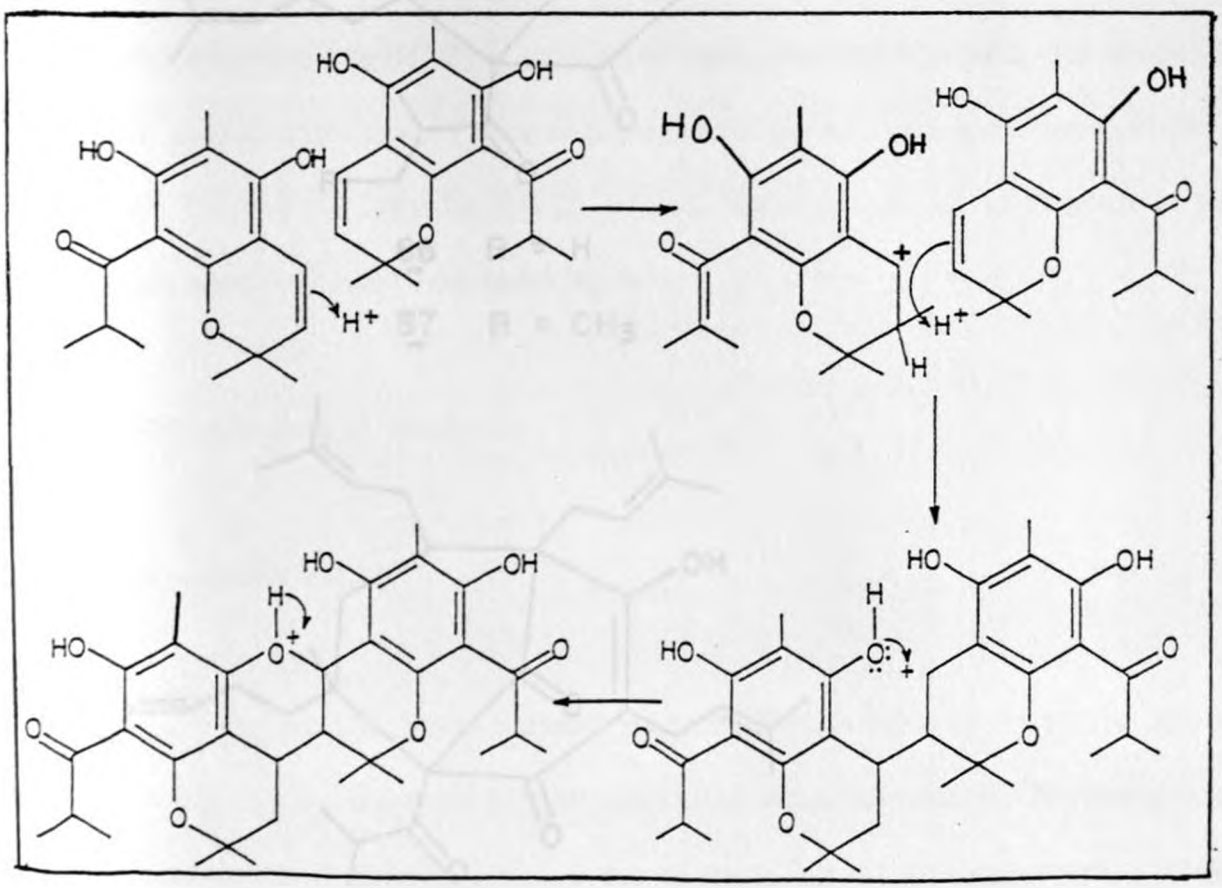


81 R = H

82 R = CH_3

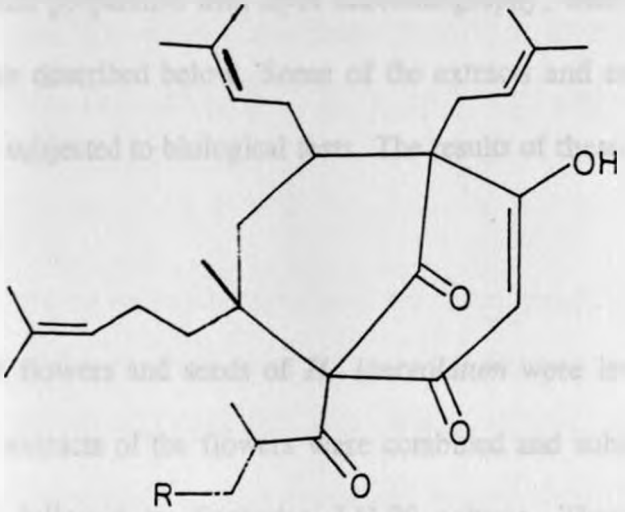


	R_1	R_2	R_3
83	H	H	H
84a	H	H	CH_3
84b	H	CH_3	H
85	H	CH_3	CH_3



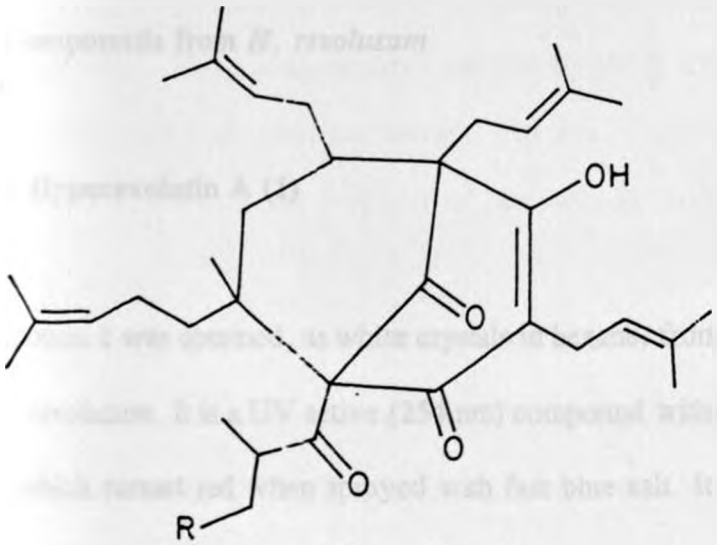
SCHEME 1.4.5.1: FORMATION OF COMPOUND 83

Hyperevolutin A (**86**) and B (**87**) were isolated from the cytotoxic petroleum ether extract of the same plant [Decosted *et al.*, 1989c]. These acylphloroglucinol derivatives appear to be related to hyperforin (**88**) and adhyperforin (**89**) [Maisenbacher and Kovar, 1992] which were isolated from *H. perforatum*. The biogenesis of these compounds originate from the polyketide mevalonate pathway with the activated precursor of isobutyryl CoA or 2-methylbutyryl CoA [Maisenbacher and Kovar, 1992].



86 R = H

87 R = CH_3



88 R = H

89 R = CH_3

CHAPTER TWO

2.0 RESULTS AND DISCUSSION

The dry powdered barks of *H. revolutum* and *H. lanceolatum* were separately extracted with hexane, dichloromethane and methanol successively. The extracts were subjected to column chromatography, both on silica gel (untreated and treated with oxalic acid) and Sephadex LH-20 columns and preparative thin layer chromatography, which led to the isolation of some of the compounds described below. Some of the extracts and compounds isolated from these plants were also subjected to biological tests. The results of these experiments will be discussed in this chapter.

The dried flowers and seeds of *H. lanceolatum* were investigated as well. The acetone and methanol extracts of the flowers were combined and subjected to column chromatography on silica gel followed by Sephadex LH-20 column. These process led to isolation of three flavonoids which will also be discussed below.

2.1 Compounds from *H. revolutum*

2.1.1 Hyperevolutin A (1)

Compound **1** was obtained, as white crystals in hexane, from the hexane extract of the stem bark of *H. revolutum*. It is a UV active (254 nm) compound with an Rf value of 0.38 (solvent system S-2) which turned red when sprayed with fast blue salt. It had a melting point of 140-142°C

(uncorrected).

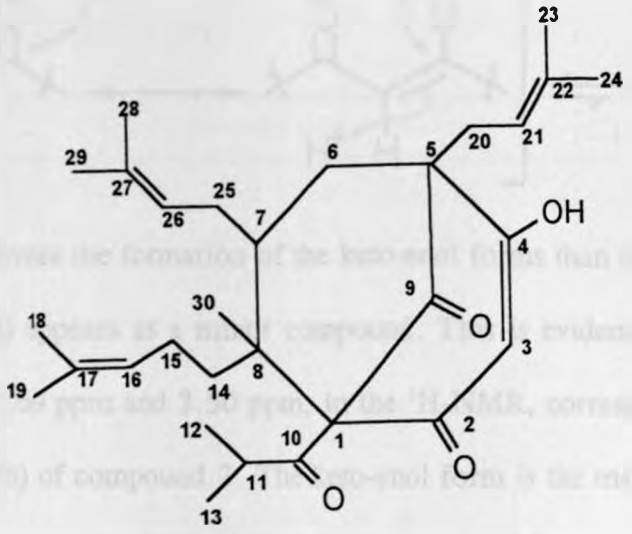
The ^{13}C-NMR signals indicated the presence of 30 non equivalent carbon atoms. Among them four carbonyl groups at 210.4, 206.6, 189.2, and 184.5 ppm; three quaternary vinylic groups at 135.2, 133.6 and 131.6 ppm; three tertiary vinylic groups at 124.5, 122.2 and 118.9 ppm. A signal at 109.8 ppm corresponding to an olefinic carbon. The rest of the ^{13}C-NMR signals correspond to three quaternary carbons (80.1, 60.2 and 48.3 ppm), two methine carbons (42.3 and 42.0 ppm), five methylene carbons (39.4, 37.2, 29.2, 27.8 and 24.9 ppm) and eight methyl carbons (26.0, 25.7, 25.6, 21.7, 20.9, 18.1, 18.0 and 17.9 ppm) deduced from the DEPT spectrum.

The EIMS gave a molecular ion at *m/z* 468, corresponding to $C_{30}H_{44}O_4$, and a base peak at *m/z* 69 indicating the presence of prenyl group(s). The presence of an enolic 1,3-diketo system is inferred from the broad absorption at 3000 cm^{-1} together with an intense peak at 1650 cm^{-1} in the infrared spectrum [Jayasuriya and McChesney, 1989].

The presence of prenyl group(s), and 1,3-diketo system indicated the possibility of hyperforin derivatives. Furthermore hyperforin derivatives had been isolated form *Hypericum* species [Decosted *et al.*, 1989; Maisenbacher and Kovar, 1992]. Comparison of the spectroscopic data of this compound with literature showed that this compound is hyperevolutin A, which was previously isolated from the root bark of Malawian *H. revolutum* [Decosted *et al.*, 1989].

In addition the mass spectrum showed a fragment ion at *m/z* 425 [M^+ - C_3H_7] resulting from the alpha cleavage of the isopropyl ketone. The cleavage of one or two isoprene units from the molecular ion through McLafferty rearrangements led to *m/z* 399 [M^+ - C_5H_9] and *m/z* 331 [M^+

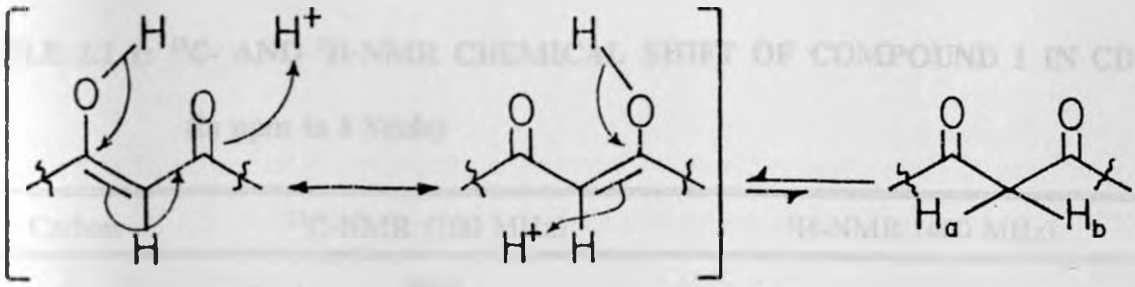
- C_5H_9 - C_5H_8]]. The fragment at *m/z* 357 [M^+ - C_5H_9 - C_3H_6] resulted from the cleavage of one prenyl group together with the cleavage of isopropyl ketone.



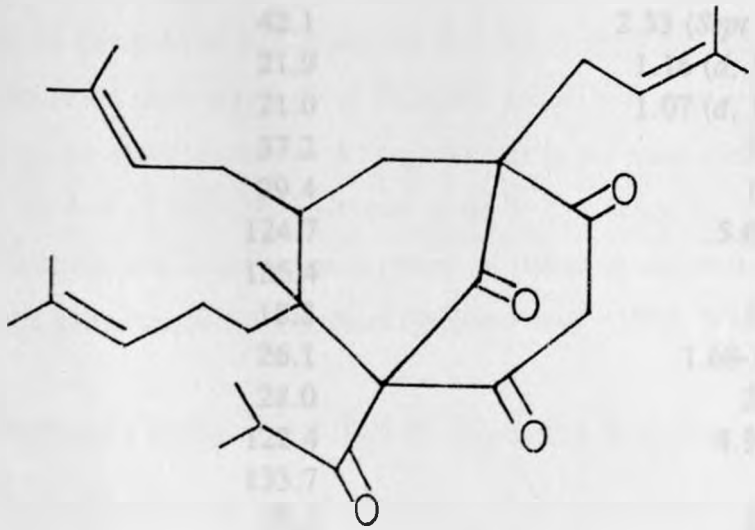
1

The ^{1}H-NMR displayed signals corresponding to: the six methyl groups of the three prenyl groups at 1.68-1.67 ppm (CH_3(19), CH_3(24), CH_3(29)), 1.64 ppm (CH_3(23)), 1.60 ppm (CH_3(28)) and at 1.58 ppm (CH_3(18)); the two methyl groups of the isobutyrl ketone at 1.16 ppm (CH_3(12), d, J = 6.5 Hz) and at 1.07 ppm (CH_3(13), d, J = 6.5 Hz); CH_3(30) at 1.06 ppm. While the three double bonds of the prenyl groups appeared at 5.04 ppm (H-26, t), 5.02 ppm (H-16, t) and at 4.98 ppm (H-21, t), H-11 appeared as a septet at 2.33 ppm (J = 6.4 Hz). These hydrogens were assigned using the H-C direct correlation (HCCOBI) spectrum.

The ^{1}H-NMR further displayed one olefinic singlet at 6.14 ppm and one broad singlet at 7.9-7.6 ppm belonging to a hydroxyl group. These groups obviously belong to the enolic 1,3-diketo system, which was inferred from the infrared spectrum, and such a system is usually in a tautomeric equilibrium as shown below:



This equilibrium favors the formation of the keto-enol forms than the diketo form. Due to this the diketo form (**2**) appears as a minor compound. This is evidenced by the presence of two small doublets at 3.69 ppm and 3.50 ppm, in the ^{1}H-NMR, corresponding to the two geminal protons (Ha and Hb) of compound **2**. The keto-enol form is the major compound as shown by the strong and sharp singlet of the olefinic hydrogen at 6.14 ppm.



2

The above equilibrium also indicated that none of the two oxygen bearing carbons of the keto-enol form, the major compound, are at any one moment an isolated carbonyl or hydroxyl bearing carbon atoms. This explains why the ^{13}C-NMR signals at 189.2 ppm and 184.5 ppm corresponding to C-2 and C-4 respectively appeared up field compared to the isolated carbonyl groups C-9 and C-10 which appeared down field at 210.4 ppm and 206.6 ppm, respectively.

TABLE 2.1.1: ^{13}C- AND ^{1}H-NMR CHEMICAL SHIFT OF COMPOUND 1 IN $CDCl_3$

(in ppm in δ Scale)

Carbon	^{13}C-NMR (100 MHz)	^{1}H-NMR (400 MHz)
1	80.1	-
2	189.0	-
3	110.1	6.14 (*s*)
4	185.0	7.9-7.6 (*br,s*)
5	60.2	-
6	39.6	1.82
7	42.5	1.83
8	48.5	-
9	210.5	-
10	206.8	-
11	42.1	2.33 (*Sept*, J = 6.5 Hz)
12	21.9	1.16 (*d*, J = 6.5 Hz)
13	21.0	1.07 (*d*, J = 6.5 Hz)
14	37.2	1.80
15	29.4	1.93
16	124.7	5.02 (*m*)
17	135.4	-
18	18.3	1.57
19	26.1	1.68-1.67 (*m*)
20	28.0	2.13
21	122.4	4.98 (*m*)
22	133.7	-
23	18.2	1.64
24	26.0	1.68-1.67 (*m*)
25	25.0	2.52 (*m*), 2.49 (*m*)
26	119.2	5.04 (*t*)
27	131.5	-
28	17.9	1.58
29	25.9	1.68-1.67 (*m*)
30	14.3	1.05 (*s*)

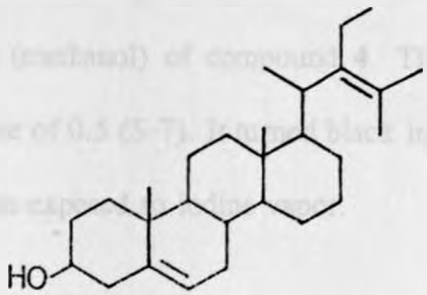
2.1.2 Stigmasterol (3)

The chloroform extract of *H. revolutum* after various chromatographic analysis gave colorless needle (acetone) like crystals of compound **3**. This compound has melting point of 168-170°C and an Rf value of 0.41 [$CHCl_3$: EtOAc(6:1)]. It gave orange color when sprayed with concentrated sulfuric acid on analytical tlc plate, after heating at 110°C.

The mass spectrum of this compound showed a prominent molecular ion at *m/z* 412, $C_{29}H_{48}O$, with a fragmentation pattern characteristic for sterols. The fragment ion at *m/z* 394 corresponds to the loss of water from the molecular ion. A doublet of peaks at *m/z* 271 and at *m/z* 273 initiated by allylic cleavage of the 17-20 bond is in agreement with the presence of a Δ^{22} unsaturation and a double bond in the nucleus of the molecule. A fragment ion at *m/z* 300 caused by the scission at 22-23 bond and ions at *m/z* 369 [M^+ - isopropyl] and *m/z* 351 (M^+ - (isopropyl + H_2O)), evident in the mass spectrum of the sterol are all confirmation of the presence of a Δ^{22} unsaturation in the sterol molecule. A fragment ion in the mass spectrum of the molecule at *m/z* 255 for the loss of the side chain and water is significant for Δ^5 and Δ^7 unsaturated sterols. This compound was identified as stigmasterol based on spectroscopic data which were in agreement with those reported in literature [Holland *et al.*, 1978; Waller, 1972].

The ^{1}H-NMR displayed a multiplet at 5.37-5.35 ppm which is attributed to the vinylic proton at H-6. The two doublets of doublets of peaks at 5.16-5.14 ppm were attributed to the allylic protons H-22 and H-23. A septate at 3.56-3.50 ppm was attributed to the hydroxyl methine proton H-3. Two singlets at 0.69 and 1.02 ppm were due to the methyl protons of C-18 and C-19, respectively. A doublet at 0.94-0.92 ppm was assigned to the methyl protons of C-21. It further displayed a doublet at 0.84-0.83 ppm which were assigned to the two equivalent methyl protons of C-26 and C-27. While a triplet at 0.83-0.80 ppm was assigned to the methyl protons of C-29.

The DEPT experiment showed the presence of a total of 29 non equivalent carbon atoms. Among them 12 are quaternary and secondary carbons while the rest are teritary and primary carbons. It showed four signals in the double band region and one hydroxyl group, which tallied with those of stigmasterol. The assignment of the carbons of this compound is shown in Table 2.1.2.



3

TABLE 2.1.2: ^{13} C-NMR CHEMICAL SHIFT VALUE OF STIGMASTEROL (3)

Carbon	Chemical Shift (ppm)	Carbon	Chemical shift (ppm)
1	37.5	16	28.5
2	31.9	17	56.3
3	72.0	18	12.1*
4	42.6	19	19.3
5	141.0	20	40.0
6	121.9	21	20.0
7	31.9	22	138.5
8	32.1	23	129.4
9	50.4	24	51.5
10	36.4	25	32.2
11	21.3	26	19.0
12	40.0	27	19.6
13	42.6	28	24.5
14	57.0	29	12.2*
15	24.5		

Chemical shifts marked with asterisks (*) may be interchanged.

2.2 Compounds of *H. lanceolatum*

2.2.1 Compounds of the stem bark of *H. lanceolatum*

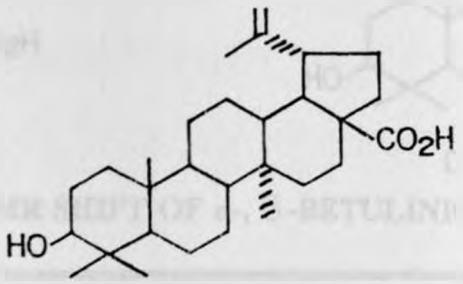
2.2.1.1 β-Betulinic acid (4)

The hexane extract of the stem bark of *H. lanceolatum* after successive chromatographic analysis afforded white crystals (methanol) of compound **4**. This compound had a melting point of >250°C and an Rf value of 0.5 (S-7). It turned black in vanillin-sulfuric acid, after heating at 110°C, and yellow when exposed to iodine vapor.

The ^{13}C-NMR signals indicated the presence of 30 non-equivalent carbon atoms. Among them are a carbonyl group (177.3 ppm), a quaternary vinylic carbon (150.4 ppm), a secondary carbon (109.7 ppm) and a tertiary alcohol (76.8 ppm). The rest of the ^{13}C-NMR signals correspond to five quaternary carbons (37.6, 40.4, 36.7, 42.0 and 55.4 ppm), ten methylene carbons (38.3, 27.1, 18.0, 33.9, 20.5, 25.1, 30.1, 31.7, 29.2, and 36.3 ppm) and six methyl carbons (28.1, 18.9, 15.9, 15.8, 15.7, and 14.4 ppm). This was deduced from the DEPT spectrum.

The EIMS gave a molecular ion peak at *m/z* 456 corresponding to $C_{30}H_{48}O_3$. The ir spectrum showed among other functional groups a hydroxyl (3420 cm^{-1}) and a carboxyl (3380-3090 and 1680 cm^{-1}) groups, confirming the presence of a hydroxyl and a carbonyl group in this compound. Comparison of these spectroscopic data with literature showed that this compound is either α- or β-betulinic acid [Mahato and Kundu, 1994]. A closer comparison of the chemical shifts of C-3 of this compound with those of α- and β-betulinic acid showed that this compound

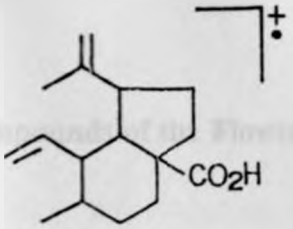
is β-betulinic acid. The ^{13}C-NMR of α- and β-betulinic acid and compound **4** is summarized in Table 2.1.1.1. Optical activity measurements confirmed that this compound is β-betulinic acid. The optical rotation value of β-betulinic acid is positive ($[\alpha]_D = +0.15$) while that of α-betulinic acid is negative [Herz *et al.*, 1972].



