STRUCTURE ELUCIDATION OF HYDROPHILLIC FLAVONOIDS OF *POLYGONUM SENEGALENSE* AND THEIR COMPARATIVE ANTIFEEDANT ACTIVITY WITH HYDROPHOBIC COMPONENTS.

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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ABSTRACT

Polygonum senegalense is one of the eleven *Polygonum* species growing in Kenya highlands. *P. senegalense* finds use in ethno-medical mode of therapy in this country along with other species of the Polygonaceae family.

In this work, the aerial parts of the plant were first washed with acetone, then the dried powdered material was sequentially extracted with cold 70 % and 50 % aqueous methanol for two days each and the combined aqueous extracts were partitioned into ethyl acetate. This was followed by chromatragraphic (analytical, preparative t.l.c. and column) analysis of the ethyl acetate fraction using silica gel, impregnated with 3 % oxalic acid solution and Sephadex LH-20. Finally the isolates were characterised using spectroscopic methods (IR, UV, NMR and MS).

The secondary metabolites isolated and characterised from the plant material were 2',4'-dihydroxy-6'-methoxychalcone (1), kaempferol (4',5,7-trihydroxyflavonol) (2), quercetin (3',4',5,7-tetrahydroxyflavonol) (3), quercetin-3-ß-O-galactoside (4), quercetin-3-ß-O-glucoside (5), 2'-ß-O-gluco-4'-hydroxy-6'-methoxydihydrochalcone (6) and quercetin-3-ß-O-glucoside-2"-gallate (7).

Antifeedant activity was done on the mid fifth instar nymphs of two species of locust (*Locusta migratoria* and *Schistocerca gregaria*). Various concentrations of the samples (surface exudate, chloroform partition, ethyl acetate partition, quercetin and quercetin-3-ß-O-galactoside) were applied on Whatman No. 1 filter paper. The surface exudate were found to have high antifeedant activity at all concentrations tested while the inner tissue compounds showed phagostimulatory activity towards the two locust species.

UV analysis of the surface exudates and some of its pure compounds within the uv-visible range indicated the possible use of the surface exudates by the plant as a shield especially for the most dangerous UV-A and UV-B radiations without interfering with the electromagnetic region of photosynthesis.

CHAPTER ONE

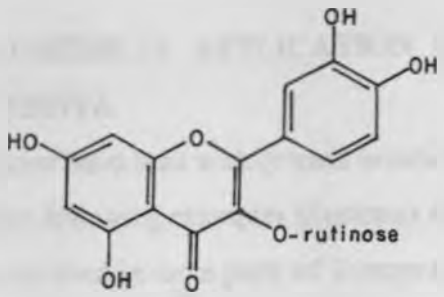
1.0.0 INTRODUCTION

Plants have been used as a source of medicine since the dawn of history of mankind in the remedy of various kinds of diseases. Phytochemical research into the traditional medicinal plants has greatly facilitated the discovery and synthesis of new kinds of modern medicines which in turn have greatly multiplied their specific applications against a number of man's ailments. The recent upsurge of interest in the traditional medicinal plants being used by different ethnic groups all over the world is partially attributed to the success obtained from the people of China who use the traditional medicines alongside modern medicines as a part of their health-care program. They have well documented data about the effective and safe use of medicinal plants (Agnew, 1974). However, in Africa the situation is the reverse as the knowledge of the African medicinal plants has been missing in the scientific journals. Nevertheless, the use of traditional medicinal plants in Africa is known to have been successful despite of isolated cases of poisoning due to concentration variation of the active ingredients and the amount of infusion administered to the patient. Infusion poisoning has also been attributed to the lack of knowledge of the chemical constituents in the plant used. A knowledge of chemical components of medicinal plants is therefore desirable.

In East Africa especially in Kenya the use of traditional medicines was vilified with the adoption of the Western medicines and the medicine men

known in Kiswahili as "Bwana Mganga" often arrested when caught administering traditional plant medicines. But of late, there has been a change in the general attitude towards the traditional medicines, with a lot of focus being directed to the plant medicines. Kokwaro (1976) in his book entitled "Medicinal plants of East Africa", recorded about 120 plants to have anthelmintic properties in the traditional therapy in East Africa. These plants include the *Rumex* species of the *Polygonaceae* family found in Kenya which contain large amounts of 1,8-dihydroxyanthraquinones (Midiwo and Rukunga, 1985). They are exploited traditionally for their laxative activity. Interestingly these 1,8-dihyroxyanthraquinones are applied for the same purpose in British Pharmacodex.

The importance of plant natural product derived medicines in modern medicine has been unjustifiably underestimated. Useful compounds such as rutin (8) and 8-methoxypsoralen (9) are plant extractives with many pharmacological activities. The medicines most commonly used by the majority of the world's population aspirin (10) (acetyl salicylic acid) was extracted from willow bark as salicylic acid and then converted to its acetate. This compound is now obtained purely synthetically. Other medicines of great importance in modern medicine today are insulin and thyroid extract used for the replacement chemotherapy are derived from the animal sources. The insulin and thyroid extract are obtained from the pancreas and thyroid glands respectively (Sofowora, 1982).



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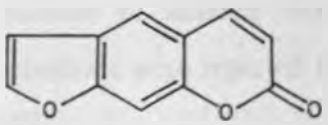

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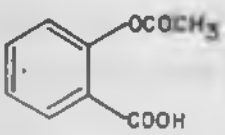

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Several approaches are used to select plants for investigation of their chemotherapeutic value. The most common is picking on plants that are used locally for medicine. A second method is screening randomly selected plants for one or more biological activity, while the third method involves phytochemical screening to establish the presence of certain chemical groups of interest (Farnsworth, 1973). In this project, the third criteria was employed. Thus this project is an effort to study the chemical constituents of *Polygonum senegalense* with an emphasis on the internal tissue flavonoids and their comparative bioassay.

1.1.0 ETHNO-MEDICAL APPLICATION OF POLYGONUM SPECIES IN KENYA

Polygonum species have been widely used around the world for ethno-medicinal purposes. The following examples illustrates the fact. *P. aviculare*, a perennial herb has been used in some parts of Europe as a home remedy for hemorrhoids and lung complaints. In Australia and German tea derived from the plant was used for asthma and bronchitis ailments (Upholf, 1968). The Chinese herbal plant *P.salchinese* has been used as a laxative, diuretic and for treatment of athletes foot and gonorrhea. Anthraquinone and flavonoid derivatives were reported to be the main components of the plant (Chi *et al.*, 1983).

In this country, *Polygonum* species are also reputed as useful for anthelmintic purposes in the traditional medicine as shown in Table 1 (Kokwaro, 1976). Of the eleven Kenyan *Polygonum* species, four are recorded in the traditional medicine as efficacious. These are *P. pulchrum, P. salicifolium, P. senegalense* and *P. setosulum.* The general application of these species are summarised in Table 1.

Table 1: Medicinal Application of Polygonum species in Kenya.

Plant origin	Medicinal Application
P. pulchrum	Leaves used for syphilis. The leaf infusion is drunk about three times daily by the patients.
P. salicifolium	A salt is obtained from the plants burnt in bundles, and this is licked as a cure for sore throat. Leaf decoction is used as a purgative, and extract from fresh leaves is used for skin problems.
P. senegalense	Reported as a medicine for cows but no specific details about how it is used or for what kind of disease.
P. setosulum	The Masai witch doctors bathe the dying patients with an infusion of the leaves in order to revive them.

1.2.0 BOTANICAL BACKGROUND

Polygonaceae is mainly a temperate family of plants bearing very inconspicuous hermaphrodite flowers. The leaves have stipules which unite to form a ring around the stem. The family of the Polygonaceae contain well-known genera; *Rumex, Polygonum, Rheum* and *Fagopyrum* and about a quarter of the 800 species in the Polygonaceae family are members of the largest genus, *Polygonum*. In Kenya the family is widely represented by *Rumex and Polygonum* among other genera. Many of the species get vast application in the ethnobotanical mode of therapy (Kokwaro, 1976; Midiwo and Rukunga, 1985). The genus *Polygonum* is represented in Kenya by eleven species written here in the order in which they occur in the "Key to species": *P. baldschuriacum* Regel, *P. convolvulus* L, *P. nepalense* Meisn, *P. capitatum* Ham, *P. afromontanum* Greenway, *P. aviculare L, P. strigosum R. Br, P. salicifolium* Wild, *P. senegalense* Meisn, *P. pulchrum* Blume and *P. setosulum* Rich (Agnew, 1974).

The occurrence of *Polygonum* genera in this country is chiefly localized within the highlands, at the foot of the mountains and swampy areas. Their height varies from one genus to another ranging from creepers to shrubs as one goes down the "key". *P. senegalense* is an erect variable hairy plant and its stem is often covered with conspicuous slightly inflated brown sheath. Leaves are lanceolate acute glabrous or densely white tomentose. The racemes number 2-6 usually with white or pink flowers. It is a common plant on the riversides, streamsides and marshes upto a height of 3000 metres above the

sea level. *P. senegalense* may occur as two extreme forms the glabrous *P. senegalense forma senegalense* or tomentose *P. senegalense forma albotomentosum*, intermediates between these two extremes are also known to exist showing different degrees of hairiness. It has been surmised that the difference in the leaf surface structure in the two extreme forms of *P. senegalense* is moisture dependent with the tomentose leaves being observed mostly in the xeric habitats while the glabrous leaves are found under aquatic conditions (Midiwo *et al.*, 1990).

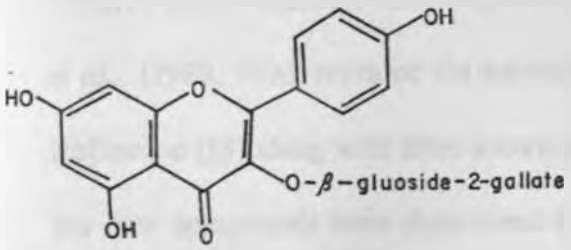
1.3.0 CHEMISTRY OF POLYGONUM GENUS

Review of *Polygonum* chemical literature reveals that as a member of Polygonaceae the most commonly reported secondary metabolites are flavonoids and anthraquinones. However, other secondary metabolites some of which have a considerable bioactivity for example stilbenes and sesquiterpenes from *P. cuspidatum* and *P. hydropiper* have also been isolated (Kimura *et al.*, 1983).

Isobe *et al.*, (1979, 1980) reported the identification of known flavonoids, **2**, **5** and **7** along with a novel compound assigned kaempferol-3-O-ß-glucoside-2"-gallate (**11**) after chemical and spectral analysis from the aerial parts of *P. nodosum*. Re-investigation of *P. nodosum* by Kuroyanagi *et al.*,(1982) revealed the presence of new cyclobutane formed from dehydrokawan by [2+2] cycloaddition and a new flavanonol (**12**) together with **7**. The structure of the new compounds were established on the basis of

physicochemical evidence.

In a separate study Yoshizaki *et al.*, (1987) examined *P. multiflorium* for its phenolic constituents. They isolated flavonoid, anthraquinone glycosides, emodin glucoside and physcion (13) together with a new acetophenone glucoside (14) (2,3,4,6-tetrahydroxyacetophenone-3-O-ß-glucoside).



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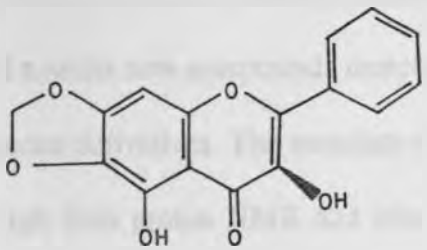

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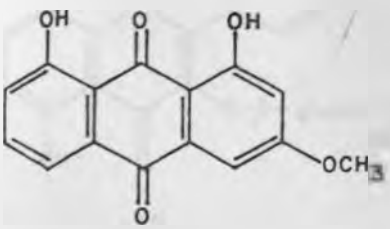

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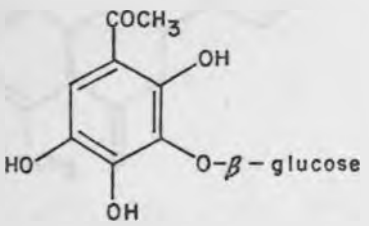

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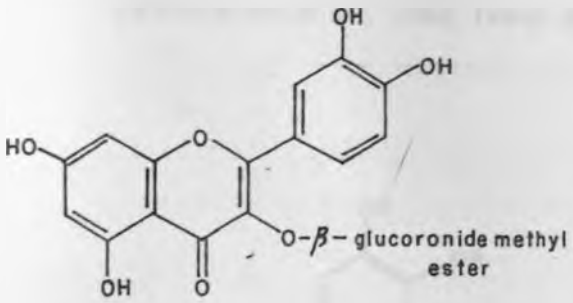
P. perlifolium commonly used as an antihypertensive plant was shown to contain known flavonoid aglycones, 2, 3 and quercetin-3-O-ß-glucuronide methyl ester (15). These compounds were identified by direct comparison with authentic markers, chemical and spectral data. This was the first report on 15 as a natural product (Lin, 1983). From the extracts of the aerial parts of *P.*

lapathifolium, Kulpina *et al.*, (1986) isolated compounds **2** and **3** in addition to fourteen flavonoid glycosides. Six of the latter were identified as quercetin-3-O-ß-glycoside and kaempferol-3-O-ß-glycoside derivatives. In the same year Rathore and co-workers characterised two new flavonoids from *P. nepalense* as 4',5-dimethoxy-6,7-methylenedioxyflavonone (**16**) and 2',3',4',5,5',6,7-heptamethoxyflavonone (**17**) together with a known flavanone (Rathore *et al.*, 1986). Further chemical screening of the leaves of the same plant by Ahmed *et al.*, (1988, 1990) revealed the presence of a series new compounds namely isoflavone (**18**) along with three known chalcone derivatives. The structure of the new compounds were determined by high field proton NMR and other spectroscopic techniques.



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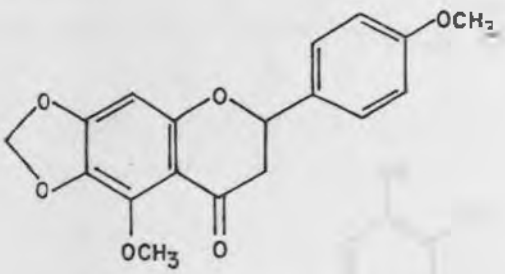

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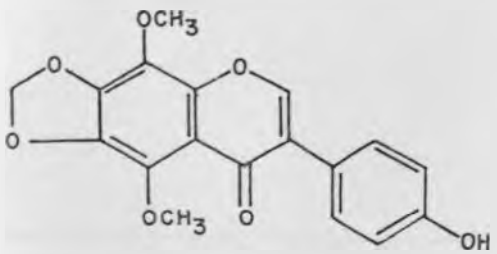

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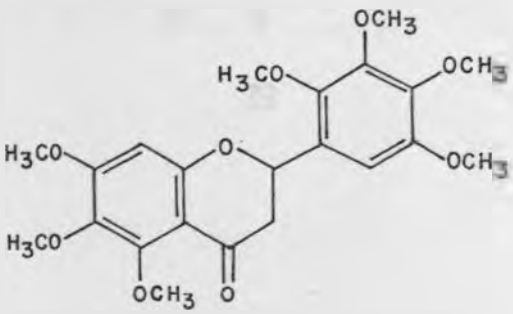

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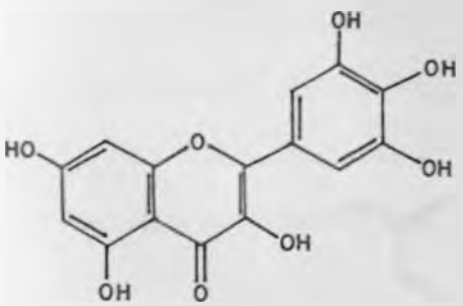
Vysochina and co-workers (1971, 1987) discussed the flavonoid content of *Polygonum* species found in the Siberian flora. Among the 57 *Polygonum* species screened all contained 3 and myricetin (19). Isorhamnetin (20) and 2 were present in only a few species. It was also noted that the aerial parts of *P. scrabrum, P. lapathifolium* and *P. tomentosum* showed higher levels of flavonoids than those of *P. amphibian, P. persicaria* and *P. hydropiper.*

The leaves, stems and flowers of *P. glabrum* are applied in the Eastern part of India for anthelmintic purposes. The major polyphenolic components in the plant were reported to be 4 (0.01% in stem and flowers), 3 (0.20% in leaves), 2 (0.003% in flowers) and 20 (0.12% in leaves and stems). Compound 5 and 8 were also reported to constitute a reasonable portion of the polyphenolic contents 0.05% and 0.01% of the plant parts respectively, (Adinarayana *et al.*, 1980; Tiwari *et al.*, 1979).



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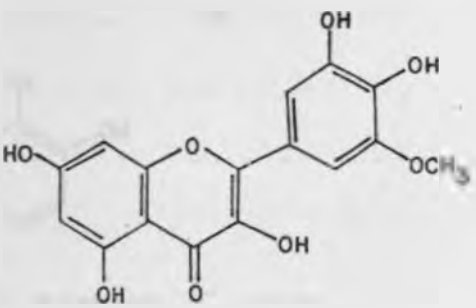

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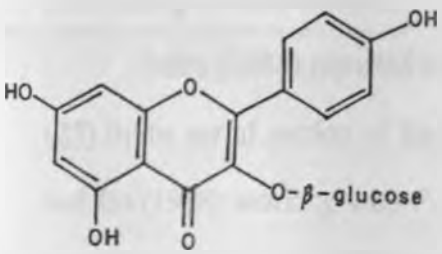
A chemotaxonomic study of flavonoids from the Japanese flora *Polygonum* species showed the presence of kaempferol-3-O-ß-glucoside (21), myricetin-3-O-ß-glucoside (22) and 5 along with 3 as the common flavonoid components of the species with 5 having the highest occurrence. It was also further reported that the plant *P. nodosum* contained flavone-3-O-ß-glucoside-2"-gallate as the main component and quercetin-3-(2-O-galloylrhamnoside) (23) was widely distributed in the *P. filiforme neofiliforme* and *P. flaccidum* (Isobe and Noda, 1987a,b).



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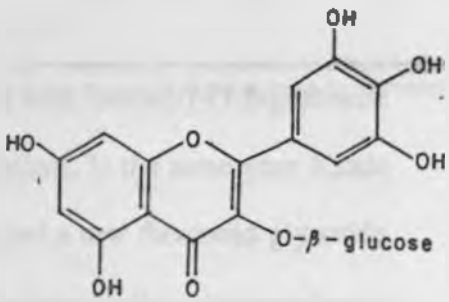

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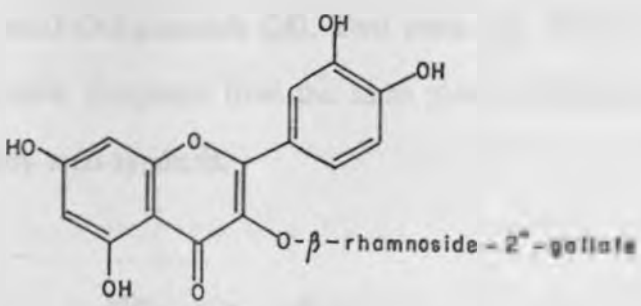

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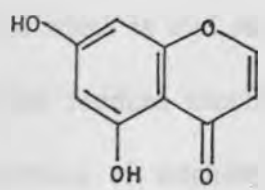
Romussi and Ciavallo, (1974) reported 5,7-dihydroxychromone (24) together with 3 and 5 in the seeds of *P. persicaria*. The compound 5,7-dihydroxychromone has been previously isolated only from *Arachus hypigaca* (Ratna *et al.*, 1973).

Kukenov and Mikhailova (1971) discussed the intraspecific variation and chemical diversification of *P. cariarium, P. bucharicum, P. sachalinense* and *P. persicaria*. It was reported that the flavonoid content of the species correlated to the growth and development with maximum flavonoid diversity occurring during the flowering period. Variation in the flavonoid accumulation in the same species also depends not only on their age but also on the meteorological conditions.

Balba (1988) reported compound 4 along with orobol-7-O-ß-glucoside (25) in the aerial section of Egyptian *P. salicifolium*. In the same year Kanda and Ito (1988) working with *P. tinctorum* reported a new flavonoid glycoside from the aerial parts of the plant. The structure of the compound was elucidated by means of spectroscopic methods to be 3,4',5-trihydroxy-6,7-methylenedioxyflavone-3-O-ß-glucoside (26). Two years later Khoda et al., (1990) isolated the same compound from the same plant and this time its structure confirmed by total synthesis.



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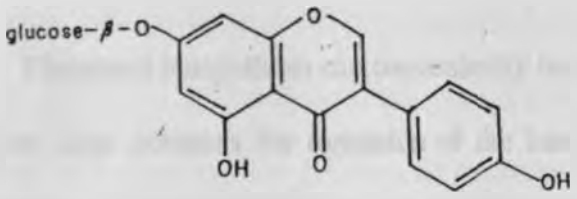

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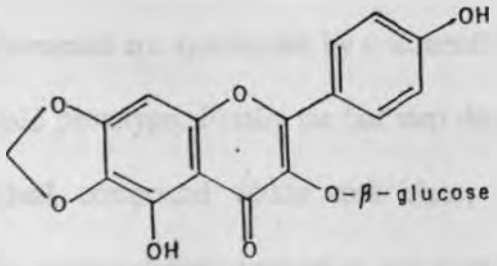

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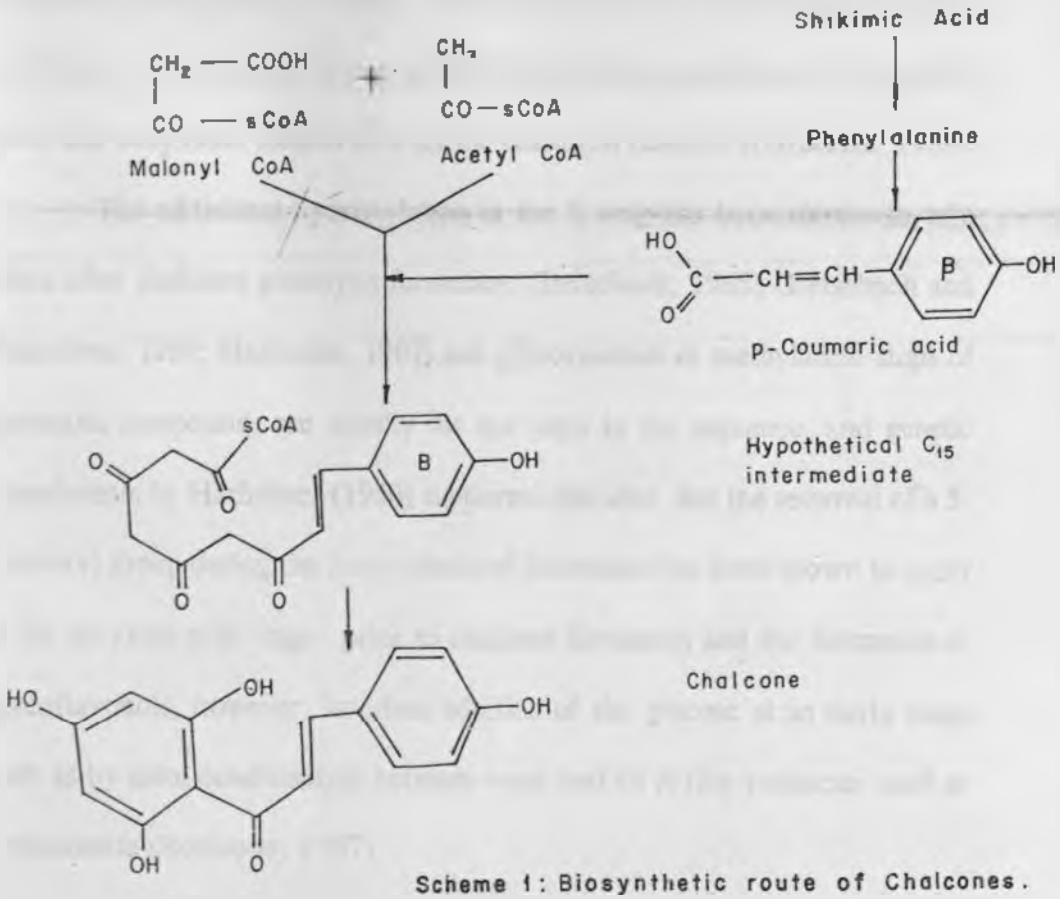
1.4.0 BIOSYNTHESIS OF FLAVONOIDS

It is estimated that about 2 % of all carbon compounds photosynthesised by plants is converted into flavonoids and related compounds. Flavonoids thus constitute one of the largest group of naturally occurring plant phenolics. In plants flavonoids exists as aglycones, O- or C-glycosides, sulphates and biflavonoids. In some cases flavonoid glycosides may either occur as O- or C- glycosides or both. Such glycosides are distinguished by the ease with which they are hydrolysed by the acid. C-glycosides are flavonoids in which sugars are attached directly to the aromatic ring by a carbon-carbon bond which is acid resistant. Flavonoid sulphates contain one or more sulphates residues attached to a phenolic or sugar hydroxyl moiety. The occurrence of sulphate flavonoids appears to be restricted to the plants which have ecological association with aquatic habitat (Markham, 1982).

Flavonoid biosynthesis can conveniently be considered in three stages. The first stage concerns the formation of the basic $C_6C_3C_6$ skeleton from a combination of acetate-malonate and shikimic acid pathways to aromatic compounds. The second step is concerned with the ways by which the various classes of flavonoids are synthesised by a sequential and parallel routes from a C_{15} flavonoid prototype. Finally the last step deals with the elaboration of each individual compound within each class, involving steps such as glycosylation, hydroxylation, acetylation and methylation (Wong, 1976).

Insights into the biosynthetic pathway of flavonoids have come from the studies of comparative anatomy, chemical genetic studies and feeding experiments with radioactive tracer and enzymatic studies (Harborne, 1967). Tracer experiments by a number of workers have established that the B and C rings of flavonoids come from the shikimic acid pathway and the A ring is formed by head-to-tail condensation of acetate-malonate molecules. The experiments further ruled out the formation of ring A from inositol or phloroglucinol as has sometimes been suggested and also established that C_6C_3 compounds such as phenylalanine, cinnamic acid and ferulic acid are efficient precursors of the C_6(B)-C_3 portion of flavonoids (Robinson, 1967). The flavonoid initially formed in the biosynthesis is thought to be a chalcone and all other forms are derived from it. The transformation leading to the formation of chalcones consists of three successive condensation steps with acetate units which results in the elongation of aliphatic side chain of *para*-coumarate by six carbon atoms which then cyclizes to give aromatic ring A.

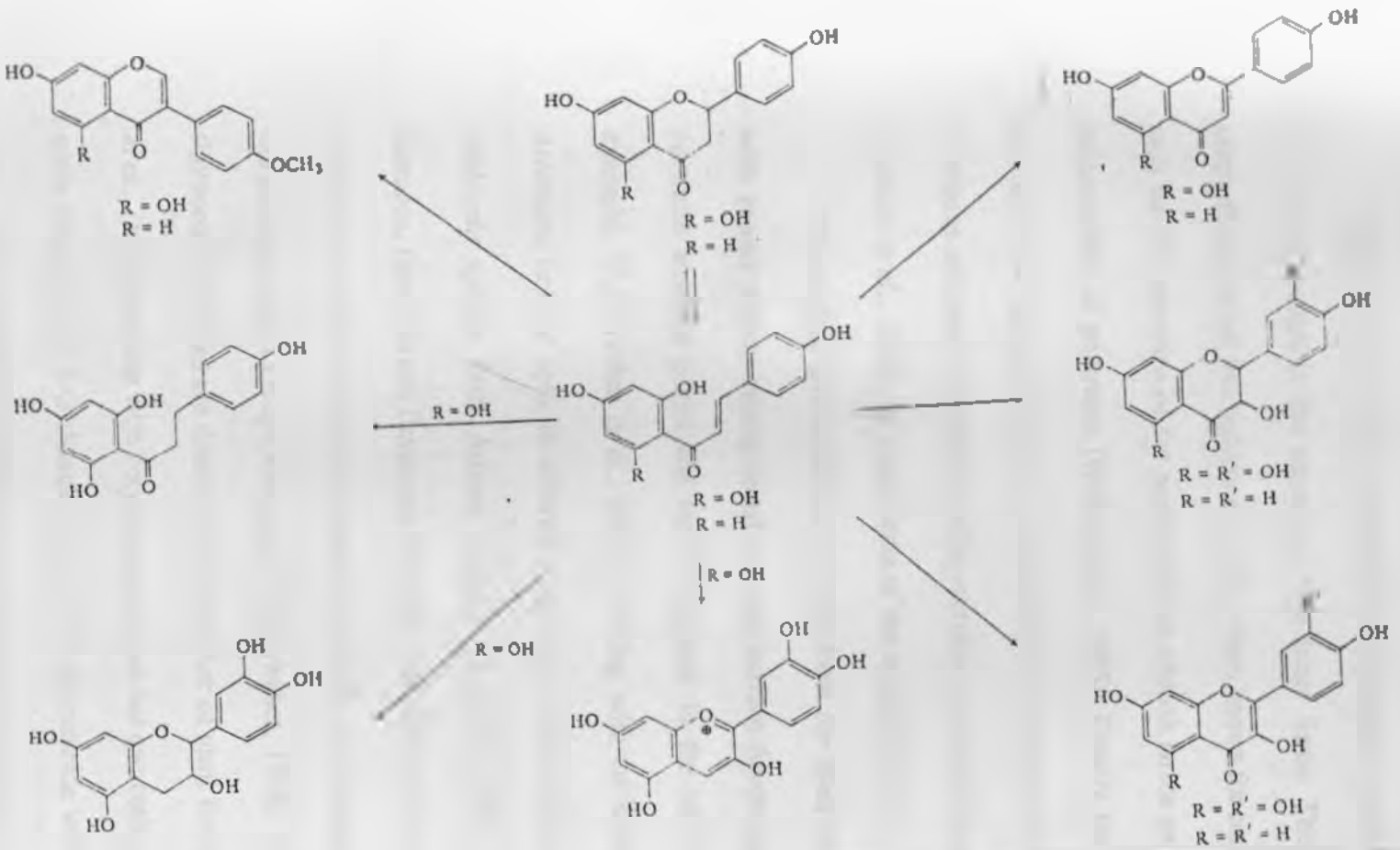
Some researchers on the biosynthesis had earlier suggested that since flavanones are thermodynamically more stable than chalcone, it was presumed that they must be the primary heterocyclic intermediate substrate in the pathway towards the biosynthesis of other classes of flavonoids (Greisebach 1965). But feeding experiments studies carried out by Wong (1968); Wong and Greisebach (1969) confirmed the contrary. It was observed that flavanones and chalcones are biologically interconvertible and the reaction chalcone-flavanone catalysed by the isomerase is not directly involved in the pathway from chalcone to other forms of flavonoids. Therefore flavanone formation is then parallel rather than an intermediate step in the biosynthesis of other classes of flavonoids.