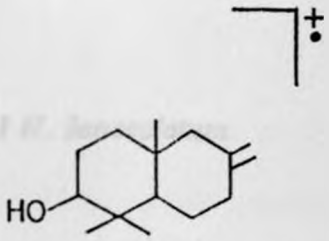
4

The ^{1}H- NMR displayed a doublet at 4.68 ppm and a broad singlet at 4.55 ppm corresponding to the terminal vinylic methylene protons. The vinylic methyl group appeared at 1.61 ppm, while the methyl groups resonate as sharp singlets at 0.91 ppm (3H), 0.85 ppm (6H), 0.75 ppm (3H) and 0.65 ppm (3H).

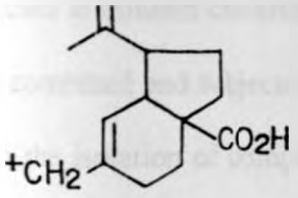
The EIMS of this compound was in accordance with these results and exhibited diagnostically important peaks at *m/z* 456 [M^+], 438 [$M^+ - H_2O$], 423 [$M^+ - 33$], 410 [$M^+ - 46$], 248 [A], 220 [B], 219 [C], 207 [D], 203 [A - 45] and 189 [D - 18]. This fragmentation pattern strongly confirmed that this compound is of the lup-20(29)-ene type and the allocation of the carboxyl group to rings A/B [Budzikiewicz *et al.*, 1963].



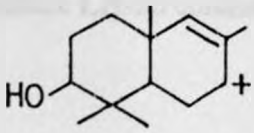
A



B



C



D

TABLE 2.1.1.1: ^{13}C-NMR SHIFT OF α-, β-BETULINIC ACID AND COMPOUND 4

Carbon	Chemical shift α-betulinic acid	β-betulinic acid	4
1	34.0	38.7	38.3
2	23.2	27.4	27.1
3	75.5	78.9	76.8
4	39.0	38.8	37.6
5	49.3	55.3	54.9
6	18.6	18.3	18.0
7	34.8	34.3	33.9
8	41.2	40.7	40.4
9	50.7	50.5	49.9
10	37.7	37.2	36.7
11	21.0	20.8	20.5
12	26.1	25.5	25.1
13	38.5	38.8	38.5
14	42.9	42.4	42.0
15	31.2	30.5	30.1
16	32.8	32.1	31.7
17	56.6	56.3	55.4
18	47.7	46.8	46.6
19	49.7	49.2	48.5
20	151.2	150.3	150.4
21	29.9	29.7	29.2
22	37.5	37.0	36.3
23	29.2	27.9	28.1
24	15.3	22.5	15.7
25	16.0	16.4	15.9
26	16.1	16.4	15.8
27	14.7	14.9	14.4
28	180.5	178.7	177.5
29	109.6	109.8	109.7
30	19.4	19.4	18.9

2.2.2 Compounds of the Flowers and Seeds of *H. lanceolatum*

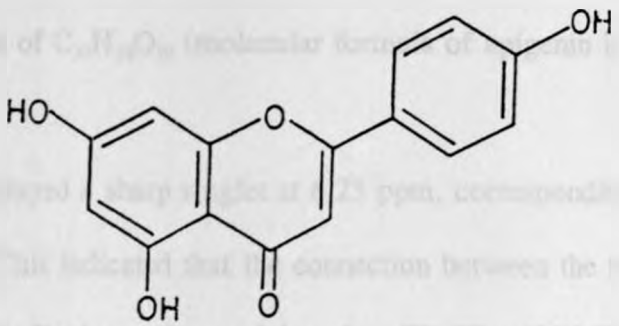
The combined acetone and methanol extract of the flowers and seeds of *H. lanceolatum* were subjected to column chromatography on silica gel. The eluents containing phenolic compounds were combined and subjected to successive Sephadex LH-20 column [MeOH:EtOH (1:1)]. This led to the isolation of compounds **5**, **6** and **7**.

2.2.2.1 I3, II8 - Biapigenin (5)

Compound **5** has an Rf value of 0.35 (solvent system S-4) and a melting point of 258-260°C. This bright yellow compound intensified more yellow on exposure to ammonia vapor and turned dark brown when sprayed with ferric chloride solution indicating that it is a flavonoid.

The UV spectrum of compound **5** in methanol solution exhibits two maximum absorption peaks at 330 nm (band I) and 274 nm (band II), which is typical for flavonoids [Markham, 1982]. The ^{1}H-NMR spectrum showed a singlet at 6.52 ppm which is a typical character of H-3 of flavones. Addition of 0.2 M aqueous sodium hydroxide solution caused a bathochromic shift of 58 nm in band I, without a decrease in intensity, in the UV spectrum. This indicated the presence of 4'-hydroxyl group in this compound. While addition of NaOAc caused a bathochromic shift in band II, which indicated the presence of a hydroxyl group at C-7. Furthermore, the UV spectrum underwent bathochromic shift with $AlCl_3$, this spectrum being unchanged on adding hydrochloric acid, indicating the presence of a 5-hydroxyl group. From the above results it was clear that

compound 3 is a flavone with hydroxyl groups at C-4', C-5 and at C-7. The UV spectral characteristics of this compound resembles those of apigenin (**90**) [Mabry *et al.*, 1970].



90

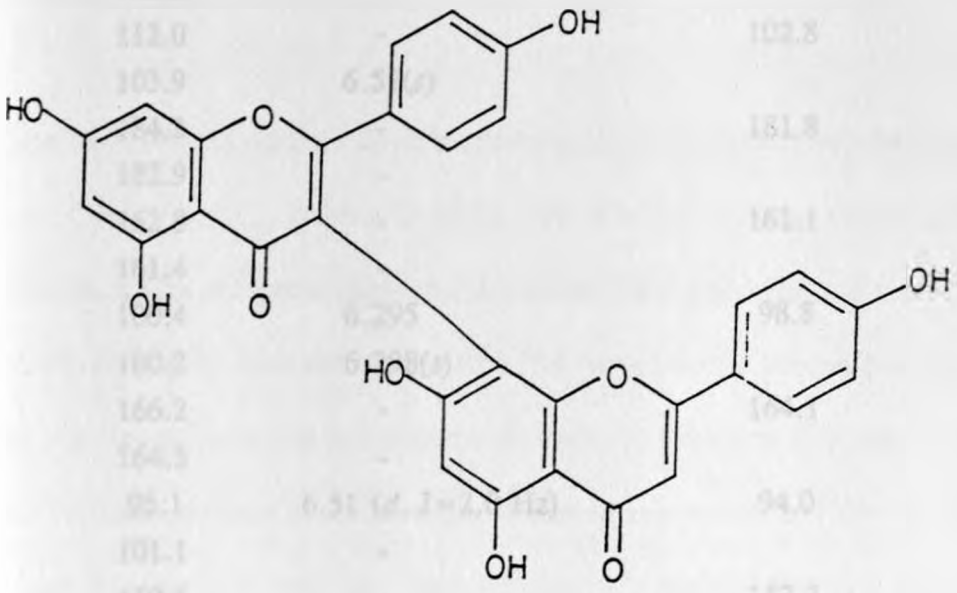
The ^{13}C-NMR signals of compound **5** indicated the presence of 26 non equivalent carbon atoms among them two carbonyl groups at 184.2 and 182.9 ppm; two oxygenated quaternary vinylic carbons at 166.3 ppm and 166.2 ppm and six hydroxyl substituted aromatic carbons at 166.2, 164.2, 163.9, 163.6, 162.9 ppm and 161.4 ppm. Where as apigenin has one carbonyl group at 182.8 ppm, one oxygenated quaternary vinylic carbon at 166.9 ppm, and three hydroxyl substituted aromatic carbons at 165.3, 163.1 and 161.2 ppm [Berghofer and Holzl, 1986]. These are half the number (but in similar chemical environment) of these groups in compound **5**. Furthermore, while the ^{1}H-NMR spectrum of apigenin [Mabry *et al.*, 1970] displayed one AA'BB' system. This compound displayed two AA'BB' systems. The above analogy showed that there are twice the number of the same groups, as there are in apigenin, in compound **5**. Apigenin has 13 non equivalent carbons, two symmetrical protons and the corresponding two carbons (see Table 2.2.1.1), making its total number of carbons to fifteen. However, compound **5** has twenty six non equivalent carbons, four symmetrical protons and using the same analogy

the corresponding four carbon atoms, making its total number of carbons to thirty. The total number of non-hydroxyl hydrogens in this compound is twelve (from integration of ^{1}H-NMR) while in apigenin they are seven. Therefore, compound **5** is a dimer of apigenin with a molecular formula of $C_{30}H_{18}O_{10}$ (molecular formula of apigenin is $C_{15}H_{10}O_5$).

The ^{1}H-NMR displayed a sharp singlet at 6.25 ppm, corresponding to H-3, integrating for one proton, not two. This indicated that the connection between the two apigenin is between IC-3 of one (I) and A- or B- ring carbons of the other (II). The 400 MHz ^{1}H-NMR spectrum showed the presence of two AA'BB' systems with signals at 7.53 ppm (J = 8.8 Hz), 7.33 ppm (J = 8.8 HZ), 6.76 ppm (J = 8.8 Hz) and 6.65 ppm (J = 8.8 Hz) each integrating for two protons, corresponding to two *para*-hydroxyl substitution in the two B-rings. This leaves the connection to be between IC-3 and one of the A-ring carbon of II. The ^{1}H-NMR further displayed two *meta*-coupled protons at 6.51 ppm (J = 2.1 Hz) and 6.295 ppm which were assigned to the two *meta*-coupled protons of IA-ring. It also showed a singlet at 6.298 ppm which was assigned to the only other proton of IIA-ring. Therefore, the connection between the two apigenin groups should be either IC-3 and IIC-8 or IC-3 and IIC-6. However, a comparison between the chemical shift of apigenin (H-6 and H-8) and this compound showed the proton at 6.298 ppm belongs to IIH-6. Therefore compound **5** is I3, II8-Biapigenin.

The structure of this compound was assigned and confirmed using DEPT, COSY 45, NOESY, HCCOBI and HMBC. The signals for the unsubstituted carbon atoms I8 and II3 appeared at the expected values (compared with apigenin) of 95.1 ppm and 103.9 ppm respectively, while II8

and I3 had their resonances at 101.1 ppm and 112.0 ppm. The down field shifts of 6 and 8.1 ppm experienced by the latter carbons are due to the substitution effect of the interflavone linkage.



5

The assigned structure (see Table 2.2.1) was further confirmed through literature correlation [Berghofer and Holzl, 1986]. I3, II-8-Biapigenin had been isolated from *Hypericum perforatum* [Berghofer and Holzl, 1986]. Biflavonoids are very common in Guttiferae especially in the genus Gerainia [Cotterill *et al.*, 1978, Crichton, 1980]. This is the first report of this compound from this plant.

TABLE 2.2.1.1: ^{13}C-NMR DATA OF 5 (CD_3OD) AND APIGENIN (DMSO-d_6) AND ^{1}H-NMR DATA OF 5 (CD_3OD) (in ppm, δ scale).

Carbon	^{13}C-NMR (100 MHz)	^{1}H-NMR (400 MHz)	^{13}C-NMR Apigenin (22.63 MHz)
2	166.3	-	163.8
2"	162.2	-	
3	112.0	-	102.8
3"	103.9	6.52(*s*)	
4	184.2	-	181.8
4"	182.9	-	
5	162.8	-	161.1
5"	161.4	-	
6	100.4	6.295	98.8
6"	100.2	6.298(*s*)	
7	166.2	-	164.1
7"	164.3	-	
8	95.1	6.51 (*d*, J=2.0 Hz)	94.0
8"	101.1	-	
9	159.6	-	157.3
9"	156.9	-	
10	105.8	-	103.7
10"	105.0	-	
1'	125.2	-	121.3
1'''	123.3	-	
2'	131.2	7.53 (*d*, J=8.8 Hz)	128.4
2'''	129.3	7.33 (*d*, J=8.8 Hz)	
3'	117.1	6.76 (*d*, J=8.8 Hz)	116.0
3'''	116.2	6.65 (*d*, J=8.8 Hz)	
4'	163.6	-	161.5
4'''	163.4	-	
5'	117.1	6.76 (*d*, J=8.8 Hz)	116.0
5'''	116.2	6.65 (*d*, J=8.8 Hz)	
6'	131.1	7.53 (*d*, J=8.8 Hz)	128.4
6'''	129.3	7.33 (*d*, J=8.8 Hz)	

2.2.2.2 Quercetin (6)

Compound **6** has an Rf value of 0.45 (solvent system S-4) and a melting point of 314-315°C. This yellow compound intensified on exposure to ammonia vapor and turned dark green when sprayed with ferric chloride solution.

The UV spectrum of compound **6** run in methanol solution exhibited two absorption maxima at λ_{max} 372 nm (band I) and λ_{max} 255 nm (band II). This is a typical feature of flavonoids. The ^{13}C-NMR spectrum of this compound displayed a peak at 138.4 ppm which is a characteristic ^{13}C-NMR spectral feature of flavonols (C(3)-OH). The presence of a second peak (a shoulder) in band II of this flavone indicated the presence of hydroxyl groups at C-3' and C-4' [Markham, 1982]. The infrared spectrum of this compound showed absorptions at 3390 cm^{-1} corresponding to a free hydroxyl group, at 3260 cm^{-1} corresponding to a chelated hydroxyl group and at 1650 cm^{-1} corresponding to a conjugated carbonyl group. Other absorption frequencies at 1250 cm^{-1} and 1140 cm^{-1} were attributed to an ether group [Cardona and Seoan, 1982]. The presence of a chelated carbonyl group is confirmed by the presence of peaks at 13.32 ppm (OH) and 177.8 ppm (C=O) in the ^{1}H- and ^{13}C-NMR spectra, respectively. The EIMS spectrum of this compound showed a molecular ion peak at *m/z* 302 corresponding to the empirical formula $C_{15}H_{10}O_7$. These observations led to the conclusion that compound **6** is a flavonol with hydroxyl groups at C-3, C-3', C-4' and C-5.

The continual reduction in intensity of band I when the UV spectrum of this compound was run

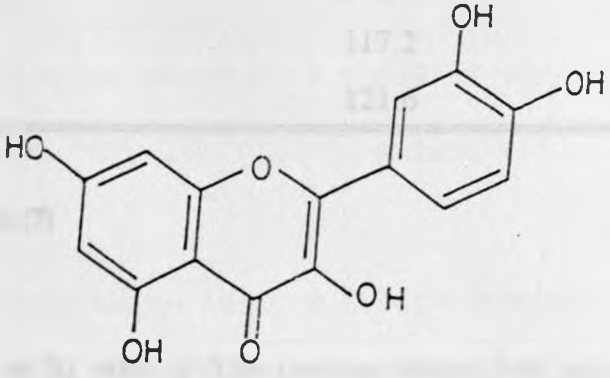
after addition of 0.2 M aqueous sodium hydroxide solution indicated the presence of alkaline-sensitive hydroxyl groups at C-3 and C-4' of the flavonoid skeleton. While a bathochromic shift in band II of the NaOAc UV spectrum indicated the presence of a hydroxyl group at C-7. Addition of $NaOAc/H_3BO_3$ caused a bathochromic shift of 20 nm, indicating the presence of *ortho*-dihydroxyl groups on B-ring, that is 3',4' - dihydroxyl group. From the foregoing it was possible to conclude that compound **6** is a flavonol with hydroxyl groups at C-3, C-3', C-4', C-5 and at C-7.

The ^{1}H-NMR spectrum displayed a broad singlet at 8.63 ppm and *ortho*-coupled doublets at 8.13 ppm (J = 8.4 Hz) and at 7.40 ppm (J = 8.4 Hz). These were assigned to H-2', H-5' and H-6' of the B-ring, respectively. The above observation in addition to a fragment ion at *m/z* 137 clearly showed that the aromatic B-ring had no other substituent besides the two hydroxyl functions, which are at C-3' and C-4' positions. The ^{1}H-NMR spectrum further displayed two broad singlets at 6.8 ppm and 6.7 ppm, each integrating for one proton. These were assigned to the A-ring protons H-8' and H-6' respectively. A fragment ion at *m/z* 153 and the above observation clearly indicated that this ring had no other substituents besides the two hydroxyl groups, which are at C-5 and C-7 positions.

The ^{13}C-NMR spectrum signals indicated the presence of 14 non equivalent carbons. The H-C NMR direct correlation (HCCOBI) spectrum showed a direct correlation between the carbon at 117.2 ppm and the protons H-2' and H-5'. This indicated that C-2' and C-5' are equivalent and, therefore, the total number of carbons of this compound is fifteen. Normally hydroxyl substituted

aromatic carbons appear around 160 ppm on ^{13}C-NMR spectra [Markham, 1982]. However, two of these groups of this compound appeared at 148.2 ppm and 147.6 ppm up field by about 12 ppm, which is a characteristic feature of aromatic *ortho*-dihydroxyl substituted carbons [Markhamm, 1982]. This further confirmed the presence of C-3' and C-4' *ortho*-dihydroxyl substituent.

Using all the above data compound **6** was assigned 3,3',4',5,7-Pentahydroxyflavone or quercetin. The structure was confirmed by DEPT, HCOBI and HMBC techniques. The assigned structure (see Table 2.2.2.2) was further confirmed through literature correlation [Mabry *et al.*, 1970; Wenker and Gottlieb, 1977]. Quercetin had been isolated from several plant species, but this is the first report from *H. lanceolatum*.



6

TABLE 2.2.2.2: ^{13}C- AND ^{1}H-NMR CHEMICAL SHIFT OF 6 IN PYRIDINE-d_5 (ppm in δ Scale)

Carbon	^{13}C-NMR (100 MHz)	^{1}H-NMR (400 MHz)
2	150.4	-
3	138.4	-
4	177.8	-
5	162.9	13.32 (*s*)
6	99.7	6.73 (*br,s*)
7	166.0	-
8	94.8	6.78 (*br,s*)
9	156.0	-
10	105.0	-
1'	124.4	-
2'	117.2	8.63 (*br,s*)
3'	147.6	-
4'	148.2	-
5'	117.2	7.40 (*d*, J = 8.4 Hz)
6'	121.6	8.13 (*d*, J = 8.4 Hz)

2.2.2.3 Myricetin (7)

Compound **7** has an Rf value of 0.56 (solvent system S-4) and a melting point of 357-60°C (uncorrected). This yellow compound changed to purple on exposure to ammonia vapor and turned light brown when sprayed with ferric chloride solution.

The UV spectrum of this compound run in methanol exhibited absorption maxima at 370 nm

(band I, cinnamoyl moiety) and at 256 nm (band II, benzoyl moiety), which is a typical feature of flavonoids. The ^{13}C-NMR displayed a peak at 137.1 ppm which is typical of flavonols (C(3)-OH). The EIMS of this compound showed a molecular ion peak at *m/z* 318 corresponding to the molecular formula $C_{15}H_{10}O_8$. The presence of a peak at 177.4 ppm in the ^{13}C-NMR spectrum confirmed the presence of the carbonyl group.

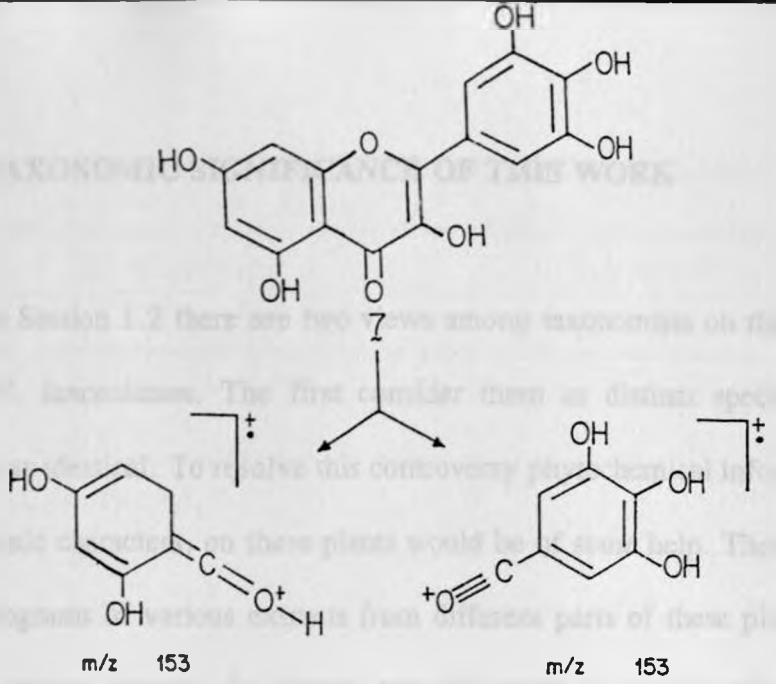
The presence of 3',4'-dihydroxyl substituent is evidenced by a second peak in band I of the UV (MeOH) spectrum of this flavone [Markham, 1982; Mabry, 1969; Jurd, 1962]. Addition of aqueous sodium hydroxide solution caused a continual reduction in intensity in the UV spectrum indicating the presence of alkaline-sensitive hydroxyl groups at C-3 and C-4'. Furthermore, band I underwent a bathochromic shift with $AlCl_3$ + HCl relative to the methanol spectrum, indicating the presence of a 5-hydroxyl group. While the bathochromic shift in band II caused when the UV spectrum was run in NaOAc/MeOH indicated the presence of a hydroxyl group at C-7. From the foregoing discussion it is possible to conclude that compound 7 is a flavone with hydroxyl groups at C-3, C-3', C-4', C-5 and at C-7.

The ^{1}H-NMR displayed a broad singlet at 7.36 ppm integrating for two protons which were assigned to H-2' and H-6' of the B-ring. Therefore, the three substituent of this ring should be located such that these protons are symmetrical, that is identical chemical shift. The results from the UV experiment had taken care of two of these substituent (hydroxyl groups at C-3' and C-4') leaving only one unknown substituent. The presence of the fragment ion at *m/z* 153 in the mass spectrum showed that this ring is trihydroxyl substituted. Therefore, the unknown substituent is

a hydroxyl group located at C-5'. The ^{1}H-NMR further displayed two *meta*-coupled protons at 6.37 ppm (J = 1.6 Hz) and 6.18 ppm (J = 1.6 Hz) which were assigned to H-6 and H-8 protons of the A-ring. A fragment ion at *m/z* 153 confirmed the dihydroxylation of this ring.

The DEPT experiment indicated the presence of 13 non equivalent carbons and two sets of equivalent carbons (C-2' and C-6'; C-3' and C-5'), making the total number of carbons of this compound to be fifteen. The ^{13}C-NMR signals at 165.7 ppm and 162.6 ppm were assigned to the hydroxyl groups at C-5 and C-7. Usually hydroxyl substituted aromatic carbons appear at around 160 ppm [Markham, 1982] on the ^{13}C-NMR spectrum. However, three of these carbons appeared at 146.9 ppm, 146.9 ppm and 137.1 ppm, up field by about 13.1 and 23 (2 x 11.5) ppm, respectively. This is a ^{13}C-NMR spectral feature of *ortho*-trihydroxyl substituted aromatic carbons [Markham, 1982]. This further confirmed presence of 3', 4',5'-trihydroxyl substituent on B-ring.

This compound was therefore identified as 3,3',4',4,5',7-hexahydroxyflavone, myricetin, based on these data and spectroscopic correlation with literature [Markham, 1970] and direct comparison with authentic sample (similar Rf values and response to ammonia vapor and ferric chloride solution). The basic fragmentation pattern of this compound is shown in Scheme 2.2.1 below. The total assignment of the protons and carbons of this compound is shown in Table 2.2.2.3.



SCHEME 2.2.1 PRINCIPAL INITIAL IONS PRODUCED IN THE MASS SPECTRAL FRAGMENTATION OF COMPOUND 7

TABLE 2.2.2.3: ^{13}C- AND ^{1}H-NMR CHEMICAL POSITIONS FOR COMPOUND 7 IN CD_3OD (in ppm in δ Scale)

Carbon	^{13}C-NMR (100 MHz)	^{1}H-NMR (400 MHz)
2	148.2	-
3	128.9	-
4	177.4	-
5	162.6	-
6	99.4	6.18 (*d*, J = 1.6 Hz)
7	165.7	-
8	94.5	6.38 (*d*, J = 1.6 Hz)
9	158.3	-
10	104.6	-
1'	123.6	-
2'	108.7	7.35 (*br,s*)
3'	146.9	-
4'	137.1	-
5'	146.9	-
6'	108.7	7.35 (*br,s*)

2.3 CHEMOTAXONOMIC SIGNIFICANCE OF THIS WORK

As discussed in Section 1.2 there are two views among taxonomists on the relationship of *H. revolutum* to *H. lanceolatum*. The first consider them as distinct species while the other recognize them as identical. To resolve this controversy phytochemical information, in addition to other taxonomic characters, on these plants would be of some help. Therefore, in this study the tlc chromatograms of various extracts from different parts of these plants were compared using different solvent systems. An attempt was also made to consider variations due to place of collection.

Thus *H. lanceolatum* samples (twigs and leaves, stem bark and flowers and seeds) were collected from Gakoe, while *H. revolutum* samples were collected from Mt. Kenya (stem bark) and the Aberdare (twigs and leaves and stem bark) ranges during the same week. These samples were extracted with hexane, dichloromethane and acetone. Their tlc chromatograms were obtained using solvent system S-5, S-2, S-4, and S-7 (see Section 3.3).

The Rf values in different solvent systems, colour under visible (VIS) and ultra violet (UV) lights, colour developed when exposed to ammonia vapour (NH_3), and colour developed when sprayed with fast blue B salt (FBS) were used as guidelines for identification. The t.l.c properties of some of the chromatographic zones are summarized in Table 2.3.1. Five of these zones were identified as as **1**, **4**, **5**, **6**, and **7** as discussed previously (Sections 2.1.1, 2.1.3, 2.2.2.1-2.2.2.3).

TABLE 2.3.1 : TLC PROPERTIES OF CHROMATOGRAPHIC ZONES

*	Rf values under				Colour under**				Compound
	S-5	S-2	S-4	S-7	UV	VIS	NH_3	FBS	
A	0.25	0.61	-	-	P	-	-	R	unknown
B	0.21	0.55	-	-	P	-	-	B	unknown
C	0.17	0.45	-	-	P	-	-	R	1
D	-	0.41	-	-	P	-	-	R	unknown
E	-	0.35	-	-	P	-	-	O	unknown
F	-	0.29	-	-	P	-	-	R	unknown
G	-	0.25	-	-	P	-	-	R	unknown
H	-	-	-	0.50	-	-	-	-	4
I	-	-	0.56	-		Y	Y		6
J	-	-	0.45	-		Y	P		7
K	-	-	0.35	-		Y	Y		5
L	-	-	0.30	-		Y	Br		unknown

* Chromatographic zones

** P=purple; R=red; O=orange; Y=yellow; Br=brown

Table 2.3.2 (below) shows the chromatographic zones which were useful to tell the similarities and differences of the three plant samples which were collected from different localities.

TABLE 2.3.2 : COMPOUNDS WITH TAXONOMIC SIGNIFICANCE IN THESE TAXA

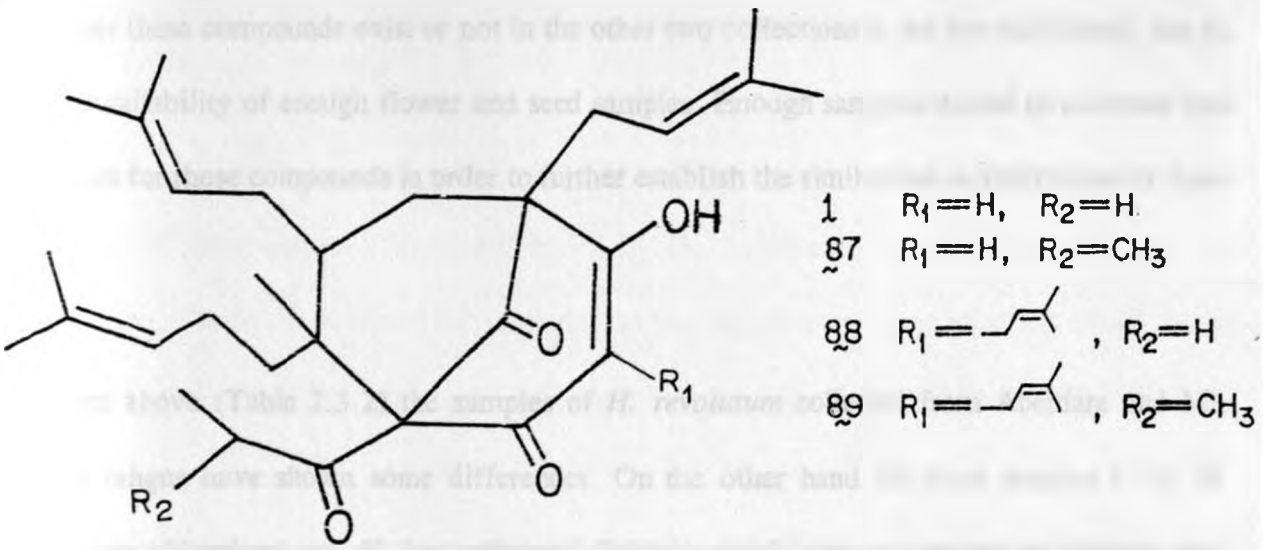
Plant sample (collected from) -Part	Compound											
	A	B	1	D	E	F	G	4	5	6	7	L
H. revolutum (Aberdare)												
-Twigs and leaves	+	+	+	-	-	+	+	-	-	-	-	-
-Stem bark	+	+	+	-	-	+	+	+	-	-	-	-
H. revolutum (Mt. Kenya)												
-Stem bark	+	±	+	+	+	+	+	+	-	-	-	-
H. lanceolatum (Gakoe)												
-Twigs and leaves	+	+	+	-	-	+	+	-	-	-	-	-
-Stem bark	+	+	+	-	-	+	+	+	-	-	-	-
-Flowers and seeds	-	-	-	-	-	-	-	-	+	+	+	+

The constituents of the stem bark and the twigs and leaves of *H. revolutum* (Aberdare) and *H. lanceolatum* (Gakoe) showed similar tlc pattern with the exception of compound **4** which occurs only in the stem bark of these plants. However, the constituents of the stem bark of *H. revolutum* (Mt. Kenya) were different from the above collections. Chromatographic zones D and E are unique to *H. revolutum* (Mt. Kenya), while chromatographic zones A, C, F and G are common to the samples collected from the three different localities.

Chromatographic zones A and B and compound **1** are common to the constituents of the twigs and leaves and stem bark of *H. revolutum* (Aberdare) and *H. lanceolatum* (Gakoe). However, the data available from this work is not enough to conclude that they are identical. Compounds **5**, **6**, and **7** and chromatographic zone L are not detected in these parts. They are unique to the

flowers and seeds.

Compound **1** (hyperevolutin A) was detected in all the three plant samples examined and occurs in large quantities. It also showed a strong red colour with fast blue B salt making it easily detectable even when the plant sample available is small. Therefore, this compound could be used as a chemotaxonomic marker in order to establish the relationship among the various species of this genus. This compound had been reported from the root bark of a Malawian *H. revolutum* (see Section 1.8). There are four such acylphloroglucinol derivatives reported from the genus *Hypericum* (Section 1.8). These are hyperevolutin A (**1**) and B (**87**) from *H. revolutum* and hyperforin (**88**) and adhyperforin (**89**) from *H. perforatum*. These compounds have in common the basic skeleton shown below:



As shown in Section 1.8 the biosynthesis of these homologues involve the polyketide mevalonate path way with the activated precursor of isobutyryl-CoA or 2-methylbutyryl-CoA. Thorough

phytochemical investigation of these plants must be carried out in order to establish the precursors involved in the biogenesis of these compounds.

Chromatographic zones A and D, which were not characterized in this work, had shown similar tlc properties as hyperevolutin A. Identifying these compounds might help in establishing the possible biosynthetic relationship and hence the chemotaxonomic significance of these compounds.

About nine monoflavonoids and two biflavones have been isolated from some *Hypericum* species (Section 1.7). In this work I3,II8-biapigenin (**5**), quercetin (**6**) and myricetin (**7**) are isolated. This appears to be the first report on the occurrence of flavonoids in *H. lanceolatum* (Gakoe). Whether these compounds exist or not in the other two collections is not yet established, due to the unavailability of enough flower and seed samples. Enough samples should be collected and analyzed for these compounds in order to further establish the similarities and difference of these taxa.

As seen above (Table 2.3.2) the samples of *H. revolutum* collected from Aberdare and Mt. Kenya ranges have shown some differences. On the other hand the plant samples (i.e. *H. revolutum* (Aberdare) and *H. lanceolatum* (Gakoe)) which were recognized as different taxa showed similar tlc pattern. These results support the view which considers *H. revolutum* and *H. lanceolatum* to be identical. However, comprehensive phytochemical and chemotaxonomic (in addition to other taxonomic characters) information is required in order to reach a strong

conclusion.

As discussed in Section 1.4.1 xanthones occur in at least 22 species of the genus *Hypericum*. It is actually the occurrence of xanthones which led to the re-classification of the family Hypericaceae (in which *Hypericum* used to belong) as a sub-family of Guttiferae, the Hypericoideae (the genus *Hypericum* is currently classified under this sub-family). The family Guttiferae is characterized by the occurrence of xanthones. However, there are many other members of this genus which do not elaborate xanthones. For example, the extracts of *H. uliginosum, H. drumondii,* and *H. japonicum* elaborate fillicinic acid and phloroglucinol derivatives (Section 1.8) and not any other class of compounds. The extracts of *H. perforatum* elaborates anthraquinones in addition to xanthones (Section 1.5). Furthermore, xanthones, fillicinic acid and phloroglucinol derivatives and anthraquinones were not detected in *H. revolutum*. *H. revolutum* elaborates the unique nine-carbon bicyclo acylphloroglucinol derivatives. It is highly probable that there could be other members of this genus which elaborate this type of compounds. This is evidenced by the occurrence of such compounds in *H. perforatum*. Therefore, it would be interesting to investigate some other members of this genus in order to appreciate the chemotaxonomic significance of these class of compounds.

From the above discussion one can infer that there are certain types of compounds belonging to some species of this genus and not others. This specificity among the various members can be utilized to classify the big genus into groups (there are 400 species in this genus (Section 1.2)). That is members of this genus elaborating xanthones as one group, those elaborating

acylphloroglucinols as another group and those elaborating fillicinic acid and phloroglucinol derivatives as yet another group, e.t.c..

2.4 BIOASSAYS RESULTS

The brine shrimp lethality of the hexane and chloroform extract of *H. revolutum* and some of the isolated pure compounds were tested. The results for the active extracts and pure compounds is summarized in Table 2.4.1 below.

TABLE 2.4.1 : LC_{50} Values

Substance tested	LC_{50} (ppm) Values
Hexane extract	6
Chloroform extract	5
Hyperevolutin A	0.5

The antimicrobial activities of the isolated pure compounds were tested against the organisms mentioned in Section 4.7.2.1. The results showed that all the compounds tested, except hyperevolutin A, did not show activity against these organisms. Hyperevolutin A showed an inhibition zone of 7.5 mm (radius) at 100 μg on *Trichophyton mentanogrophyte*, the activity of this compound against *Saccharomyces cerevisae* is shown in Table 2.4.2. Hyperevolutin A did not show activity against the other organisms. Chloramphenicol showed an inhibition zone of

12.5 mm at 25 μg against *Saccharomyces cerevisae*, under the same condition.

TABLE 2.4.2 : ANTIMICROBIAL ACTIVITIES OF HYPEREVOLUTIN A AGAINST *SACCHAROMYCES CEREVISAE*

Sample	Quantity (μg)	Inhibition zone (radius in mm)
Hyperevolutin A	50	10
	25	8
	12.5	6
	6	5.5

CHAPTER THREE

3.0 CONCLUSIONS AND RECOMMENDATIONS

The conclusions reached at and the recommendation made in this work are presented follows:

3.1 CONCLUSIONS

1. Phytochemical analysis of the stem bark of *H. revolutum* (Mt. Kenya) led to the isolation of the triterpenoid stigmasterol (**3**) and the acylphloroglucinol derivative hyperevolutin A (**1**) and its diketo form (**2**). Analysis of the stem bark of *H. lanceolatum* (Gakoe) yielded -betulinic acid (**4**). Hyperevolutin A has also been isolated from the same plant.

2. The combined methanol and acetone extract of the flowers and seeds of *H. lanceolatum* (Gakoe) afforded two flavonols, i.e. quercetin (**6**) and myricetin (**7**), and one biflavone, I3,II8-biapigenin (**5**).

3. Analysis of the tlc chromatograms of the three collections showed that there is close relationship between *H. revolutum* (Aberdare) and *H. lanceolatum* (Gakoe) than between *H. revolutum* (Mt. Kenya) and *H. revolutum* (Aberdare).

4. The crude hexane and chloroform extract of *H. revolutum* were shown to be toxic to brine shrimp. The activity observed in these extracts is due to hyperevolutin A, which is the major compound in these extracts. Hyperevolutin A has an LC_{50} value of 0.5 ppm. Such *in vivo* lethality against brine shrimp, *Artemia salina*, has been used in the discovery of new bioactive natural products [McLaughlin *et al.*, 1991]. There is usually a positive correlation between brine shrimp lethality and cytotoxicity on some cancer cells. Due to its high toxicity to brine shrimp this compound is a potential cytotoxic compound. Therefore, cytotoxicity of this compound against various cancer cells should be determined.

5. This compound has also shown anti-fungal activity against *Trichophyton mentanogrophyte* and *Saccharomyces cerevisiae*.

3.2 RECOMENDATIONS

1. The chemical structures of most of the chromatographic zones observed in this work were not established. Further phytochemical work should be done in order to characterize the compounds.

2. Most of the uncharacterized compounds were unstable in both normal and deactivated

silica gel. Rapid chromatographic techniques such as flash chromatography and/or preparative HPLC or other chromatographic techniques such as droplet countercurrent chromatography, which do not involve active solid support, should be used.

3. A very comprehensive phytochemical and chemotaxonomic, in addition to other taxonomic characters, study is required, however, before reaching conclusions on the relationship or non-relationship of these taxa.

4. Hyperevolutin A is found in large quantities in these taxa. This fact together with its easy detectability with fast blue B salt make this compound a very suitable chemotaxonomic marker. Chromatographic zones A and D, which were not identified in this work, has shown similar tlc pattern as hyperevolutin A. Characterization of these compounds might help in establishing the precursors of hyperevolutin A and hence the chemotaxonomic significance of these compounds.

5. Establishing whether the flavonoids isolated from *H. lanceolatum* (Gakoe) exist or not in *H. revolutum* (Aberdare) and *H. revolutum* (Mt. Kenya) will throw light on the relationship or non-relationship of these taxa.

6. There are certain member of the genus *Hypericum* which specialize in elaborating certain class of compounds than the other. An attempt should be made to establish that this is indeed the case, for all members of this genus, so that this information could be utilized in order to classify this genus into small groups.

7. The biological activity of this compound and the other compounds, which did not

show activity against the organisms tested, should be determined against other organisms.

CHAPTER FOUR

4.0 EXPERIMENTAL SECTION

4.1 GENERAL

The ^{1}H - and ^{13}C - NMR spectra were recorded on a Brucker AMX-400 spectrometer in $CDCl_3$, C_6D_5 and CD_3OD at the University of Strathclyde, in Scotland. Chemical shifts were measured in ppm in δ values relative to the internal standard tetramethyl silane (TMS). EI-Mass were recorded on a high resolution Varian 300 spectrometer at ICIPE.

Melting points were determined using a Gallenkamp melting apparatus with capillary tubes and are uncorrected. Merck Polyethylene plastic and aluminum precoated silica gel 60 PF 254 were used for analytical thin layer chromatography (tlc). Merck silica gel 60 PF 254 and silica gel 40 (70-230 mesh) were used for preparative tlc and column chromatography respectively.

4.2 PLANT MATERIAL

The bark, flowers and seeds of *H. lanceolatum* were collected from Gakoe along the roadside in November, 1995. The bark of *H. revolutum* was collected from Mt. Kenya in February 1995. The plant samples were colected and identified by Mr. Mathenge of the Department of Botany ,University of Nairobi. The plant samples were allowed to dry in open air and then ground to powder using a Willy mill machine.

4.3 CHROMATOGRAPHY

All solvents were distilled using an all glass fractional distillation apparatus before use. Silica gel and sephadex LH-20 were used for chromatographic analysis and separation. The silica gel used is either normal silica gel or silica gel impregnated with 3% oxalic acid solution in deionized water. The solvent systems used for both analytical and preparative tlc plates are shown below:

S-1 : C_6H_{14} : EtOAC : AcOH (75:20:5)

S-2 : C_6H_{14} : EtOAC : AcOH (65:30:5)

S-3 : CH_2Cl_2 : $(CH_3)_2CO$: MeOH (75:15:10)

S-4 : C_6H_{14} : EtOAC : AcOH (30:65:5)

S-5 : C_6H_{14} : EtOAC : AcOH (85:10:5)

S-6 : 2% MeOH in CH_2Cl_2

S-7 : 5% MeOH in CH_2Cl_2

4.3.1 Impregnation of the Silica Gel

4.3.1.1 Column Chromatography

About 1000 ml of 3% oxalic acid in deionized water was added on 500 g of silica gel G 40 (70-230 mesh). The mixture was stirred to a uniform slurry with a glass rod and allowed to stand for about 2 hours. The excess oxalic acid solution was filtered and the resulting wet silica gel was placed in an oven set at 120°C for 30 minutes.

4.3.1.2 Preparative tlc

About 20 g of preparative tlc grade silica gel was used to prepare a 20 x 20 cm^2 preparative tlc plate. The plates were prepared manually using exactly 2.5 ml of 3% oxalic acid solution in deionized water for every one gram of preparative tlc grade silica gel. The mixture was shaken thoroughly till a uniform slurry was formed. The uniform slurry was poured on to a 20 x 20 cm^2 glass plate and uniformly spread to 2 mm thickness with a spreading machine. The plate was allowed to dry overnight at room temperature, placed in an oven set at 120°C for 30 minutes and allowed to cool to room temperature before use.

4.3.1.3 Analytical tlc

Factory made analytical tlc plates were cut into the right sizes and dipped in 3% oxalic acid solution in methanol for about 5 minutes. The plates were then air dried and placed on a hot plate for about five minutes before use.

4.4 EXTRACTION OF THE STEM BARK OF *H. REVOLUTUM*

The stem bark of *H. revolutum* were broken manually into smaller pieces and allowed to dry in shade. The dried stem bark was ground into powder using a Willy mill. About 800 g of the powder was exhaustively extracted with hexane (3 x 2000 ml), chloroform (3 x 2000 ml) and methanol (2 x 2000 ml).

4.4.1 Isolation of Compounds from the Stem Bark of *H. revolutum*

The hexane extract was dried using rotary evaporator *in vacuo*. The extract showed a number of unresolved UV-active and non UV-active spots on analytical tlc plate (Solvent System S-2). Three major spots were observed (analytical tlc).

About 15 g of the hexane extract of *H. revolutum* was dissolved in chloroform and adsorbed on equivalent weight of silica gel. It was then dried *in vacuo* and transferred to a mortar and made into powder using a pestle. The powder was then subjected to column chromatography in a 4 cm diameter column packed with 300 g of silica G 60 (70-230 mesh) packed in hexane. Elution was performed starting from hexane with increasing amounts of dichloromethane and then of methanol. Eluents of 500 ml each were collected initially and then changed to 250 ml of eluents. The eluents collected were spotted on analytical tlc plates and developed with Solvent Systems S-5, S-1 and S-2.

Eluents containing the same components were pooled together and concentrated *in vacuo*. The first six fractions basically contained fatty materials.

The eluents eluted with 50% dichloromenthane in hexane were combined, dried *in vacuo* and subjected to column chromatography on silica gel impregnated with 3% oxalic acid solution in deionized water. This column was eluted with hexane/dichloromethane solvent system in order of increasing polarity. The eluents were monitored on an analytical tlc using [C_6H_{14}:CH_2Cl_2(3:2)] as a solvent system. The eluents eluting with 50% dichloromethane in hexane contain one major compound and some other minor compounds. These eluents were combined, dried *in vacuo* and were subjected to further purification on a deactivated silica gel column. This led to the isolation

of 25 mg of a yellow uncharacterized compound SDW-15K. SDW-15K intensifies in ammonia vapor and has an R_f value of 0.34 [C_6H_{14}:CH_2Cl_2(3:2)].