Scheme 1: Biosynthetic route of Chalcones.

From the chalcone C_{15} prototype, different classes of flavonoid compounds encountered in nature are obtained either by oxidation, reduction and group migration processes. Radioactive studies by Dewick (1978); Goodwin (1976) demonstrated that isoflavonoid skeleton results from the C_{15} precursors by 1,2 aryl migration of the B ring and this shift takes place after the formation of the chalcone intermediate. Thus the proposal that chalcones are intermediate precursors of flavonoids biosynthesis enjoys general acceptance indeed, it is 2',4',6'-trihydroxychalcone and 2',4,4',6'-tetrahydroxychalcone that normally act as a substrate for the aryl migration (Dewick, 1978) and the presence of a *para* hydroxyl group on the B ring of the chalcone precursor is essential for the rearrangement to isoflavonoids (Harborne and Mabry, 1982). The intervention of chalcones in aurone synthesis has not been fully established by feeding experiments but, both *in vitro* and enzymatic studies favours the oxidation reaction (Harborne, 1967).

The additional hydroxylation in the B ring has been shown to take place after chalcone prototype formation (Greisebach, 1965; Greisebach and Grambow, 1968; Harborne, 1967) and glycosylation or methylation steps of flavonoid compounds are usually the last steps in the sequence, and genetic experiments by Harborne, (1960) supported this idea. But the removal of a 5-hydroxyl group during the biosynthesis of flavonoids has been shown to occur at the polyketo acid stage prior to chalcone formation and the formation of glycoflavonols, however, involves addition of the glucose at an early stage such as by aldol condensation between sugar and an A ring precursor such as acetoacetate (Robinson, 1967).



Scheme 2: Interconversion of chalcones to other flavonoids

1.5.0 Epicuticular Vs internal tissue flavonoids

Flavonoids are widely distributed as glycosides dissolved in the cell sap, that is located in the vacuoles of the inner tissue. Their existence as externally deposited free aglycones has long been known for conspicuous cases such the bud excretion of the poplars, or the whitish farina on the leaves and inflorescence of primroses (Wollenweber, 1985). Usually the occurrence of the flavonoid aglycones is also correlated with the existence of secretory structures and production of lipophillic excretion, mostly of terpenenoid origin (Midiwo *et al.*, 1990), as constituents of the epicuticular layer.

The surface flavonoids are different from the inner tissue flavonoids with greater variety usually found with the surface flavonoids and they are common amongst plants that have originated in the xeric habitats. For example, Wollenweber *et al.*, (1987) working with the surface exudate of *Asteracea* isolated upto 40 different compounds from *Baccharis spp.* From *Ambrosia* species from Arizona a series of methyl ether derivatives of flavones, flavonols and flavanones were also isolated. The structural diversity of the surface flavonoids has prompted their use in chemotaxonomy studies in the *Achillea nobilis* group (Valant and Wollenweber, 1988). The inner tissues flavonoid glycosides are distributed in a number of plant families . Kawasaki *et al.*, (1976) working with Polygonaceae found that quercetin glycosides were quite frequent with 3-O-rhamnoside and 3-O-glucuronide widely distributed.

The existence of flavonoids on the aerial surface of *P. senegalense* is intriguing but what is more striking is the array of compounds that have been

characterised from it. Midiwo *et al.*, (1990; 1992) working with *P. senegalense* isolated several flavonoid aglycones from the exudate. Of these twelve were identified as 2'-hydroxy-3',6'-dimethoxychalcone (27), 2',4'-hydroxy-3',6'-dimethoxychalcone (28), 2',6'-hydroxy-3',4'-dimethoxychalcone (29), 2',6'-dihydroxy-4'-methoxydihydrochalcone (30), 2',4'-dihydroxy-6'-methoxychalcone (31), 5-hydroxy-7-methoxyflavanone (32), 7-hydroxy-5,8-dimethoxyflavanones (33), 7-hydroxy-5-methoxyflavonone (34), 3,7-dihydroxy-5,8-dimethoxyflavonone (35), 7-hydroxy-5,8-dimethoxyflavonone (36), 2',4'-dihydroxy-6'-methoxydihydrochalcone (37) and 3',6'-dihydroxy-2',4',5'-trimethoxychalcone (38).

Gikonyo (1991) working with the methanol hydrolysate extract of the same plant reported the presence of known aglycones 2 and 3 and hence suggested the possible existence of various glycosidic derivatives of these aglycones in the plant. Therefore it was the aim of this project to isolate and characterise the inner tissue components of *P. senegalense* in their native nature that is the glycosidic forms.

1.6.0 PHARMACOLOGICAL ACTIVITIES OF FLAVONOIDS AND POLYGONUM EXTRACTS

Some of the functions of flavonoids in plants may be to protect them against diseases caused by micro-organisms and to act as feeding deterrents to insects and other herbivorous animals. Since flavonoids are phenolic compounds, they may interact with enzymes and this makes them toxic to certain animals, or inhibit their growth, or give unpleasant taste sensation to

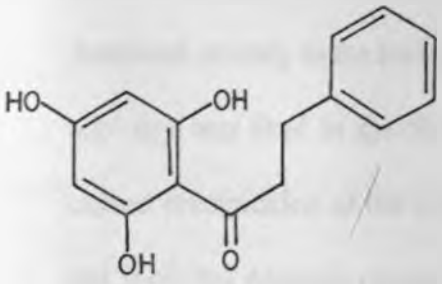
animals. For instance, Dreyer and Jones (1981) investigated the feeding deterrence of a number of flavanones and dihydrochalcones against the aphid *Schizaphis graminun*. Flavanone glycosides appeared to be less active at all concentrations tested but the corresponding flavanone aglycones showed activity. Of the dihydrochalcone tested, phloretin (**39**) showed a high deterrence, but the semi-synthetic dihydrochalcones derivatives were less active.

Quite a number of *Polygonum* plants are employed invariably in the ethno-pharmacological treatment of many kinds of human ailments in various countries all over the world. This effect is related mainly to flavonoids, anthraquinones, terpenes and other secondary metabolites in the plant species (Adinarayana *et al.*, 1980; Chi *et al.*, 1983; Kokwaro, 1976; Tiwari et al., 1979).

The Chinese plant *P. perfoliatum* extract is known to be antihypertensive (Lin, 1983). A total of twenty one crystalline compounds were isolated from this plant amongst which 3,3'-dimethylellagic acid (**40**) which when administered to renal hypertensive rats produced a significant effect on the blood pressure and heart contractile force (Lin, 1983).

Harborne (1967) reported biological activities of compound **8** together with other related flavonoids. These compounds have been shown to decrease fragility of the capillaries in guinea pig and they were thought to possess vitamin-like activity in human. Also when taken in large doses compound **8** acts as an antioxidant to adrenaline and ascorbic acid. Other research on

compound 8 by Efigenia (1951) reported that a mixture of 8 and 4 is administered to expecting mothers to prevent haemorrhage. But an overdose of the medicine may produce harmful effects on both the mother and child. Honda *et al*, (1980) reported that tryphanthrin (41) isolated from *P. tinctorum* possessed antimicrobial activity against dermatophytes. Also isolated from *P. tinctorum* was compound 26 which showed antiplatelet activity on human blood. Further tests on the compound showed that at a concentration of 0.2mg/mL it had IC_{50} of platelet aggregation inhibitory activity in the human blood plasma (Kanda and Ito, 1987; Khoda *et al.*, 1990).



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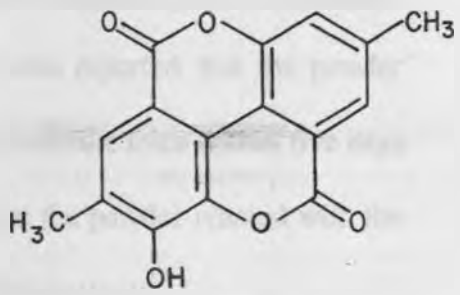

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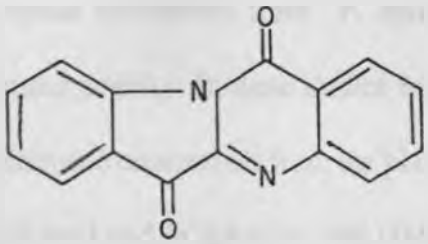

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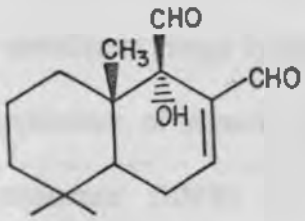
It has been reported that the infusion of the roots of *P. hydropiper* showed antifertility action (East, 1955; Garg and Mathur, 1972). Garg and Mathur, (1972), reported the effect of various infusion of the plant against pregnant mice. The infusions were given orally at 100 mg/Kg doses on days 1 to 7 of pregnancy, it was found out that petroleum infusion prevented pregnancy in 8 out of 10 mice tested while both petroleum/benzene (1:1 v/v) and benzene/chloroform (1:1 v/v) infusions prevented pregnancy in 6 out of 10 and at the same time they caused resorption of the foetus. In another study (East, 1955), tested the effect of the dry powder of aerial organs of the same plant against the fertility in mice and guinea pigs. He observed that the powder temporarily impaired the fertility of the male and female mice and produced sterility in the female guinea pig when 1.0 g/mouse and 9.0 g/guinea pig/ day was feed in the formal diet. It was also reported that the powder caused precipitation of the vaginal opening in immature mice within five days and inhibited oestrous cycle. It was thought that the powder reacted with the pituitary gland thereby inhibiting its gonadotropic activity.

Sesquiterpene derivatives from *P. hydropiper* have been found to display anti-tumour activity. In these studies Matsumoto and Tokuda (1990) tested the sesquiterpene compounds from the plant *in vitro* [Epstein-Birr virus activation in Rafi cell] and in the mice with DMBA induced papillomas. The results indicated that the sesquiterpene dialdehyde warburganal (**42**) and polygonal (**43**) inhibited tumour growth considerably. Other work on **42** as a possible medicine for althete's foot by Matsumoto and Kageyana (1986) also

indicated that a transdermal pharmaceutical medicine comprising of 1.0 % 42 was effective in clinical trials for treatment of patients with althete's foot as compared to the common available medicine containing imidazole derivatives.



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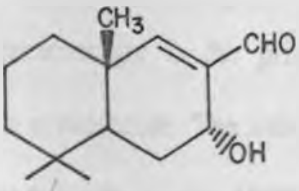

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The plant, *P. hydropiper* L., was also reported to be used against cancer. The sesquiterpene aldehydes; warburganal (42) has been reported to have intense cytotoxic (0.01ug/ml, KB) and antifeedant properties (0.1 ppm/cm^2 against African Army worms) also, polygodial another drimane-type sesquiterpene dialdehyde was also reported to have shown intense antifeedant and plant growth inhibitory activities (Fukuyama *et. al.*, 1983a,b).

1.7.0 SPECTRAL PROPERTIES OF FLAVONOIDS

Recent developments in the structural study of flavonoids have been centred largely on the applications of spectroscopic methods. The main steps generally followed in the structure elucidation of flavonoids are recognition of the class to which the compound belongs and determination of the nature and orientation of the substituent groups in the aromatic rings. These steps are well served by the application of infra-red (IR), mass spectroscopic (MS), nuclear magnetic resonance (NMR) and ultraviolet-visible (UV-Vis) techniques.

1.7.1 INFRA-RED SPECTROSCOPY

Infra-red spectroscopy is the primary probe for the detection of functional groups in a molecule. The infra-red spectra of all the flavonoids show absorption bands in the region 1500 - 1600 cm^{-1} due to aromatic ring, along with a carbonyl band at 1620 -1670 cm^{-1}. The presence of hydroxyl group in the hydroxyl flavonoids is evidenced by absorption in the region 3300 - 3450 cm^{-1}. An absorption at *ca.* 925 cm^{-1} is indicative of a methylenedioxy group and the presence of a *geminal* dimethyl group is indicated by the appearance of a band at *ca* 1400 cm^{-1}. The glycosidic nature of a flavonoid is reflected by broad bands at *ca.* 3250 cm^{-1} and 1060 cm^{-1} (Agrawal, 1989).

1.7.2 PROTON NMR SPECTROSCOPY

Proton NMR spectroscopy is one of the tools widely used for the structural elucidation of organic compounds. Structure elucidation of flavonoids also rely on proton NMR technique to a considerable extent. Chemical shifts as well as coupling pattern and coupling constant values provide information about the substitution pattern of flavonoids.

The protons on ring A of a flavonoid molecule located at C-6 and C-8 of flavone, flavonol and isoflavone which contain 5- and 7-dihydroxyl substitution pattern give rise to two doublets (J= 2.5 Hz) in the range of 6.0 - 6.5 ppm due to the *meta* coupling whereas the protons of the B ring usually appear in the range of 6.7 -7.9 ppm which is downfield from the region where the A ring protons absorb. The signal pattern observed for the B-ring is characteristic for the substitution pattern of the ring. In addition it suggests the oxygenation level of ring C (Mabry *et. al.*, 1970). If ring B is in conjugation with a double bond of ring C as in the case of flavone, isoflavone and chalcone H-2' and H-6' appear together and likewise H-3' and H-4' with H-5'. If ring B is oxygenated at C-4' a typical four peak pattern of two doublets (J= 8.5 Hz) is observed (Mabry *et. al.*, 1970; Markham, 1982). The doublets for the H-3' and H-5' always appear upfield from the H-2' and H-6' protons because they will be shielded more by the C-4' oxygen substitution. With flavonoid glycosides the chemical shift of the anomeric proton of the sugar attached to the flavonoid hydroxyl group depends both on the nature of the flavonoid and on the position and stereochemistry of attachment to the

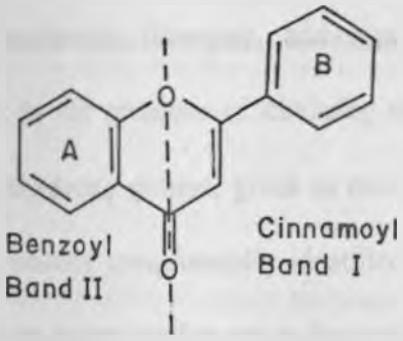
flavonoid nucleus. In C-4, C-5 and C-7 types of flavonoid-O-glycosides the anomeric proton signal occurs near 5.0 ppm while in the 3-O-glucoside the anomeric proton signal appears downfield at about 5.8 ppm (Mabry *et al.*, 1970; Markham, 1982).

1.7.3 CARBON-13 SPECTROSCOPY

Carbon -13 nuclear magnetic resonance (NMR) spectroscopy is an important aid for elucidation of the structure of flavonoids and related compounds. All the fifteen signals due to a flavonoid nucleus resonate in the region 90-200 ppm, in the case of 2,3-unsaturated flavonoids whereas 2,3-saturated flavonoids possess only thirteen signals in this region, the two additional signals resonate at a higher field. The flavonoids; pterocarpanoids and chalcones possess three aliphatic (carbon) resonances and twelve aromatic resonances in the above mentioned chemical shift range. The presence of many signals in the 60-80 ppm region is generally indicative of glycosidic carbons (Agrawal, 1989) and 55 ppm resonance is very likely due to a 4'-methoxy group while all other methoxy groups of flavonoids resonate near 56 ppm (Iha *et. al.*, 1980). The carbonyl signals of both flavones and isoflavones resonate in the region 174-178 ppm but C-2 and C-3 are sufficiently different in the two series to permit an immediate distinction. In flavones the C-2 signal appears at 160-163 ppm and that of C-3 occurs at 104-108 ppm whereas in isoflavones the C-2 resonance is seen at 149-155 ppm and that of C-3 at 122-123 ppm. A carbonyl resonance of 181+1 ppm clearly indicates the presence of a hydroxyl moiety at C-5 (Pelter *et. al.*, 1976).

1.7.4 UV-VISIBLE SPECTROSCOPY

UV-visible spectroscopy is particularly applicable to flavones and flavonols because of the direct conjugation of both the A and B rings to the carbonyl. The methanol spectra of flavonoids exhibit two major absorption bands or peaks in the region 240 - 400 nm. These two peaks are commonly referred to as band I (300-380nm) and band II (240 - 280nm). Band I is considered to be associated with absorption due to the B ring, cinnamoyl system and band II with absorption involving the A ring system (Mabry et al., 1970).



Flavonoids oxygenated in ring A but not in ring B, tend to give spectra in methanol with pronounced band II and a weak band I; in similar compounds which possess ring B and oxygenation at position 3, band I is more pronounced and appears at longer wavelengths. Thus the position of band I can be used to some extent to distinguish between 3-hydroxyflavones and flavones.

On increasing the oxygenation pattern of the B ring in flavonols and flavones about a 3 - 8 nm bathachromic shift in band I occurs with each additional oxygen function. On the other hand changes in the B ring oxygenation pattern usually do not produce a shift in band II but it may appear as either one peak or more peaks (degeneracy) depending on the B ring oxygenation pattern. For example, the 3',4'-oxygenated flavonols and flavones usually exhibits two absorption peaks between 250 - 270nm while the 4'-oxygenated equivalent have only one.

The "ordinary" uv-visible spectra of flavonoid compounds in methanol solutions are extremely informative as to class and possibly the number of hydroxyl groups in the molecule. However, additional measurements of spectra at varying pH, or in the presence of chelating metal ions or anion capable of binding *ortho* dihydroxy groups, gives so much extra information that the compound can be usually unequivocally identified. Some of the shift reagents used in the structure determination are sodium methoxide (NaOMe), aluminum trichloride /hydrochloric acid ($AlCl_3$/HCl) and sodium acetate/boric acid ($NaOAc/H_3BO_3$).

Sodium methoxide is a strong base and ionizes to some extent all hydroxyl groups on the flavonoid nucleus. The addition of NaOMe to flavonols and flavones in methanol usually produces bathochromic shifts in all the absorption bands. However, a large bathochromic shift of band I of about 45-60nm, without a decrease in intensity, is diagnostic for the presence of a free 4'-hydroxyl group. Flavonols lacking a free 4'-hydroxyl group but having

a free 3-hydroxyl group also give a 50-60nm bathochromic shift in band I with a decrease in intensity of the peak.

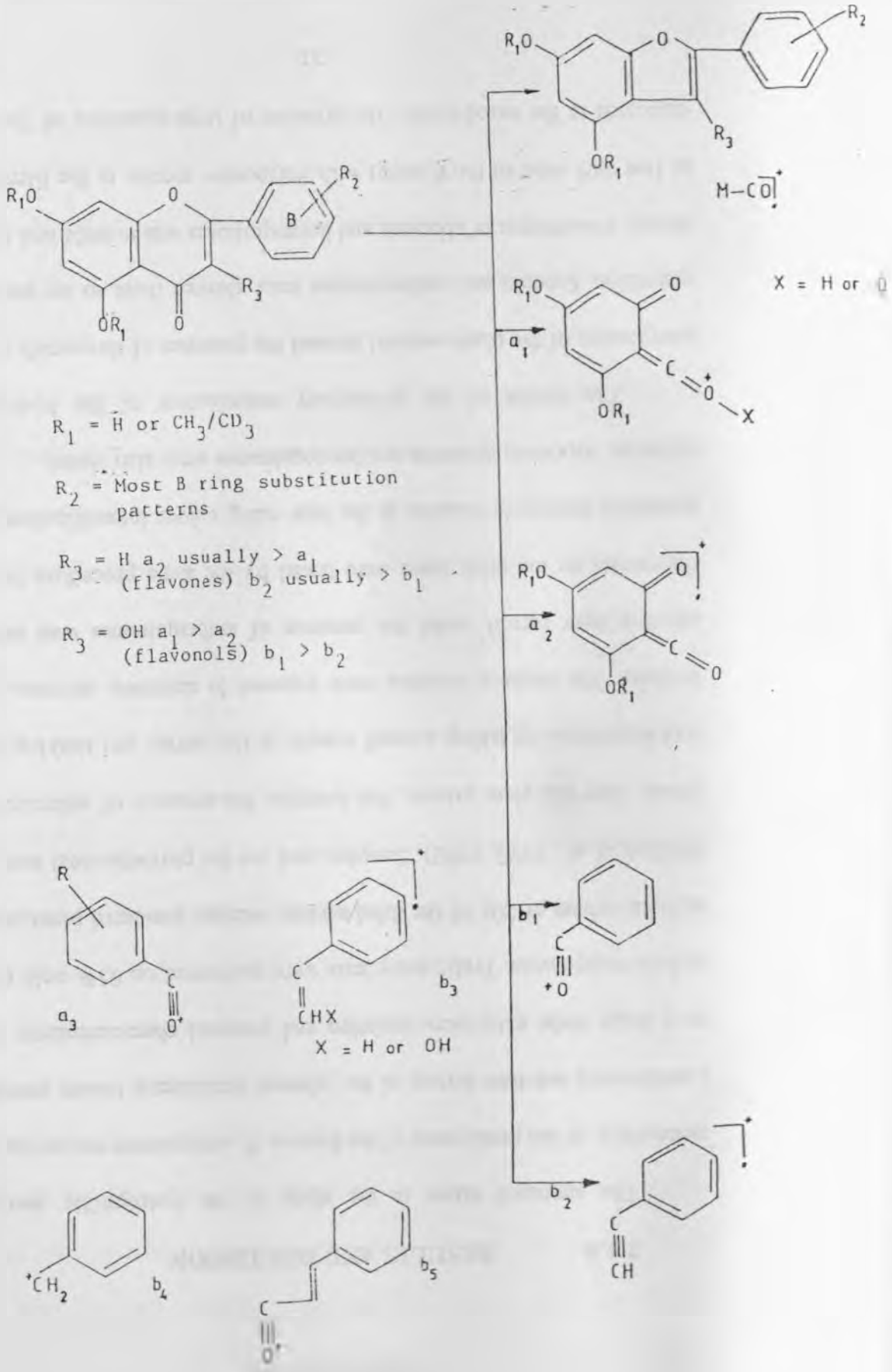
Sodium acetate is a weaker base than NaOMe, and, as such ionizes only the more acidic protons in the flavonoid nucleus that is 3,4',7-hydroxyl groups. Because ionization of the 7-hydroxyl group mainly affects band II, thus NaOAc is a particularly useful diagnostic reagent for the specific detection of 7-hydroxyl group. The uv-visible spectra of flavones and flavonols containing a free 7-hydroxyl group usually exhibit a diagnostic 5 - 20 nm bathochromic shift of band II in the presence of NaOAc. However, when 6,8-oxygenated substituents are present in flavones, the bathochromic shift with NaOAc is often small or imperceptible. In the presence of NaOAc, boric acid will chelate with *ortho*-dihydroxyl groups at all locations on the flavonoid nucleus except at 5,6,8 positions. Thus a flavonoid nucleus containing a B- ring *ortho*-dihydroxyl group shows a consistent 12-30 nm bathochromic shift of band I in the presence of $NaOAc/H_3BO_3$. With aluminum chloride, flavonoids containing hydroxyl groups at C-3 or C-5 (Markham, 1982) form acid stable complexes, in addition $AlCl_3$ forms acid labile complexes with flavonoids which contain ortho-dihydroxy systems (Markham and Mabry, 1968). For flavonols and flavones, the presence of non *ortho*-dihydroxy groups in the flavonoid moiety can be detected by comparison of the spectra of the flavonoid in the presence of $AlCl_3$ and with that obtained in $AlCl_3$/HCl; a hypochromic shift of 30-40 nm is observed in band I of the $AlCl_3$ spectrum with the *ortho*-dihydroxy group on addition of HCl. The

presence of three adjacent hydroxyl groups in the B-ring gives a 20nm hypochromic shift on the addition of acid to the $AlCl_3$ solution. While if the shifted spectrum is not removed, then the presence of 3- or 5-hydroxy groups are either absent or substituted (Mabry, 1969).

1.7.5 MASS SPECTROMETRY

Mass spectrometry is another useful spectroscopic tool for flavonoid structure elucidation. The main application in the structural analysis of flavonoids is the determination of the molecular weight of the compound, establishment of the distribution of the substituent groups between the A and B rings and lastly the determination of the nature and the site of attachment of the sugar moiety in flavonoid C- and O-glycosides. Therefore the objective in interpreting a flavonoid mass spectrum is to identify the unfragmented molecular ion peak (M^+), and to relate other major fragments to it by rationalising the loss in molecular weight using recognised fragmentation pathways (Markham, 1982).

Fission of the M^+ into ions of rings A and B containing a number of fragments also aid in the identification of the substitution pattern of the flavonoids. These fragmentations usually involve one of the two competing pathways I (Retro-Diels-Alder, RDA) and II (see the scheme below). The dominant pathway is determined by the flavonoid type although in some occasions neither pathway produces significant fragments (Agrawal, 1989).



Scheme 3: Fragmentation pattern of flavonoids

CHAPTER TWO

2.0.0 RESULTS AND DISCUSSION

The approach taken in the study of the hydrophillic secondary metabolites of the aerial parts of the Kenyan *P. senegalense* was to carry out a preliminary test-tube survey of the inherent constituents before embarking on a large scale extraction, isolation and eventual characterisation of the various components. Preliminary tests were performed on 95% cold ethanol aqueous extract of 20g of the dried acetone washed powdered plant material (Midiwo *et al.*, 1990, 1992). Samples used for the phytochemical tests were drawn from this plant extract. For example the presence of anthraquinones was determined by taking a small sample of the extract and shaking it with benzene. The benzene solubles were exposed to ammonia solution, if the aqueous layer turned violet the presence of anthraquinones was inferred. Flavonoids on the other hand were tested by the same procedure but with potassium hydroxide solution as the base using colour intensification as the criterion. Saponins, alkaloids and benzoquinones were also tested.

The results of the preliminary examination of the hydrophillic components of the plant material showed the presence of flavonoids in large quantities. Saponin and anthraquinone tests showed them to be present in traces. The absence of alkaloids and benzoquinones was notable and this was in line with most of the findings with *Polygonum* species in the literature as discussed in the introduction. The presence of large quantities of flavonoids

in the inner tissues of the plant prompted us to isolate, characterise them and evaluate them for insect antifeedant activity in comparison with the surface exudate (acetone wash). The procedure employed for the large scale extractions of the hydrophillic components of *P. senegalense* entailed sequential cold extractions of the ground powdered plant material previously washed with acetone with 70% and 50% aqueous methanol for two days each (Goodwin, 1976). The methanol extract was concentrated *in vacuo* using a rotatory evaporator. The remaining aqueous extract was partitioned into dichloromethane to remove the remaining flavonoids from the surface exudate. The aqueous was then partitioned into ethyl acetate and finally freeze dried. The freeze dried material was exhaustively triturated with ethyl acetate. Solvent removal from the partitioned sample materials were carried out *in vacuo* (see Experimental section). The acetone wash of the surface exudate was also concentrated and kept for use in the uv-visible and insect antifeedant experiments.

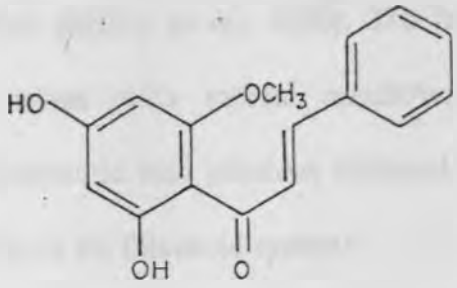
Column chromatography of the ethyl acetate partitioned material from the aqueous extracts was performed on both impregnated silica gel and Sephadex LH-20/silica gel columns and led to isolation and characterisation of several naturally occurring flavonoid aglycones and glycosides; among them 2',4'-dihydroxy-6'-methoxychalcone (1), kaempferol (2), quercetin (3), quercetin-3-ß-O-D-galactoside(4), quercetin-3-ß-O-D-glucoside(5), 2'-ß-O-D-glucosy-4'-hydroxy-6'-methoxydihydrochalcone (6) and quercetin-3-ß-O-D-glucoside-2"-gallate (7). Identification of the compounds was achieved through

their chemical and physical data analysis, literature correlation as well as analogy with authentic marker compounds. Substitution patterns have been established through uv-visible shift data.

The uv-visible spectrum of compound (1) when run in methanol showed one maximum absorption at 341.6 nm. Such an absorption band is characteristic of a chalcone chromophore (Markham, 1982). The proton NMR displayed doublets at 7.82 ppm (J= 15.71 Hz) and 7.64 ppm (J= 15.71 Hz) which were attributed to the C-ß and C-α protons respectively (Table 5). This was confirmed by comparison of proton NMR spectra of chalcones isolated from *Alnus viridus* (Wollenweber *et al.*, 1974) and published spectra for chalcones (Mabry *et al.*, 1970). Also the high value of the coupling constant is characteristic of *trans* coupling of an olefinic moiety (Bhatiya and Gupta, 1981). These observations led to the suggestion that compound (1) could be a chalcone. The two multiplets centred at 7.44 ppm and 7.43 ppm in the proton NMR spectrum integrating for 2H and 3H, respectively, and the ion peak at m/z 91 in the mass spectrum implied an unsubstituted B ring. The observed molecular ion peak at m/z 270 suggested two hydroxyl groups and a methoxy group in the molecular skeleton. The ion peak at m/z 167 and 140 suggested that both the substituents to be on ring A.