About 3 g of the other set of fractions eluted with 50% dichloromethane in hexane were subject to flash column chromatography. The eluents containing one major yellow compound (analytical t.l.c) were combined, dried *in vacuo*, and was applied on sephadex LH-20 column and eluted with [$CHCl_3$:MeOH (1:1)]. This was then further purified on preparative tlc plate (solvent system,[C_6H_6:CH_2Cl_2(1:1)] this led to the isolation of 30 mg of an unidentified compound, SDW-22B. SDW-22B is a yellow compound with an Rf value of 0.2 [C_6H_6:CH_2Cl_2(1:1)].

The eluents eluted with 4-10% MeOH in CH_2Cl_2 contain a major compound and some other minor compounds. These were pooled together and concentrated *in vacuo* and subjected to column chromatography on a silica gel impregnated with 3% oxalic acid in deionized water. Thirteen eluents of approximately 150 ml each were collected. The first three eluents did not contain the major compound. The other eluents contained mainly the major compound and two other minor compounds and therefore were combined. About 1.3 g of this fraction was subjected to column chromatography on deactivated silica gel using hexane, dichloromethane solvent system with increasing order of polarity.

The eluents eluting with 40-100% dichloromethane in hexane showed to contain the major compound (tlc analysis). These were pooled together and dissolved while heating in small amount of dichloromethane and then a small amount of hexane was added till it just clouds. It was then heated in a water bath till the cloud disappeared and then was left to crystalize. About 270 mg of crystalline compound, the major and minor compounds **1** and **2** respectively, crystallized out, with a melting point of 140-142°C (uncorrected). Compound **1** and **2** are UV

(254 mm) active compounds with Rf value of 0.38 in S-2.

Compound **1** showed infra-red absorption peaks (cm^{-1}) in nujol at 3100-2900, 1700, 1640, 1510, 1500, 1420 and 1290. The mass spectrum showed peaks at *m/z* values of 468 (M^+), 425, 357, 277, 263, 209, 109, 69 (base peak), 43, 41. The ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 Mhz) data of this compound is shown in Table 2.1.1. While compound **2** showed two additional small doublets peaks at 3.69 and 3.50 ppm on the ^{1}H-NMR spectrum.

About 17 g of the chloroform extract of *H. revolutum* has been subjected to column chromatography on deactivated silica gel. The column was eluted with hexane/dichloromethane solvent system. The eluents eluting with hexane were combined (analytical tlc) and was applied on sephadex LH-20 column and eluted with [$CHCl_3$:MeOH (1:1)]. About 20 mg of white crystalline compound, compound **3**, crystallized in acetone. Compound **3** is a non UV active compound which turns yellow in iodine chamber with an Rf value of 0.41 in [($CHCl_3$:EtOAC(6:1)]. The mass spectrum of this compound showed peaks at *m/z* 412 (M^+, 6.31%), 394 ($(M-H_2O)^+$, 0.87%), 351 ($(M-C_3H_7-H_2O)^+$, 1.47%) 300 (1.97%), 273 ((M-Side chain)$^+$, 5.43%), 255 ((M-Side chain-H_2O)$^+$, 8.65%) 213 (9.97%), 159 (10.29%), 105 (10.10%), 83 (16.41%), 55 (78.79%). The chemical shift for the ^{1}H- and ^{13}C-NMR of this compound is shown in Table 2.1.2.

4.5 EXTRACTION OF THE STEM BARK OF *H. LANCEOLATUM*

The stem bark of *H. lanceolatum* was cut into smaller pieces, allowed to dry in shade and was ground into powder using a Willy mill machine.

Approximately 1.4 Kg of the powder was exhaustively extracted with (3 x 2500 ml) of hexane, (3 x 2500 ml) of chloroform and (2 x 2500 ml) of methanol.

4.5.1 Isolation of Compounds from the Stem Bark of *H. lanceolatum*

About 12 g of the hexane extract was subjected to column chromatography on a 3 cm diameter column packed with deactivated silica gel. The column was eluted with hexane/ethyl acetate solvent system with increasing polarity. The eluents were spotted on analytical tlc plates and developed using Solvent System S-5, S-1 and S-2. Eluents with similar tlc patterns were combined together and concentrated *in vacuo*.

The first fraction which was eluted with 2% ethyl acetate in hexane contained a white non UV active precipitate and a colorless UV active compound. This fraction was washed with chloroform under suction using a buchner funnel to get rid of most of the white non UV active compound, which was kept for further analysis. The mother liquor was dried *in vacuo* and applied on sephadex [$CHCl_3$:MeOH,(1:1)] column. The UV active compound was further purified using preparative tlc [C_6H_4:CH_2Cl_2 (1:1)] and afforded 22 mg of a compound labelled as SDW-26Q. This uncharacterized compound had an Rf value of 0.3 (CH_2Cl_2) it turned yellow initially when exposed to ammonia and turned brown after sometime.

The last fraction eluted with 2% EtOAC in hexane had a white precipitate in a yellow mother liquor. This was washed similarly with chloroform under suction. This white precipitate was combined with the previous one. The yellow mother liquor was dried *in vacuo* and subjected to column chromatography on a deactivated silica gel column and eluted with hexane/dichloromethane/methanol solvent system with increasing order of polarity. The eluents

eluting with 80% dichloromethane in hexane were dried *in vacuo* and applied on sephadex LH-20 [($CHCl_3$: EtOH (1:1)] column. This led to the isolation of about 30 mg of the uncharacterized compound SDW-29J. SDW-29J had an Rf value of 0.26 (1% MeOH in CH_2Cl_2).

The above non-UV active white precipitate was thoroughly washed under suction with chloroform. It was then dissolved in hot methanol and allowed to crystalize. About 800 mg of white needle-like crystals of compound **4** crystallized out. Compound **4** had an Rf value of 0.5 (5% MeOH in CH_2Cl_2). It turned yellow in iodine vapor and black when sprayed with vanillin sulfuric acid. This compound showed infra-red absorption peaks (cm^{-1}) in nujol at 3420, 3400-3100, 1670, 1630, 1230. Its mass spectrum showed peaks at *m/z* values of 456 (M^+,10.24%), 438 (5.28%), 423 (4.57%), 410 (3.38%), 248 (28.35%), 220 (14.24%), 219 (13.98%), 207 (34.54%) . The ^{1}H-NMR displayed peaks at 4.67 (1H, d), 4.53 (1H, d), 4.25 (1H, dd), 1.6 (3H, s), 0.9 (3H, s), 0.8 (6H, s), 0.7 (3H, s), and 0.6 ppm (6H, s). The ^{13}C-NMR of this compound is shown in Table 2.1.3.

About 30 g of the chloroform extract was subjected to column chromatography in a 4 cm diameter deactivated silica gel column. The column was eluted with hexane/dichloromethane/ethyl acetate/methanol solvent system in order of increasing polarity. The eluents were spotted on an analytical tlc plate and developed using Solvent Systems S-5, S-1 and S-2. Eluents with similar tlc patterns were combined together and concentrated *in vacuo*.

The eluents eluted with 70-100% dichloromethane in hexane were combined, dried *in vacuo* and subjected to Vacuum Liquid Chromatography (VLC) starting from hexane to remove some fatty materials. The fractions eluted with 30% ethyl acetate in hexane contained mainly an oily compound. This was further purified using preparative tlc (ptlc) [(C_6H_{14}:CH_2Cl_2 (1:1)] and

afforded about 25 mg of SDW-38H. SDW-38H has an Rf value of 0.67 [C_6H_{14}:CH_2Cl_2(3:7)]. This an unidentified compound is UV active at 254 nm but it does not intensify on exposure to ammonia vapor.

The eluents eluted with 5% ethyl acetate in dichloromethane were combined and dried *in vacuo*. This fraction was subjected to column chromatography on silica gel using a dichloromethane/ethyl acetate solvent system. Eluents containing one major UV active compound and some other minor compounds were combined and applied on sephadex LH-20 [($CHCl_3$:MeOH (1:1)] column. This was then further purified on preparative tlc (ptlc) [S-5] and this led to the isolation of 25 mg of an uncharacterized compound SDW-34L. SDW-34L is a UV active compound with an Rf value of 0.67 [S-5].

4.6 EXTRACTION OF THE FLOWERS AND SEEDS OF *H. LANCEOLATUM*

The flowers and seeds of *H. lanceolatum* were allowed to dry in shade. The dried seeds and flowers were ground into powder using a Willy mill . About 400 g of the powder was exhaustively extracted with dichloromethane (3 x 1500 ml), acetone (3 x 1500 ml) and methanol (2 x 1500 ml).

4.6.1 Chromatography of the Extracts of the Flowers and Seeds of *H. lanceolatum*

All the extracts were dried using rotary evaporator *in vacuo*. The dichloromethane extract contains mainly fatty materials and chlorophyll (tlc analysis) and therefore was not analyzed any further. The acetone and methanol extracts showed similar tlc patterns and therefore were

combined. This combined extract was spotted on analytical tlc plate and was developed using Solvent System S-3. A number of UV active spots were observed, out of which four of them intensified when exposed to ammonia vapor and turned permanently to a different color when sprayed with ferric chloride solution. This is a peculiar property of phenolic compounds, and therefore these compounds were targeted.

About 10 g of this extract was adsorbed on equivalent amount of silica gel impregnated with 3% oxalic acid solution in deionized water. This was then subjected to column chromatography on a 3 cm diameter column packed with silica gel impregnated with 3% oxalic acid solution in deionized water. The column was packed in 5% ethyl acetate in hexane and then was run at a high flow rate since the less polar compounds were not targeted. It was run in this manner until 800 ml of 30% ethyl acetate in hexane has run through the column. Monitoring of the eluents collected up to now with analytical tlc using Solvent System S-3 showed that none of these eluents contained the phenolic compounds sought.

The column was then run at a normal rate with 40% of ethyl acetate in hexane. This continued with increasing order of polarity until 2 liters of 100% of ethyl acetate has run through the column. All the eluents collected were monitored using analytical tlc plate developed using Solvent System S-3. The phenolic compounds were located using ammonia vapor and/or ferric chloride solution. Eluents containing these phenolic compound were combined, concentrated *in vacuo* and applied on sephadex LH-20 column and eluted using [MeOH:EtOH (1:1)] as a solvent system.

About 14 eluents of 100 ml each were collected. These were spotted on analytical tlc plate and

developed with Solvent System S-3. The result showed that eluents 1 and 2 are blank, eluent 3 contained about 150 mg of a yellow phenolic compound, compound **5**. Compound **5** had an Rf value of 0.35 (Solvent System S-4) and a melting point of 258-260°C. The chemical shift for the ^{1}H and ^{13}C-NMR of this compound is shown in Table 2.2.1. The UV spectrum showed UV max(MeOH, nm) 330, 295sh, 274; 0.2 M NaOH 388, 320, 270; $AlCl_3$ 276, 304sh, 350, 385sh; NaOAc 285, 373; NaOAc/H_3BO_3 274, 330. Eluent 4 was a mixture, eluent 5 contained about 70 mg of a yellow pure phenolic compound, Compound **6**. Compound **6** had an Rf value of 0.56 (Solvent System S-4) and a melting point of 314-315°C. The chemical shifts for the hydrogens and carbons of this compound is shown in Table 2.2.2. It has the following UV spectroscopic characteristics: UV max(MeOH, nm) 372, 304sh, 270sh, 255; 0.2 M NaOH 325(dec), 251sh; NaOAc 390, 330, 275, 255sh; NaOAc/H_3BO_3 392, 305sh, 260. Eluents 6 to 11 contained a mixture, while eluents 12 to 14 contained a pure phenolic compound, Compound **7**. Compound **7** had an Rf value of 0.45 (Solvent System S-4) and a melting point of 357-360°C. The chemical shift for the ^{1}H- and ^{13}C-NMR of this compound is shown in Table 2.2.3. This compound showed is characterized by the following UV characteristics: UV max (MeOH/nm) 370, 304sh, 270, 256; 0.2 M NaOH 420(dec), 325, 280sh; $AlCl_3$ 450, 410sh, 270; $AlCl_3$ + HCl 425, 358sh, 270sh, 265; NaOAc 330, 270.

4.7 BIOLOGICAL ACTIVITY TESTS

4.7.1 Brine shrimp lethality test

Artificial sea water was prepared by dissolving 38 g of sea salt in one liter of water. This was added to a perforated tank which is divided into two portions. *Artemia salina* (brine shrimp) eggs were added to one side of tank which is covered from light.

This is kept in a dark cabin. The nauplii hatched from the eggs (after 48 hours) swim through the perforations into the part of the tank which is not covered and therefore exposed to a 25 watt lamp light. Test solutions were prepared at 0.5, 1, 5, 10, 100, and 1000 ppm in 3 ml vials from a stock solution of 10 mg of sample dissolved in one milliliters of chloroform. A control experiment was performed in vials with no samples, other components remaining constant. The chloroform was allowed to dry over night and 10 nauplii were added into each vial. The vials were then topped with the artificial sea water to a 3 ml level mark. The number of nauplii alive were counted after 24 hours. The experiment was considered successful when the percentage of lethality in controls is lees than 10%. This bench top lethality test is developed by McLaughlin *et al.*[1991].

4.7.2 Antimicrobial tests

4.7.2.1 Media preparation

For the antifungal tests 62 g of sabouraud dextrose agar was dispersed in 1 liter of deionized water and soaked for 10 minutes. This was swirled and the mixture sterilized at 121°C for 15 minutes. It was then allowed to cool to 47°C and transferred into petridishes (10 ml each) and left to cool in a laminar flow cabinet before use. The procedure was repeated for the anti-bacterial tests but using Mueller Hinton agar (38 g/l).

Exactly 1 mg of each of the compounds isolated, that is compound 1, 3, 4, 5, 6 and 7 were dissolved in 1 ml of suitable solvent. From this stock solutions 100μl, 50μl and 10μl were aliquoted corresponding to 100 μg, 50 μg and 10 μg of samples respectively.

The compounds were tested (in triplicate) against two gram positive bacteria; *Bacillus subtilis*

and *Staphylococcus aureus*, and four species of fungus; *Candida albicans*, *Trichophyton mentanogrophyte*, *Microsporium gypsum* and *Saccharomyces cerevisae*.

The appropriate medium was inoculated with the test micro-organisms on radial axes starting from the center of the medium. Blank discs (5 mm diameter) were placed on the surface of the medium and the various concentrations were injected into the discs using a syringe and then incubated. The incubation for the fungi was run at 26°C and the fungal growth was monitored for 72 hours. The incubation temperature for the bacteria was 30°C and it was run for 24 hours after which the plates were checked for anti-bacterial activity.

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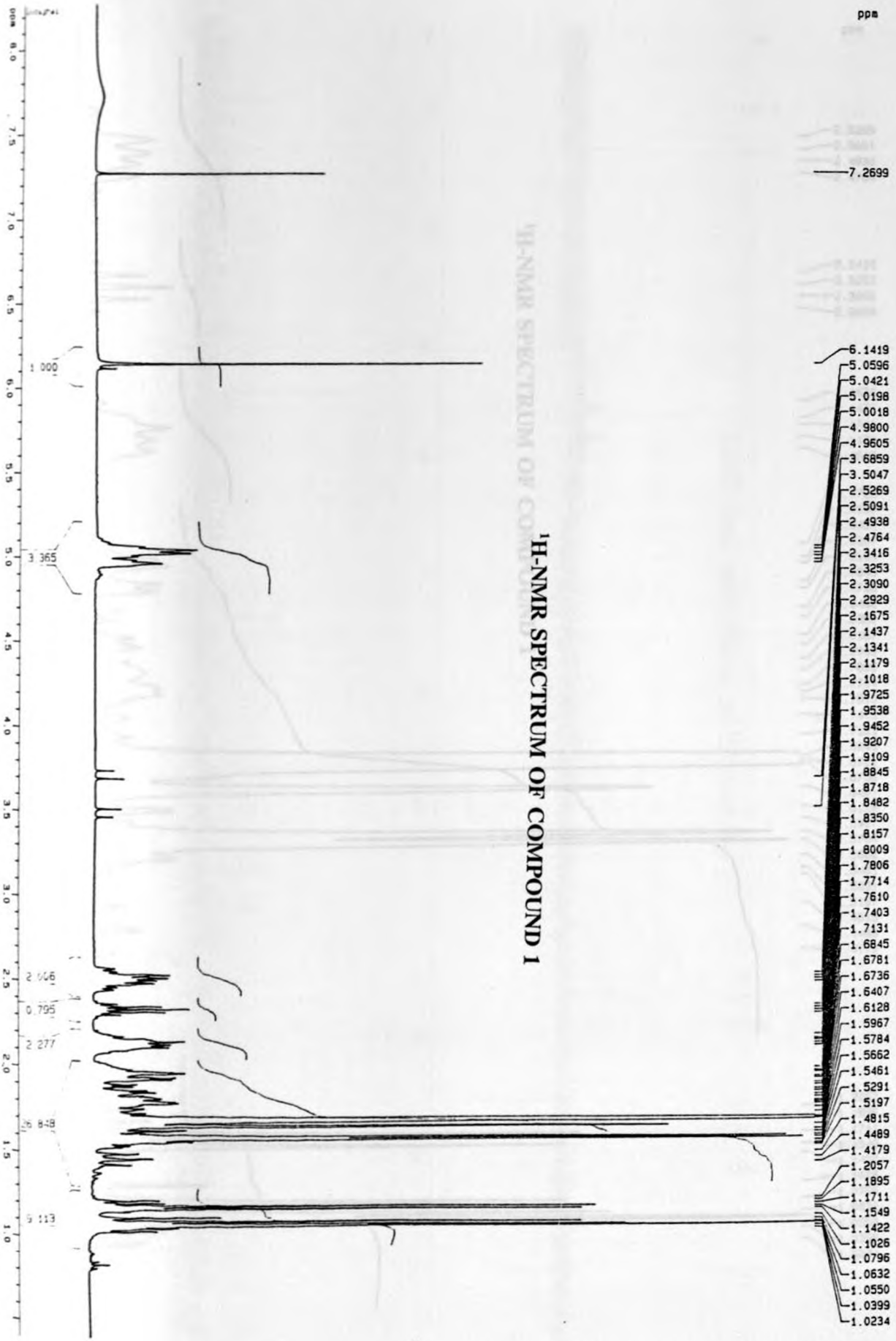
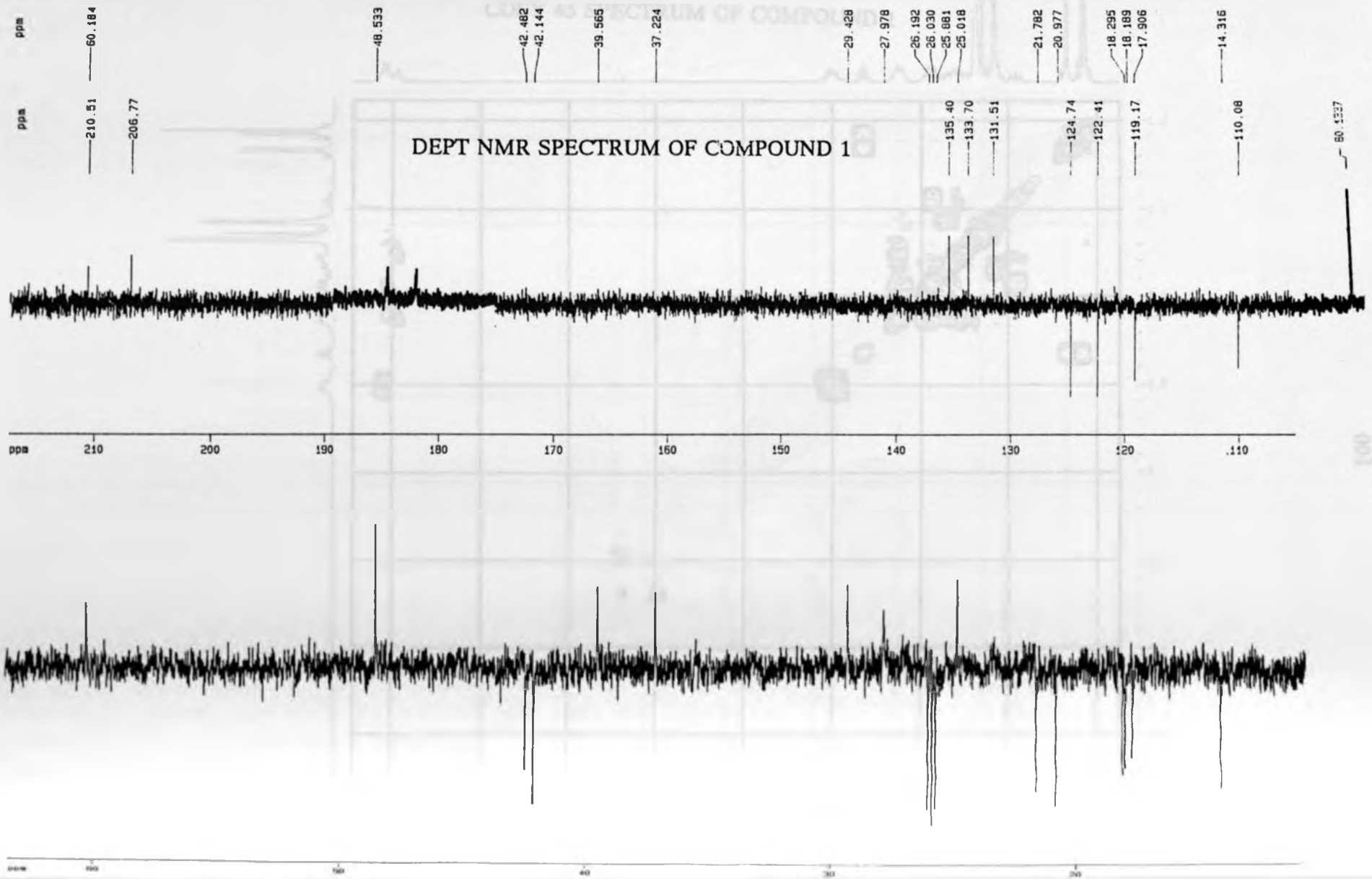


^{1}H-NMR SPECTRUM OF COMPOUND 1

^{1}H-NMR SPECTRUM OF COMPOUND 1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20.977
18.295
18.189
17.906
14.316
ppm
210.51
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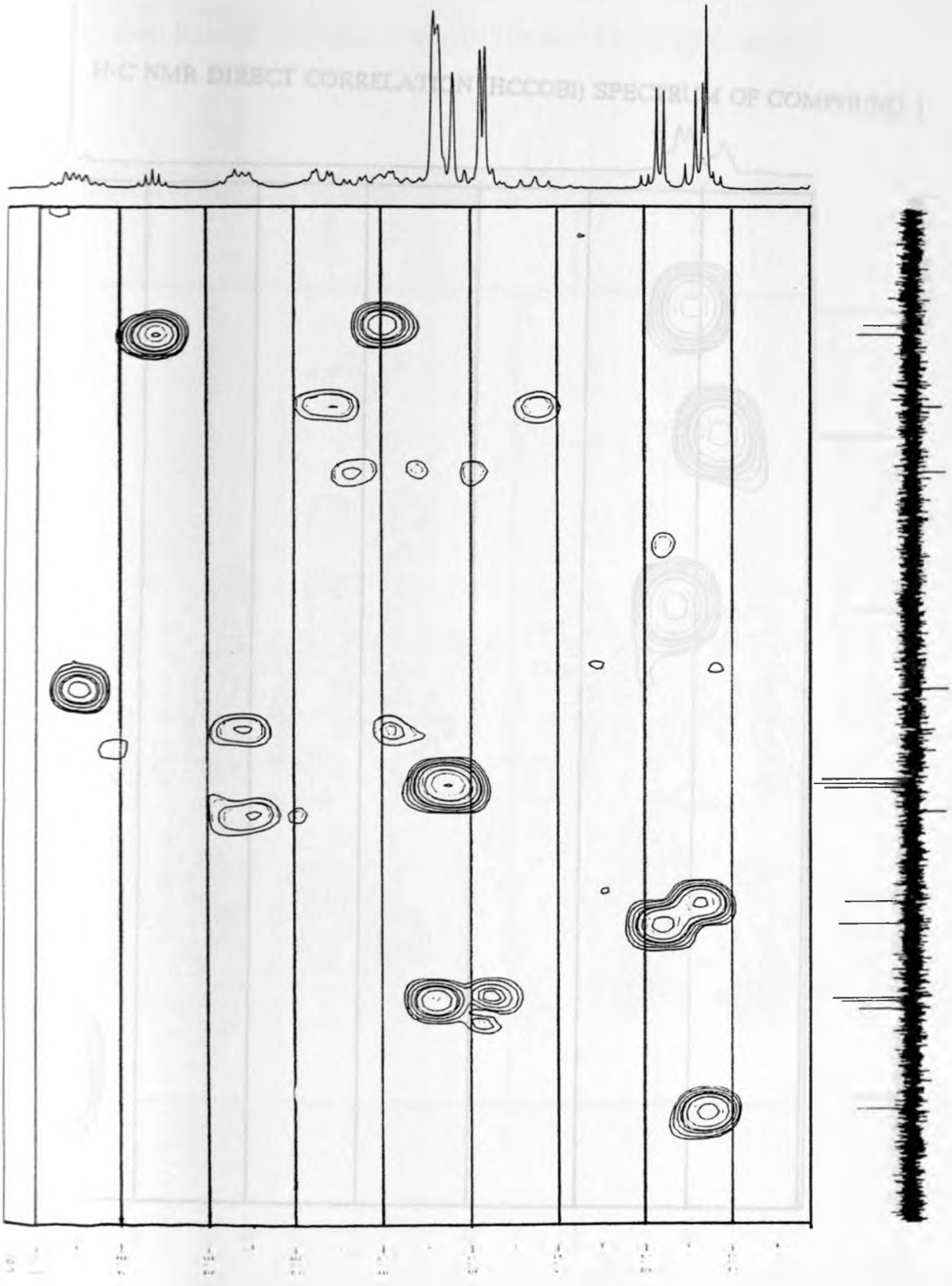
DEPT NMR SPECTRUM OF COMPOUND 1
ppm
210
200
190
180
170
160
150
140
130
120
110

COSY 45 SPECTRUM OF COMPOUND 1

COSY 45 SPECTRUM OF COMPOUND 1

H-C NMR DIRECT CORRELATION (HCCOBI) SPECTRUM OF COMPOUND 1



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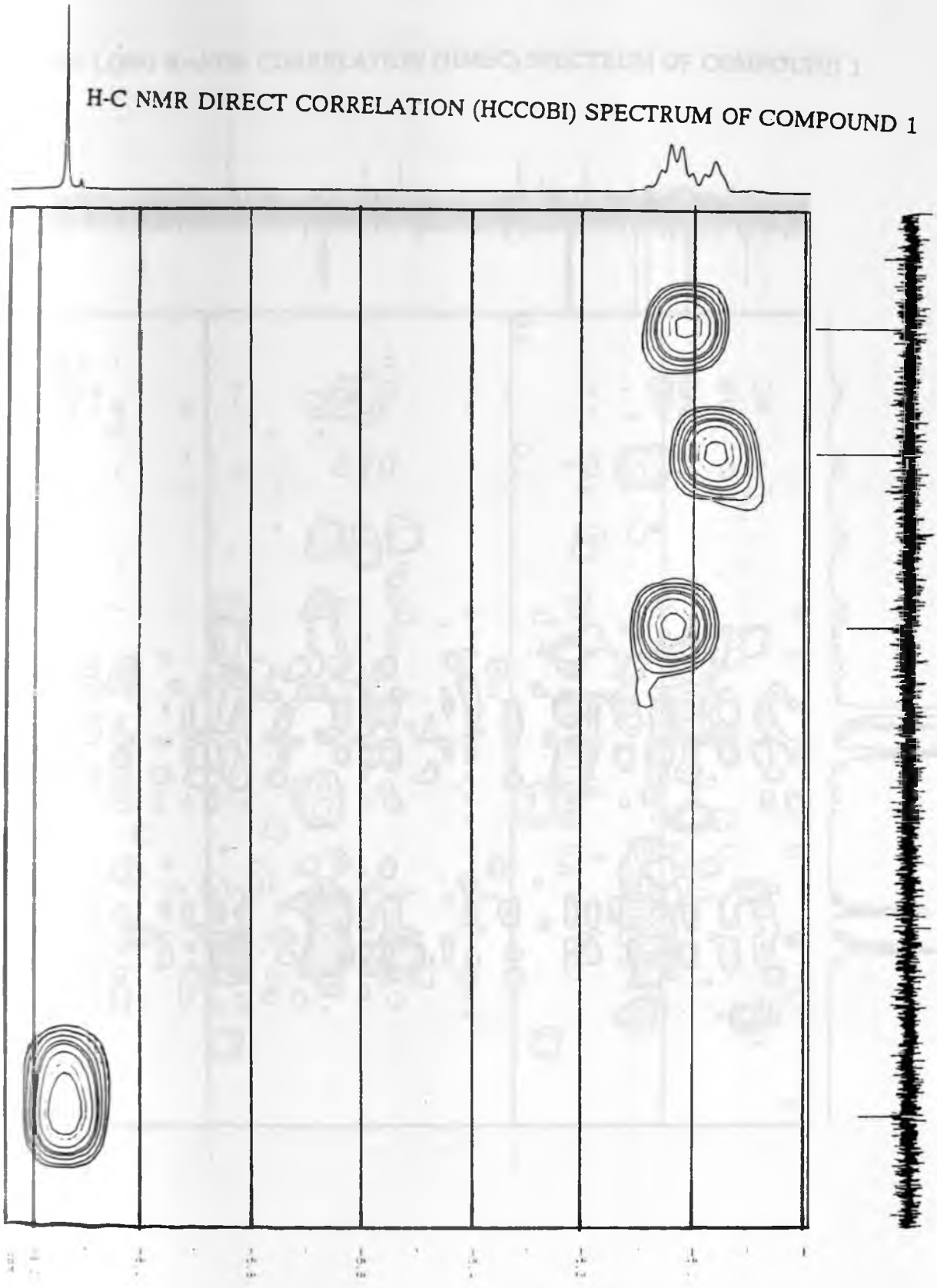

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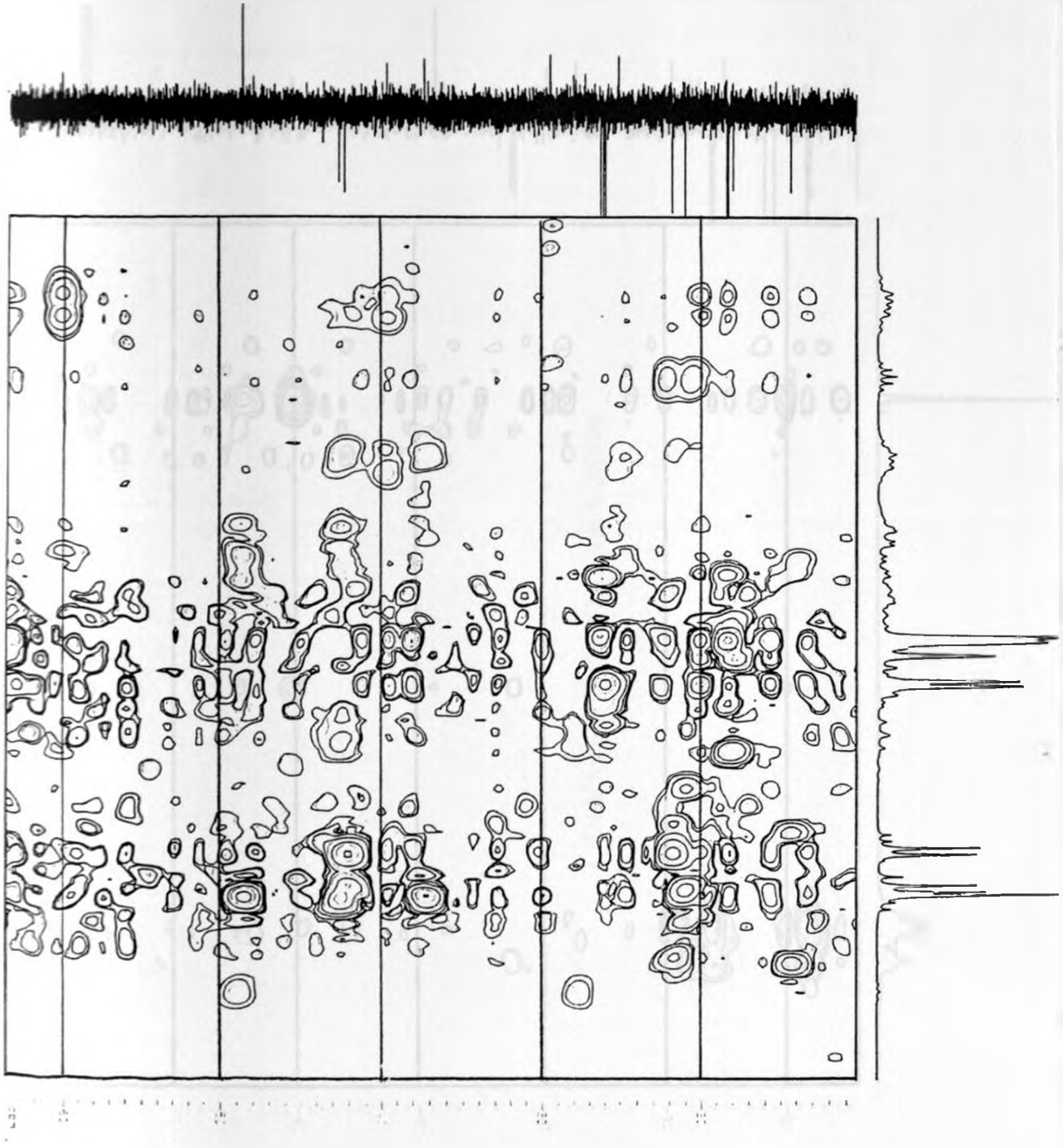

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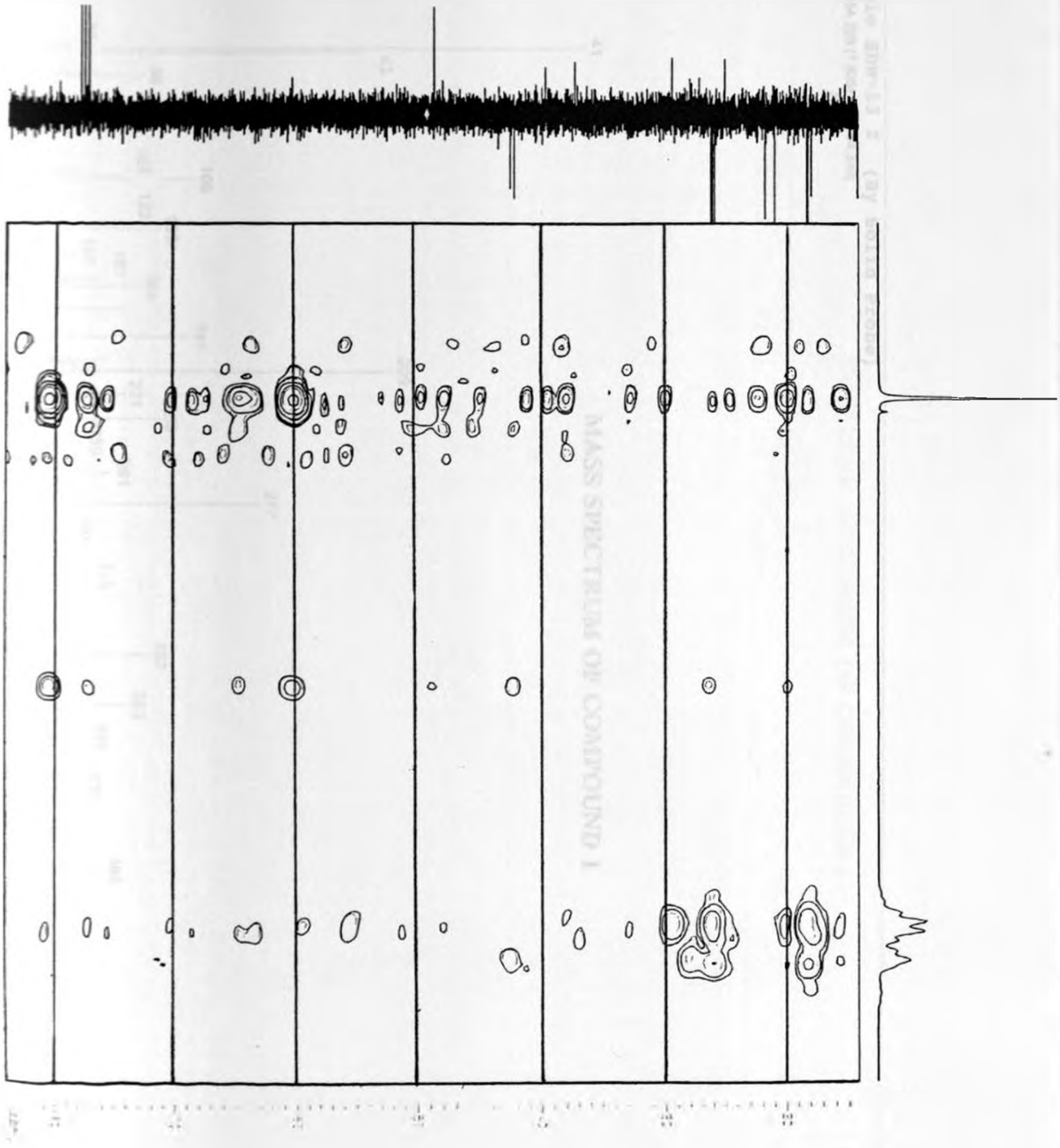
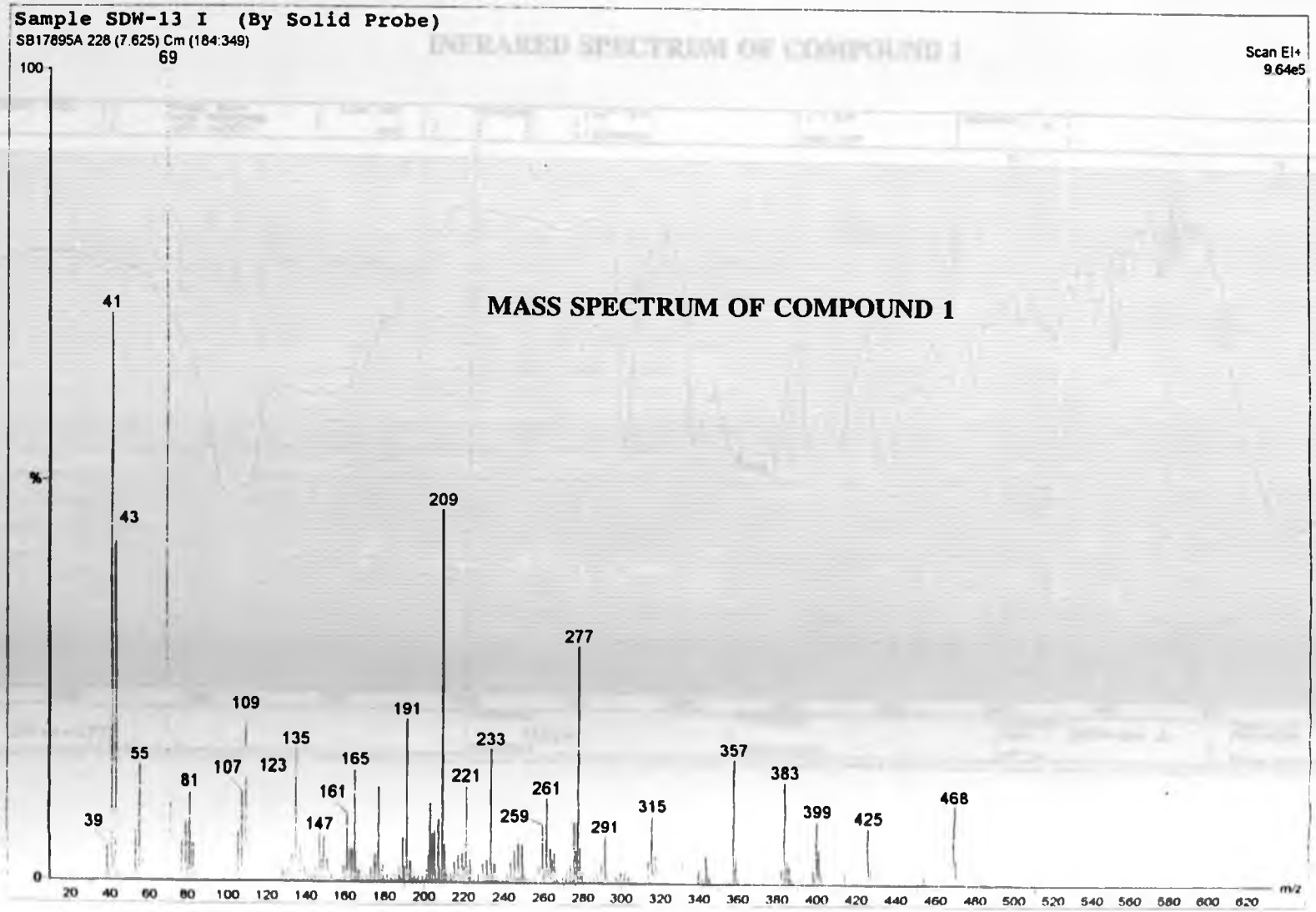
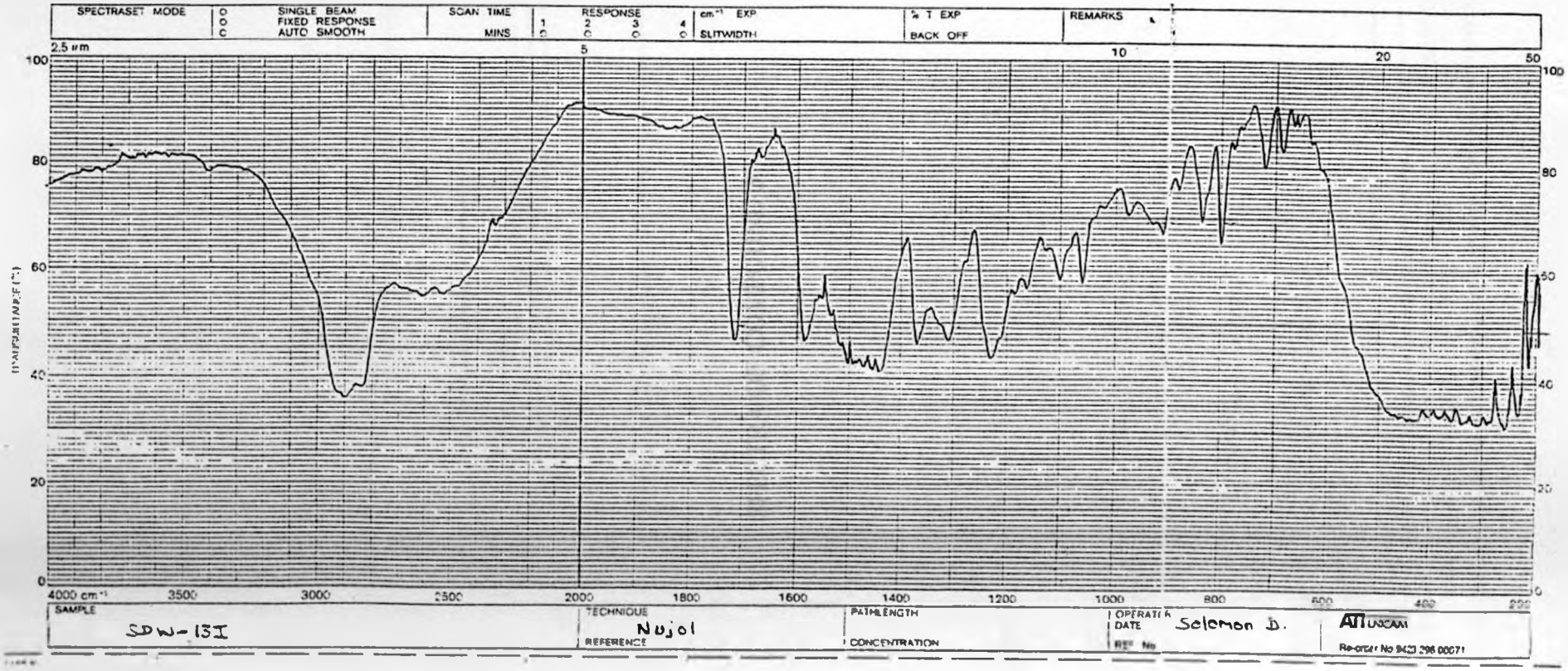