Further analysis of the uv-visible spectra of compound 1 using standard methods (Mabry *et al.*, 1970; Markham, 1982) results in a bathochromic shift of band I by 28.5 nm and 37.2 nm upon addition of sodium acetate and aluminium trichloride solutions, respectively, to a methanolic solution of

compound 1 led to the assigning of the hydroxyl groups at positions 4' and 2', respectively. Lack of a shift in the uv-visible spectrum on addition of hydrochloric acid solution to a methanol/aluminum trichloride solution of the compound further confirmed the positioning of a hydroxyl group at C-2' position and of the conclusion that the two hydroxyl groups are not *ortho* to one another. Furthermore, the proton NMR spectrum showed the presence of *meta*-coupled doublet signals at 5.92 ppm (J= 2.06 Hz) and 6.01 ppm (J= 2.06 Hz) integrating for one proton each for the A ring showing that the set of protons are in non-equivalent environments, implying a trisubstituted system in the A ring of the chalcone moiety. A three proton singlet at 3.83 ppm was assigned to the methoxy group (Markham, 1982). Based on these data and through literature correlation (Mabry *et al.*, 1970; Wollenweber *et al.*, 1974) compound **1** was assigned the structure 2',4'-dihydroxy-6'-methoxychalcone. The assigned structure of compound 1 was further supported by the carbon-13 NMR data (see Experimental) which showed the presence of the methoxy carbon atom at 56.0 ppm, two olefinic and carbonyl carbon atoms at 127.5, 141.7 and 197.7 ppm, respectively, in addition to the aromatic carbon atom signals. Compound 1 (2',4'-dihydroxy-6'-methoxychalcone) was first isolated from the seeds of *Alpinia speciosa* where its constitution was established by hydrolytic cleavage, isomerization to the corresponding flavanone and by comparison with a synthetic sample (Krishma and Chaganty, 1973) and later from the leaves of *Alnus viridus* (Wollenweber, et al., 1974).



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Compound **2** was eluted from the impregnated silica gel column with 5 % methanol/chloroform mixtures. It had a melting point 242-243 °C and R_f value of 0.51 with solvent system 5. The infrared spectrum of the compound run as potassium bromide pellet showed absorption bands at 3440 and 1680 cm^{-1} for hydroxyl and carbonyl functions in the molecular structure. Other peaks at 1640, and 1290 cm^{-1} were attributed to a phenolic moiety were also structurally indicative of a flavonoid (Briggi and Locker, 1956).

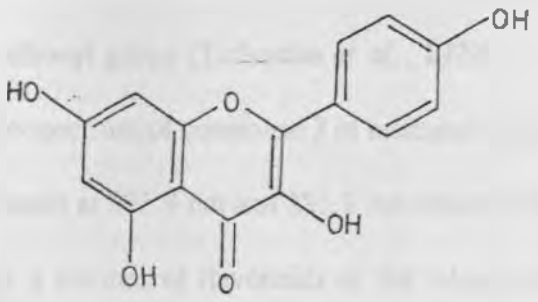
The uv-visible spectrum of compound **2** run in methanol showed strong absorption bands at 362.8 nm (band I) than that at 268.0 nm (band II) suggesting a flavonoid skeleton lacking a sugar residue on the molecular structure (Rao *et al.*, 1983). The bathochromic shift of band I with aluminium trichloride/hydrochloric acid solutions is a characteristic feature of a free 3- or 5-hydroxyl group (Mabry *et al.*, 1970). The bathochromic shift of 16.5 nm with sodium acetate solution indicated the presence of unsubstituted 7-hydroxyl group. Furthermore in the presence of sodium methoxide solution band I exhibited a bathochromic shift of only 48.5 nm with an increase in the

intensity in accordance with a free 4'-hydroxyl group in a flavonol system or flavone derivative (Mabry *et al.*, 1970). The lack of response when the compound is mixed with sodium acetate/boric acid and aluminum 2trichloride/hydrochloric acid solutions indicated the absence of an *ortho*-dihydroxyl group in the flavonoid system.

The mass spectrum of compound 2 showed a prominent molecular ion peak at m/z 286 and elemental analysis indicated its molecular formula as $C_{15}H_{10}O_6$; the molecular ion peak showed 100% abundance while the other ion peaks had relatively low abundance. Low intensity ion peaks at m/z 153 and 121 are derived from RDA fragmentations and other peaks at m/z 105, 93, 77 and 69 were attributed to the secondary fragmentation after the major RDA fragmentation.

The proton NMR spectrum of compound 2 determined in DMSO-d_6 (400 MHz) (Table 6) substantiated the presence of a 4'-hydroxyl group in ring B of the flavonol skeleton due to the two well-resolved doublets at 8.03 ppm (J= 8.06 Hz) and 6.91 ppm (J= 8.06 Hz) integrating for two protons each showing that the two sets of coupled protons are characteristic of an A_2B_2 pattern (Markham, 1982). The ion peak at m/z 121 in the mass spectrum plus the reaction of the compound with sodium methoxide solution reconfirmed the presence of a free hydroxyl group at 4'-position. The occurrence of the two doublets at 6.39 ppm (J= 1.98 Hz) and 6.19 ppm (J= 1.98 Hz) integrating for one proton each with *meta*-coupling and the ion peak at m/z 153 in the mass spectrum further demonstrated the existence of free hydroxyl groups at

positions 5 and 7 of the A ring previously suggested by the uv-visible shift data. When compound **2** was mixed with an authentic sample of kaempferol, the melting point was not depressed (242-243 °C). Based on these observation compound **2** was assigned the structure of 4',5,7-trihydroxyflavonol or kaempferol. The assigned structure of compound **2** was further confirmed through literature correlation of its carbon-13 NMR shift values with those reported for kaempferol (Harborne and Mabry, 1982). Besides the signal at 175.9 ppm for the carbonyl group, the low field region of carbon-13 NMR spectrum further displayed resonances at 163.8, 160.7, 159.2, 156.1, 147.0 and 135.6 ppm corresponding to the quarternary ethylenic O-bond carbon atoms in comparison with those recorded for kaempferol at 135.6, 146.8, 156.2, 159.2, 160.7 and 163.9 ppm for C-3, 2, 9, 4', 5 and 7, respectively, (Harborne and Mabry, 1982; Wager et al., 1976).



2

Kaempferol is one of the flavonoids which are widely distributed in the *Polygonum* species both as aglycone and glycosidic forms, even though it occurs in small quantities in the aerial parts of *P. senegalense*; It was earlier reported in the leaf of *P. nodosum* as one of the major secondary metabolites both in the free form and glycosydated forms (Isobe *et al.*, 1979; 1980).

Further elution of the oxalic acid impregnated silica gel column with 10 % methanol/chloroform mixture gave compound 3. Compound 3 had a melting point of 313-314 °C and an R_f value of 0.26 with the solvent system 5 on analytical silica gel t.l.c. plate impregnated with 3 % oxalic acid solution. On exposure to ammonia solution the spot intensified its yellow colouration. The infrared spectrum of compound 3 run as potassium bromide pellet showed absorption frequencies at 1620 and 3400 cm^{-1} identified as chelated carbonyl and hydroxyl functions of a flavonoid chromophore respectively (Munay and McCabe, 1979). Other absorption frequencies at the region 1386 and 1200 - 1100 cm^{-1} were attributed to the aromatic moiety in conjugation to the carbonyl group (Tschesche *et al.*, 1979).

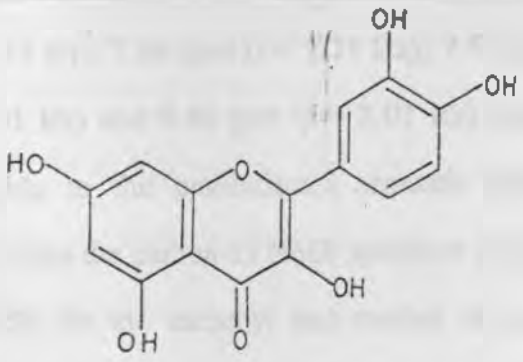
The uv-visible spectrum of compound 3 in methanol exhibits two major maxima absorption bands at 371.4 nm and 250.1 nm which were very similar to those reported for a number of flavonoids of the 3-hydroxylflavone type (Mabry et al., 1970). The bathochromic shifts of the uv-visible absorption of both bands I and II with sodium methoxide solution indicated the presence of hydroxyl groups in the flavonoid skeleton and the degeneracy of the band I, confirmed the alkaline-sensitive hydroxyl groups in positions 3, 3' and 4'

within the molecule. Further investigation of the uv-visible absorption spectrum of compound **3** following standard procedures (Mabry et al., 1970; Markham, 1982); confirmed a free hydroxyl group at C-7 (sodium acetate 387.8 nm and a new peaks at 324.2 nm with sodium methoxide solutions). The presence of an *ortho*-dihydroxyl groups on the B ring was also deduced (reactions of the compound with sodium acetate/boric acid solutions). A bathochromic shift of 55.0 nm in band I relative to the methanol after addition of hydrochloric acid to methanol/aluminum trichloride solutions of compound **3** was attributed to the presence of a free 5-hydroxyl group.

The mass spectrum of **3** showed a molecular ion peak at m/z 302 which fits the molecular formula $C_{15}H_{10}O_7$ and was confirmed by elemental analysis. A peaks at m/z 285 was attributed to the loss of hydroxyl group and peaks at m/z 153 and 137 were initiated by the RDA fragmentation. The other peaks in the mass spectrum are due to secondary fragmentations after the major RDA fragmentations.

The ABC type aromatic protons of compound **3** (Table 7) was evidenced by the presence of a doublet at 7.67 ppm (J= 2.13 Hz), 6.87 ppm (J= 8.50 Hz) and 7.53 ppm (J= 2.13; 8.50 Hz) integrating for one proton each and attributed to the protons of the B ring showing that the three sets of protons are in different environments. This observation, the reaction of the compound with sodium acetate/boric acid and aluminium trichloride/hydrochloric acid solutions and the ion peak at m/z 137 in the mass spectrum reconfirmed the presence of two hydroxyl groups in the B ring at

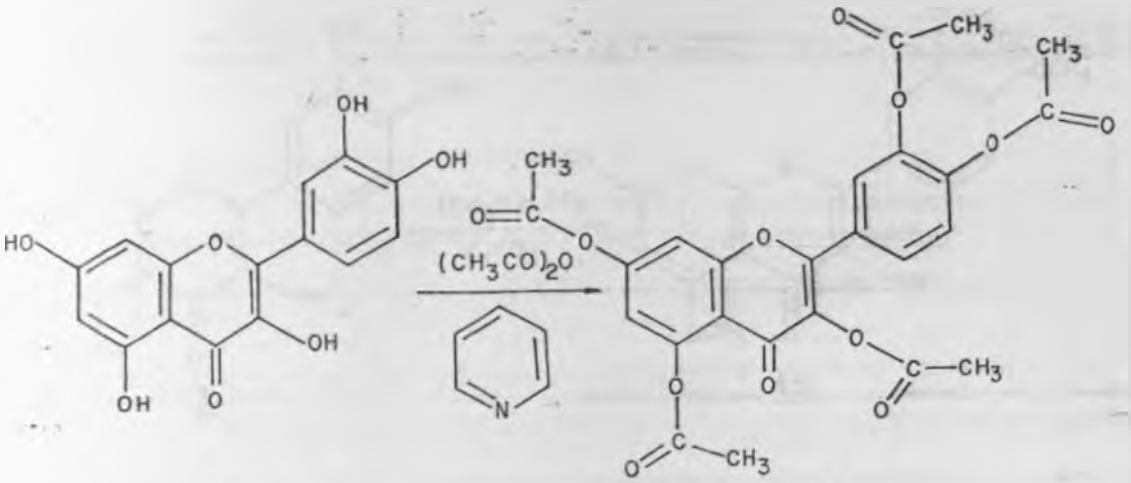
positions 3' and 4'. The proton NMR further displayed *meta*-coupled doublets at 6.17 ppm (J=1.97 Hz) and 6.40 ppm (J= 1.97 Hz) integrating for one proton each indicating the compound is unsubstituted at positions H-6 and H-8 respectively on the A ring and the two sets of protons are in different chemical environments. This evidence, the ion peak at m/z 153 in the mass spectrum and the reaction of the compound with sodium acetate solution reconfirmed the presence of the two free hydroxyl groups at postions 5 and 7 in the flavonoid skeleton. With all these data (IR, UV, NMR and MS) compound 3 was assigned 3',4',5,7-tetrahydroxyflavonol or quercetin. The assigned structure was further confirmed through literature correlation of the carbon-13 NMR spectrum of compound 3 and those of quercetin reported in the literature (Harborne and Mabry, 1982; Wager *et al.*, 1976).



3

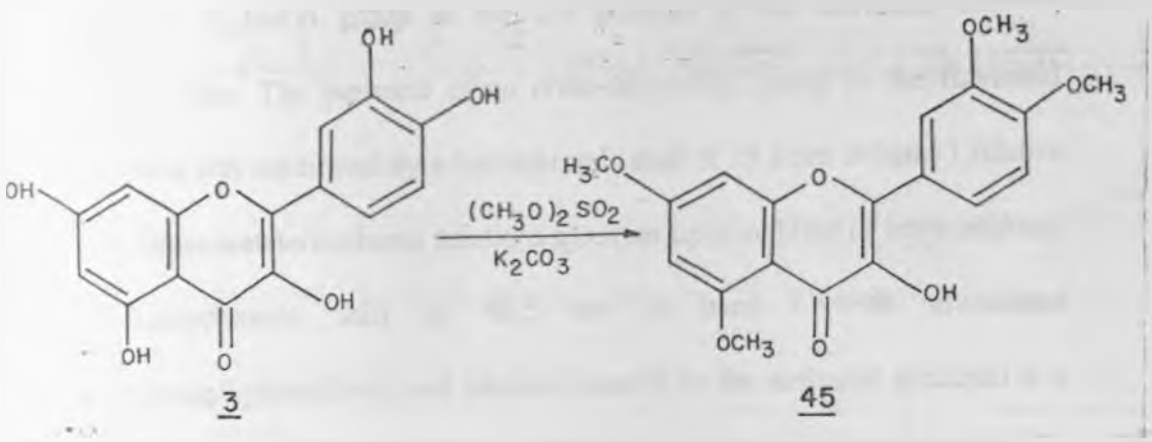
Compound 3, therefore had a pentahydroxylated flavonoid skeleton which was further confirmed by penta-acetylation reactions. Compound 3 is one of the commonest compounds in the *Polygonum* species and occurs both as aglycone and glycoside in combination with several sugar residues, such as quercetin-3-α-arabinoside from *P. weyrichii* (Varnite, 1982); quercetin-3-ß-D-glucoside and quercetin-3-ß-D-glucoside-2"-gallate from *P. nodosum* a native plant of Japan (Isobe *et al.*, 1979; 1980). In a free state, Horhammer and Kriesmair, (1955) reported a similar compound from *P. polystachy* plant. The compound had a melting point of 315 °C and displayed similar spectroscopic data.

Acetylation of compound 3 was carried out using acetic anhydride and pyridine. The acetylation product compound 44 was obtained as white crystals melting point 188-190 °C (lit 186 °C Horhammer and Kriesmair, 1955) and an Rf value of 0.48 with solvent system 4. The proton NMR spectrum of compound 44 (Table 12) displayed multiple signals at 2.30-2.40 ppm attributed to the methyl protons of the acetyl groups and doublet at 7.86 ppm (J= 2.21; 8.74 Hz); 7.64 ppm (J= 2.21 Hz); 7.51 ppm (J= 8.74 Hz); 7.26 ppm (J= 2.01 Hz) and 6.88 ppm (J= 2.01 Hz) integrating for one proton each assignable to the unsubstituted aromatic protons of the flavonoid skeleton. Whereas the carbon-13 NMR spectrum showed the presence of five chemical shifts for the carbonyl and methyl carbon atoms in the region between 167.8-169.3 ppm and 20.9-20.4 ppm respectively in addition to the other flavonoid chemical shifts. These observations further confirmed the presence of five hydroxyl groups on compound 3 and completion of the acetylation reaction.



3 44

Methylation of compound 3 was carried out using dimethyl sulphate in anhydrous acetone and potassium carbonate gave 45 as yellow crystals melting point 156-158°C and R_f value of 0.73 with solvent system 4. The proton NMR spectrum of 45 (Table 13) displayed four singlets in the region between 3.84-3.85 ppm integrating for 3H each corresponded to the 3',4',5 and 7 methoxy groups by literature correlation and doublets integrating for one proton each due to the unsubstituted aromatic protons of a flavonoid skeleton. The carbon-13 NMR of 45 displayed four peaks with equivalent intensities in the region between 50-60 ppm assignable to C-3', 4', 5 and 7 methoxy carbon atoms by literature correlation with reported data for quercetin methyl ether derivatives (Harborne and Mabry, 1982; Kup2chan and Bauschmidts, 1971). Thus based on these data, compound 45 was assigned the structure quercetin-3',4',5,7-methyl ether 45.



Compound 4 was obtained as a yellow crystalline compound and recrystallization of the compound yielded yellow crystals melting at 230-232 °C. It gave a homogenous spot on analytical t.l.c. plate, R_f value of 0.32 with solvent system 4 and appeared deep purple under UV light. It turned yellow with concentrated ammonia solution suggesting compound 4 could be a flavonol glycoside with a sugar moiety on the C-3 hydroxyl group.

The infrared spectrum of compound 4 run as a potassium bromide pellet exhibited absorption frequencies at 3410 cm^{-1} and 1650, 1605 cm^{-1} which are due to chelated hydroxyl group and α, ß-unsaturated ketone moiety stretches. Other peaks at 960, 860 and 770 cm^{-1} were associated with the aromatic ring (Tschesche *et al.*, 1979). The uv-visible spectrum of the compound in methanol displayed bands at 359.5 nm (band I) and 258.0 nm (band II), suggesting that it is a flavonol glycoside (Mabry *et al.*, 1970; Markham, 1982). Its uv-visible spectrum was shifted bathochromically on the addition of both sodium acetate and aluminum trichloride solutions (see Experimental) indicating the presence of a free hydroxyl group at C-7 and a

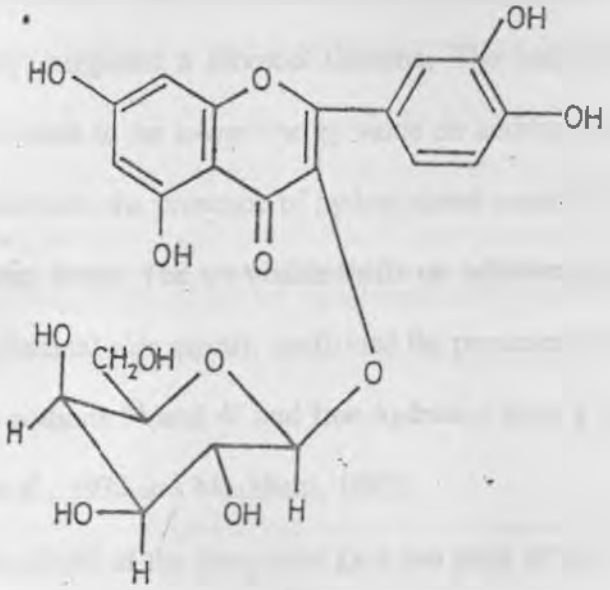
chelated hydroxyl group at the C-5 position of the flavonoid skeleton respectively. The presence of an *ortho*-dihydroxyl group in the flavonoid skeleton was confirmed by a bathochromic shift of 15.0 nm in band I relative to sodium acetate/methanol solutions spectrum upon addition of boric acid and a bathochromic shift of 40.5 nm of band I with aluminium trichloride/hydrochloric acid solutions relative to the methanol spectrum is a characteristic feature of 5-hydroxyl-3-substituted flavonols (Mabry et al., 1970).

The proton NMR spectrum of the compound (Table 8) showed the existence of the aromatic protons as an ABC system and sugar protons in the compound. An anomeric proton signal at 5.37 ppm (d, J= 7.69 Hz) indicated that the sugar residue was attached to the aglycone moiety of the compound by a ß-linkage (Ariya, *et al.*, 1992). The position of the anomeric proton in the proton NMR and reaction of the compound with sodium acetate solution indicated the C-3 position of the aglycone was occupied by the sugar residue (cf 5.00 ppm for C-7 sugar residue which forms complex signals due to the interaction of the anomeric protons with the H-6 and H-8 protons, Markham, 1982). The occurrence of the other sugar protons at 3.63 ppm (d, J= 3.72 Hz), 3.55 ppm (dd, J= 7.69; 9.27 Hz), 3.44 ppm (d, J= 5.93 Hz) and multiple signals at 3.40-3.20 ppm suggested the presence of a galactoside residue rather than a glucose which normally forms broad multiple signals for the non-anomeric protons (Bennin *et al.*, 1992).

The aromatic protons of an ABC system in compound 4 was evidenced by the presence of doublets at 7.51 ppm (J= 2.17 Hz), 7.66 ppm (J= 2.17; 8.50 Hz) and 6.80 ppm (J= 8.50 Hz) integrating for one proton each showing *meta-ortho* and *ortho-ortho* coupling implying that the three sets of protons in the B ring are in non equivalent environments. This observation and the observed 20.0 nm hypochromic shift in band I with aluminium trichloride/hydrochloric acid relative to aluminium trichloride/methanol solutions of compound 4 confirmed the presence of unsubstituted protons in the 2', 5' and 6' on the B ring. The 5,7-disubstituted pattern in the A ring of the flavonoid skeleton was evidenced by the presence of well-resolved meta-coupled protons shifts at 6.19 ppm (J= 1.98 Hz) and 6.39 ppm (J= 1.98 Hz) integrating for one proton each implying that the two sets of protons are in different chemical environments. Based on these data and by literature correlation (Bennin *et al.*, 1992), compound 4 was identified as a quercetin-3-ß-O-galactoside. Acid hydrolysis of compound 4 further suggested the assigned structure because it enabled the isolation of quercetin identified by comparison with an authentic marker on silica gel t.l.c. plate and galactose identified by paper chromatography against an authentic sample.

The carbon-13 NMR spectrum (400 MHz DMSO-d_6) also supported this conclusion as it fit satisfactorily with the superimposition 8of the expected signals for the quercetin and galactose fragments (Bennin *et al.*, 1992). The melting point of compound 4 was identical with those reported for quercetin-3-ß-O-galactoside from *Rumex acetosa* 230-233 °C (Horhammer and Volf,

1955). Finally the confirmation of the molecular weight of compound 4 was established by FAB-MS at 464 and this was associated with the elemental analysis of the assigned structure assigned ($C_{21}H_{19}O_{12}$). The EIMS of the compound 4 displayed a number of peaks attributed to the loss of a sugar unit and fragmentation of the M^+ ion.



5

Compound 5 was obtained after successive fractionations of the 20 and 30 % methanol/chloroform eluants from the Sephadex column. It had a melting point of 220-222 °C and an R_f value of 0.26 with solvent system 5. It flouresced deep purple with ammonia solution indicating that it is a flavonol glycoside with a substituted-3-hydroxyl group (Brasseur and Angenot, 1986; Nasr *et al.*, 1987).

The infrared spectrum of the compound run as a potassium bromide pellet showed absorption frequencies at 3300 cm^{-1} and 1640 cm^{-1} identified as due to a chelated hydroxyl and carbonyl functions of a flavonoid skeleton respectively. Other absorption frequecncies at 1365 cm^{-1} and 1100-1000 cm^{-1} were assigned to the C-O and aromatic stretches (Sakushima and Nishibe, 1988). The uv-visible spectrum of the compound run in methanol displayed absorption bands at 360.1 nm and 255.9 nm attributed to bands I and II, respectively, suggested a flavonol skeleton. The bathochromic shifts of the absorption bands to the lower energy value on addition of sodium methoxide solution indicated the presence of hydroxylated aromatic moiety (Sakushima and Nishibe, 1988). The uv-visible shifts on addition of diagnostic reagents (see Experimental *vide supra*); confirmed the presence of free *ortho*-hydroxyl groups at postions 3' and 4' and free hydroxyl groups at 5 and 7 positions (Mabry *et al.*, 1970 and Markham, 1982).

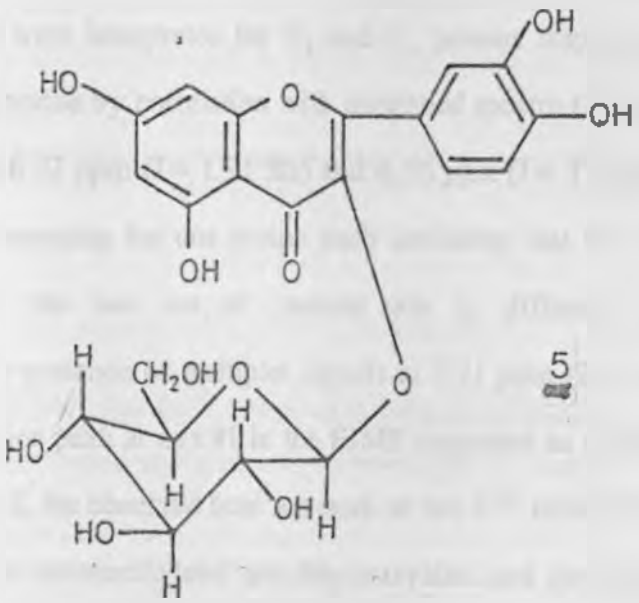
The EIMS of the compound gave ion peak at m/z 302 (98 % relative intensity to the base peak) evidence of loss of C-3-O-glycosyl moiety (Sakushima and Nishibe, 1988) and the proton NMR spectrum of the compound (Table 9) indicated the presence of sugar protons and the aromatic protons of the ABC type. The anomeric proton chemical shifts occured at 5.38 ppm and the configuration of the sugar anomeric centre was determined to be ß from the proton NMR coupling constant of 7.66 Hz. The multiple signals at 3.57-3.330 ppm integrating for six protons of the non-anomeric protons of the sugar moiety suggested the presence of a glucose residue (Bennin et al., 1992).

The ABC type aromatic protons of the compound occurred as doublets at 7.67 ppm (J= 2.24; 8.50 Hz), 7.53 ppm (J= 2.24 Hz) and 6.81 ppm (J= 8.50 Hz) integrating for one proton each showing *meta* and *ortho* coupling of the three protons in the ring B. This observation and the reaction of the compound with sodium acetate/boric acid, aluminium trichloride/hydrochloric acid solutions led to the positioning of the *ortho* dihydroxyl groups on the B ring at positions 3' and 4'. Furthermore, B ring *ortho* dihydroxylation was confirmed by the presence of fragmentation ion peak at m/z 137 in the EIMS. Two prominent doublets integrating for one proton each at 6.20 ppm and 6.40 ppm with a coupling constant 2.07 Hz plus an ion peak at m/z 153 in the EIMS and bathochromic shift of band II with sodium acetate solution strongly suggested a *meta* orientaion for the two sets of protons and confirmed a free C-7 hydroxyl group. Acid hydrolysis of compound 5 afforded only glucose and 3 (UV, NMR).

A comparison of the effect of the sodium methoxide solution (under spectral analysis conditions) upon the aglycone (quercetin) and compound 5 reconfirmed the presence of the glucose moiety at C-3 position. Because compound 5 remained stable to sodium methoxide solution whereas the aglycone residue rapidly decomposed, meant the presence of alkaline-sensitive 3, 3' and 4' hydroxylation pattern in the aglycone (Howard and Mabry, 1970). By comparison with published values for the IR, UV and NMR spectra (Bennin *et al.*, 1992) compound 5 was assigned the structure of quercetin-3-ß-O-glucoside.

The presence of the ß-glucoside moiety was further confirmed by the carbon-13 NMR chemical shifts of six aliphatic carbon atoms signals which were identical with those observed in quercetin-3-ß-O-glucoside isolated from

Erica cinerea (Bennin et al., 1992), the other signals had been assigned according to literature data (Markham *et al.*, 1978; Markham and Ternai, 1976; Ternai and Markham, 1976). Finally the FAB-MS of the compound gave a molecular formula of m/z 464, which required the molecular formula of the compound to be $C_{21}H_{20}O_{12}$ further confirmed by elemental analysis. Quercetin-3-ß-O-glucoside (5) is a very common compound in plants and it had been reported in a number of *Polygonum* species (see literature survey) from a number of countries but had never been described in the Kenyan *Polygonum* species.



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Compound 6 was eluted with 10 % methanol in chloroform mixture from the Sephadex column. It had a melting point of 191-193 °C and an R_f value of 0.48 with solvent system 2. Its spot assumed greenish colouration on exposure to ammonia solution suggesting a flavonoid skeleton. On illumination of the compound with UV light it appeared dull yellow. The infrared spectrum of compound 6 run as potassium bromide pellet showed the presence of a

hydroxyl and carbonyl functions stretches at 3300 cm^{-1} and 1620 cm^{-1}, respectively, suggesting intra-molecular hydrogen bonding. Other characteristic peaks were the aromatic stretches observed in the region of 1400-1100 cm^{-1} (Mabry et al., 1970).

The single major absorption band in the uv-visible spectrum of the compound at 323.0 nm in methanol suggested a flavonoid skeleton lacking conjugation of A and B ring. The proton NMR spectrum of the compound (Table 10) indicated the existence of proton signals in the aromatic and sugar proton regions. A pair of triplets at 3.12 ppm and 2.91 ppm integrating for two protons each were interpreted for C_β and C_α protons respectively of a dihydrochalcone residue by correlation with published spectra (Mabry et al., 1970). Centred at 6.32 ppm (J= 1.91 Hz) and 6.16 ppm (J= 1.91 Hz) were doublet signals integrating for one proton each indicating that the A ring is trisubstituted and the two set of protons are in different chemical environments. The presence of multiplet signals at 7.21 ppm (2H) and 7.20 ppm (3H) and the ion peak at m/z 91 in the EIMS suggested an unsubstituted B ring. From EIMS, the observed base ion peak at m/z 272 indicated that the aglycone residue is monomethylated and dihydroxylated and the peak at m/z 153 required both substitutions to be on the A ring.

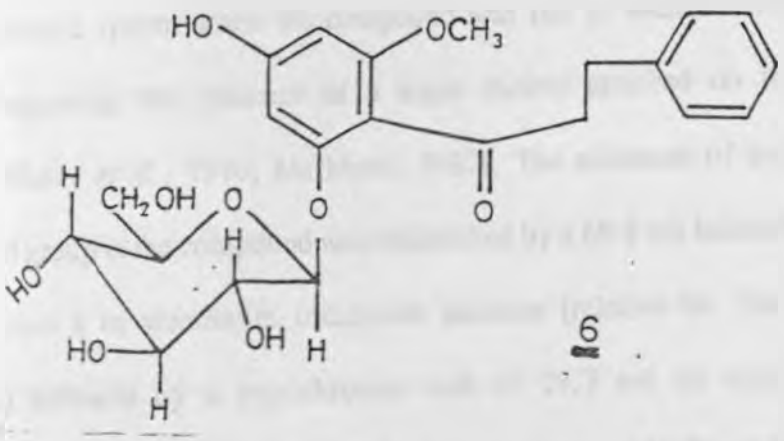
The appearence of a doublet at 4.83 ppm (J= 7.76 Hz) assignable to an anomeric proton and the reaction of the compound with aluminium trichloride solution indicated that the sugar moiety was linked to the flavonoid skeleton through its 2' position. The high value of the coupling constant pointed to the *trans* diaxial relationship with the neighbouring proton hence to ß-stereo-chemistry at the anomeric centre in the pyranoside (Marco *et al.*,

1986). The other non-anomeric sugar protons were observed as multiple signals at 3.69-3.30 ppm integrating for six protons only was consistent with the existence of a glucose unit in the compound. The compound had only one methoxy group in the A ring which appeared as a singlet at 3.71 ppm integrating for three protons.

A sample of the compound was hydrolysed with dilute hydrochloric acid. After removal of aglycone, the aqueos mixture was analysed for sugars. The only sugar indentified was glucose (PC, authentic marker) and the aglycone identified as 2',4'-dihyroxy-6'-methoxydihydrochalcone (37) (UV, IR and NMR) and correlation with literature (Midiwo *et al.*, 1990). It was therefore concluded from these results that the compound was a monoglycoside. The position of the attachment of the substituents on the flavonoid skeleton was further determined by examination of the uv-visible characteristic data of compound **6** and its aglycone residue. A bathochromic shift band I as obtained in the methanol when sodium acetate solution was added to compound **6** suggested a free 4'-hydroxyl group. In the presence of aluminuim trichloride solution the aglycone residue exhibited a typical bathochromic shift of an *ortho*-hydroxyl acetophenone (Bohm and Glennie, 1969) whereas compound **6** showed no reaction. These observations led to the conclusion that the naturally occurring compound **6** is 2'-ß-O-gluco-4'-hydroxy-6'-methoxydihydrochalcone.

Additional confirmation of this structural assignment was sought from carbon-13 NMR. The possible carbon atoms chemical shift assignments around the A ring was also obtained from the calculated shift values on the basis of structure **6**. The calculated shift values were found as C-1' at 109.5

ppm, C-2' at 154.0 ppm, C-3' at 107.7 ppm, C-4' at 161.9 ppm, C-5' at 99.1 ppm and C-6' at 162.7 ppm and could be matched to the peaks in the carbon-13 NMR spectrum at 114.1, 157.8, 97.0, 162.3, 94.7 and 162.4 ppm. All other signals have been assigned according to literature correlation (Mabry *et al.*, 1970; Markham *et al.*, 1978, Markham and Ternai, 1976, Ternai and Markham, 1976). The DEPT carbon-13 NMR spectrum of compound 6 at $\Theta =$ 90° displayed negative signals at 62.6, 47.5 and 31.2 ppm and at 135 the signal at 56.2 disappeared which was in agreement with the assigned structure. Finally the molecular weight determined by FAB-MS showed the ion peak at m/z 435 which fits $C_{22}H_{26}H_9$. Glycosidation at 2'-position in the dihydrochalcone skeleton is not very usual. We have not been able to trace this structure in the literature. It is plausible that the external exudate non-polar flavonoids of *P. senegalense* could be made from the internal form through such an intermediate. Such intermediates should really only have fleeting existence in the cell vacuoles and yet compound 6 accumulates fairly large quantities to be isolated. Interestingly no other non-polar aglycone glycoside have been found. Whether compound 6 is the intermediate of all the other epicuticular flavonoids or not, needs further investigation.



6

Compound 7 was the most polar substance in this series of natural products from the Kenyan *P. senegalense*. It had a melting point of 203-204 °C, R_f value of 0.24 with solvent system 3 on analytical t.l.c plate and darkened on illumination with UV light. The infrared spectrum of the compound run as potassium bromide pellet showed absorption frequencies at 3300 cm^{-1} and 1620 cm^{-1} assignable to a chelated hydroxyl and carbonyl functions respectively of a flavonoid system (Munay ans McCabe, 1969). Two other absorption frequencies at 1650 cm^{-1} and 1510 cm^{-1} are characteristic of aromatic rings in conjugation with a carbonyl function (Tschesche *et al.*, 1979). A C-O vibration in the region of 1100-1000 cm^{-1} suggested a glycosidic nature of the compound (Sakushima and Nishibe, 1988). Other absorption frequencies at 1460, 1360, 1300 and 1200 cm^{-1} are due to aromatic vibrations.

The uv-visble spectrum of the compound run in methanol showed stronger absorption maxima at 359.9 nm (band I) than 259.2 nm (band II) indicating the presence of a sugar moiety in the compound and the absence of an important alkaline decomposition feature of 3, 3'and 4'hydroxyl fuctions on the flavonoid system when the compound was run in sodium methoxide solution suggesting the presence of a sugar moiety attached on the C-3 position (Mabry *et al.*, 1970; Markham, 1982). The existence of an *ortho*-dihydroxyl group in the compound was established by a 68.8 nm bathochromic shift of band I in aluminuim trichloride solution (relative to band I in methanol) followed by a hypochromic shift of 29.7 nm on addition of hydrochloric acid solution; also the bathochromic shift of 18.7 nm for band

I in sodium acetate/boric acid solutions reconmfirmed the presence of the *ortho*-dihydroxyl system in the naturally occurring compound (Markham, 1982). Bathochromic shifts of 21.9 nm and 40.1 nm of band I with sodium acetate and aluminium trichloride/hydrochloric solutions are characteristic features of unsubstituted 7-hydroxyl group and 5-hydroxy-3-substituted flavonols (Mabry *et al.*, 1970; Markham, 1982).

From the proton NMR spectrum (Table 11), the presence of a doublet at 7.77 ppm (J= 2.21 Hz), 7.55 ppm (J= 2.21, 8.50 Hz) and 6.81 ppm (J= 8.50 Hz) integrating for one proton each and the ion peak at m/z 137 in the EIMS confirmed the presence of an ABC type aromatic protons and an *ortho*-dihydroxylation pattern in the B ring of the flavonoid skeleton. The existence of two more doublets at 6.44 (J= 2.00 Hz) and 6.17 ppm (J= 2.00 Hz) integrating for one proton each assignable to the *meta*-coupled protons and ion peak at m/z 153 in the EIMS plus the reaction of the compound with sodium acetate confirmed that the A ring is unsubstituted at positions 6 and 8. The presence of a galloyl moiety in the compound was deduced from a two proton singlet at 6.88 ppm and an ester absorption band at 1690 cm^{-1} in the proton NMR and IR spectra respectively (Isobe *et al.*, 1990; Nonaka *et al.*, 1982). A doublet at 5.10 ppm (J= 7.87 Hz) was attributed to the anomeric proton indicating that the sugar moiety was linked to the flavonoid unit through its C-3 position and the high value of the coupling constant between H-1'" and C-2'" evidenced that the sugar moiety was linked in a diaxial orientation (ß-configuration, Markham, 1982). The other non-anomeric sugar protons were

observed as multiplets in the region between 3.80-3.40 ppm integrating for six protons thus suggesting a glucose moiety (Bennin *et al.*, 1992).

From IR, UV, NMR spectra and the observed base ion peak at m/z 302 in the EIMS suggested a pentahydroxylated aglycone system which was confirmed by elemental analysis to be $C_{15}H_{10}O_7$. Other ion peaks in the EIMS were observed at m/z 318, 182 and 170, but the nature of the glycosidic unit could not be obtained. Acid hydrolysis of compound 7 enabled the identification of quercetin (3) by comparison (UV, IR and NMR) with an authentic marker, glucose was identified by paper chromatography (whatman No.1) with authentic samples and the presence of gallic acid in the aqueous portion was established due to the formation of a black precipitate between the aqueous layer and ferric chloride solution (Finar, 1963; Schmidt, 1955). Through NMR, IR and UV literature correlation (Isobe *et al.*, 1979; 1980; Markham *et al.*, 1978), compound 7 was assigned the structure of quercetin-3-β-O-glucoside-2''-gallate.

The identity of the galloyl group carbon atoms in the carbon-13 NMR spectrum of the compound at 167.74 ppm (CO_2), 145.72 (C-2''/C-5''), 135.60 (C-4''), 122.74 (C-1'') and 110.08 (C-2''/C-6'') was further confirmed through literature correlation with the carbon-13 NMR of compound 11 (Isobe *et al.*, 1980). The FAB-MS showed a protoned molecular ion peak at m/z 616 $[M+H]^+$ and finally the melting point 203-204 °C was also identical with the melting point reported for the same compound from a Japanese plant *P. nodosum* by Isobe *et al.*, 1979, 1980).

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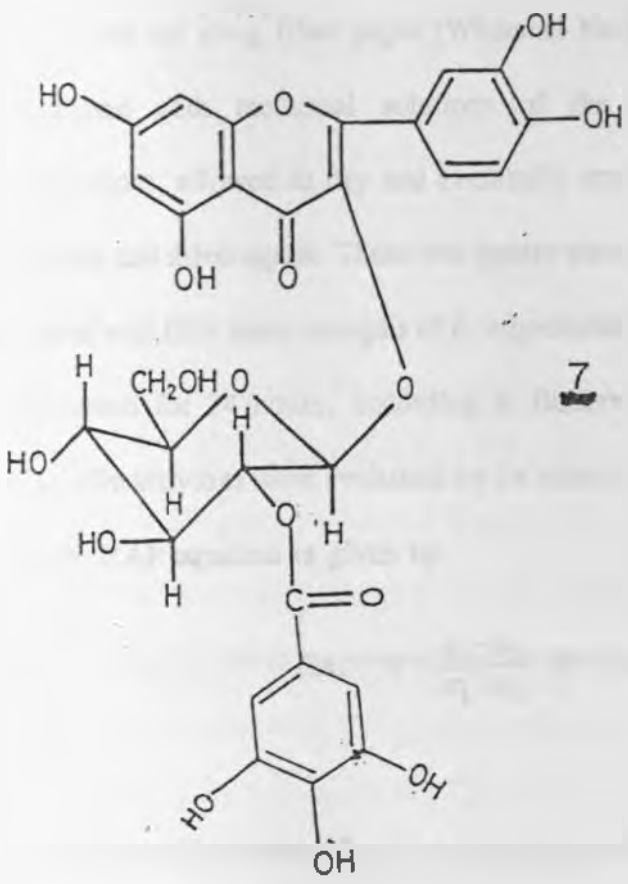

7

In conclusion it can be seen that the structure variability found amongst the external flavonoids is lacking compared with internal tissue forms. They seem to be predominantly glycosides of quercetin possibly together with kaempferol and luteolin (Gikonyo, 1991). The reason for the existence of these external tissue flavonoids in the plant must be a protective function as will be seen from the circumstantial evidence presented on the comparative antifeedant activity of the internal vs external flavonoids and UV-A and UV-B absorbing ability of the exudate flavonoids.

2.1.0 ANTIFEEDANT ACTIVITY TESTS

Antifeedant tests against *Locusta migratoria* and *Schistocerca gregaria* were carried out using filter paper (Whatman No.1). These test papers were impregnated with methanol solutions of the test samples at various concentrations, allowed to dry and eventually sprayed with 0.25M solutions of sucrose and dried again. These test papers were then presented to separate groups of mid fifth instar nymphs of *L. migratoria* and *S. gregaria* which had been starved for 24 hours, according to Butterworth and Morgan method (1971). The activities were evaluated by the relative antifeedant percent (RAP) measure. RAP equation is given by:

$$RAP = \frac{X_1 - X_2}{X_1 + X_2} \; X \; 100$$

where x_1 = is the average % of the control paper consumed

X_2 = is the average % of the treated paper consumed

* The experiments were carried out in triplicates

The results of the study (Tables 2 and 3) showed that a total protection of the filter papers against both the species of locust was obtained upto a concentration of 10 μg/ml of the acetone wash extract for eight hours. Broad band chromatography of the acetone wash on silica gel was then effected. Three solvents were used, benzene, petroleum ether and chloroform. Fractionation was monitored with feeding bioassay and t.l.c. There was high relative antifeedant activity (95.6 at 10 μg/ml) in the chloroform band whereas

the other bands were found to be either slightly phago-stimulatory or non-effective towards *S. gregaria* (Table 3). Further chromatographic analysis of the chloroform band resulted in the isolation of 2',6'-dihydroxy-4'-methoxydihydrochalcone (30) as the possible compound with high antifeedant activity.

On the other hand a test for the antifeedant activity of the ethylacetate fraction of the hydrophilic extract partition (see Experimental section) against the mid fifth instar nymphs on both species showed a RAP values of -83.2 (*L. migratoria*) and -79.6 (*S. gregaria*) at 100 μg/ml. This suggests the presence of a positive feeding stumulus in the fraction. Quercetin-3-ß-O-galactoside (4) isolated from the ethylacetate partition, was also found to be highly phago-stimulatory to the fifth instar nymps of *L. migratoria* and had a RAP value of -92.7 at a concentraction of 100 μg/ml whereas quercetin (3) had a RAP value of -62.2 at the same concentraction (Table 2). Thus it is possible to suggest that the higher phago-stimulatory activity of 4 is attributed to the presence of the sugar residue in the flavonoid skeleton. The high antifeedant activity of the surface exudate of the plant was also comparable to the activities published for azadirachtin from the seeds of *Azadirachta indica* (Butterworth and Morgan, 1971) and the crude surface exudate of *Melia volkensii* (Mwangi and Mukiama, 1988) at 0.04 and 5.4 μg/ml respectively. It was interesting to note that the crude surface exudate and the most active compound 2',6'-dihydroxy-4'-methoxydihydrochalcone (30) had comparable biological activity at concentrations of 10 μg/ml and above. It is therefore likely that the total activity of the surface exudate at these concentractions may be as a result of the synergistic effect.

TABLE 2

Relative Antifeedant Percentage (RAP) of aerial parts of *P. senegalense* at various concentrations against 5th instar nymphs of *L. migratoria.*

Conc. μg/mL	Crude exudate	Ethyl acetate	3	4
100	100.0	-83.2	-62.2	-92.7
50	100.0	-58.4	-53.9	-67.9
10	100.0	-36.3	-34.8	-57.5
1	64.5	-6.7	-2.2	-20.1

TABLE 3

Relative Antifeedant Percentage (RAP) of aerial parts of *P. senegalense* at various concentrations against 5th instar nymphs of *S. gregaria*

Conc. μg/mL	Crude exudate	Chloroform	Ethyl acetae	3
100	100.0	100.0	-79.6	-89.4
10	100.0	95.6	-19.2	-23.0
1	68.7	86.5	-7.1	-2.9

2.2.0 UV-VISIBLE ANALYSIS

The uv-uisible experiments were carried out using the crude surface exudate and its pure isolates using a Pye-Unicam SP8-500 uv-visible spectrophotometer. In this method 1mg of the test samples were separately dissolved in 100 ml of double distilled methanol and the absorption maxima for each of the test sample recorded as shown in Table 4.

Clearly, the uv-visible absorption maxima value (Table 4) indicated that the surface exudate flavonoids have a potential uv-visible quenching property. It can be suggested that the surface exudate flavonoids as well as other compounds with similar absorption characteristics located on the epicuticular layer act as light filters thereby protecting the mesophyll cells from the deleterious physiological effects of UV-A and UV-B radiation.

Therefore the results obtained here tend to surport earlier hypothesis that the surface exudate flavonoids play a UV-visible protective function to the plant species especially where the light intensities are highest (Sobolevskaya and Vysochina, 1971; Wollenweber, 1985) and when the cuticle is not yet fully developed (Midiwo *et al.*, 1990).

2.3.0 CONCLUSION AND RECOMMENDATION

The inner tissue flavonoids of *P. senegalense* are found to be glycosides of quercetin (3) and possibly kaempferol(2) and luteolin. Three glycosides of quercetin (3) were characterised quercetin-3-ß-O-galactoside(4), quercetin-3-ß-O-glucoside (5) and quercetin-3-ß-O-glucoside-2"-gallate(7). Glycosides based on 2 and luteolin have not been isolated but t.l.c analysis indicate the existence of several glycosides which have not been charaterised

due to paucity of material. The other glucoside is the compound 2'-β-O-gluco-4'-hydroxy-6'-methoxydihydrochalcone (6). This structure is clearly related to the 2',4'-dihydroxy-6'-methoxydihydrochalcone (37) which is one of the constituent aglycones in the surface exudate. The glucoside **6** therefore must represent the last stages in the transformation of **2**, or **3**, or luteolin glucosides (other glycosides) into the non polar forms (37). The process of transformation of the internal flavonoids to the surface exudate is a complicated phenomenon which takes place for some reason. The ascription of the *raison-d'etre* for this process is still difficult despite the results we have here and even though the surface exudate are known to be antimicrobial (Ishitsuka *et al.*, 1982; Malterud and Faegri, 1983) and molluscicidal (Maradufu and Ouma, 1978). In all, the aglycones of the inner tissue are more polar than those of the external exudate.

The antifeedant bioassay data obtained from this study indicate that the surface exudate acts as an antifeedant towards two locust species. The surface exudate is composed mostly of chalcones. Chalcones have a wide range of biological activities which include bacteriostatic and bacteriocidal (Geiger and Conn, 1967), fungicidal (Wurm and Lachmann, 1974), acaricidal and antibacterial (Velarde, 1970) and anthelmintic (Takayanagi, 1954) activities. Panosyan *et al.*, (1986) has also pointed out that glycosides of **2** and **3** are blood platelet inhibitors or stimulants. Therefore the economic use to which the plant *P. senegalense* may be put should be explored vigorously especially in the fields of health care and crop protection. A number of pests have recently developed resistance to industrial chemicals and these chemicals pose environmental health problems since some of them are not biogradable. This

process would involve both *in vivo* and *in vitro* testing of the whole extract and purified samples. The *in vivo* testing are necessary so as to evaluate its activities and its correct dossage level so that the various active components of the plant may be incorporated in the commercial formulation.

The study of the uv-visible quenching activities on the surface exudate and its isolates carried out was promising; the surface flavonoids were found to be potential uv-visible quenchers. It would be interesting to consider making a skin UV protection cream from *P. senegalense* surface exudate because of this property.

TABLE 4

Uu-visible absorption maxima for the surface exudate flavonoids

Component	Band I (nm)	Band II (nm)
2,'4'-dihydroxy-6'-methoxychalcone (31)	341	298
2'-hydroxy-3',6'-dimethoxychalcone (27)	336	290
3',6'-dihydroxy-2',4',5'-trimethoxychalcone (38)	317	-
2',4'-dihydroxy-3',6'-dimethoxychalcone (28)	344	-
2',6'-dihydroxy-3',4'-dimethoxychalcone (29)	334	-
2',6'-dihydroxy-4'-methoxydihydrochalcone (30)	316	283
5-hydroxy-7-methoxyflavanone (32)	316	285
7-hydroxy-5,8-dimethoxyflavanone (33)	320	284
Crude exudate	400 - 240	
Photosynthesis	700 - 600	
UV-A	400 - 320	
UV-B8	320 - 290	

CHAPTER THREE

3.0.0 EXPERIMENTAL SECTION

3.1.0 General

Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. The uv-visible spectra were determined using Pye-Unicam SP8-150 and Perkin-Elmer model Lambda 3 uv-visible spectrophotometers. Solvent systems used for each compound (run) under the experimental conditions were recorded and are in the brackets. Infrared spectra were recorded on Perkin-Elmer 467 and Pye Unicam SP3-300 infrared spectrophotometers. The spectra were measured on samples dispersed in potassium bromide and pressed into pellets.

The proton and carbon-13 NMR spectra were obtained with a 400 MHz instrument in DMSO-d_6, methanol, $CDCl_3$ and MeOD-d_4 at the University of Maryland, College Park, U.S.A. The chemical shifts are given in ppm relative to the internal standard tetramethylsilane (TMS). EI-Mass and FAB-Mass spectra were also obtained at the University of Maryland and Meiji College of Pharmacy, Japan. Polyethene plastic and Merck aluminium precoated silica gel 60 PF_{254} were used for analytical thin layer chromatography (t.l.c). Merck silica gel G 60 and silica gel 40 (70-230 mesh) were used for preparative t.l.c and gradient elution respectively. Silica gel mesh 0.07-0.3 was used for flash elution.

3.2.0 Plant material

The aerial parts of the plant samples were collected from within Nairobi and the surrounding districts at two different times of the year (July, 1991 and November, 1992). The plant samples were identified at the Department of Botany, Herbarium in the University of Nairobi. The fresh plant material was acetone washed to remove the surface exudates as previously reported by Midiwo *et al.*, (1990), then dried under room temperature and powdered using a Willy Mill machine.

3.3.0 Preliminary studies

3.3.1 Surface exudate

The fresh aerial parts of *Polygonum senegalense* were acetone washed by dipping the aerial parts of the plant into a number of beakers containing acetone. This was done until the last beaker was almost clear. This process of extracting the aerial parts of the plant took approximately two to three minutes to prevent the solvent system from extracting the inner tissue compounds and chlorophyll complexes. The resultant surface exudate was filtered through vacuum suction and then concentrated in vacuo using rotary evaporator to give a brown semi-solid material which was kept for further use in bioassay's and uv-visible studies.

3.3.2 Inner tissue components

The prevously acetone washed aerial parts of *P. senegalense* were air dried then ground into powder using a Willy mill. The powder was extracted with 95 % cold aqueous ethanol for two days.

The resultant ethanol extract was filtered under vacuum suction and concentrated in vacuo using a rotary evaporator leaving a dark brown semi-solid material of mass 30 g which was used for the preliminary investigation of the secondary metabolites in the plant. The investigations of the secondary metabolites was done for the presence of flavonoids, anthraquinones, saponins, alkaloids and benzoquinones. The presence/absence of the secondary metabolites were confirmed by the colour change or precipitation of the solutions after the addition of testing reagents.

3.3.3 Test for the presence of flavonoids

Approximately 2 g of the extract was defatted several times with n-hexane. The defatted residue was dissolved in 20 ml of 80% ethanol and filtered under a vacuum suction. The resultant filtrate was handled as follows:

(i) To 3 ml of the filtrate in a test tube , 4 ml of 1N potassium hydroxide solution was added. Appearance of a dark yellow colour confirmed the presence of flavonoids.

(ii) To 3 ml of the filtrate in a test tube , 0.4 ml of concentrated hydrochloric acid and few magnesium turnings were added. Changing the colour of the filtrate to red indicated the presence of flavonoids.

(iii) To 3 ml of filtrate in a test tube, 4 ml of 1 % aluminium chloride in methanol was added. Appearance of the yellow colour reconfirmed the presence of flavonoids.

3.3.4 Test for the presence of anthraquinones

A sample of 2 g of the extract was shaken thorough with 10 ml of benzene and then filtered under a vacuum suction. To 3 ml of this filtrate in a test tube, 5 ml of 10 % ammonium hydroxide solution was added and the mixture was thoroughly shaken. A violet coloration observation in the ammonical layer was the evidence for the presence of anthraquinones.

3.3.5 Test for the presence of benzoquinones

Approximately 3 g of the extract was boiled with 30 ml of 1% hydrochloric acid for five minutes and the reaction mixture was filtered while hot under a vacuum suction. The resultant filtrate was partitioned into 10 ml of *n*-hexane. To the organic layer, 5 ml of 10 % ammonia solution was added. The absence of pink-violet coloration in the basic medium evidenced the absence of the benzoquinones.

3.3.6 Test for the presence of alkaloids

(i) About 2 g of the extract was mixed with 40 ml of 2 N hydrochloric acid and heated in a water bath for ten minutes. The resultant mixture was cooled and filtered under a vacuum suction. To 3 ml of the filtrate in a test tube, few drops of Mayer's reagent were added. Absence of turbidity (white precipitation) was an evidence of the absence of alkaloids in the plant.

(ii) Approximately 2 g of the extract was heated with 20 ml of chloroform:methanol (1:1 v/v) mixture. The resultant solution was spotted on analytical t.l.c. plate and developed with methanol/chloroform (1:9 v/v); ethyl acetate/chloroform (1:4 v/v) and ammonia/methanol (3:100 v/v). Dragendorff's reagents was sprayed on to the plate. Absence of the yellow spots on the plates reconfirmed the absence of alkaloids in the plant.

3.3.7 Test for the presence of saponins

About 2 g of the extract was vigorously shaken with water in a test tube. A persistent froth observed for appreciable period of time evidenced the existence of saponins in the plant.

3.4.0 Chromatography

All the chromatographic solvent systems were purified by distillation before use. Sephadex LH-20 and silica gel were both used for the chromatographic analysis. Silica gel for chromatography was either

impregnated or not with 3 % oxalic acid (*vide infra*) solution in deionised water. Solvent removal from the fractions was carried out *in vacuo* using a rotary evaporator. Analytical and preparative t.l.c. plates were necessary impregnated with 3 % oxalic acid solution as indicated below. The solvent systems used for both analytical and preparative t.l.c. plates were ethyl acetate/methanol/water (100:16.5:13.5 v/v), 1; chloroform/methanol (4:1 v/v), 2; chloroform/methanol/water (4.1:0.75:0.15 v/v), 3; ethyl acetate/*n*-hexane (1:1 v/v), 4; chloroform/methanol (19:1 v/v), 5;. Paper chromatography was done on Whatman No 1 filter paper using solvent systems *n*-butanol/benzene-pyridine/water (5:1:3:3 v/v), 6. A mixture of *meta*-phenylene diamine (0.5 g), stannous chloride (1.2 g), acetic acid (20 ml) and ethanol (80 ml) was used as a locating reagent.

3.5.0 IMPREGNATION OF THE SILICA GEL

3.5.1 For column Chromatography

Approximately 750 g of silica gel G 60 (mesh 70-230 ASTM) was adsorbed in 1500 ml of 3 % oxalic acid in deionised water and left to stand for five hours. Excess acid solution was filtered through a vacuum suction. The resultant wet silica gel was air dried then placed in an oven set at 120 °C until the silica gel regained their original texture. The same procedure was used in the case of flash chromatography.

3.5.2 Analytical t.l.c.

Both the polyethene plastic and Merk aluminium precoated silica gel analytical t.l.c. plates were dipped into a solution of 3 % oxalic acid in deionised water for about five minutes. The plates were air dried then placed in an oven at 120 °C for ten minutes before use.

3.5.3 Preparative t.l.c.

Approximately 20 g of silica gel 60 G was dumped into 40 ml of 3% oxalic acid solution to form a slurry of the gel. This mixture was thoroughly shaken for about seventy five seconds then poured on to a 20 x 20 cm^2 glass plate and spread uniformly to a depth of 2 mm with a spreading machine. The plate was air dried then placed in an oven at 120 °C for one hour before use.

3.6.0. Extraction and isolation

Large scale extraction and isolation of the secondary metabolites was done in two parts. The first part was done on the plant samples collected in July, 1991, and processed at the University of Nairobi, on silica gel impregnated with 3 % oxalic acid solution and the other part of this work was done on plant material collected in November, 1992, and processed at the Addis Ababa university (Ethiopia) on Sephadex LH-20 and silica gel columns.

3.6.1 Column chromatography on impregnated silica gel

Fresh leaves, previous acetone washed were dried at ordinary temperature and ground using a Willy mill. The powdered material was extracted with 70 % aqueous methanol twice. Consecutive extracts were combined, decanted into an elmayer flask and concentrated over a rotary evaporator until all the methanol was recovered.

The concentrated aqueous extract was exhaustively extracted with dichloromethane, then ethyl acetate in a separatory funnel. Qualitative t.l.c. analysis of the dichloromethane soluble partition revealed the presence of chlorophyll and residuai flavonoid aglycones previously established as present in the surface exudate (Midiwo *et al.*, 1990; 1992). While ethyl acetate partition showed a number of unresolved yellow spots which did not move on analytical t.l.c. plate impregnated with oxalic acid solution solvent system 4. Further spotting was done, and this time development with the solvent system 10 % methanol in chloroform. Four spots were observed with R_f values 0.15, 0.41, 0.72 and 0.85.

Approximately 20 g of the ethyl acetate partitioned fraction of the sample material was subjected to column chromatography in a 4 cm diameter column packed with 200 g of silica gel G 60 impregnated with 3 % oxalic acid solution. Elution was performed first with chloroform until the first yellow band started coming out of the column. At this stage there were other bands also observed in the column. This first yellow band was eluted out completely by passing 1 % and 2 % methanol in chloroform successively

through the column and collecting eluants of 150 ml each. Each eluents collected was spotted on an analytical t.l.c. plate impregnated with oxalic acid, developed with the solvent system 5 to prove that it was a single compound. Fractions containing the same components were pooled together and concentrated *in vacuo*. The first four eluents of the first yellow band were not investigated because they were shown to contain fatty material and chlorophyll (t.l.c. analysis) and they did not correspond to the flavonoid tests carried out. The other eluents of the band gave a positive test for flavonoids but the t.l.c. of these showed two spots R_f values 0.62 and 0.90 solvent system 5, when spots were visualized under the uv-visible light and exposed to concentrated aqueous ammonia solution. These eluants were pooled together and concentrated *in vacuo* to a yellow solid material which was eluted through a small column packed with impregnated silica gel using chloroform as mobile phase.