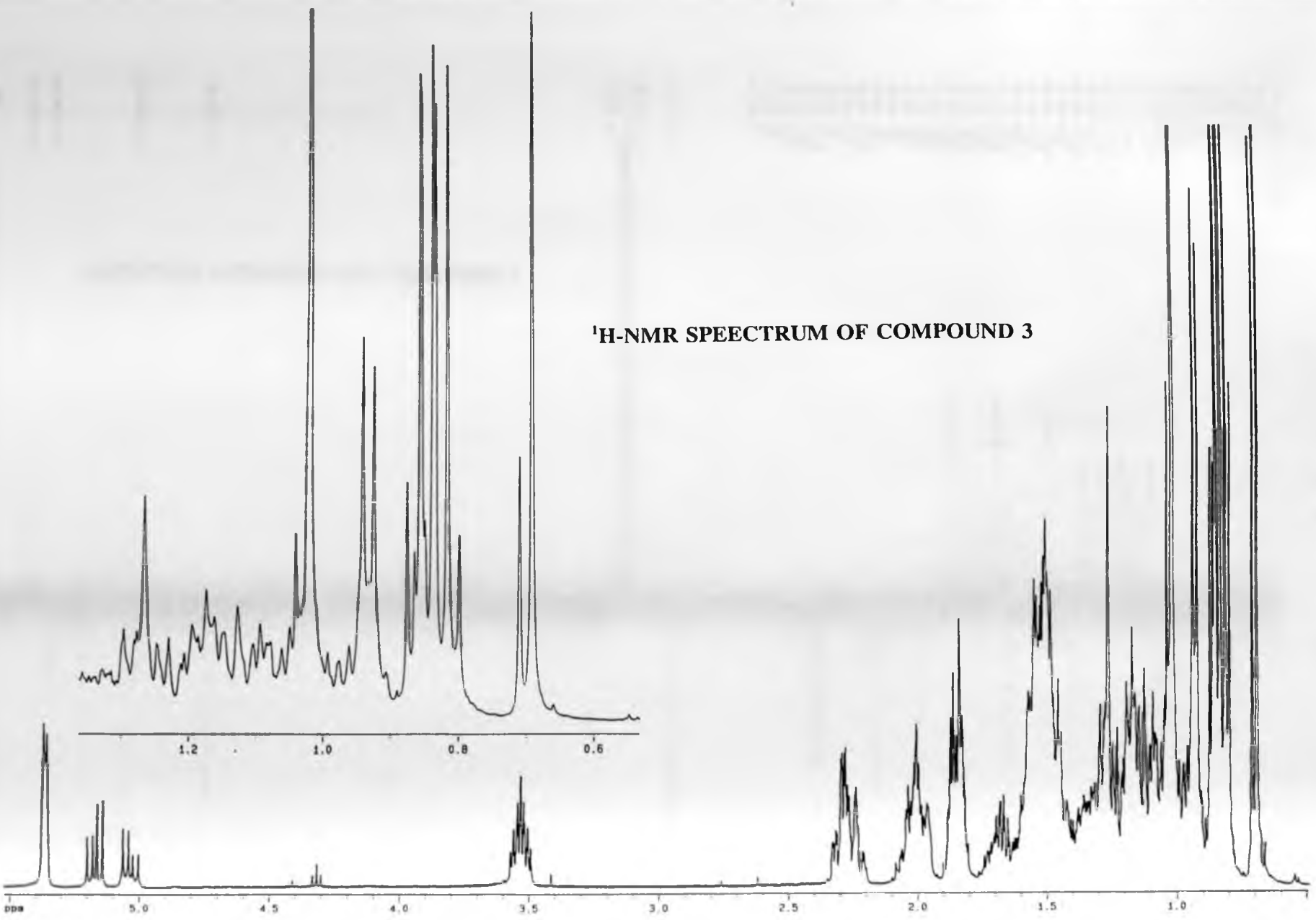
MASS SPECTRUM OF COMPOUND 1

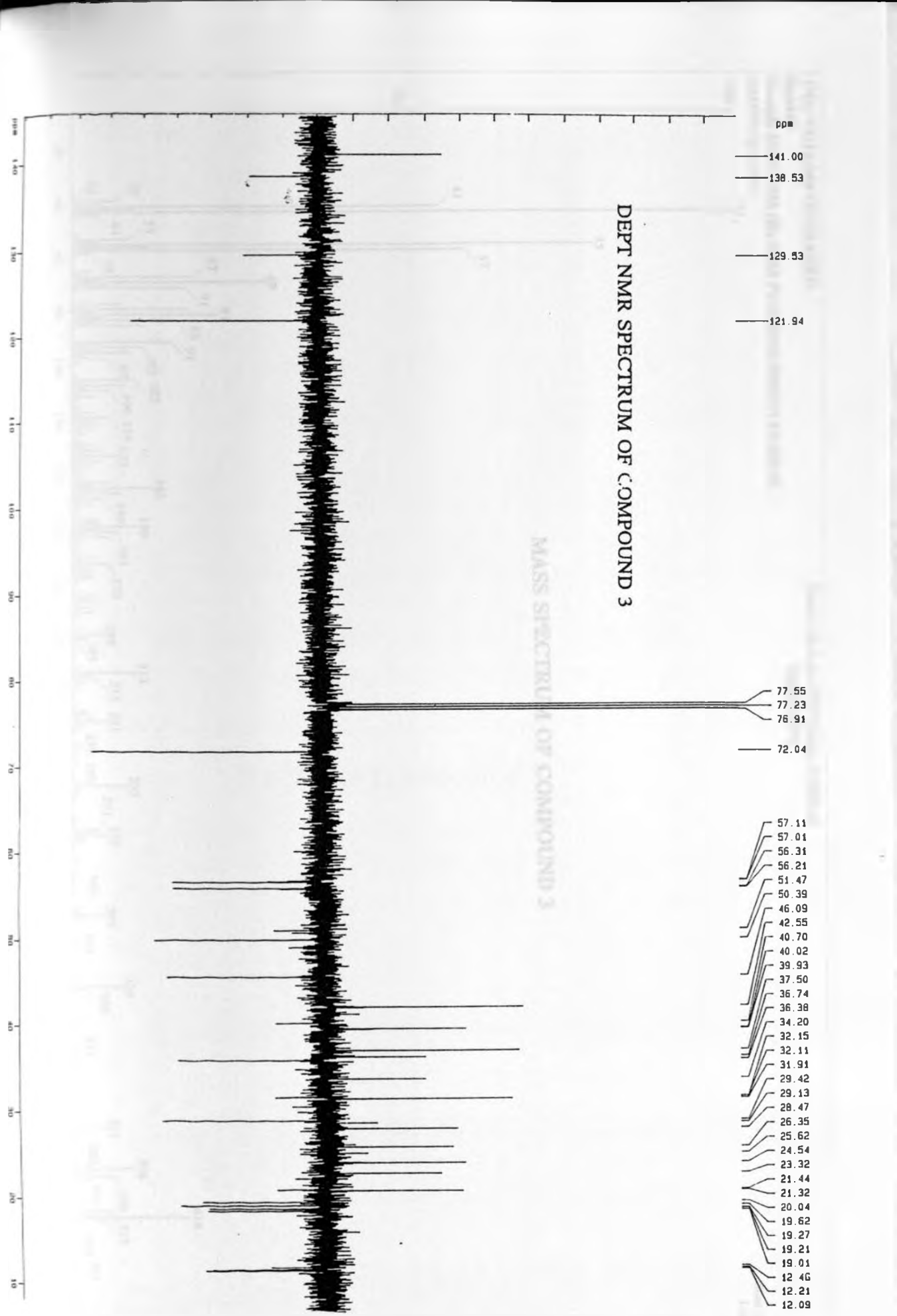
INFRARED SPECTRUM OF COMPOUND 1



SPECTRA OF COMPOUND 3

^{1}H-NMR SPEECTRUM OF COMPOUND 3

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42.55
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40.02
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37.50
36.74
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31.91
29.42
29.13
28.47
26.35
25.62
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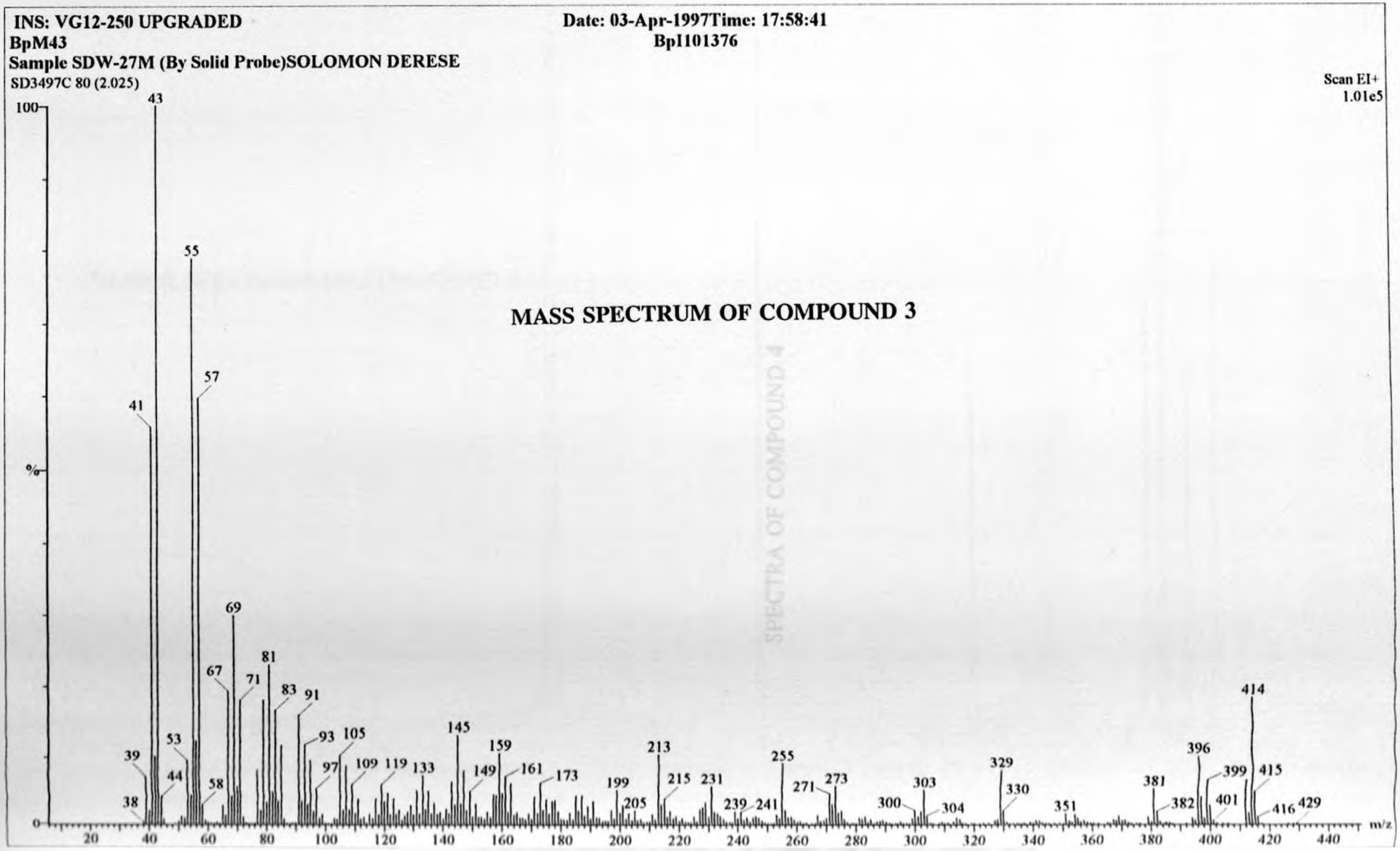

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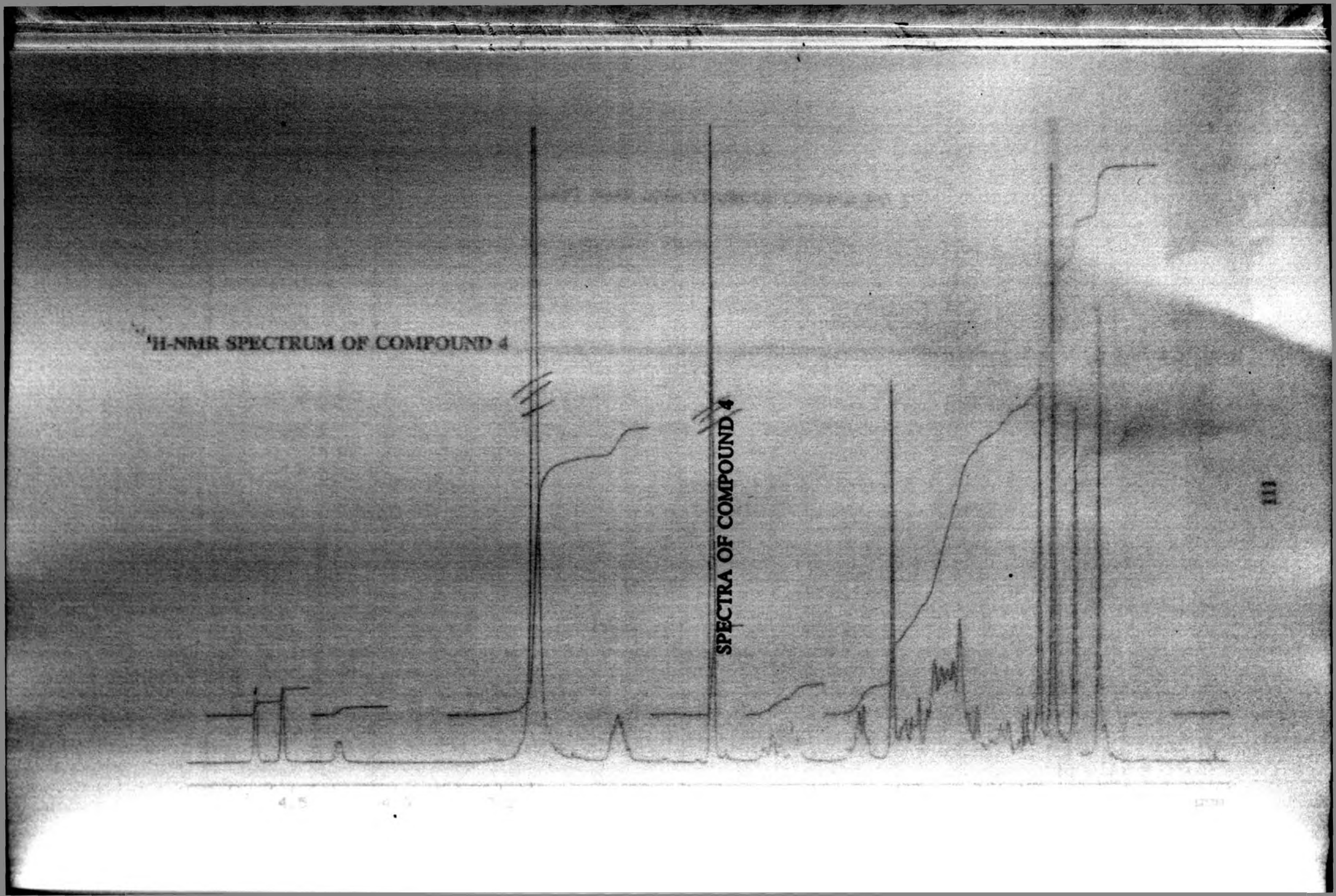
^{1}H-NMR SPECTRUM OF COMPOUND 4
SPECTRA OF COMPOUND 4

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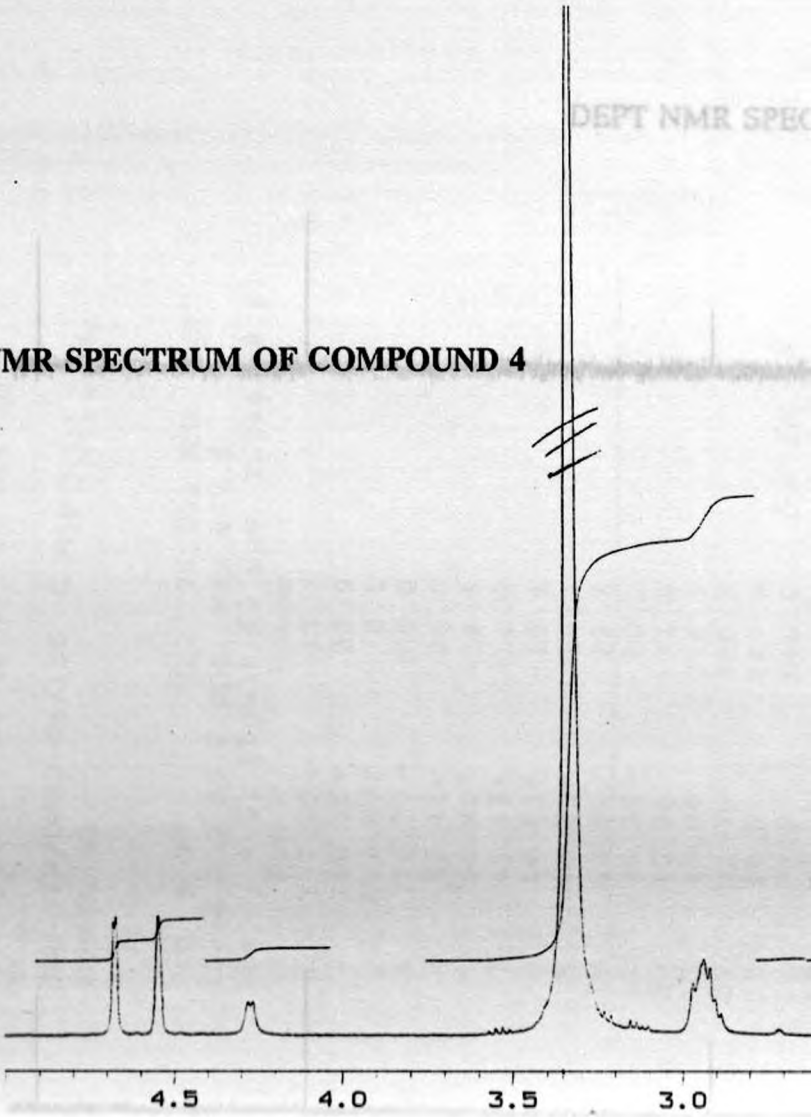

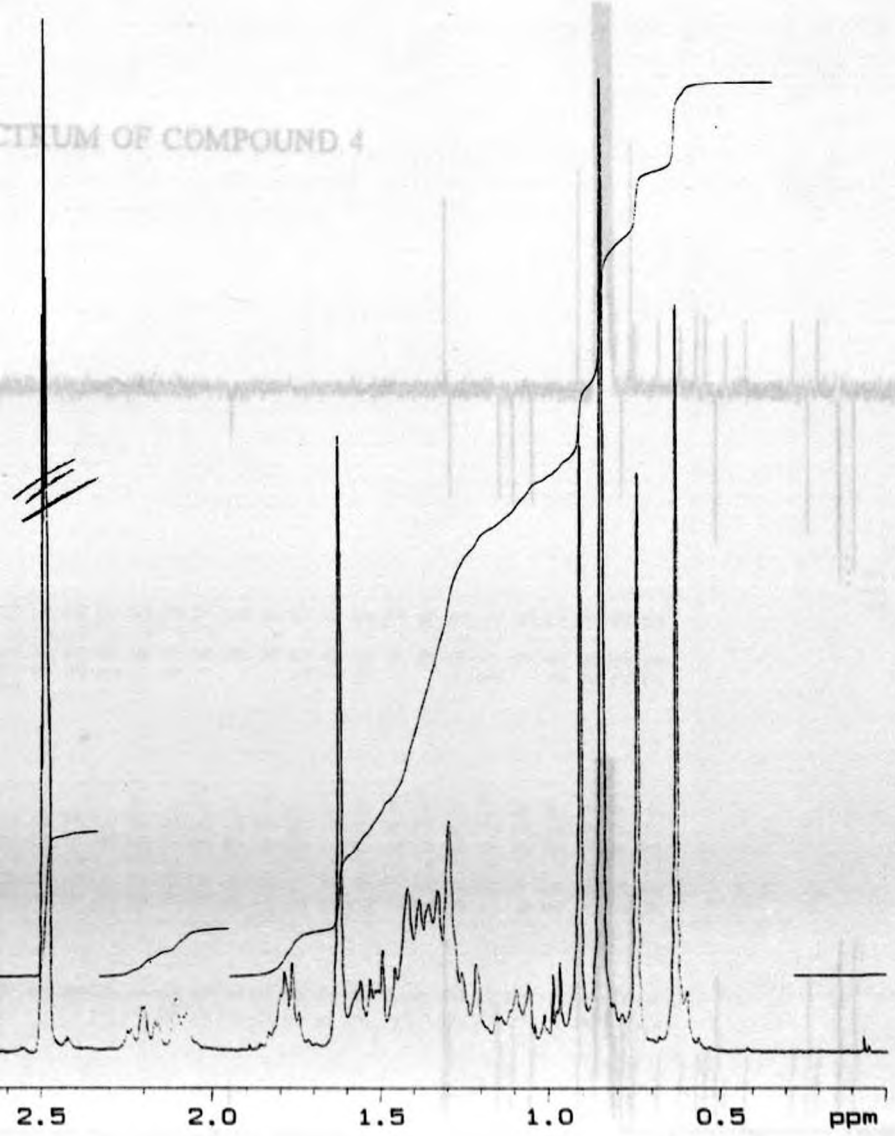
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ppm

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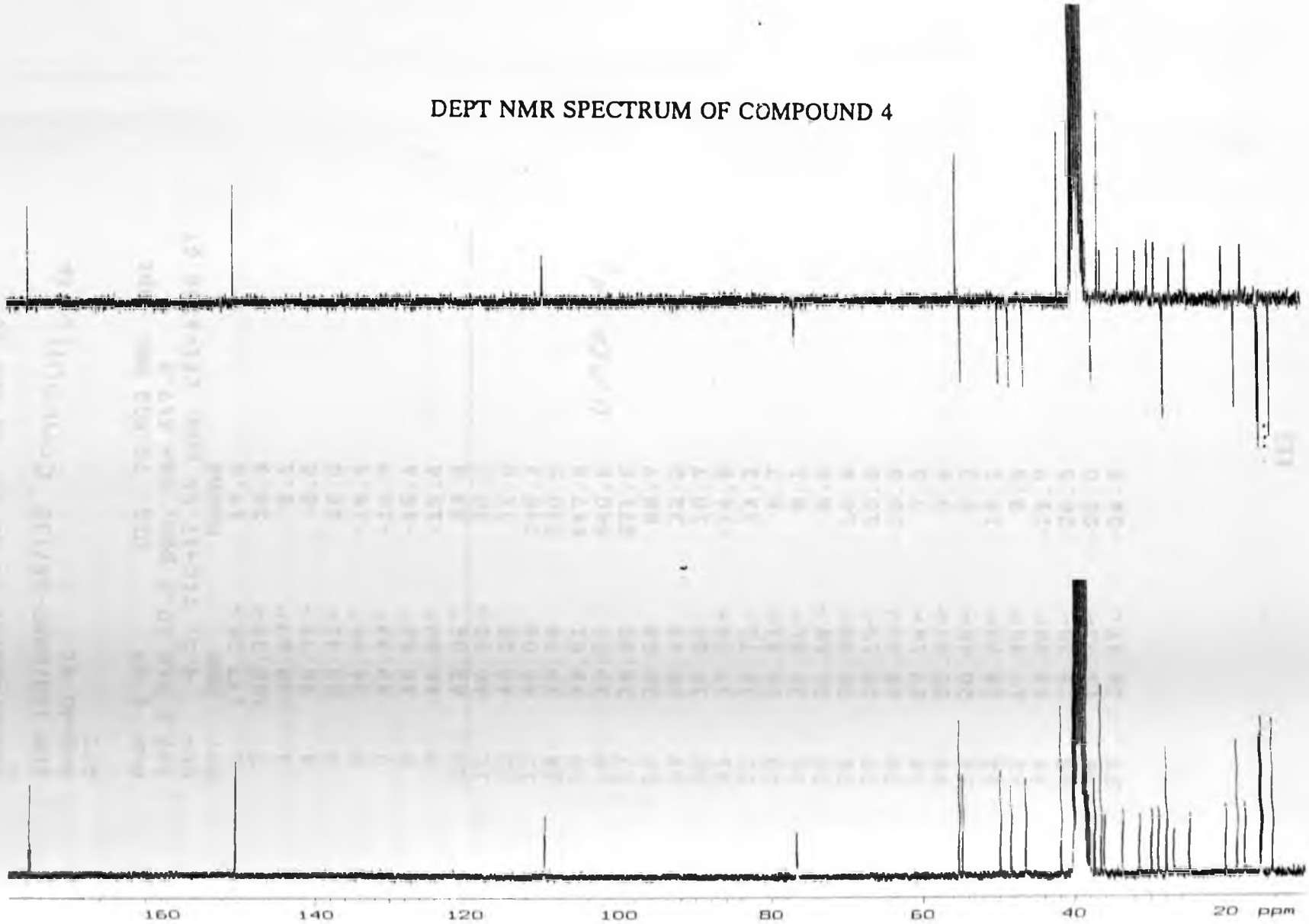