Five eluants of approximately 100 ml each were collected. The first two eluants which contained the compound with R_f value 0.90 with other minor spots turned out be the remnant of the surface components. However, eluants 3 and 4 gave only a single yellow spot on the t.l.c. plate hence they were concentrated *in vacuo* to yield a yellow solid material which gave a yellow precipitate on addition of chloroform. The supernatant was decanted to leave a yellow precipitate which was further purified by preparative t.l.c. to give a compound identified as 2',4'-dihydroxy-6'-methoxychalcone (1) melting point 187 - 189 °C. The melting point of the compound was not

depressed by mixing with the authentic sample previously isolated in our laboratory. The compound showed infrared absorption peaks (cm^{-1}) in potassium bromide pellet at 3245-3005, 1640, 1465, 1330, 1225, 1210 and 1110. The uv-visible absorption maxima peaks (nm) were observed at (MeOH), 341; (MeOH-NaOMe), 298, 382; (MeOH-$AlCl_3$), 368, 314 s; (MeOH-$AlCl_3$-HCl), 367, 314 s; (MeOH-NaOAc), 378, 296 and (MeOH-NaOAc-H_3BO_3), 396, 318 s, 332 s. The mass spectrum showed peaks at m/z values 270 [M^+],(18.4), 269 (36.4), 193 (73.1), 95 (100), 43 (37.3). The proton NMR (400 MHz, DMSO-d_6) and carbon-13 NMR data are in Table 5.

Further elution of the column was performed with increasing amounts of methanol in chloroform upto 5 %. This solvent system eluted the second yellow band. Again the eluants were collected in approximately 150 ml fractions and monitoring the purity with analytical t.l.c. plate with solvent system 5. The t.l.c. plate results showed that the eluents contained a major spot and traces of another compound. Both the spots responded to flavonoid test and were uv-visible active at 254 nm. These eluants were combined and concentrated *in vacuo* yielding 50 mg of a yellow material with R_f values 0.51 and 0.26 with solvent system 5. The major spot was separated from the mixture by using preparative t.l.c. plate solvent system 5 which was cleaned through a small column using silica gel impregnated with oxalic acid solution to give 20 mg of the major compound identified as compound **2**. This compound was recrystallized from chloroform/methanol mixture to give yellow crystals with an R_f value of 0.56 in solvent system 5 and a melting

point 242 - 243 °C. The melting point of the compound was not depressed by mixing with kaempferol (an authentic marker). Thus this compound was identified as kaempferol (2). The infrared absorption peaks (KBr disc) were observed at (cm^{-1}): 3400, 3320, 3000, 1658, 1610, 1570, 1500, 1380, 1250, 1225. The uv-visible absorption bands of the compound gave peaks at (nm): (MeOH) 363.8, 332.4 s, 268.0; (MeOH-NaOMe), 412.3 degenerated after 5 minutes, 320.5, 278.5; (MeOH-$AlCl_3$), 422.6, 303.8, 268.3; (MeOH-$AlCl_3$-HCl), 422.3, 303.5, 267.5; (MeOH-NaOAc), 388.2, 319.4, 274.5; (MeOH-NaOAc-H_3BO_3), 368.5, 322.7 and 268.0. Mass spectrum showed the ion peaks at m/z: 286 [M^+], (100.0); 258 (25.3); 229 (25.3); 167 (28.3); 153 (15.5); 121 (50.0); 105 (15.6); 93 (25.5); 77 (22.5) and 69 (45.8). The proton and carbon-13 NMR spectra data of the compound in DMSO-d_6 are given in Table 6.

Eluents 10-15 which were eluted with 10 % methanol in chloroform constituted the third yellow band, were combined together after they showed a single spot on the t.l.c. plate on illumination with uv-visible light at 254 nm and the yellow colour intensified on exposure to concentrated aqueous ammonia. Evaporation of the solvent mixture in vacuo yielded approximately 150 mg of a yellow substance which had an R_f value 0.26 on t.l.c. plate with solvent system 5. This compound also crystallised from chloroform/methanol mixture to give a yellow crystalline compound. Recrystallisation of the compound from ethyl acetate/*n*-hexane mixture gave a yellow crystalline compound with an R_f value of 0.26 in solvent system 5 which was identified

as quercetin (3) melting point of 313-314 °C. Infra-red spectrum (KBr disc) displayed the absorption bands at (cm^{-1}): 3300, 1623, 1525 and other bands at 1370, 1320, 1220, 1170, 1100, 1000 and 930. Uv-visible showed absorption maxima peaks at (nm): (MeOH) 240.1, 371.4; (MeOH-$NaOCH_3$) 319.2; (MeOH-$AlCl_3$), 269.9, 441.3; (MeOH-$AlCl_3$-HCl), 274.2, 322.9, 390.9; (MeOH-NaOAc), 260.0, 387.8 and (MeOH-NaOAc-H_3BO_3), 255.3 and 372.2. The mass spectrum displayed ion peaks at: 302 (100.00), 272 (34.09), 245 (15.91), 153 (28.03), 137 (31.82) and 69 (30.06). The proton and carbon-13 NMR spectra data of the compound in DMSO-d_6 (400 MHz) are shown in Table 7. The last yellow band of the impregnated silica gel column was eluted with 15 and 20 % of methanol in chloroform mixtures and these eluants were pooled together and concentrated to give a yellow substance. Trituration of the yellow material with methanol gave a yellow precipitate. The supernatant was decanted and the remaining solid recrystallised from methanol, filtered through a vacuum and washed with methanol to give yellow crystals identified as quercetin-3-O-ß-galactoside (4), melting point 230 - 232 °C, R_f value 0.32 solvent system 2. The uv-visible spectrum of this compound showed absorption bands at (nm): (MeOH), 359.5, 258.0; (MeOH-NaOMe), 406.5, 271.5; (MeOH-NaOAc), 371.6, 272.5; (MeOH-NaOAc-H_3BO_3), 372.0, 264.0; (MeOH-$AlCl_3$), 431.5, 273.5 and (MeOH-$AlCl_3$-HCl), 400.0, 268.5. The 2-D NMR in DMSO-d_6 showed signals due to carbon-hydrogen interaction at ppm: 98.59, 93.42, 115.90, 121.03, 101.78, 71.15, 73.15, 67.86, 75.78 and 60.08. The mass spectrum showed peaks at: 448 (3.23), 386

(13.28), 338 (8.08), 316 (8.44), 302 (100.00) 286 (81.00) and 272 (85.80). The proton and carbon-13 NMR spectral data of the compound in DMSO-d_6 (400 MHz) are showed in Table 8.

A solution of 4 in 5 % hydrochloric acid was heated under reflux for three hours. The solid aglycone obtained was crystallised from ethyl acetate/*n*-hexane mixture to give yellow crystals, melting point 313-314 °C, identified as quercetin (3) by mixed melting point, uv-visible, IR, NMR and co-t.l.c. with an authentic marker. The aqueous layer was diluted, filtered through a vacuum and the filtrate was evaporated under reduced pressure. The residue was found to contain only galactose by paper chromatography and authentic samples.

3.6.2 Sephadex LH-20 column

The dried powdered sample of mass 500 g of previously acetone washed was extracted sequentially with 5.0 litres of cold 70 % and 50 % aqueous methanol for two days each. The extracts were combined, filtered and methanol was removed *in vacuo* leaving an aqueous extract. The aqueous extract was then partitioned with ethyl acetate and the ethyl acetate was concentrated *in vacuo* leaving a semi solid of mass 50 g. Thin layer chromatography (t.l.c.) analysis of the semi-solid material with solvent system 1 showed a number of prominent yellow spots with R_f values 0.20, 0.30, 0.38, 0.54 and 0.78. On developing the t.l.c. plate with concentrated aqueous ammonia all the spots intensified their yellow / or darkened colour and they

also fluoresced on illumination with uv-visible at 254 nm. A flash column chromatography was performed using a glass column of 3.6 cm diameter packed with 150.0 gm of silica gel G 60 (mesh 0.040-0.063 mm/ 230-400 ASTM) in 5 % methanol/chloroform mixture. A mass of 15.0 gm of the crude ethyl acetate partition was preadsorbed on a small amount of the silica gel and transferred on to the column. 500 ml of 5 % methanol/chloroform mixture was the first solvent system of elution. It eluted an orange red coloured component of mass 3.8 g which did not correspond to the preliminary tests carried before for flavonoids. It also moved with the solvent front on application to thin layer chromatography plate in all the solvent systems used. It was therefore suspected to be chlorophyll complexes, fatty acid materials and/or carotenoids thus it was not purified and characterized.

Polarity of the solvent system was increased with increased volumes of methanol upto 30 % methanol/chloroform mixtures and finally the column was washed with 50 % methanol/chloroform. Analytical thin layer chromatography (t.l.c.) analysis of the eluants from the flash chromatography showed that eluants were not effectively separated and those which contained similar spots were combined together. The 10 % methanol/chloroform mixture eluents comprised of compounds with R_f values 0.78 and 0.54. Compounds with R_f values 0.38, 0.30, 0.20 and some other traces compounds not identified which intensified or darkened their yellow colours on exposure to concentrated ammonia vapour were established as the constituents of the 20 %, 30 % and 50 % methanol/chloroform mixtures after t.l.c. analysis using

solvent system 1. All the t.l.c. spots were UV active at 254 nm. After the t.l.c. analysis the solvent was concentrated *in vacuo* using a rotary evaporator leaving a sticky brown paste of masses 2.0, 2.3, 1.5 and 0.7 g, respectively. A small glass column of 2.2 cm diameter was therefore packed with Sephadex LH-20 in chloroform/methanol(1:1 v/v) mixture slurry. Approximately 1.5 g of the concentrated mass of the 10% methanol/chloroform fraction was predissolved in a minimum amount of chloroform/methanol (1:1 v/v) and introduced carefully on to the Sephadex column using a dropper to ensure a uniform distribution of the sample on top of the Sephadex. The column was eluted with chloroform/methanol (1:1 v/v) mixture. A yellow band was washed first and showed two spots R_f values, 0.33 and 0.48, on t.l.c analysis with solvent system 2. The second yellow band from this column was washed out much later with the same solvent system and t.l.c analysis showed only a single spot with R_f value 0.48 (chloroform/methanol 4:1 v/v). This compound crystallised out from the chloroform/methanol mixture as yellow crystals. The yellow crystalline compound was co-spotted with an authentic sample of quercetin previously isolated, and the two compounds gave only a single spot. Thus it was identified as quercetin (3) previously isolated from the silica gel column impregnated with oxalic acid solution. Recrystallisation of 3 from chloroform/methanol mixtures yielded yellow crystals (248 mg 0.05 % of the dry plant weight), identified quercetin melting point 313-314 °C.

Separation of the two compounds R_f values 0.33 and 0.48 from the first yellow band was effected by use of preparative t.l.c. Each preparative

t.l.c. (20 x 20 cm^2) was streaked with approximately 50 mg of the concentrated mass and subsequently developed using solvent system 2. This process resulted in the isolation of one major compound identified as compound **6** (76.3 mg) and a small amount of compound **3**. Compound **6** was further purified using a small column packed with Sephadex LH-20 and eluted with chloroform/methanol (1:1 v/v). The resultant eluants gave only a single spot R_f value 0.52 solvent system 3. Compound **6** was identified as 2'-ß-O-gluco-4'-hydroxy-6'-methoxydihydrochalcone R_f value 0.48 solvent system 2 and melting point 191-193 °C. EI-MS of this compound showed ion peaks at m/z: 435 (0.20); 369 (0.20); 303 (0.44); 277 (100.00); 272 (18.91); 255 (4.56); 241 (100.00) and 167 (100.00). The infrared spectrum (KBr disc) displayed absorptions maxima (cm^{-1}) at 3300, 2940, 1680, 1620, 1380, 1290, 1185 and 1100-1000 (broad). Uv-visible maxima absorption bands (nm) occurred at (MeOH) 343 s, 282; (MeOH-NaOMe) 323 s, 292, 250; (MeOH-$AlCl_3$) 364 s, 308, 276; (MeOH-$AlCl_3$-HCl) 364 s, 306, 277; (MeOH-NaOAc) 323 s, 284 and (MeOH-NaOAc-H_3BO_3) 328 s, and 284. The DEPT carbon-13 at $\theta = 90°$ and 135° showed negative signals at ppm: 31.17, 47.49 and 62.55. The proton and carbon-13 (400 MHz, DMSO-d_6) of the compound are shown in Table 10.

Approximately 5.0 mg of compound **6** was hydrolysed using 20.0 ml of 2N hydrochloric acid, the reaction mixtures were put in a 100 ml round bottomed flask and refluxed for two hours using a water bath. The reaction mixture was then cooled and the methanol was recovered *in vacuo* using a

rotary evaporator leaving a concentrated aqueous fraction. The aqueous fraction was diluted with water, then exhaustively partitioned with ethyl acetate to extract the aglycone moiety of the glycoside. The ethyl acetate portion was concentrated and purified through a small Sephadex LH-20 column using chloroform/methanol mixture (1:1 v/v). This process gave a dihydrochalcone as the aglycone moiety. The dihydrochalcone was identified as 2',4'-dihydroxy-6'-methoxydihydrochalcone (37), melting point 178-179 °C. This compound was positively identified through mixed melting point with an authentic sample, carbon-13 and proton NMR spectra. The carbon-13 and proton NMR of the dihydrochalcone are showed in Table 14. The aqueous residue was filtered and the filtrate was concentrated, then developed on paper chromatography (Whatman No 1 filter paper) using *n*-butanol/benzene/pyridine/water (5:1:3:3 v/v) and sprayed with a locating reagent then placed in an oven at 120 °C for one hour. The sugar moiety was identified as glucose by comparison with authentic samples, carbon-13 and DEPT carbon-13 spectra of the glucoside molecule.

The combined 20 and 30 % methanol/chloroform concentrates of the ethyl acetate fraction was subjected to a bigger glass column packed Sephadex LH-20 and eluted under pressure with chloroform-methanol mixture (1:1 v/v), still the separation of the constituents was not achieved. Therefore eluants were combined together evaporated to dryness, subjected to a smaller Sephadex column and eluted with the same solvent system. The first four eluents gave a mixture of spots on the analytical t.l.c. plate which were not further investigated.

Eluants 4-9 of the smaller Sephadex column gave one major spot on analytical t.l.c. plate. They were combined, dried and recrystallised from chloroform/methanol mixture to give 30 mg of yellow needle like crystals identified as quercetin-3-ß-O-glucoside (5); melting point 220-222 °C; R_f value 0.12 solvent system 2. Infrared spectrum of the compound (KBr disc) gave absorption peaks at (cm^{-1}): 3300 (broad), 2920, 1670, 1655, 1615, 1510, 1365. Other bands were at 1255, 1210, and 1100-1000 (broad). Uv-visible spectrum showed absorption maxima at (nm): (MeOH) nm, 255.9, 360.1; (MeOH-NaOMe), 272.0, 331.0 sh, 410.7; (MeOH-NaOAc), 273.6, 324.2 sh, 386.3; (MeOH-NaOAc-H_3BO_3), 261.9, 380.0; (MeOH-$AlCl_3$), 273.8, 421.0; (MeOH-$AlCl_3$-HCl), 269.1, 290.1 and 410.0. The proton and carbon-13 NMR spectra in methanol (400 MHz) are given in Table 9. Mass spectrum displayed ions at (FAB m/z (%)) 464 (6.00); 374 (6.80); 358 (28.57); 343 (8.89); 328 (48.63); 318 (26.30); 302 (100.00); 285.98 (27.00) and 272 (16.95).

Eluants 10-15 from the smaller Sephadex column showing two spots of R_f values 0.12 and 0.15 with solvent system 2, were pooled together, concentrated and purified by preparative t.l.c. solvent system 1. The two bands obtained from the preparative t.l.c. were further cleaned by a small Sephadex column and eluted with chloroform/methanol (1:1 v/v). This process gave two compounds, one with an R_f value of 0.38 (20.7 mg) melting point 230 - 231 °C and another an R_f value of 0.30 (18.6 mg) with solvent system 1. The compound with the R_f value of 0.38 was identified as quercetin-3-ß-O-galactoside (4) while the other one as compound 5 on the basis of an authentic marker, UV, IR and NMR techniques.

The 50 % methanol/chloroform mixtures from the big column of the ethylacetate partition showed only one spot R_f value of 0.24 with solvent system 3 on the analytical t.l.c. plate and on illumination with uv-visible light at 254 nm. This fraction was concentrated *in vacuo* to give a crude yellow material which on separation by preparative t.l.c. using solvent system 3 led to the isolation of a pure compound. This was the most polar compound in the series and it was identified as quercetin-3-O-ß-glucoside-2"-gallate (7). This compound was recrystallised from methanol R_f value 0.24, solvent system 3 to give 17.7 mg of yellow crystals, melting point 204-205 °C. The FAB-MS and EIMS spectra of the compound displayed absorption peaks at m/z: 616, 358 (6.75), 302 (100.00), 273 (12.13), 170 (5.19), 153 (41.70) and 137 (23.53). The infrared spectrum (KBr disc) showed absorption maxima at cm^{-1}: 3300, 1680, 1660, 1620, 1560, 1510, 1460, 1358, 1300, 1260, 1200 and 1100-1000. The uv-visible spectra displayed absorption bands at (nm): (MeOH) 359.9, 289.0, 259.2; (MeOH-NaOMe) 409.7, 328.0, 272.8; (MeOH-$AlCl_3$) 429.7, 300.0, 273.6; (MeOH-$AlCl_3$-HCl) 400.0, 362.9, 300.0, 270.8; (MeOH-NaOAc) 381.0, 324.0, 272.8; (MeOH-NaOAc-H_3BO_3) 379.6, 269.7, 264.0. The proton and carbon-13 NMR spectra in methanol are shown in Table 11.

Solutions of compounds 5 and 7 in 5 % hydrochloric acid in methanol were heated under refluxed for three hours separately. The solids separating on cooling were crystallised from methanol/chloroform mixture to give yellow crystals melting point 312-314 °C which was identified as quercetin (3) by mixed m.p, UV, IR, NMR and Co-t.l.c. with an authentic sample. The sugar

fractions from the acid hydrolysis of compounds 5 and 7 were neutralised, concentrated *in vacuo* and chromatographed on Whatman No 1 filter paper using *n*-butanol/benzene/pyridine/-water (5:1:3:3 v/v). Glucose was identified for the both samples by comparison with authentic samples. In addition to the above observation for the aqueous fractions, compound 7 also showed the presence of gallic acid moiety as a result of the reaction between the aqueous fraction of compound 7 and ferric chloride.

3.7.0 Derivatization Reactions

3.7.1 Acetylation of 3

A sample of 10 mg of (3) was placed in a round bottomed flask containing 2.5 ml of freshly distilled acetic anhydride and approximately 5ml of dry pyridine. This reaction mixture was stirred for twenty four hours at room temperature using a magnetic stirrer. To the reaction mixture crushed ice/water was added and a further stirring was continued. The resulting precipitation was filtered by vacuum suction and the residue dissolved in ethyl acetate, dried using anhydrous magnesium sulphate and finally concentrated in vacuo using a rotary evaporator to give a white solid. Recrystallisation of the white solid from ethyl acetate/*n*-hexane mixtures gave white crystals of mass 12 mg and melting point 188-190 °C. The white crystals were identified as penta-acetyl derivative of quercetin (44), Rf value 0.43 solvent system 4 and m.p 188-190 °C. Infrared spectrum of the compound as potassium bromide recorded absorption peaks at (cm^{-1}): 1775, 1725, 1645, 1625, 1615,

1500, 1480, 1370, 1195 and 900. The üv-visible absorption bands (nm) in methanol gave peaks at 296 and 249. The proton and carbon-13 NMR of the compound in DMSO-d_6 (400 MHz) are showed in Table 12.

3.7.2 Methylation of 3

A mixture of 20 mg of 3 in 10 ml of dimethylsulphate in 20.0 ml of dry acetone and 3 g of freshly ignited potassium carbonate in a round bottomed flask was refluxed for twenty four hours. The reaction mixture was monitored by analytical t.l.c. plate solvent system 4. This reaction mixture was then cooled and filtered using vacuum suction. The residual potassium carbonate was washed several times with acetone. The acetone washings were pooled together and then concentrated *in vacuo*. The concentrated mixture was poured into crushed ice leading to a solid mass. It was then filtered by vacuum suction, washed well and finally crystallised in ethyl acetate yielding yellow needle-like crystals identified as pentamethyl ether derivative of quercetin (45). The product was identified as tetramethylated quercetin ether Rf 0.73 solvent system 4, melting point 156-158 °C. The proton and carbon-13 NMR spectra of the compound in DMSO-d_6 are given in Table 13.

3.8.0 ANTIFEEDANT ACTIVITY TESTS

3.8.1 Antifeedant activity tests

Antifeedant activity tests were done on crude surface exudate, chloroform fraction of the broad band chromatography and ethyl acetate partition of the aqueous glycosidic extract, quercetin (3) and quercetin-3-ß-O-

galactoside (4) against two species of locust, *Locusta migratoria and Schistocerca gregaria.* Control and choice experiments were set with ten mid-fifth instar nymphs which were starved for twenty four hours and presented with the test material according to Butterworth and Morgan (1971) procedure. In this method the Whatman No 1 filter papers (2.0 x 2.0 cm^2) were impregnated with the tests solutions allowed to dry and then sprayed with 0.25 M sucrose solution and allowed to dry in an oven at 40 °C. For each test solution, the mid fifth instar insects were presented with filter papers impregnated with the sucrose only, to act as a control. After eight hours, the filter paper were removed and the area of the filter paper consumed determined.

3.9.0 UV Experiments

3.9.1 Column chromatography of surface exudate

About 20 g of the surface exudate was chromatographed on silica gel column and eluted with benzene, petroleum ether and chloroform. A total of four fractions were collected. Further column chromatography of the fractions followed by preparative t.l.c. led to isolation of the following compounds, 2'-hydroxy-3',6'-dimethoxychalcone (27), 2',4'-hydroxy-3',6'-dimethoxy-chalcone (28), 2',6'-hydroxy-3',4'-dimethoxychalcone (29), 2',6'-dihydroxy-4'-methoxydihydrochalcone (30), 2',4'-dihydroxy-6'-methoxychalcone (31). 5-hydroxy-7-methoxyflavanone (32), 7-hydroxy-5,8-dimethoxyflavanones (33) and 3',6'-dihydroxy-2',4',5'-trimethoxychalcone (38). Identification of the compounds were based on the UV, IR, NMR and t.l.c. comparisons with authentic makers. The absorption maxima values for these major surface compounds and the crude mixtures are shown in Table 4.

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APPENDIX A

TABLE 5

NMR Chemical shift positions for 2',4'-dihydroxy-6'-methoxychalcone (1) in DMSO-d_6

Position of Carbon	^{13}C NMR (ppm)	^{1}H NMR (ppm)
1'	134.89	-
2'	164.99	-
3'	95.80	5.92 (d, J= 2.06 Hz)
4'	166.20	-
5'	91.64	6.01 (d, J= 2.06 Hz)
6'	162.64	-
C=O	197.71	-
α	127.48	7.64 (d, J= 15.71 Hz)
ß	141.67	7.82 (d, J= 15.71 Hz)
1	105.10	-
2	128.97	7.44 m
3	128.28	7.43 m
4	130.21	7.43 m
5	128.28	7.43 m
6	128.97	7.44 m
6'-OCH_3	55.98	3.87 s

TABLE 6

NMR Chemical shift positions for kaempferol (2) in DMSO-d_6

Position of carbon	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)
2	147.03	-
3	135.61	-
4	175.86	-
5	160.66	-
6	98.15	6.18 (d, J= 1.90 Hz)
7	163.84	-
8	93.42	6.43 (d, J= 1.90 Hz)
9	156.13	-
10	103.00	-
1'	121.62	-
2'	128.86	6.91 (d, J= 8.06 Hz)
3'	115.59	8.03 (d, J= 8.06 Hz)
4'	159.15	-
5'	115.59	8.03 (d, J= 8.06 Hz)
6'	128.86	6.91 (d, J= 8.06 Hz)

TABLE 7

NMR Chemical shift positions for quercetin (3) in DMSO-d_6

Position of carbon	^{13}C-NMR (ppm)	^{1}HNMR (ppm)
2	146.80	-
3	135.72	-
4	175.83	-
5	160.71	-
6	98.18	6.17 (d, J= 1.97 Hz)
7	163.87	-
8	93.34	6.40 (d, J= 1.97 Hz)
9	156.13	-
10	103.01	-
1'	120.96	-
2'	115.07	7.67 (d, J= 2.13 Hz)
3'	145.05	-
4'	147.69	-
5'	115.60	6.87 (d, J= 8.49 Hz)
6'	119.98	7.53 (d, J= 2.13, 8.49 Hz)

TABLE 8

NMR Chemical shift positions for quercetin-3-O-ß-galactoside (4) in DMSO-d_6

Position of carbon	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)
2	156.19	-
3	133.47	-
4	177.43	-
5	161.17	-
6	98.59	6.19 (d, J= 1.98 Hz)
7	164.04	-
8	93.42	6.39 (d, J= 1.98 Hz)
9	156.19	-
10	103.86	-
1'	121.05	-
2'	115.90	7.51 (d, J= 2.21 Hz)
3'	144.74	-
4'	148.38	-
5'	115.90	6.80 (d, J= 8.50 Hz)
6'	121.03	7.51 (d, J= 2.21, 8.50 Hz)
1"	101.78	5.37 (d, J= 7.69 Hz)
2"	71.15	3.55 (d, J= 9.27 Hz)
3"	73.15	3.40 - 4.20 m
4"	67.86	3.63, broad
5"	75.78	3.40 - 3.20 m
6"	60.08	3.44 (d, J= 5.93 Hz)

TABLE 9

NMR Chemical shift positions for quercetin-3-O-ß-glucoside (5) in MeOH

Position of Carbon	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)
2	158.69	-
3	135.76	-
4	179.53	-
5	163.03	-
6	99.92	6.20 (d, J= 2.07 Hz)
7	166.17	-
8	94.73	6.40 (d, J= 2.07 Hz)
9	158.69	-
10	105.61	-
1'	122.92	-
2'	116.08	7.53 (d, J= 2.24 Hz)
3'	145.82	-
4'	149.95	-
5'	117.77	6.81 (d, J= 8.50 Hz)
6'	122.95	7.67 (d, J= 2.24, 8.50 Hz)
1"	105.59	5.37 (d, J= 7.66 Hz)
2"	73.17	3.57-3.30, m
3"	75.10	"
4"	70.02	"
5"	77.19	"
6"	61.95	"

TABLE 10

NMR Chemical shift positions for 2'-O-ß-glucosyloxy-4'-hydroxy-6'-methoxydihydrochalcone (6) in MeOH

Position of Carbon	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)
1	142.76	-
2	129.71	7.27, m
3	126.76	7.20, m
4	126.76	7.20, m
5	136.76	7.20, m
6	129.45	7.27, m
C=O	206.41	-
α	31.17	2.91, m
ß	47.49	3.12, m
1'	114.12	-
2'	157.77	-
3'	97.01	6.16 (d, J= 1.91 Hz)
4'	160.04	-
5'	94.71	6.32 (d, J= 1.91 Hz)
6'	162.27	-
6'-OCH_3	56.19	3.71, s
1"	103.07	4.80 (d, J= 7.76 Hz)
2"	74.78	3.69-3.30, m
3"	78.35	"
4"	71.16	"
5"	77.95	"
6"	62.55	"

TABLE 11

NMR Chemical shift positions for quercetin-3-O-β-glucoside-2"-gallate (7) in MeOH

Carbon No.	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)
2	158.30	-
3	135.69	-
4	179.48	-
5	162.10	-
6	99.91	6.17 (d, J= 1.96 Hz)
7	167.97	-
8	94.83	6.34 (d, J= 1.96 Hz)
9	158.30	-
10	105.50	-
1′	123.00	-
2′	116.10	7.77 (d, J= 2.21 Hz)
3′	145.72	-
4′	149.90	-
5′	117.76	6.81 (d, J= 8.50 Hz)
6′	123.00	7.55 (d, J= 2.21, 8.50 Hz)
1"	105.47	5.10 (d, J= 7.87 Hz)
2"	74.59	3.80-3.40, m
3"	74.59	"
4"	71.60	"
5"	75.52	"
6"	63.85	"
CO_2	167.97	-
1"′	122.74	-
2"′	110.08	6.88, s
3"′	145.72	-
4"′	135.69	-
5"′	145.72	-
6"′	110.08	6.88, s

TABLE 12

NMR Chemical shift positions for 3,3′,4′,5,7-pentaacetyl-quercetin (44) in DMSO-d_6

Position of carbon	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)
2	144.44	-
3	133.22	-
4	169.30	-
5	154.37	-
6	114.70	7.26 (d, J= 2.21 Hz)
7	156.36	-
8	114.08	7.26 (d, J= 2.21 Hz)
9	153.34	-
10	110.08	-
1′	127.13	-
2′	124.51	7.64 (d, J= 2.16 Hz)
3′	142.17	-
4′	149.49	-
5′	124.51	7.51 (d, J= 8.74 Hz
6′	126.68	7.86 (d, J= 2.16, 8.74 Hz)
3-C=O	168.73	-
3′-C=O	168.33	-
4′-C=O	168.01	-
5-C=O	168.18	-
7-C=O	167.75	-
3-OCH_3	20.85	2.33, s
3′-OCH_3	20.74	2.32, s
4′-OCH_3	20.35	2.31, s
5-OCH_3	20.38	2.32, s
7-OCH_3	20.35	2.31, s

TABLE 13

NMR Chemical shift positions for 3′,4′,5,7-tetramethylquercetin (45) in DMSO-d_6

Position of Carbon	^{13}C-NMR (ppm)	^{1}H-NMR(ppm)
2	151.37	-
3	138.31	-
4	178.10	-
5	160.92	-
6	97.85	6.37 (d, J= 2.06 Hz)
7	165.21	-
8	92.49	6.78 (d, J= 2.06 Hz)
9	156.36	-
10	105.28	-
1′	122.08	-
2′	111.35	7.64 (d, J= 2.02 Hz)
3′	148.51	-
4′	155.50	-
5′	111.64	7.15 (d, J= 8.61 Hz)
6′	122.08	7.71 (d, J= 2.02, 8.61 Hz
3′-OCH_3	56.12	3.84, s
4′-OCH_3	59.81	3.80, s
5-OCH_3	55.70	3.85, s
7-OCH_3	55.70	3.85, s

TABLE 14

NMR Chemical shift positions for 2',4'-dihydroxy-6'-methoxy-dihydrochalcone (37) in MeCH.

Position of carbon	^{13}C NMR (ppm)	^{1}H NMR (ppm)
1	142.76	-
2	128.30	7.24, m
3	125.86	"
4	128.45	"
5	125.86	"
6	128.30	"
C=O	203.74	-
α	30.13	2.87, m
β	45.07	3.22, m
1'	104.43	-
2'	164.89	-
3'	95.71	5.88 (d, J= 1.88 Hz)
4'	163.01	-
5'	91.41	5.98 (d, J= 1.88 Hz)
6'	166.00	-
6'-OCH_3	55.90	3.80, s

APPENDIX B

Spectra: UV, IR, NMR and MS

Key to Uv-visible spectra

1 max in MeOH

2 max in MeOH/NaOMe

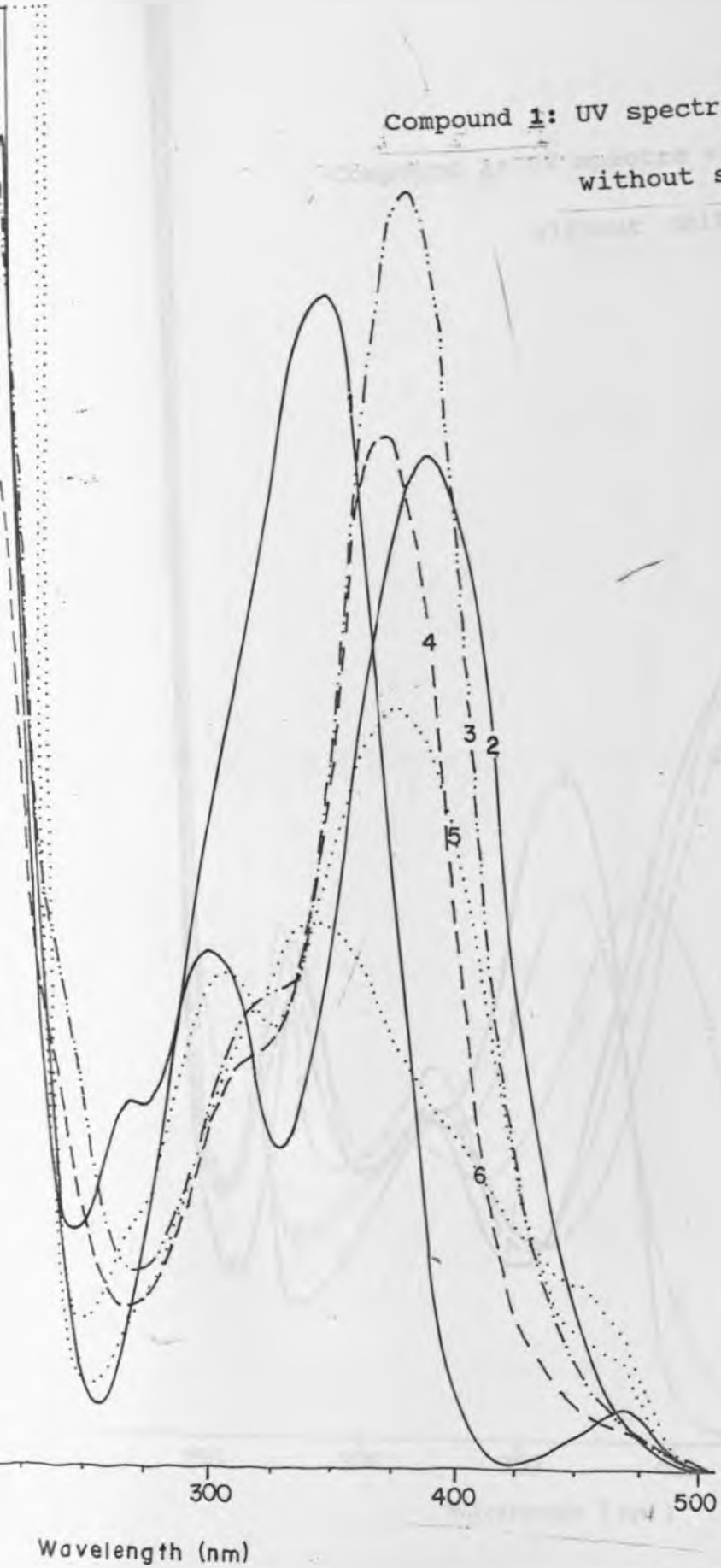
3 max in MeOH/$AlCl_3$

4 max in MeOH/$AlCl_3$/HCl

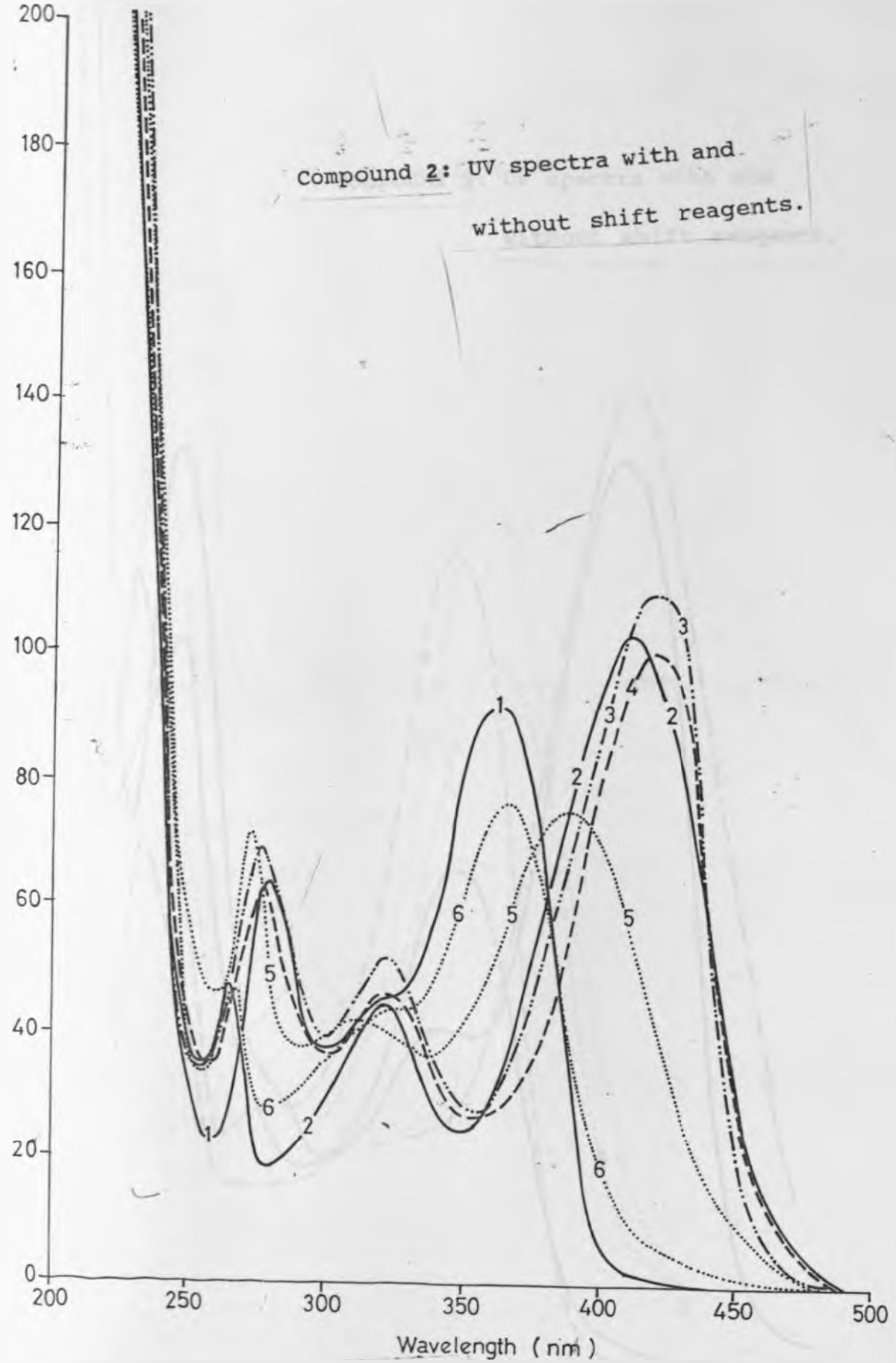
5 max in MeOH/NaOAc

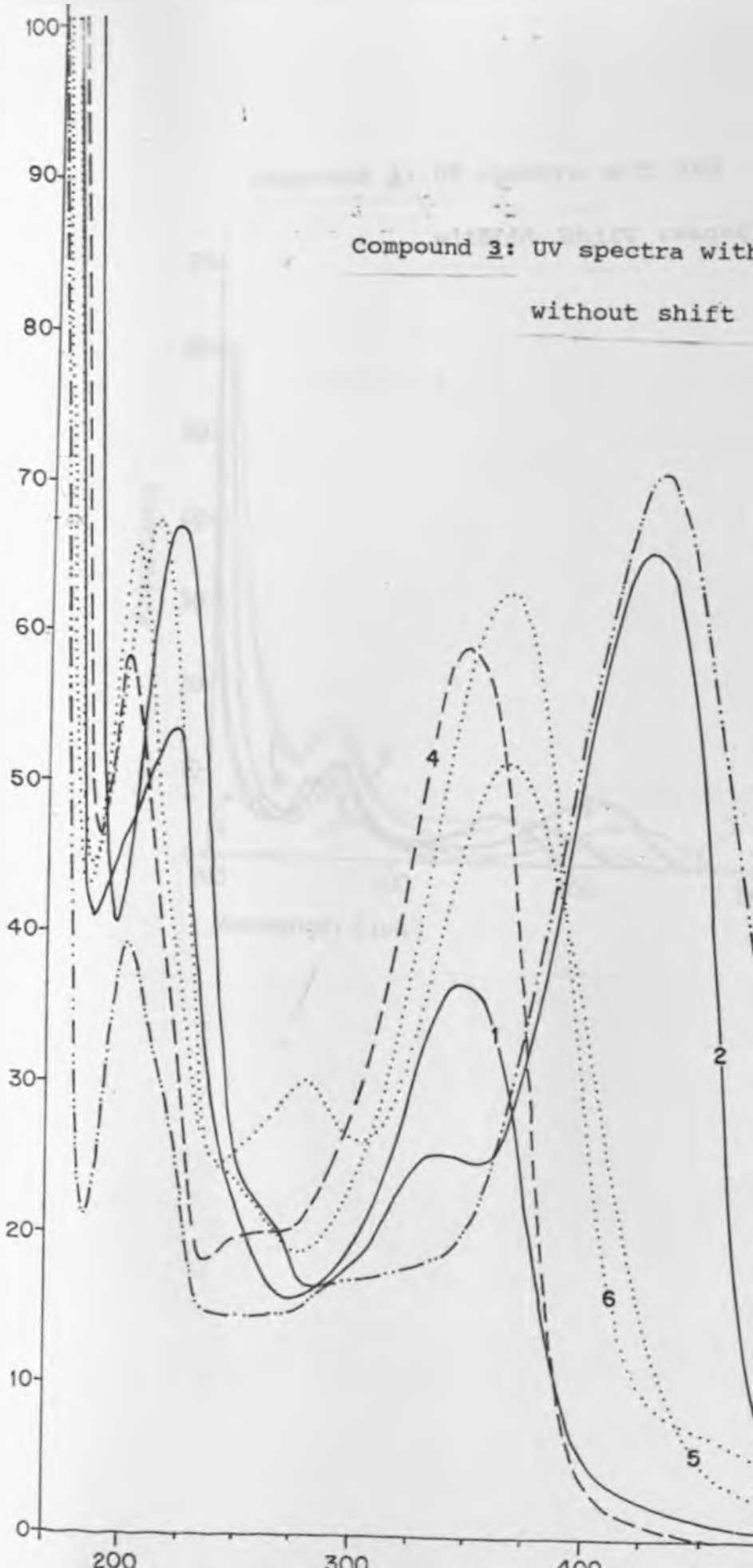
6 max in MeOH/NaOAc/H_3BO_3

Compound 1: UV spectra with and without shift reagents.

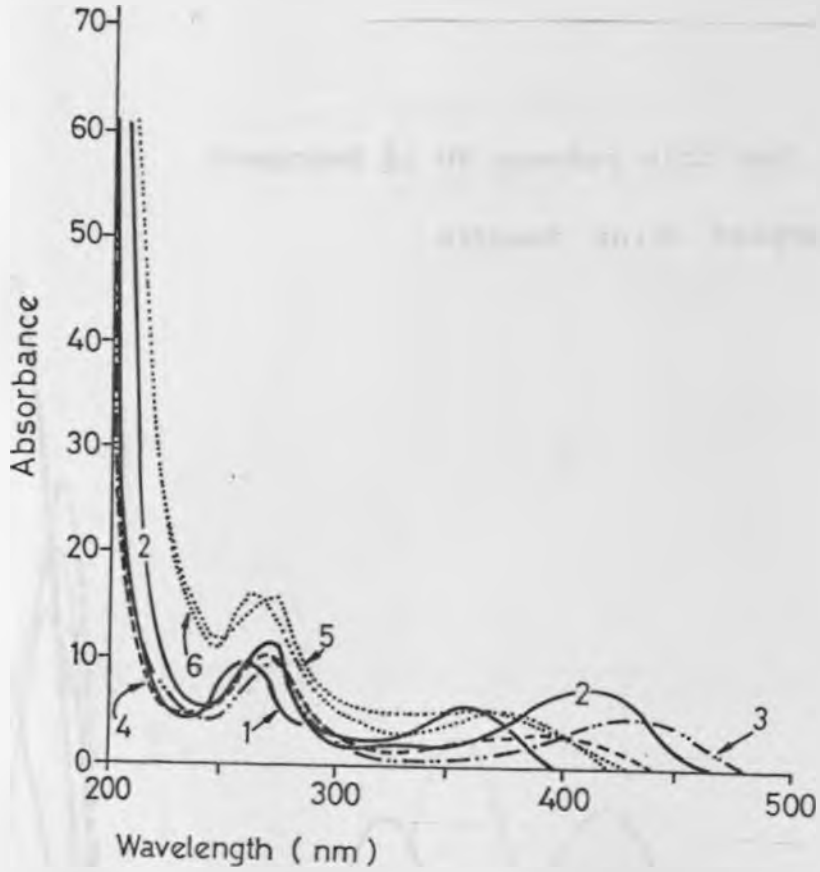


Compound 2: UV spectra with and without shift reagents.

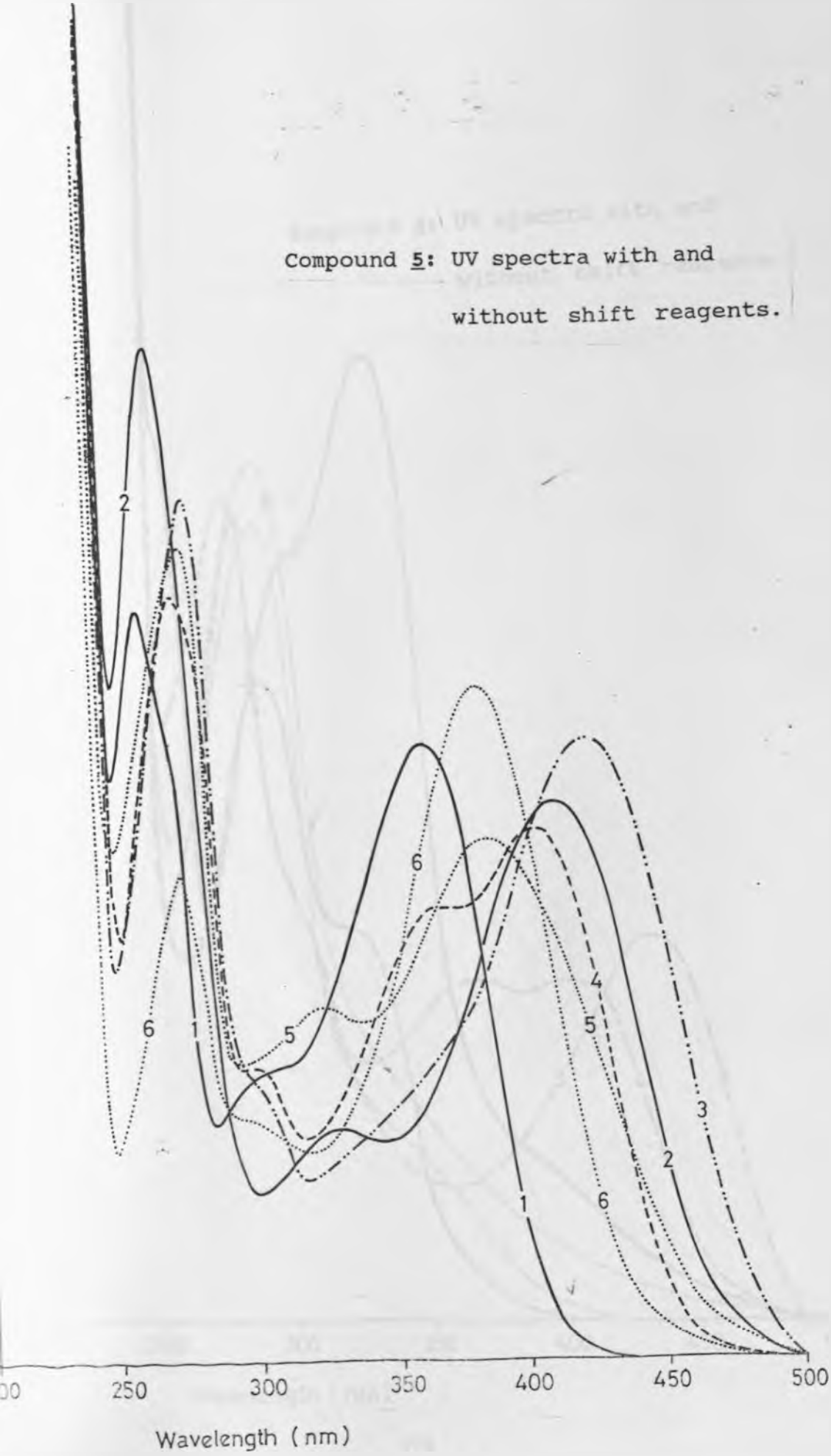


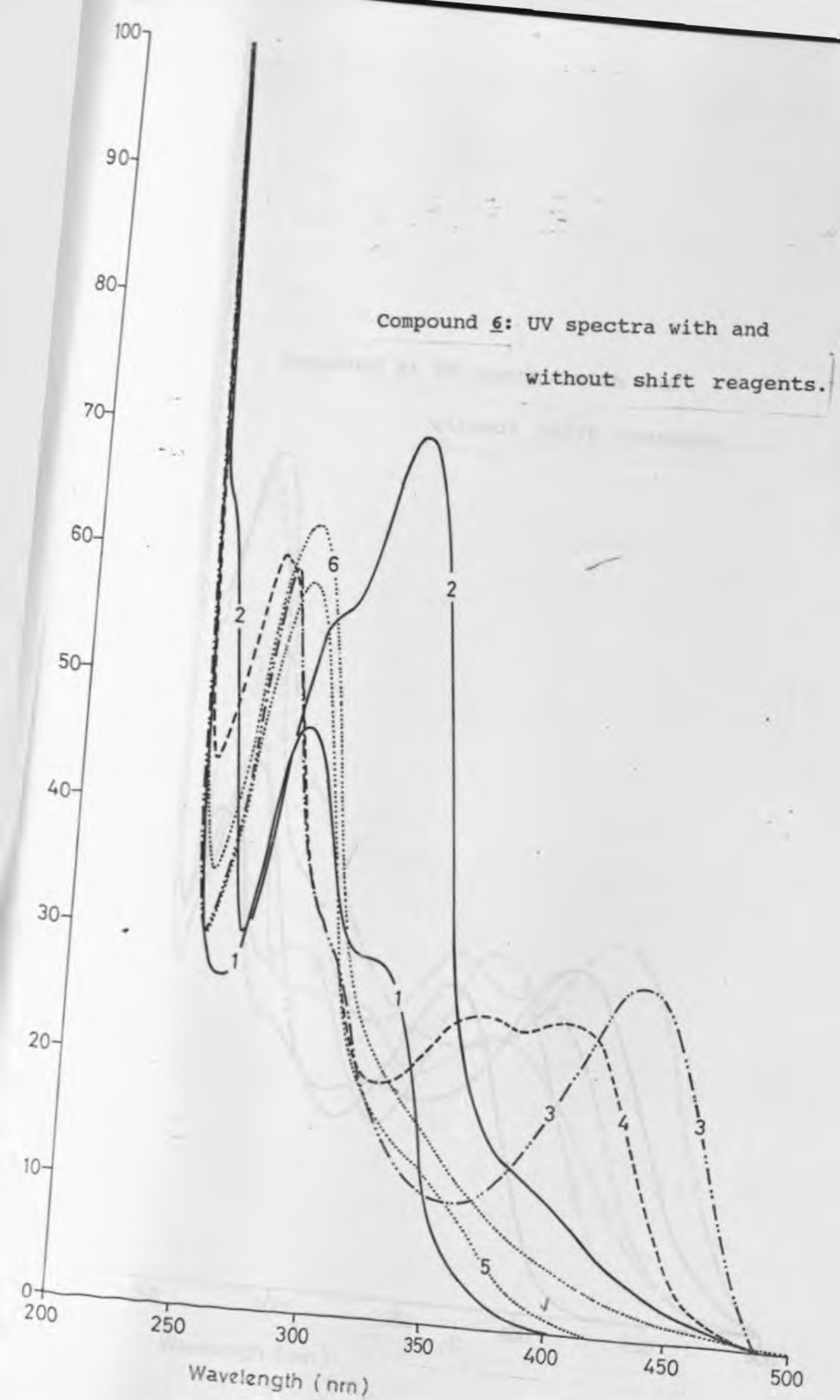

Compound 3: UV spectra with
without shift
100
90
80
70
60
50
40
30
20
10
0
200
300
400
1
2
4
5
6

Compound 4: UV spectra with and without shift reagents.

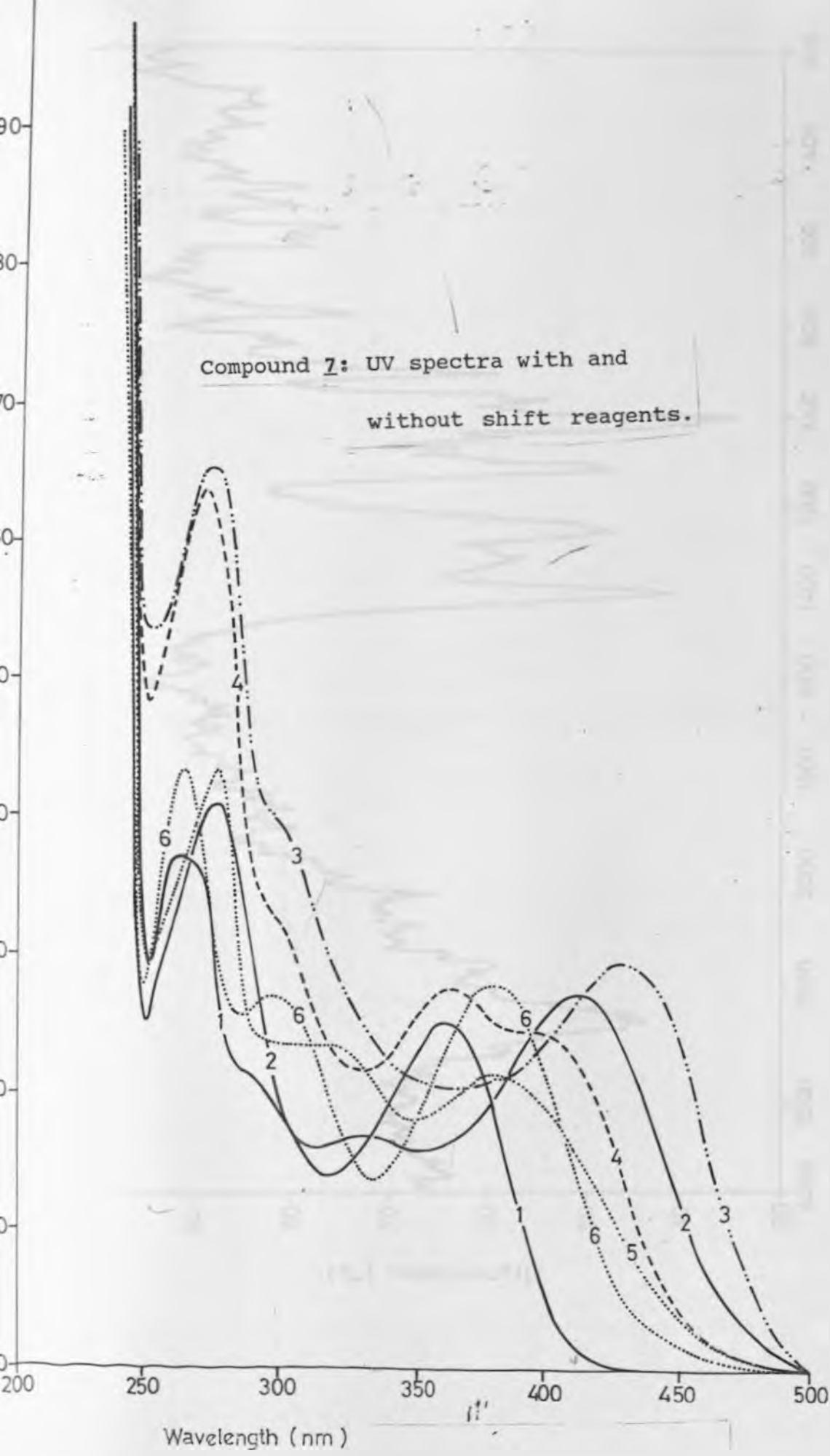


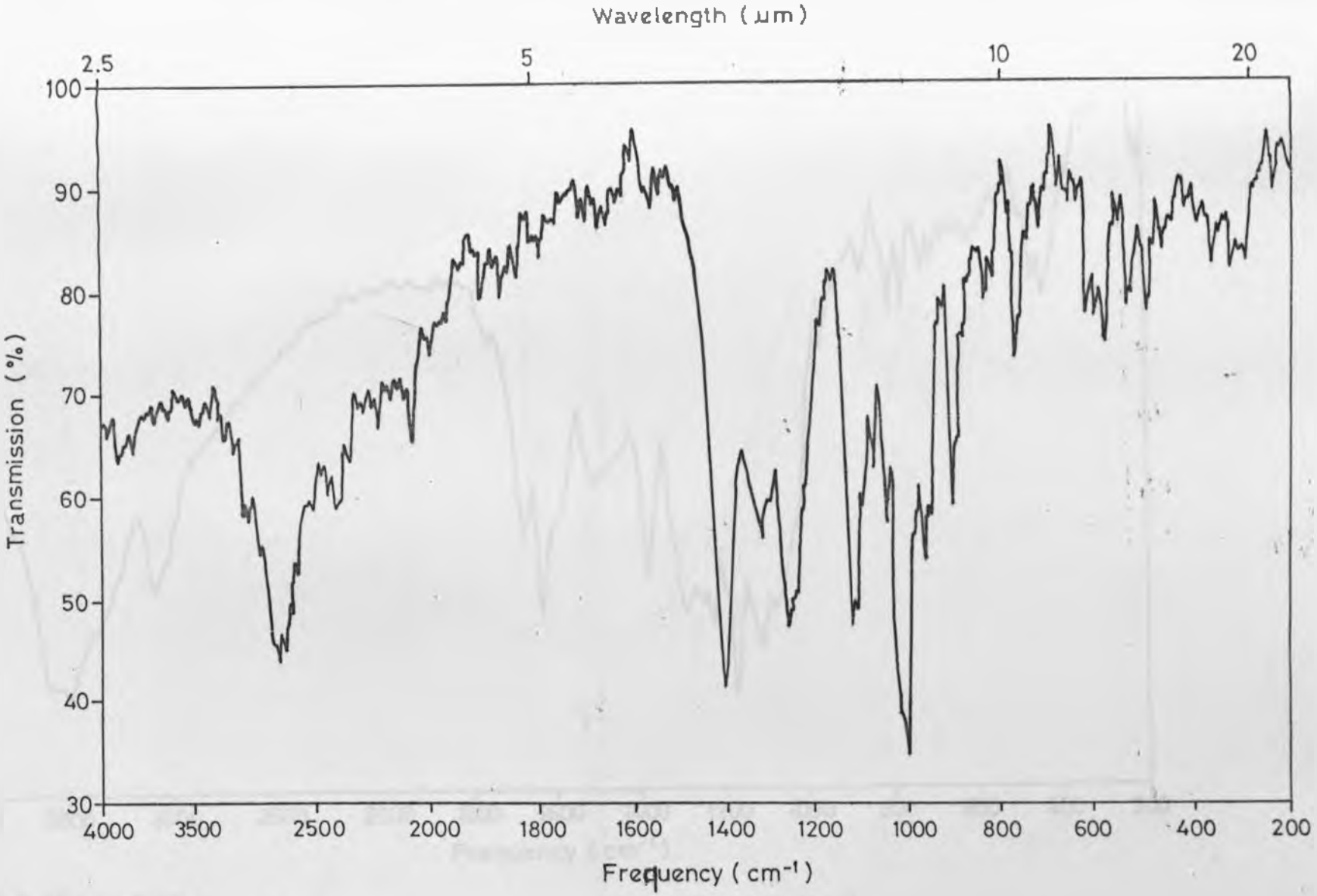
Compound 5: UV spectra with and without shift reagents.