Halle/Saale, 05-02-97 08 Uhr 30

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Angedu 41
APT

Feb 4 97 C13 75.502 MHz apt
180.0 bis 10.0 ppm, vs= 647.8
th= 4.5, rfp=39.50 ppm, rfl=4138.67

Nr.	ppm	Hoehe
1	177.25	17.6
2	150.35	20.9
3	109.67	8.1
4	76.79	-8.0
5	55.41	26.0
6	54.90	-14.4
7	49.93	-14.9
8	48.53	-15.4
9	46.62	-15.4
10	42.00	29.8
11	40.35	80.2
12	40.25	31.8
13	40.06	258.3
14	39.79	520.5
15	39.51	647.8
16	39.23	540.6
17	38.95	271.6
18	38.68	88.7
19	38.49	22.2
20	38.26	10.7
21	37.59	-14.5
22	36.72	33.2
23	36.33	8.7
24	33.91	9.1
25	31.69	8.6
26	30.09	10.6
27	29.19	10.0
28	28.09	-20.9
29	27.14	7.3
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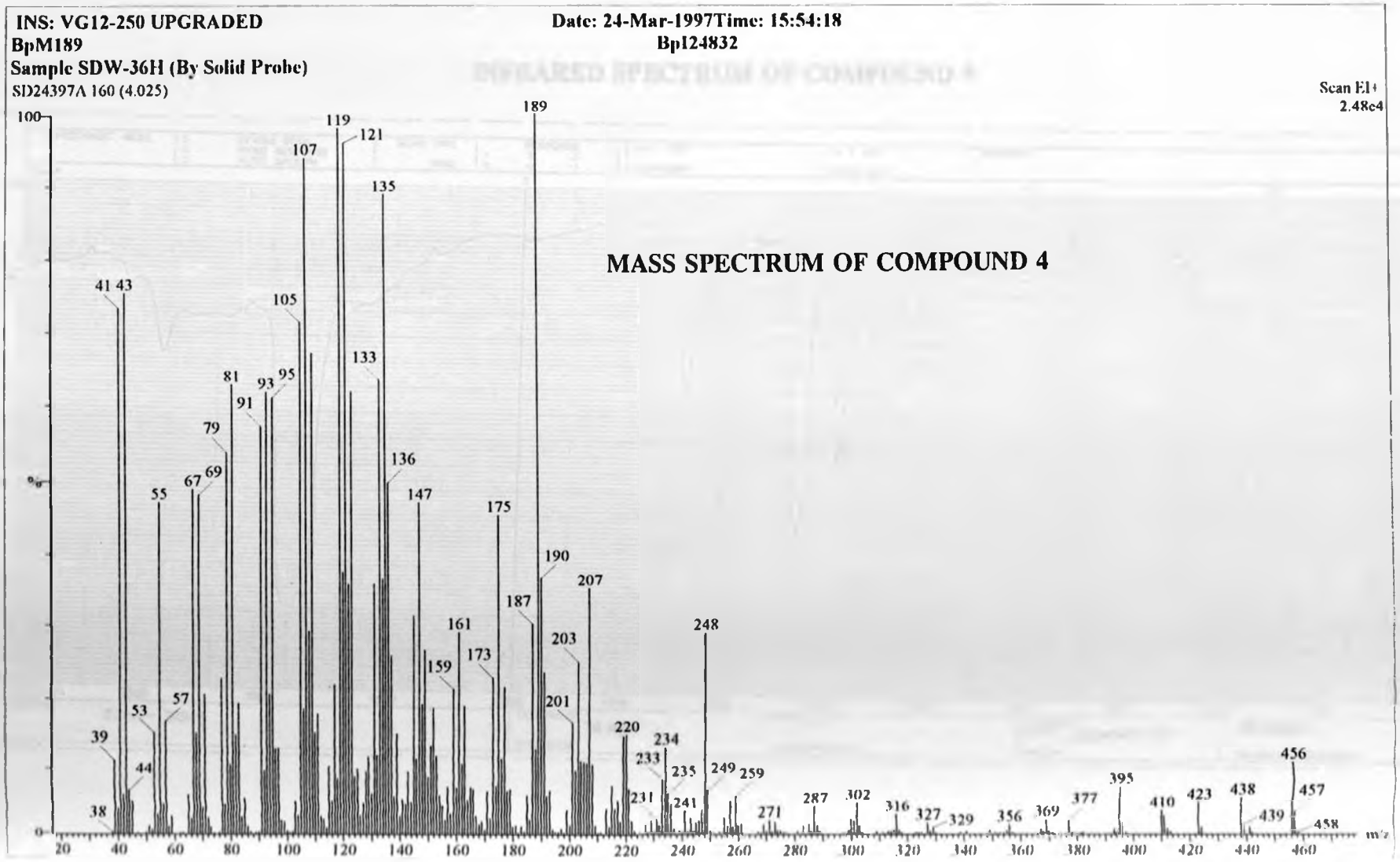
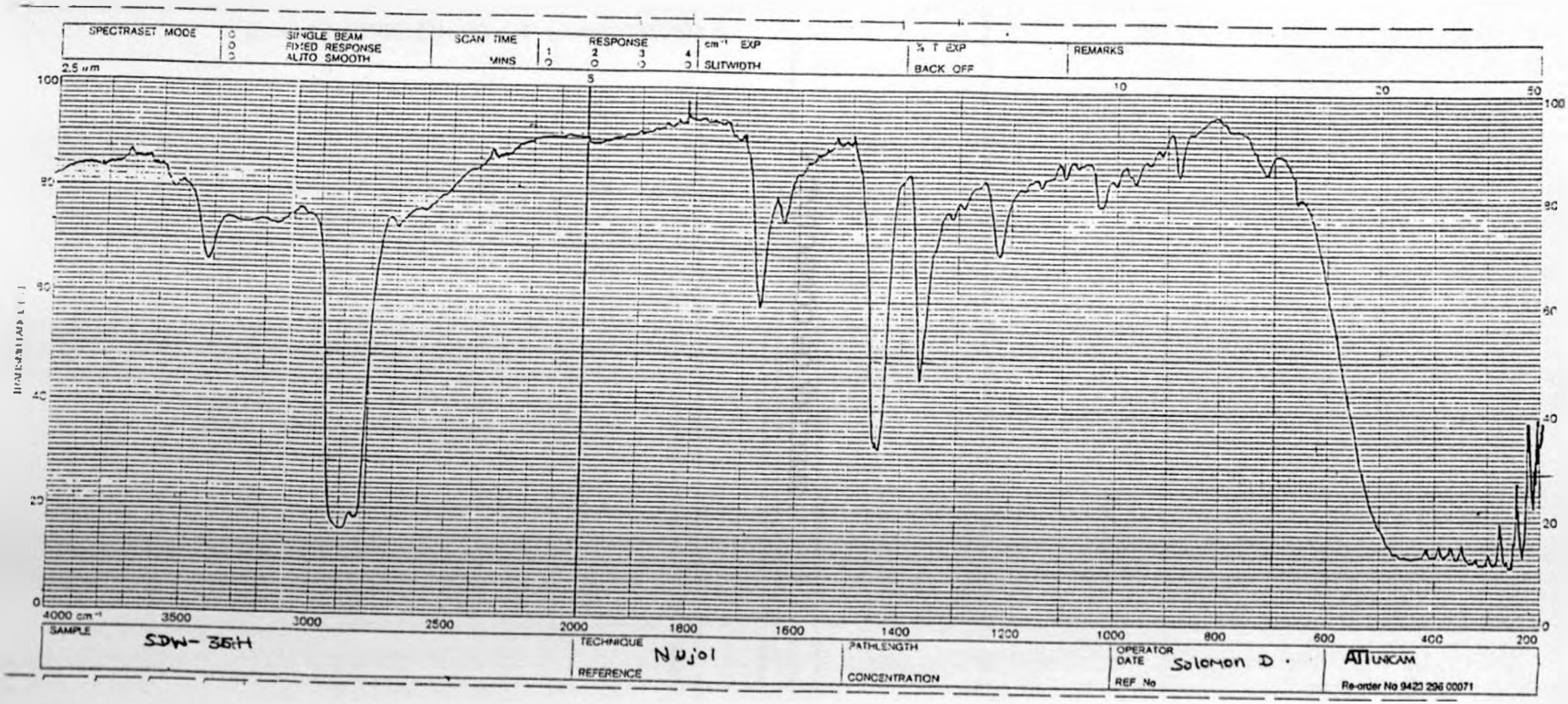
32	18.93	-19.1
33	17.95	9.5
34	15.93	-21.7
35	15.79	-26.3
36	15.72	-22.0
37	14.37	-24.5

DMSO-d_6

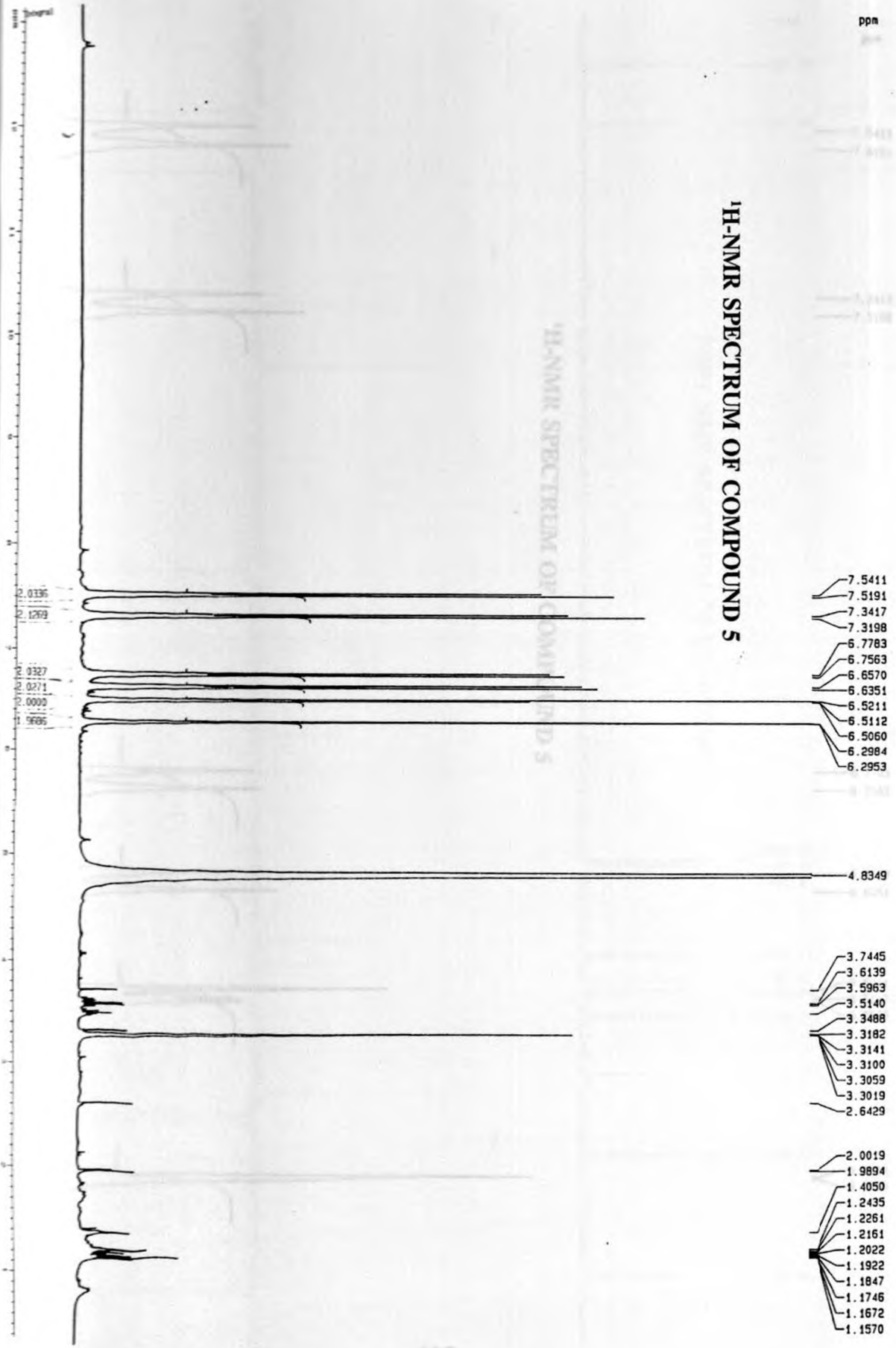

INS: VG12-250 UPGRADED
BpM189
Sample SDW-36H (By Solid Probe)
SD24397A 160 (4.025)
Date: 24-Mar-1997Time: 15:54:18
Bp124832
Scan EI+
2.48e4
MASS SPECTRUM OF COMPOUND 4
100
%
0
m/z
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460
189
119
121
107
135
41 43
105
133
81
93
95
91
79
136
67
69
55
147
175
190
207
187
161
248
203
159
173
201
220
234
233
235
249
259
53
57
39
44
38
231
241
271
287
302
316
327
329
356
369
377
395
410
423
438
456
457
439
458