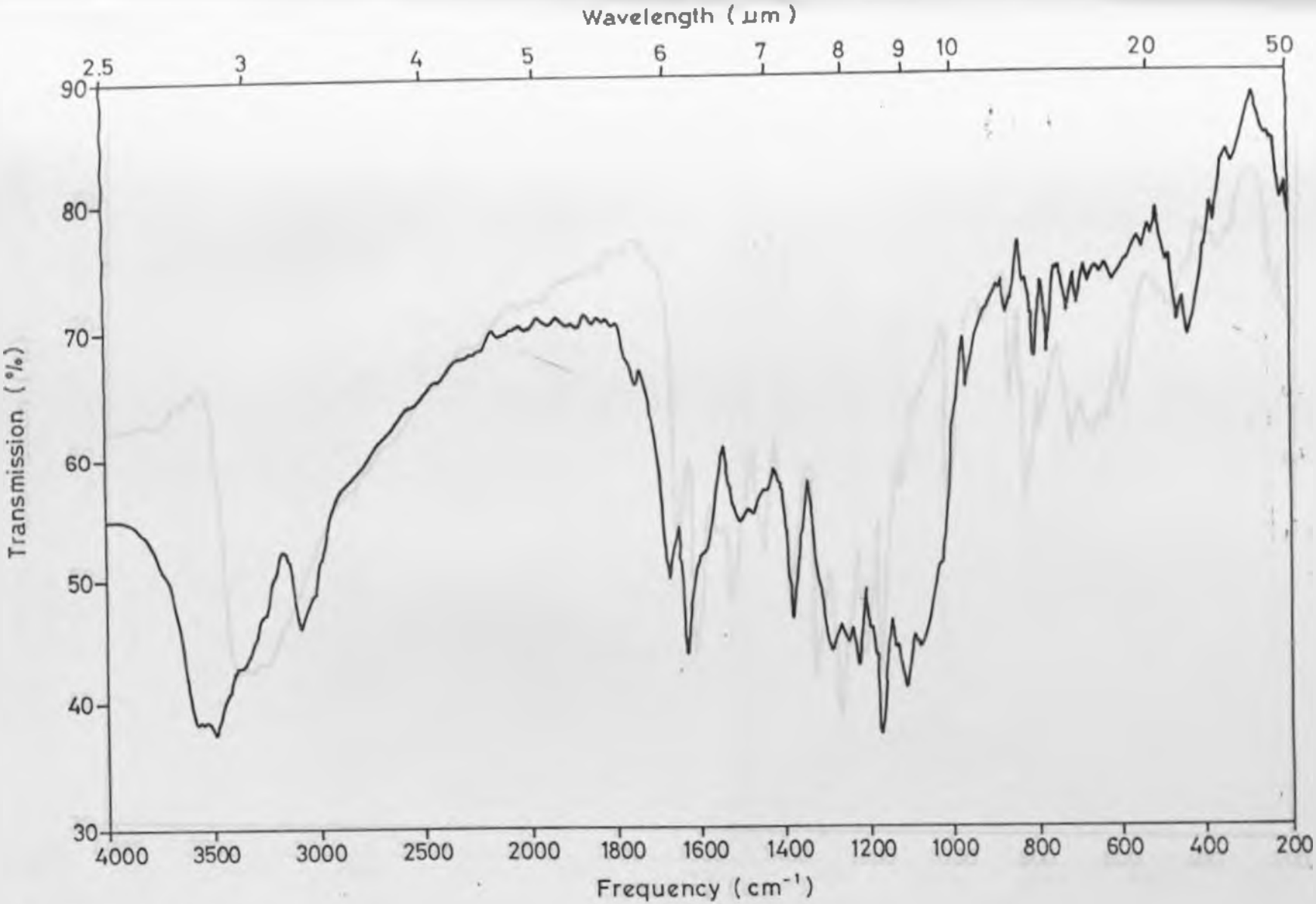


Compound 6: UV spectra with and without shift reagents.

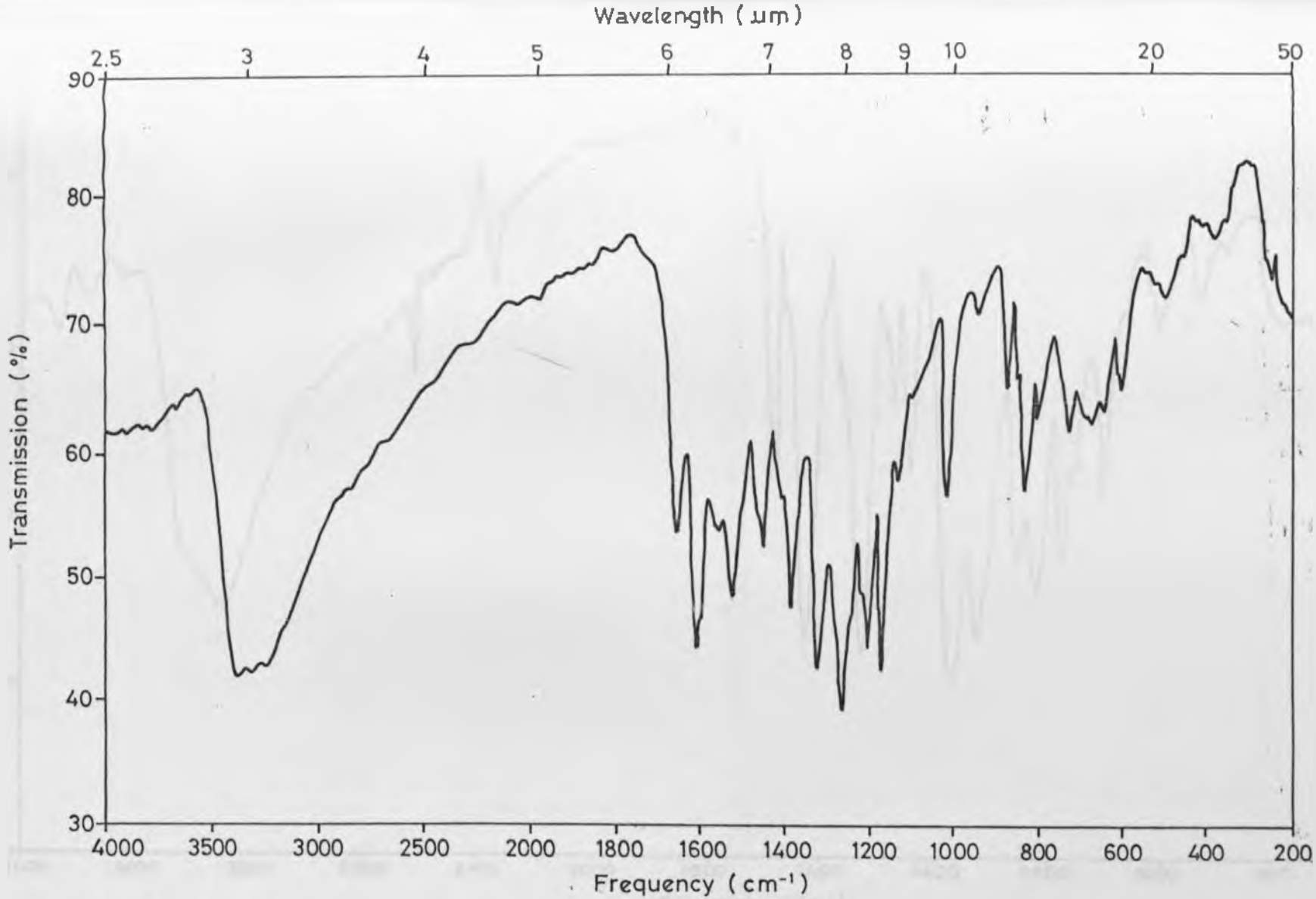

Compound 7: UV spectra with and without shift reagents.
200
250
300
350
400
450
500
Wavelength (nm)



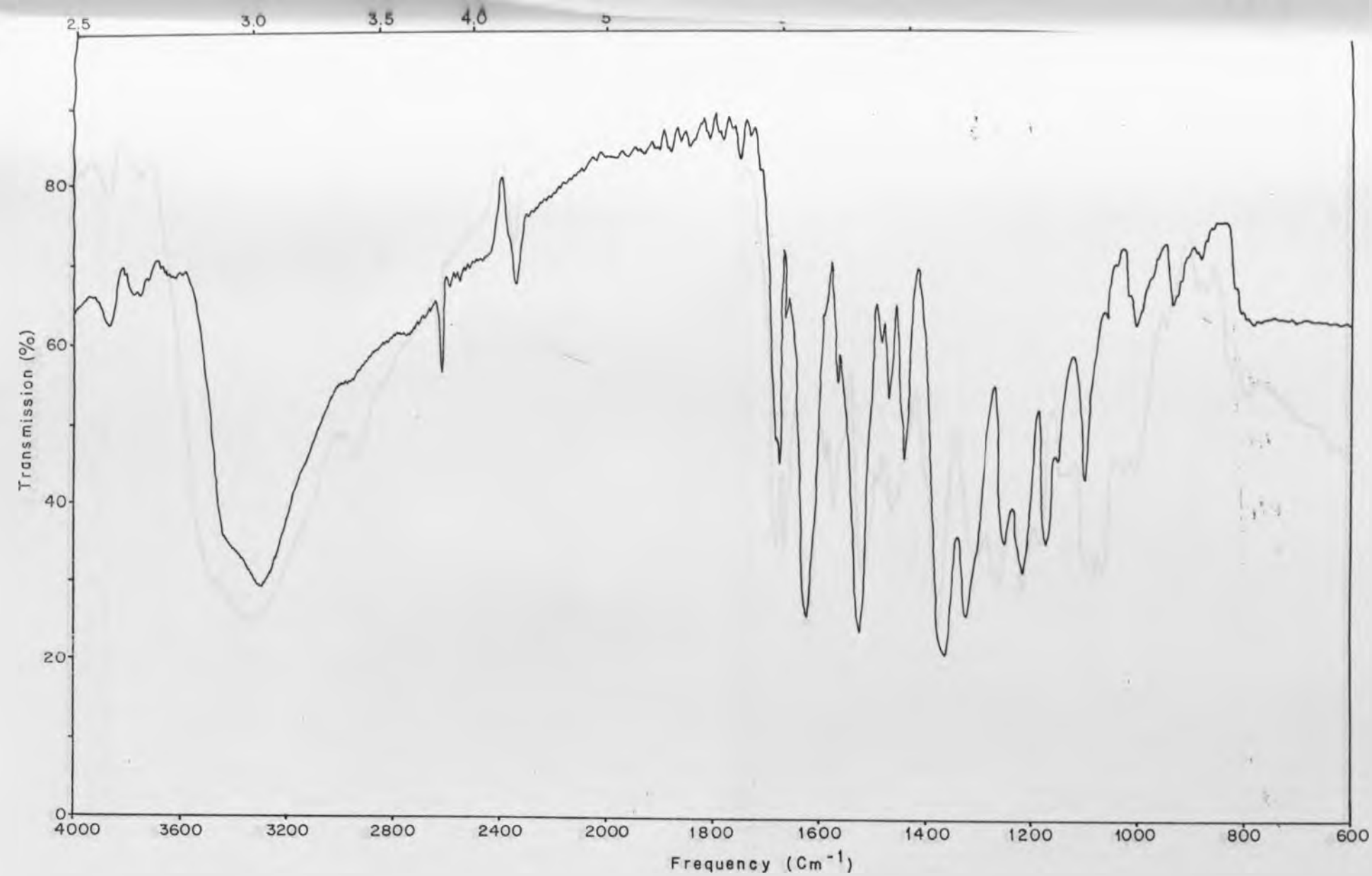
Compound 1 IR spectrum.



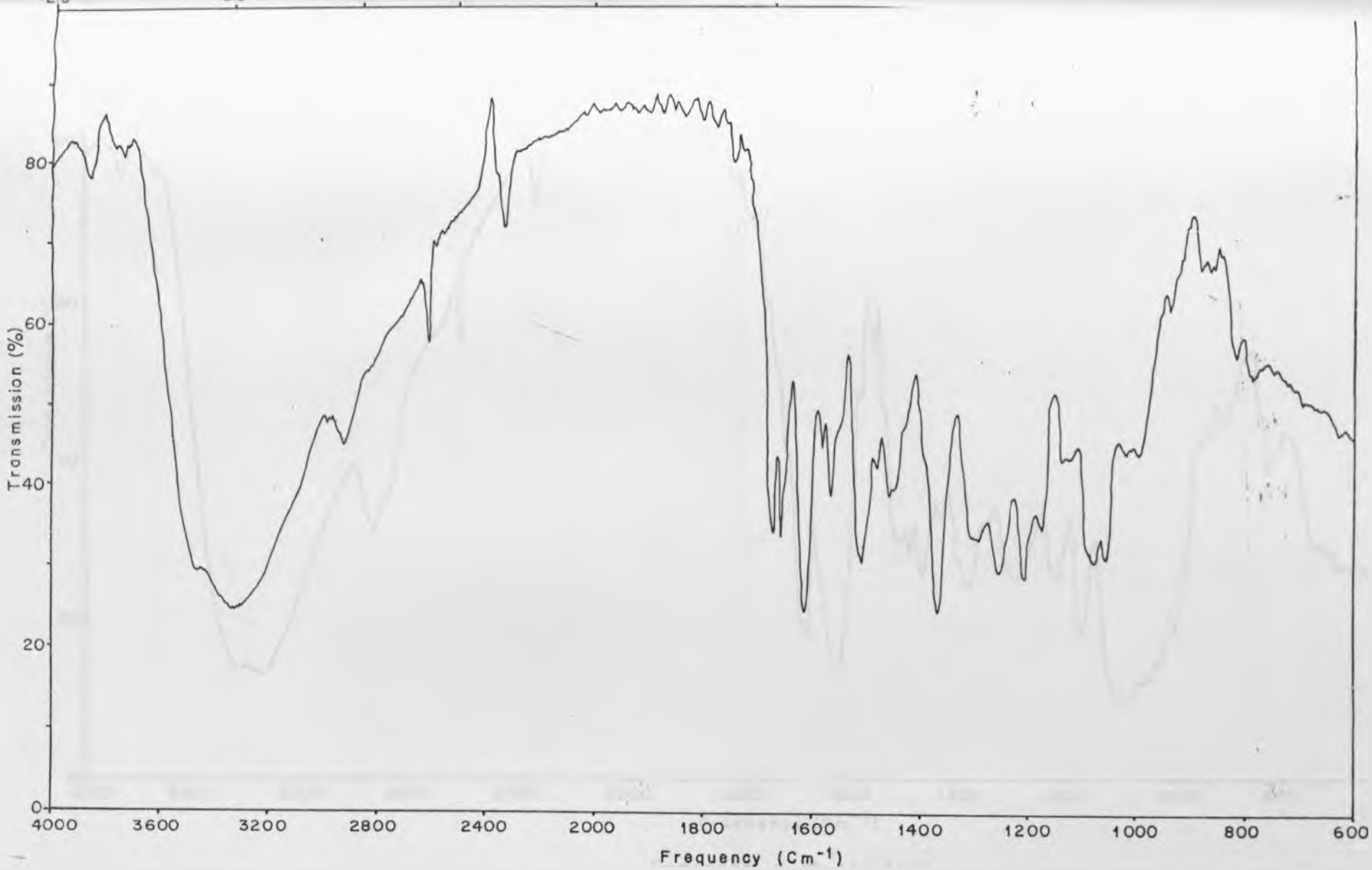
Compound 2 IR spectrum.



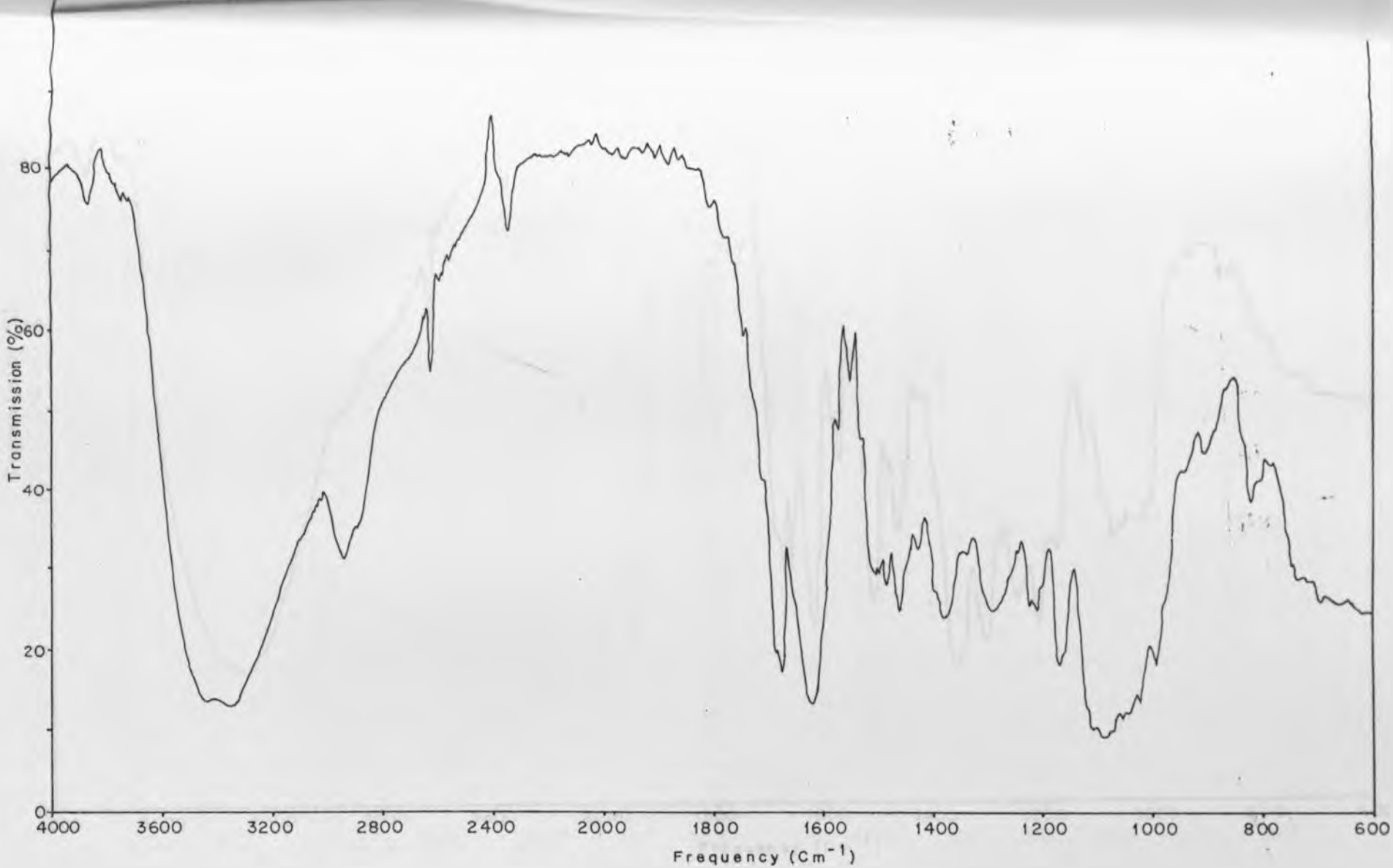
Compound 3 IR spectrum.



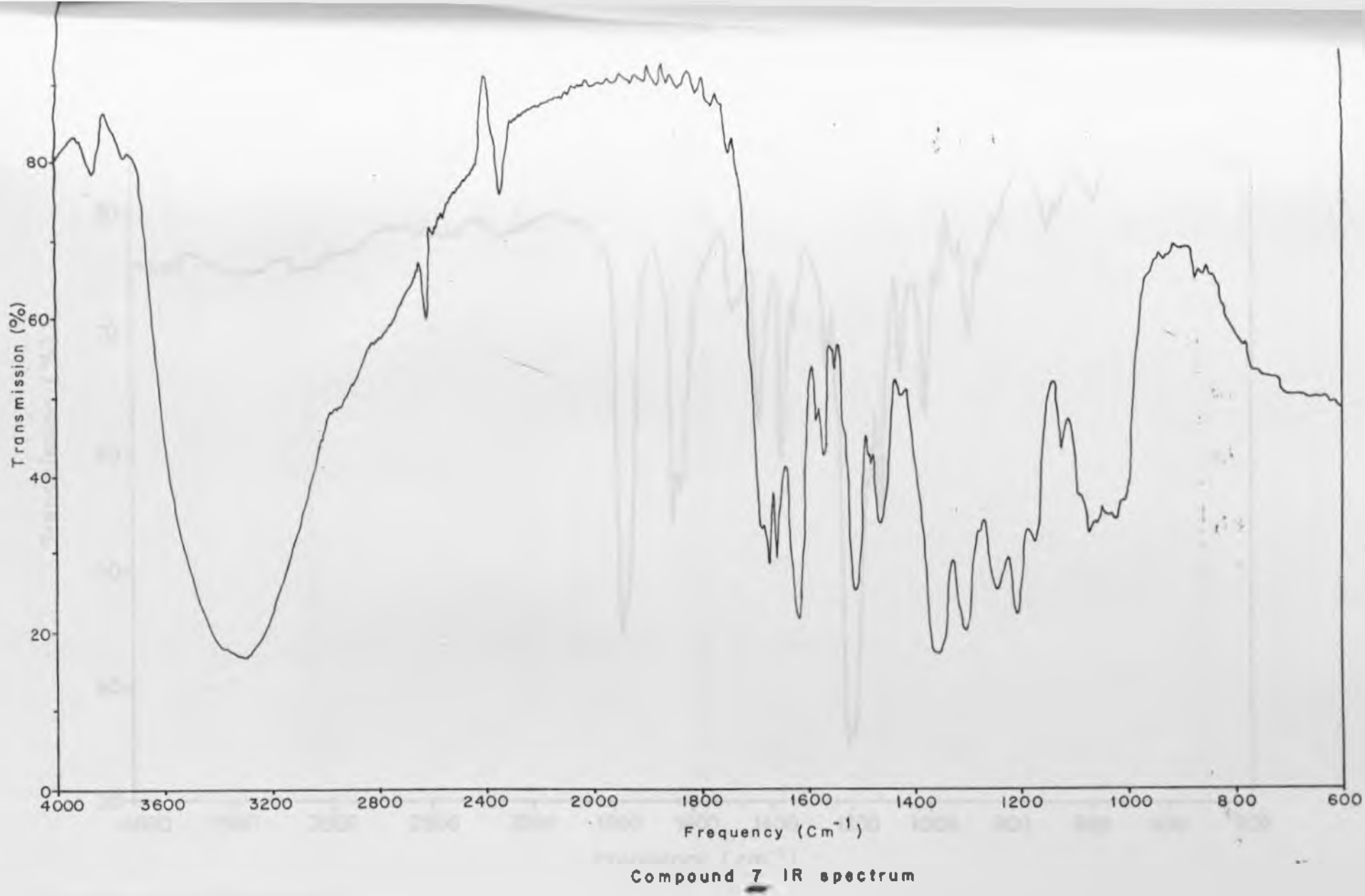
Compound 4 IR spectrum



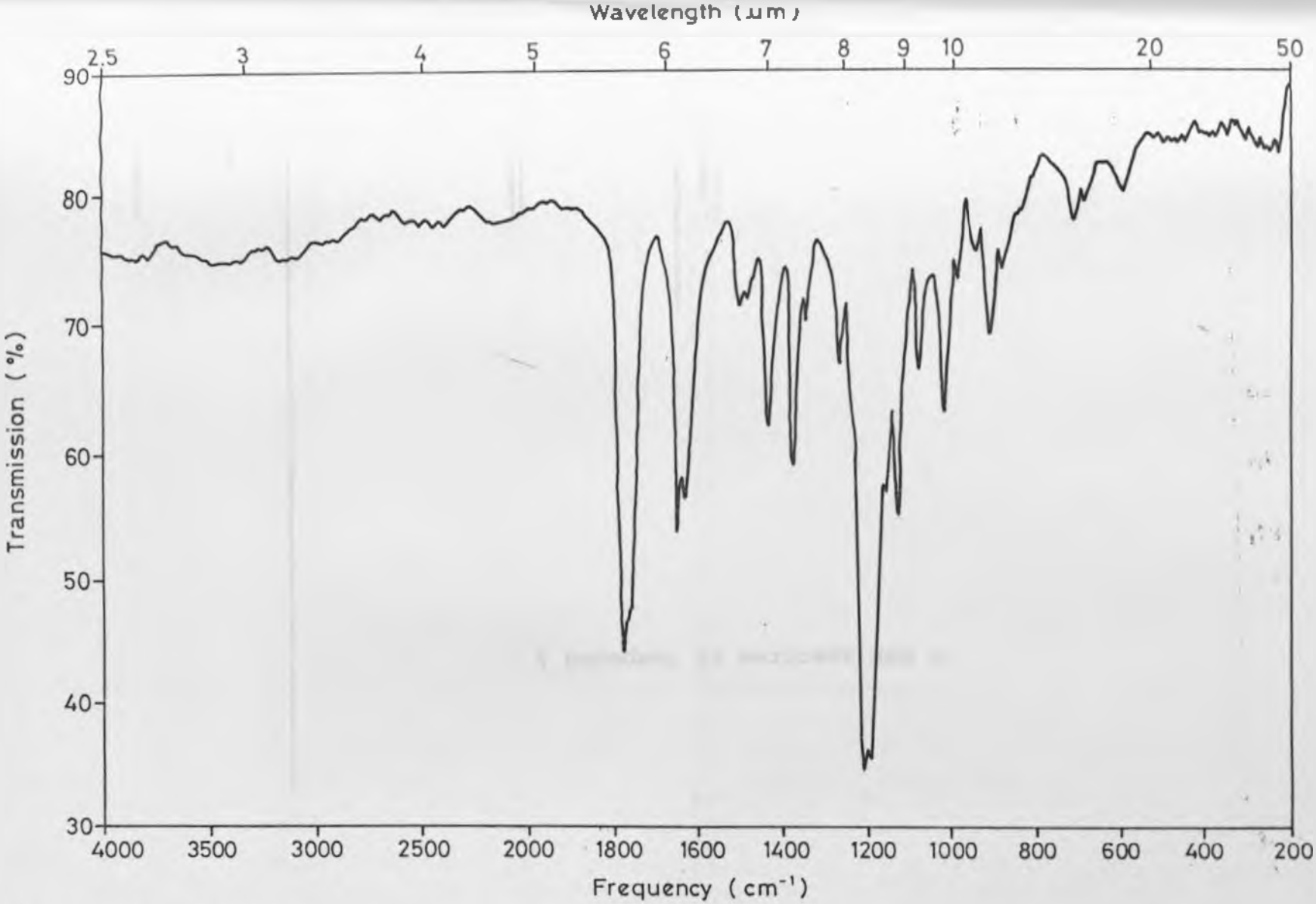
Compound 5 IR spectrum



Compound 6 IR spectrum

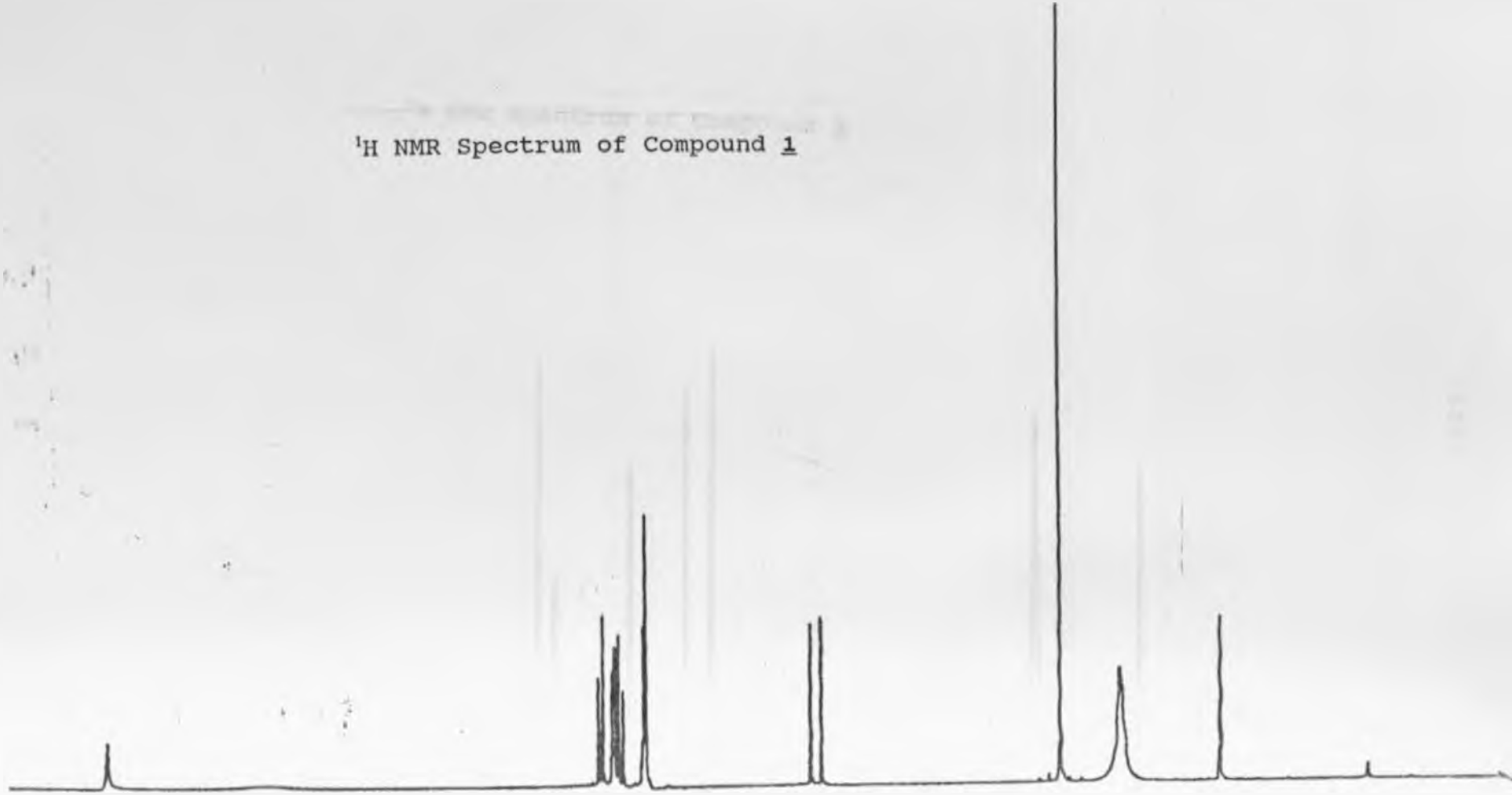


Compound 7 IR spectrum

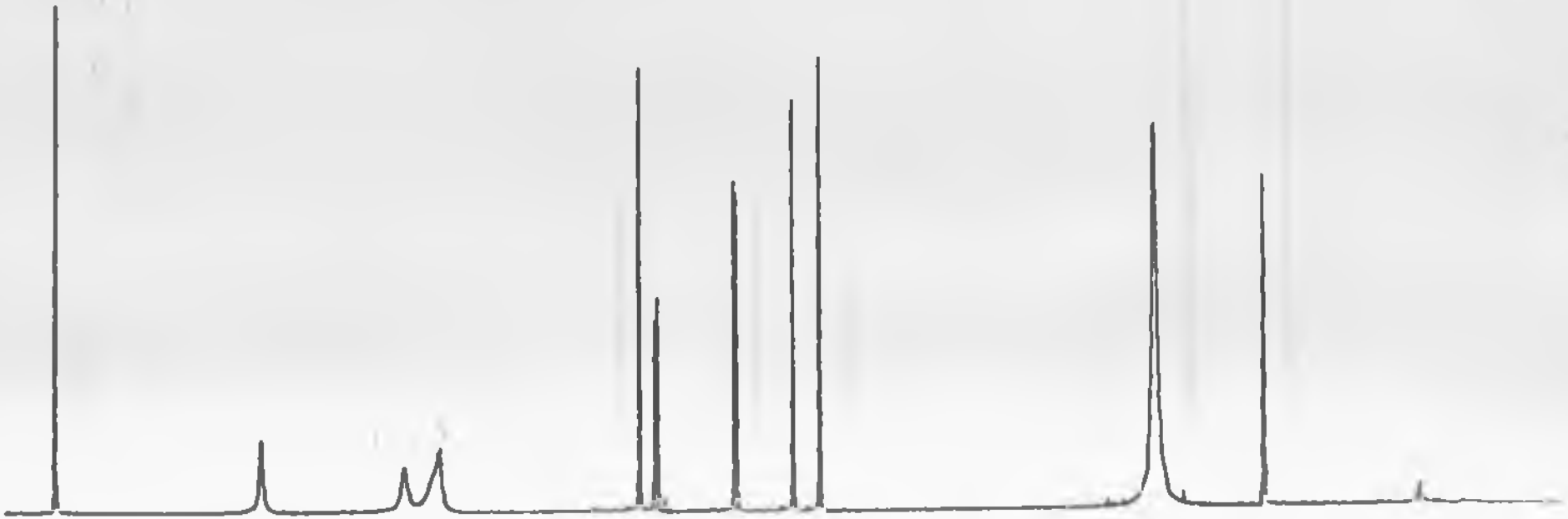


Compound 44 IR spectrum.