INFRARED SPECTRUM OF COMPOUND 4

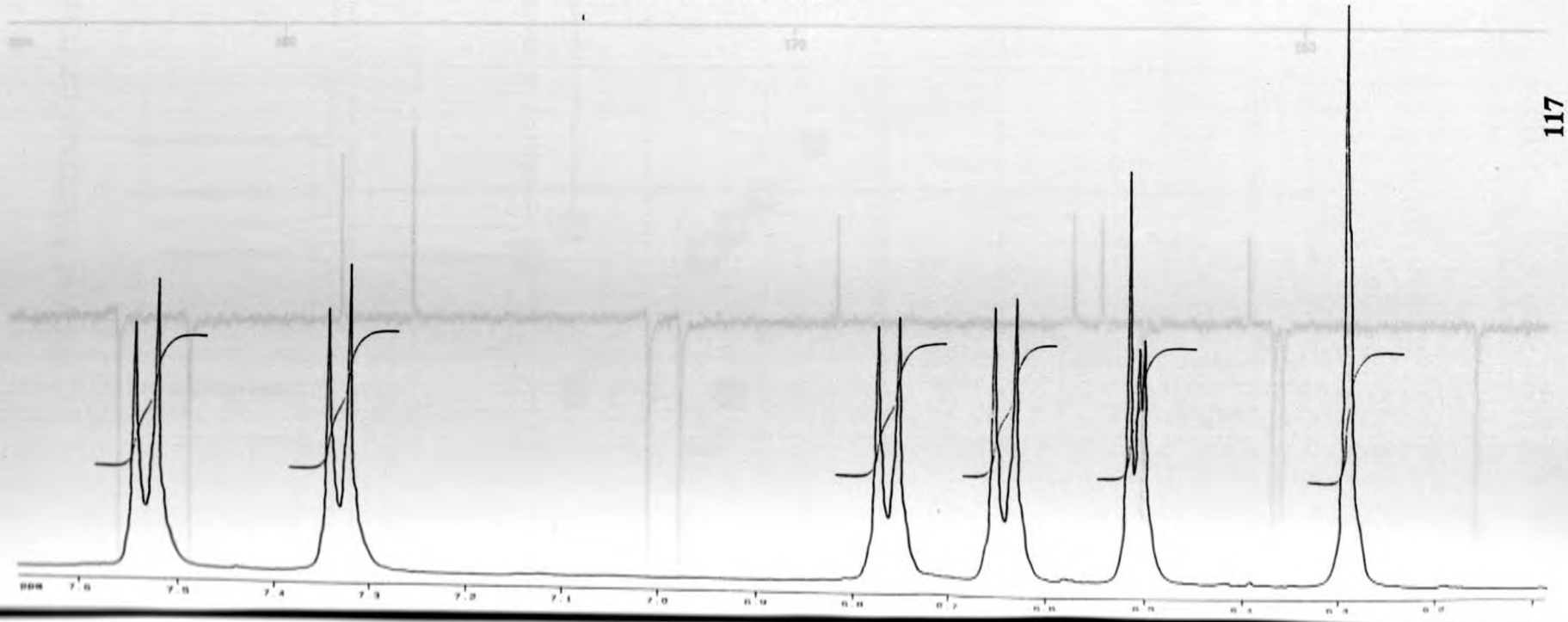


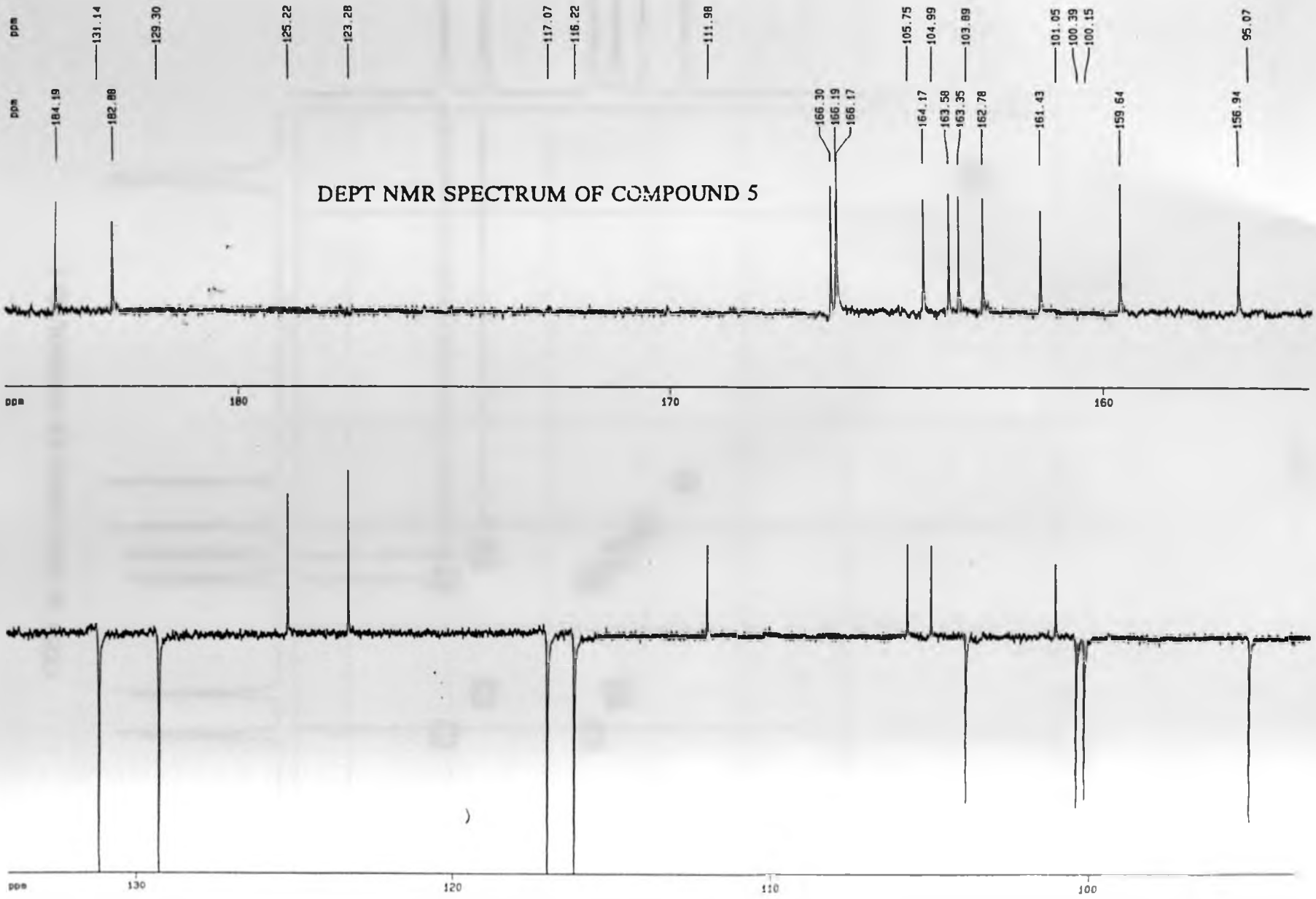
SPECTRA OF COMPOUND 5

^{1}H-NMR SPECTRUM OF COMPOUND 5



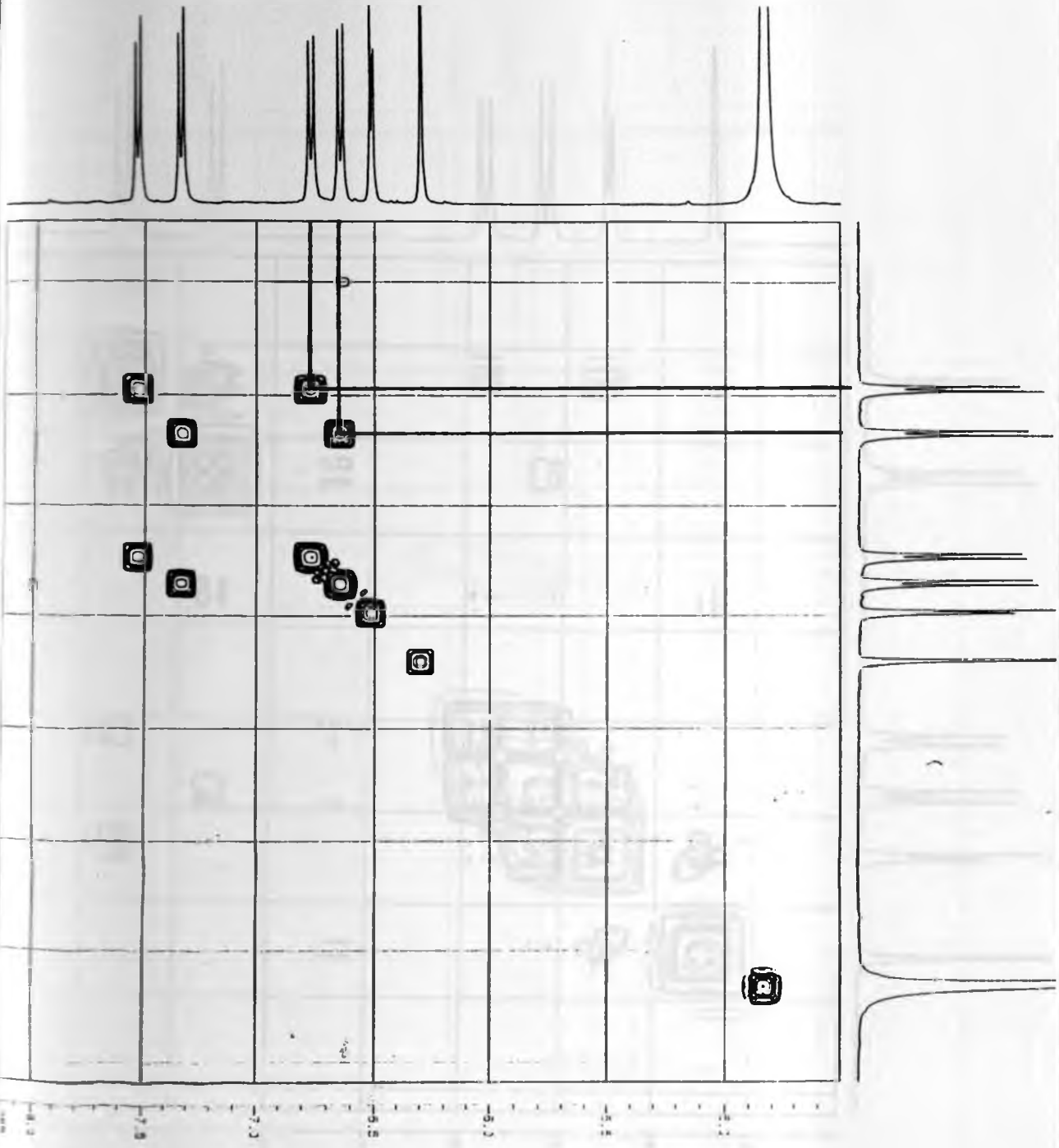
^{1}H-NMR SPECTRUM OF COMPOUND 5



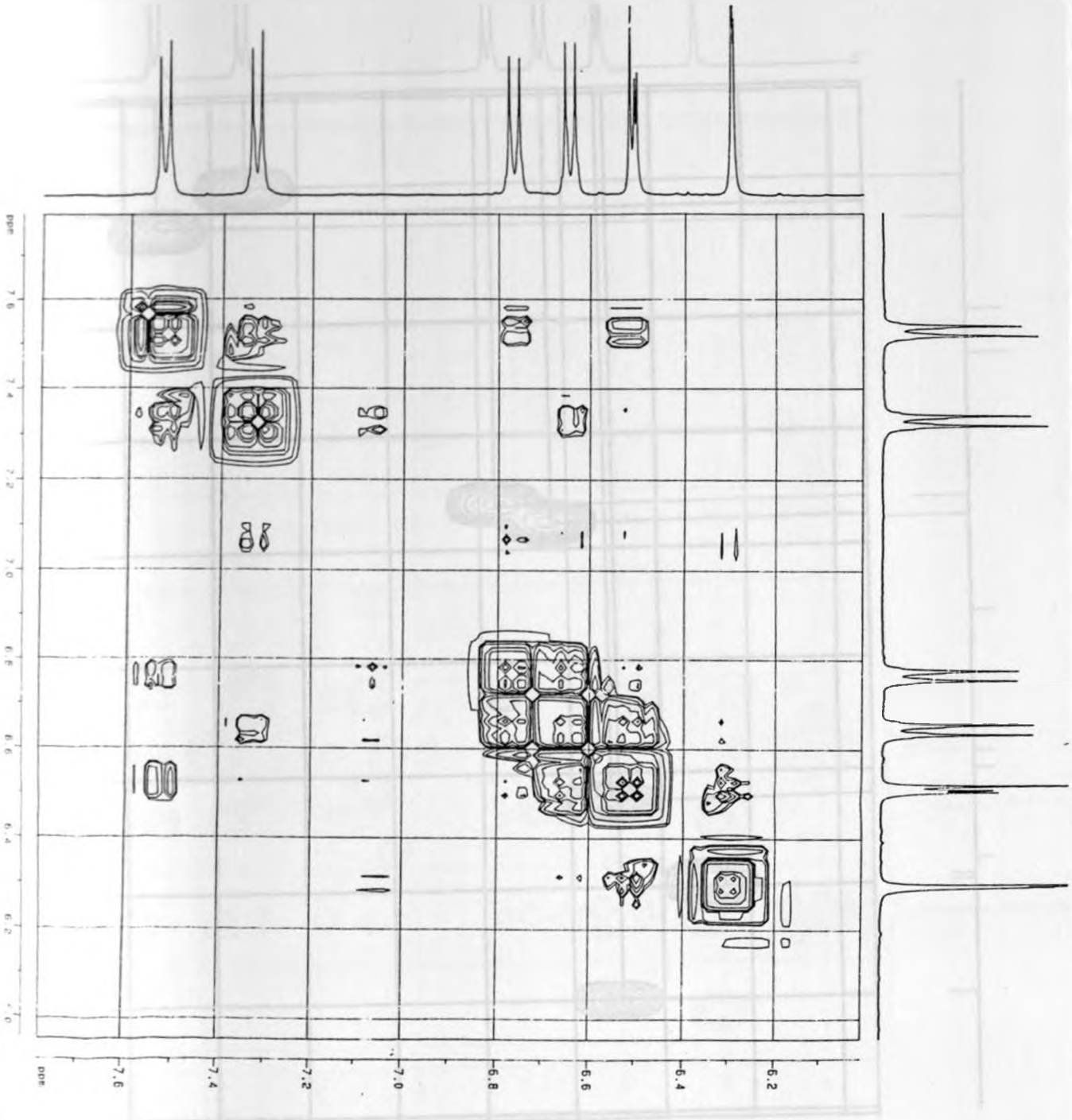


DEPT NMR SPECTRUM OF COMPOUND 5

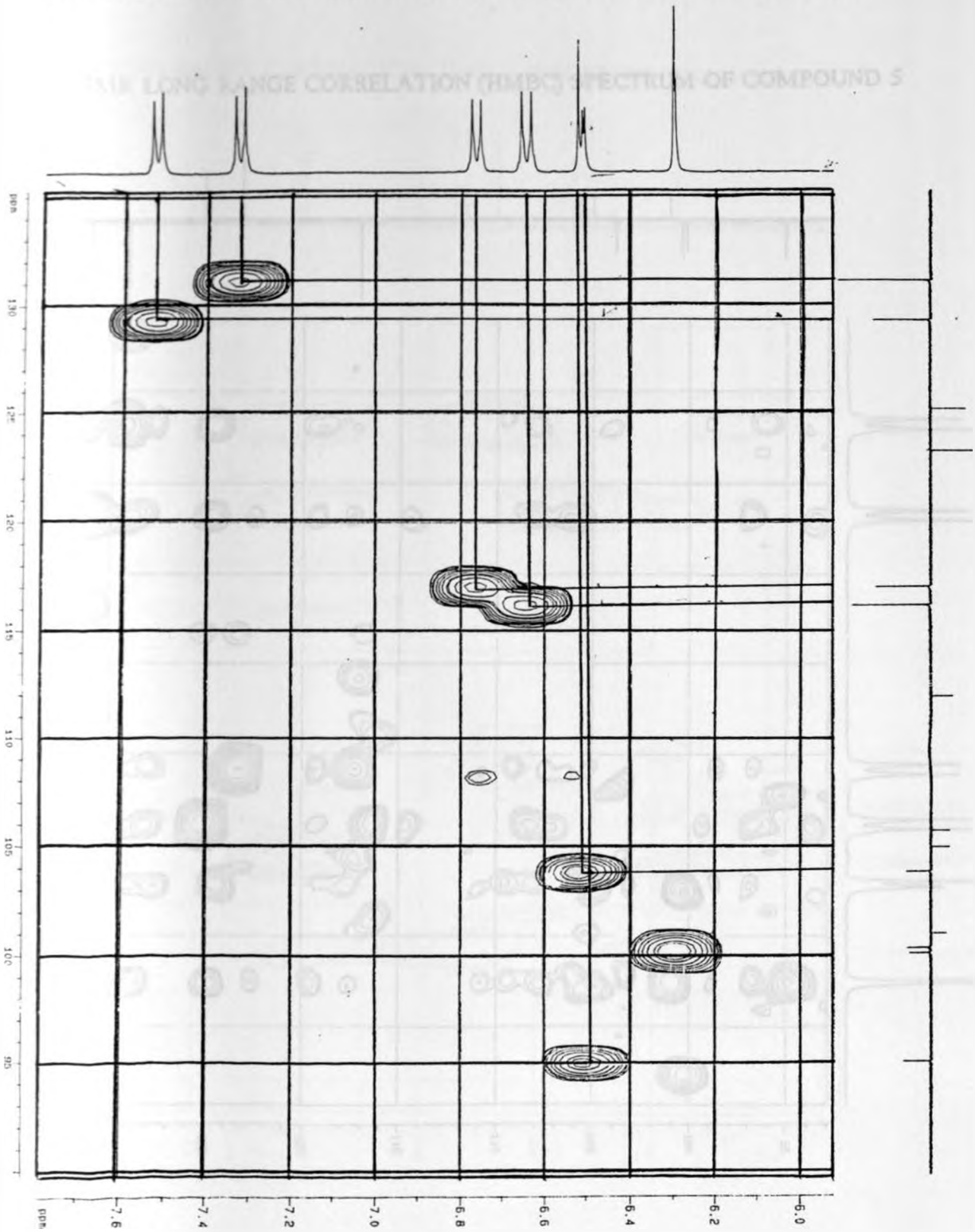
COSY 45 SPECTRUM OF COMPOUND 5



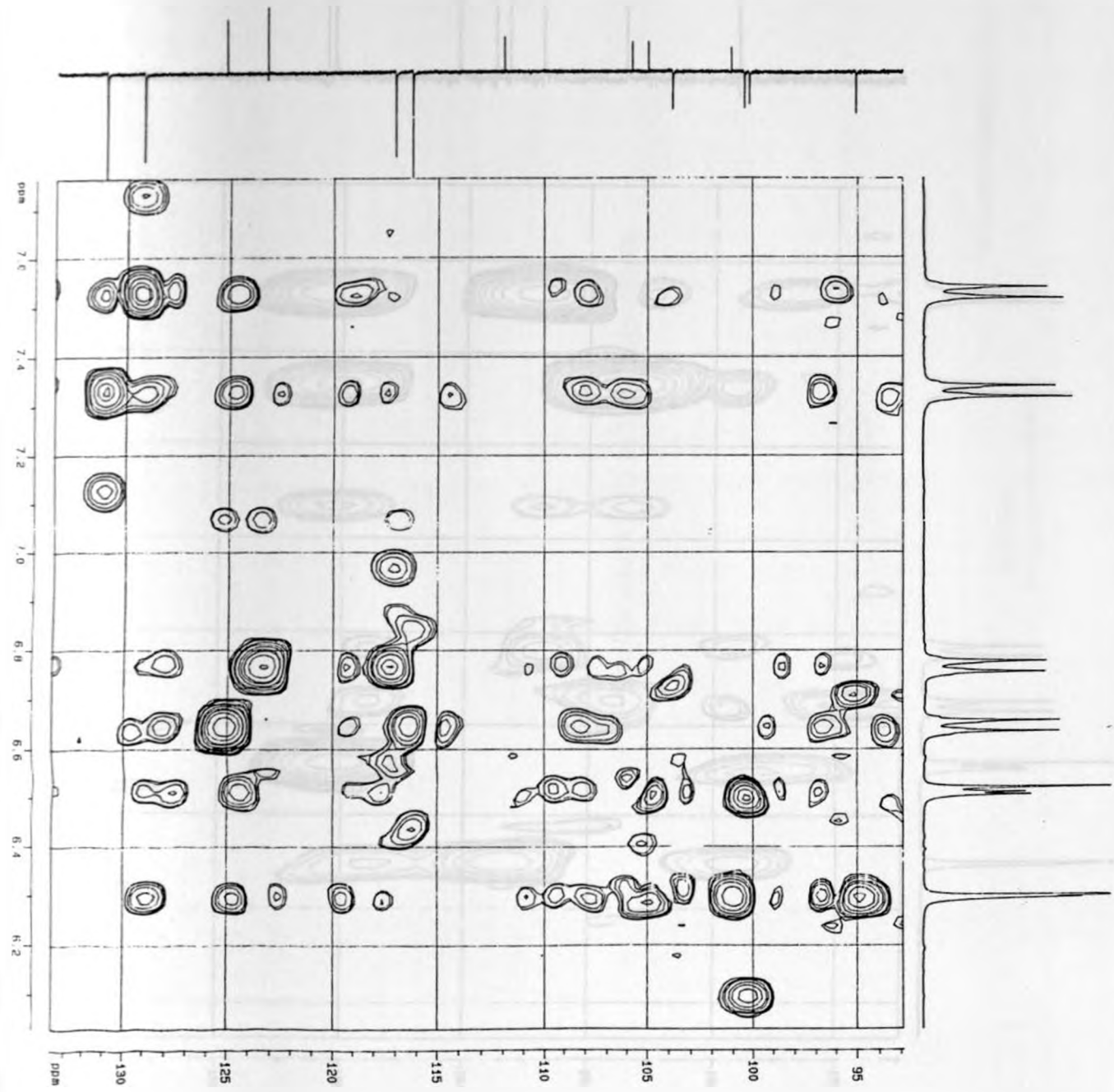
NOESY SPECTRUM OF COMPOUND 5



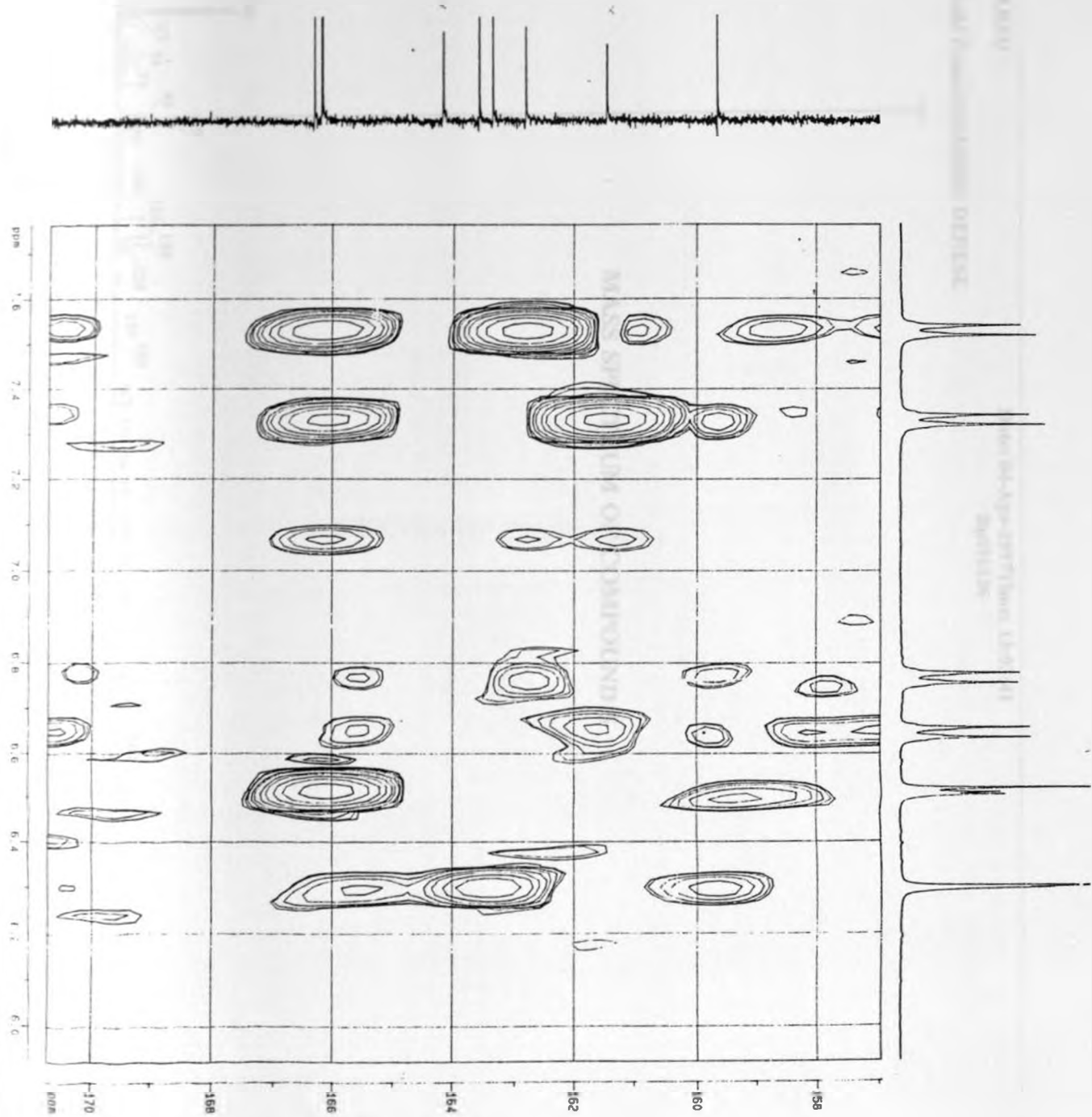
H-C NMR DIRECT CORRELATION (HCCOBI) SPECTRUM OF COMPOUND 5



H-C NMR LONG RANGE CORRELATION (HMBC) SPECTRUM OF COMPOUND 5



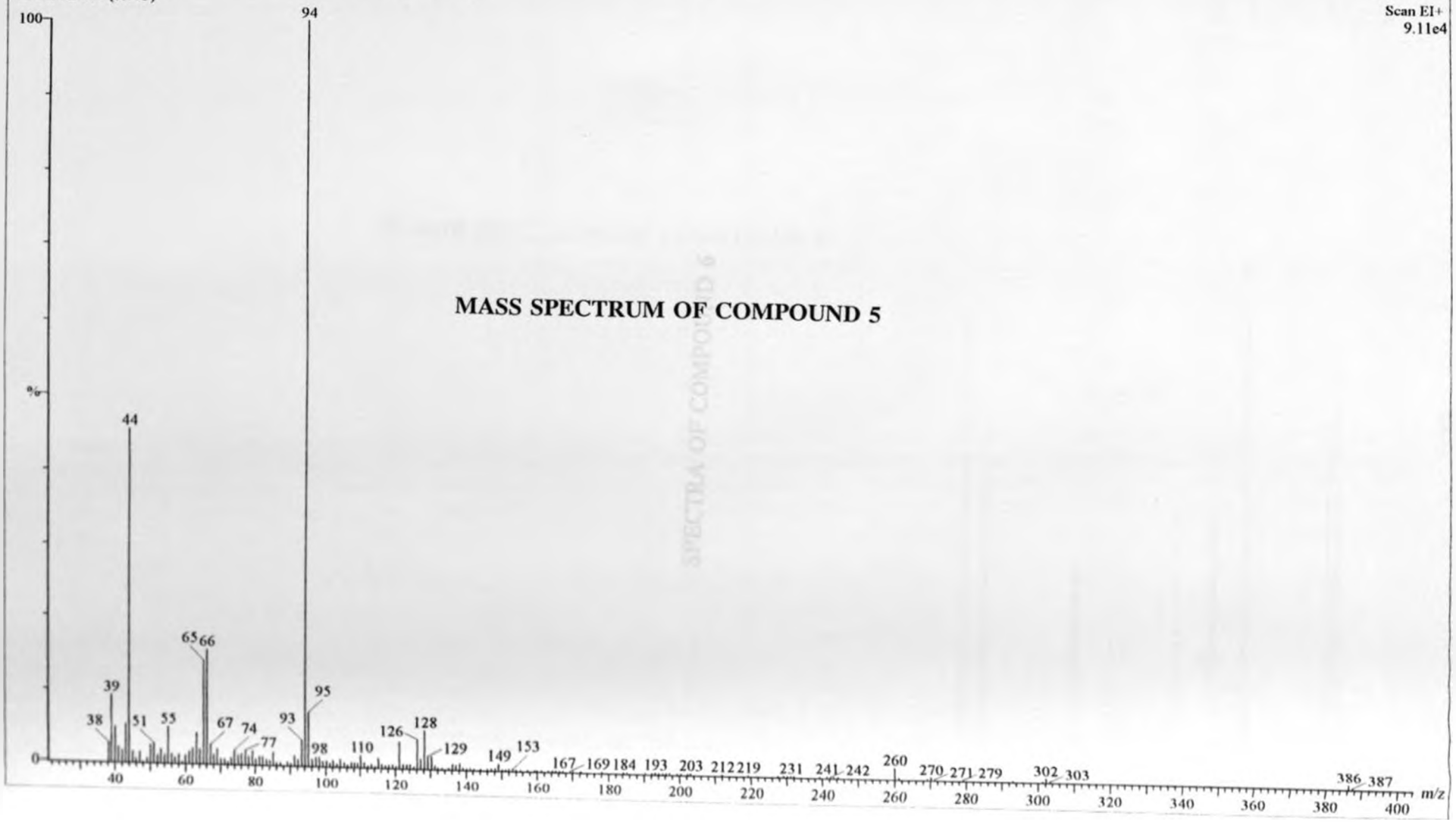
H-C NMR LONG RANGE CORRELATION (HMBC) SPECTRUM OF COMPOUND 5



INS: VG12-250 UPGRADED
BpM94
Sample SDW-41A (By Solid Probe)SOLOMON DERESE
SD4497B 150 (3.775)

Date: 04-Apr-1997Time: 13:03:41
BpI91136

Scan EI+
9.11e4



MASS SPECTRUM OF COMPOUND 5