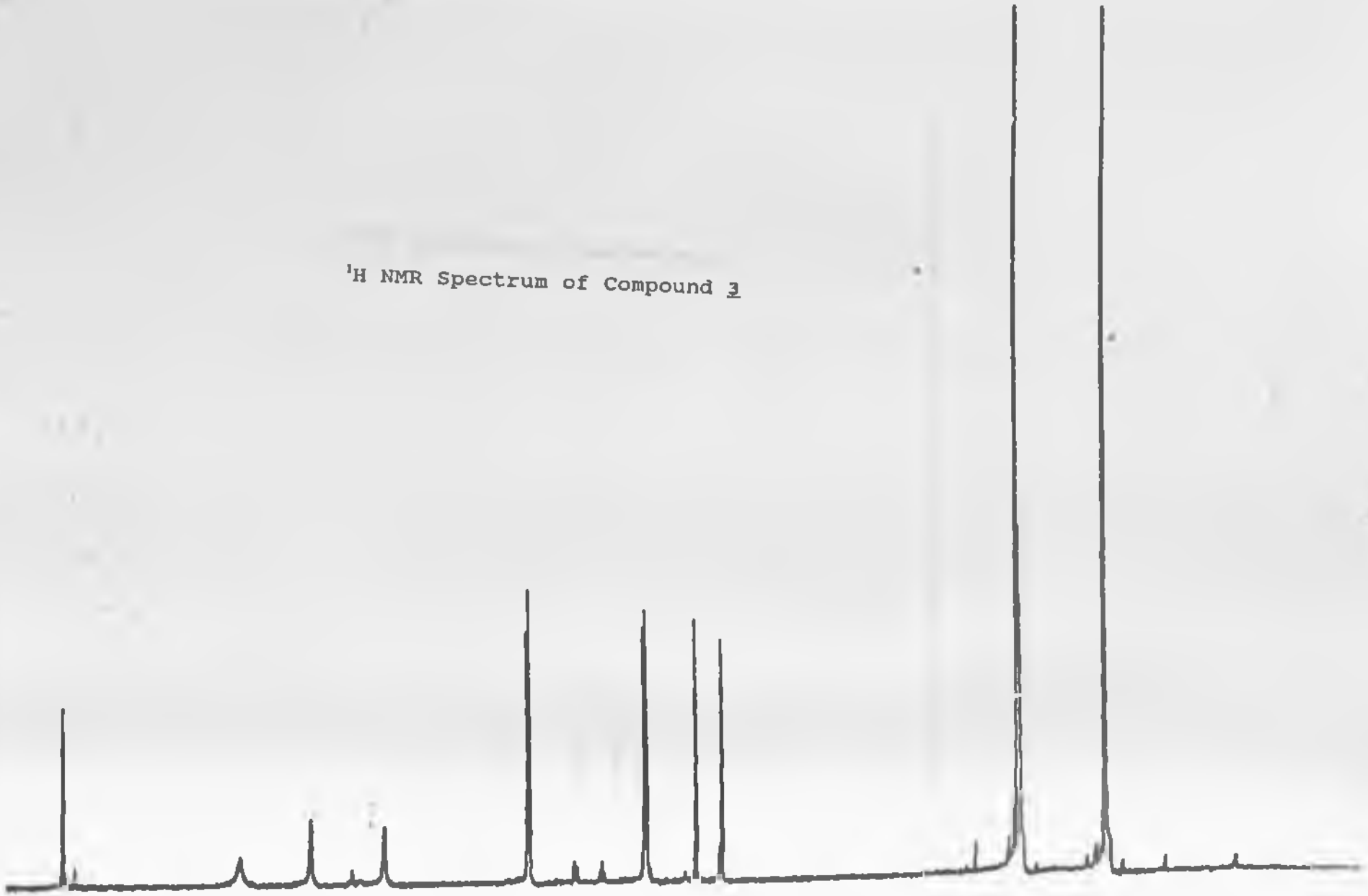
^{1}H NMR Spectrum of Compound 1



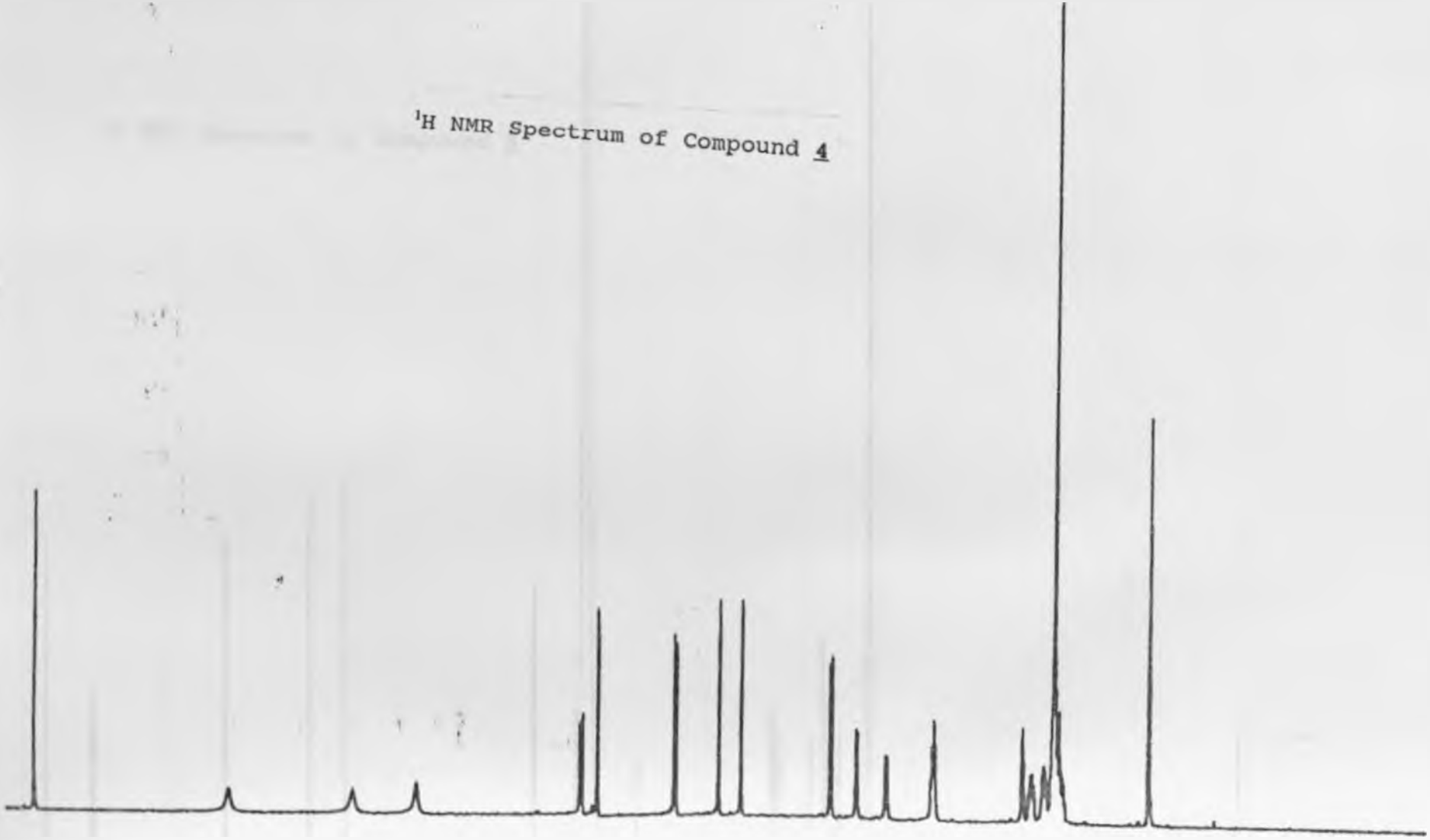
^{1}H NMR Spectrum of Compound **2**



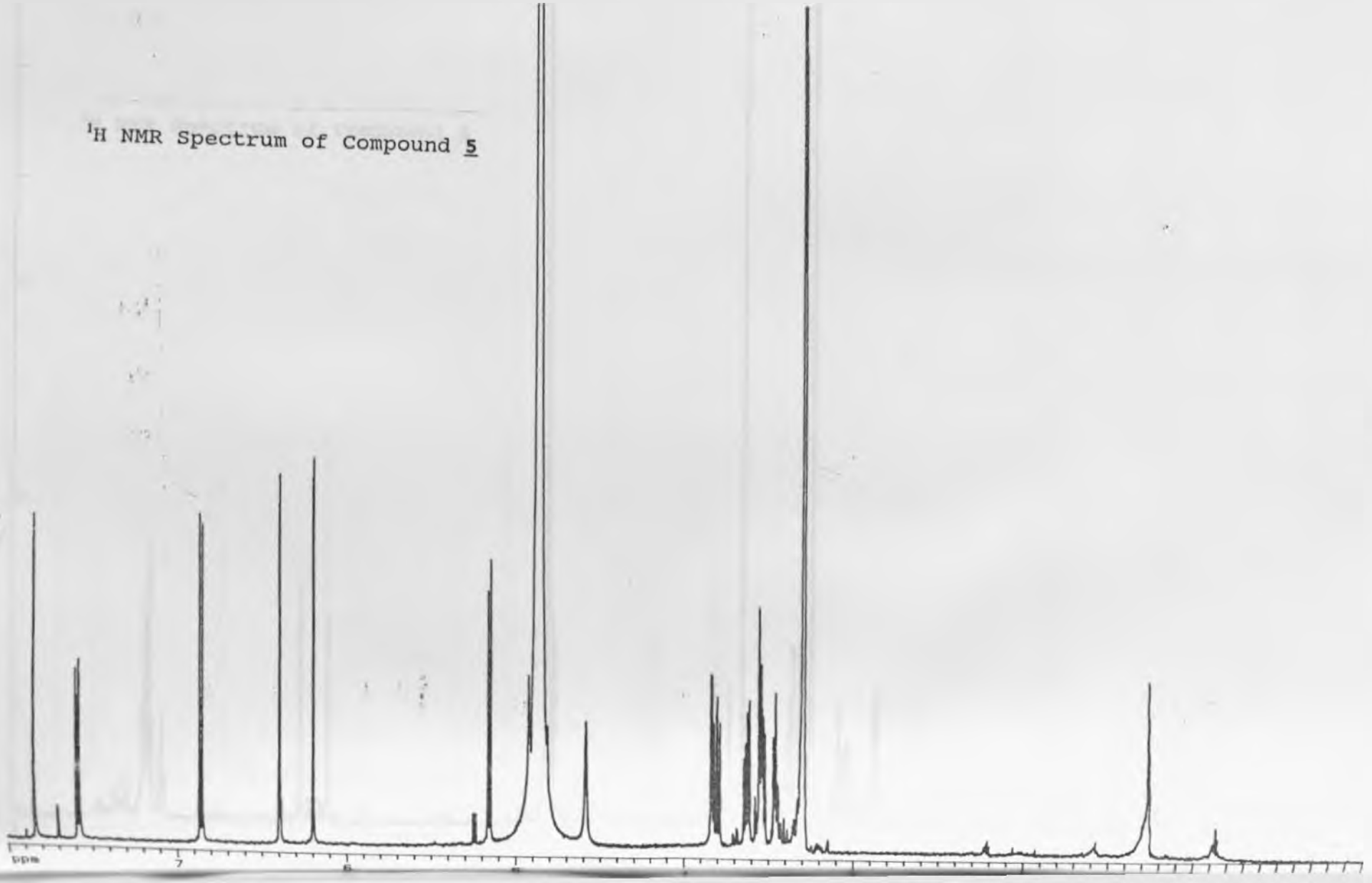
^{1}H NMR Spectrum of Compound 3



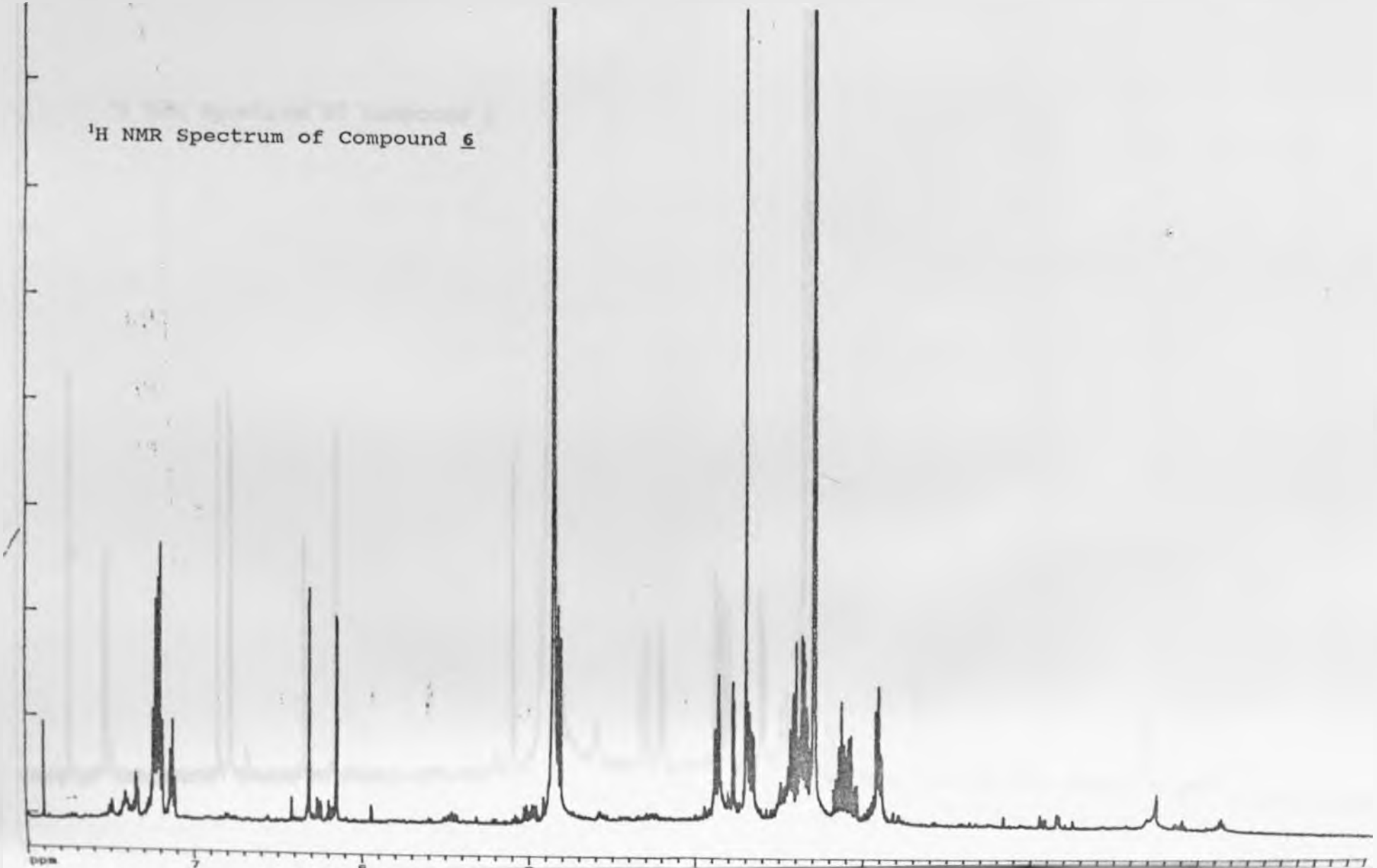
^{1}H NMR Spectrum of Compound **4**



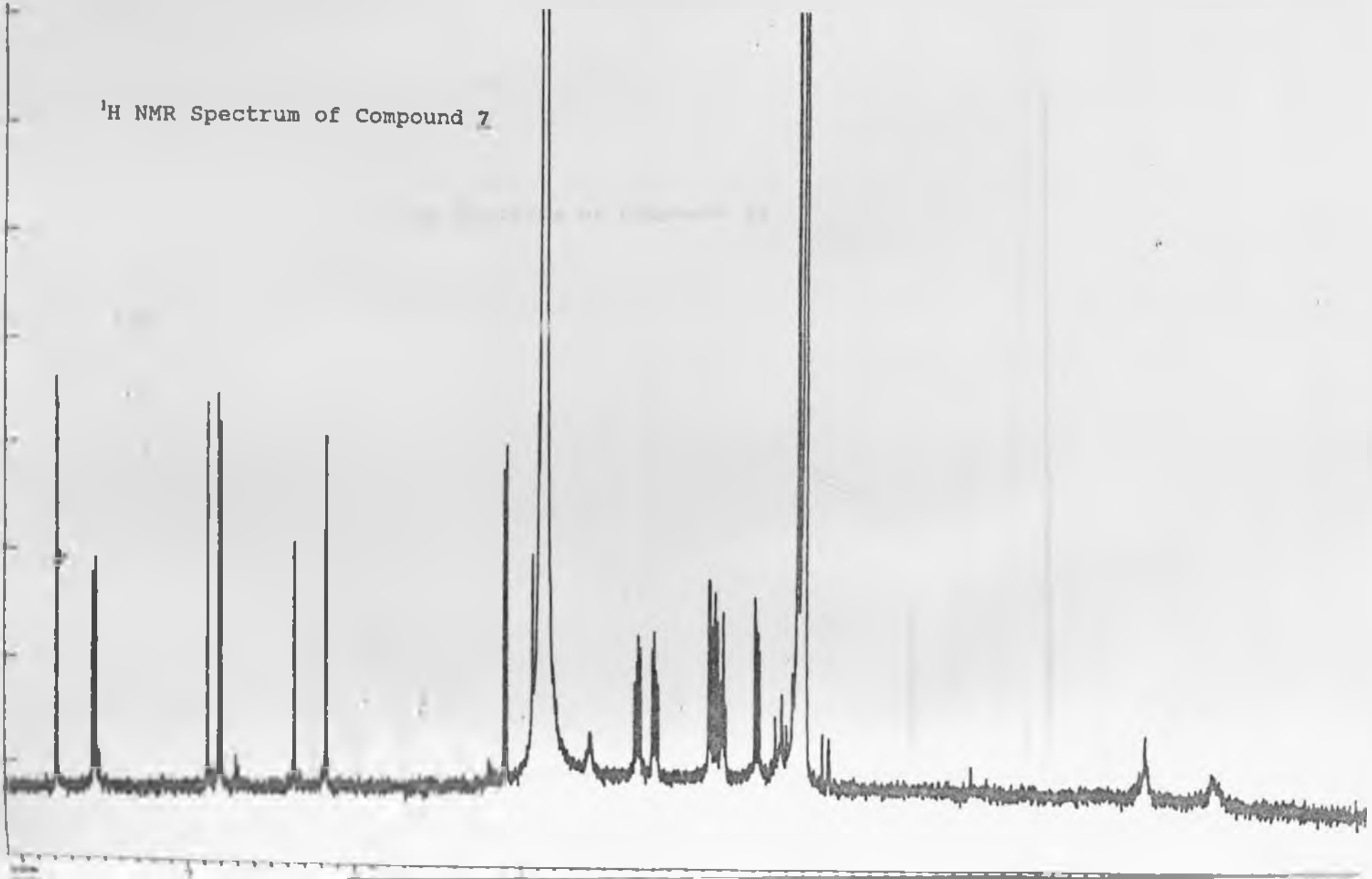
^{1}H NMR Spectrum of Compound **5**



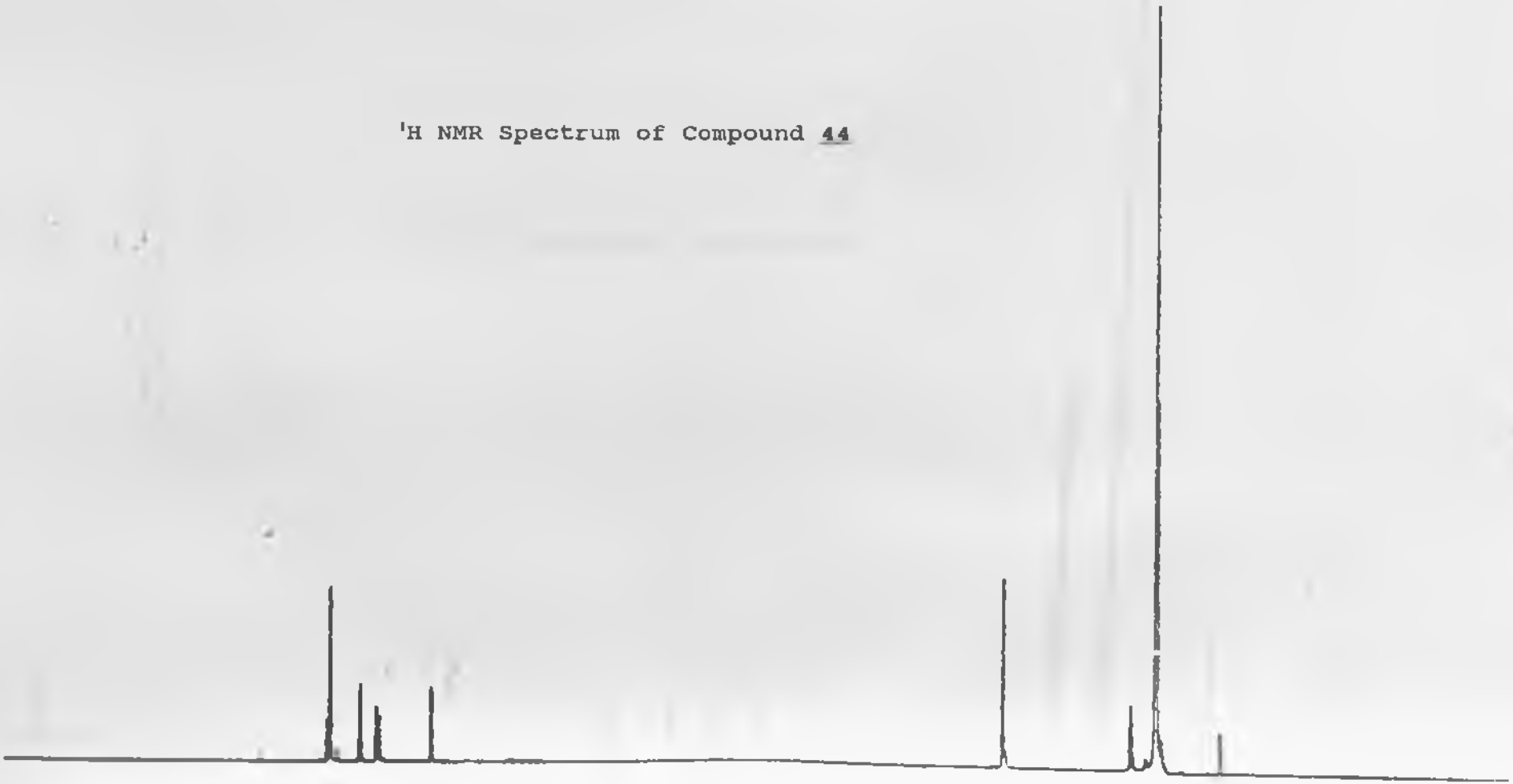
^{1}H NMR Spectrum of Compound 6



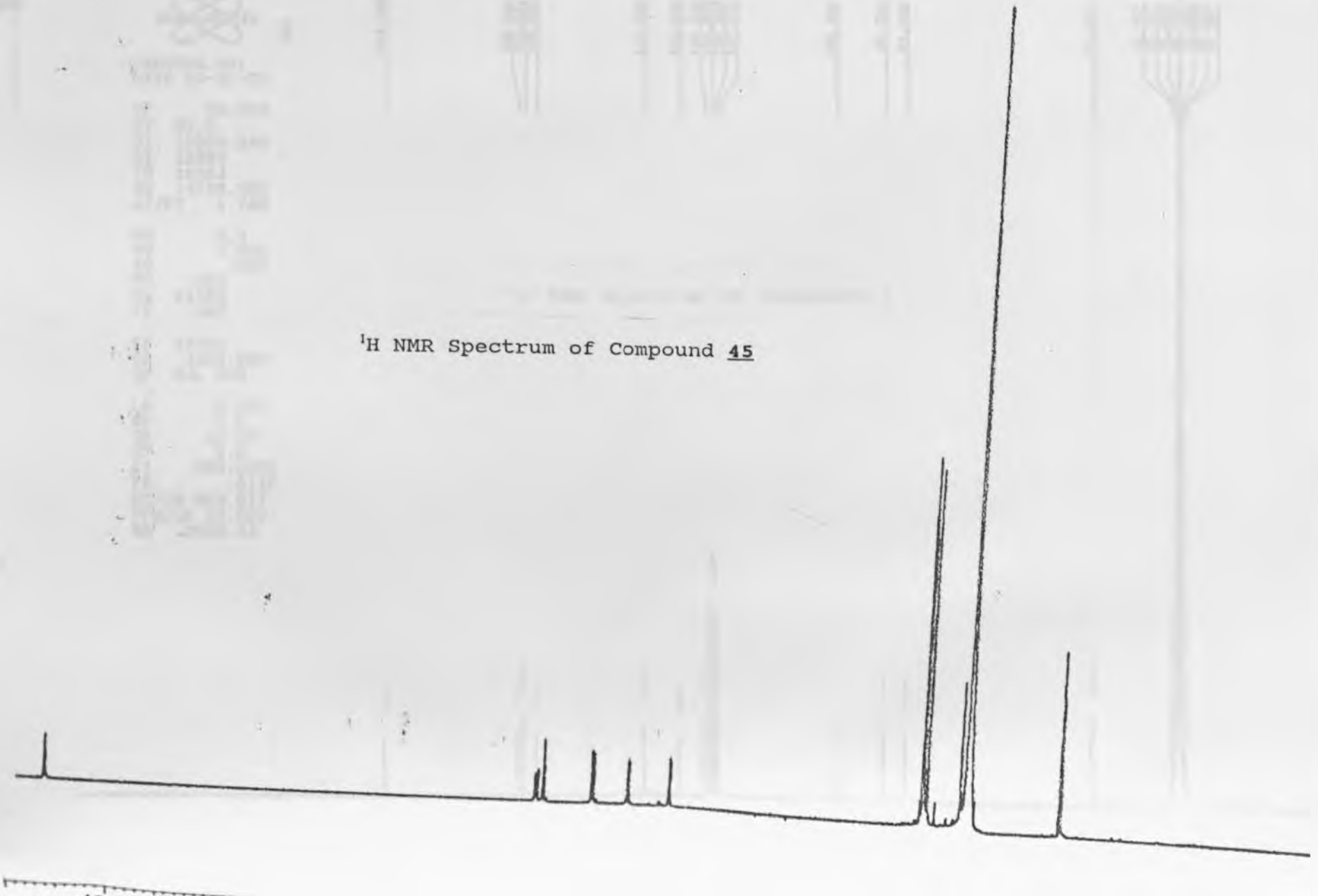
^{1}H NMR Spectrum of Compound 7

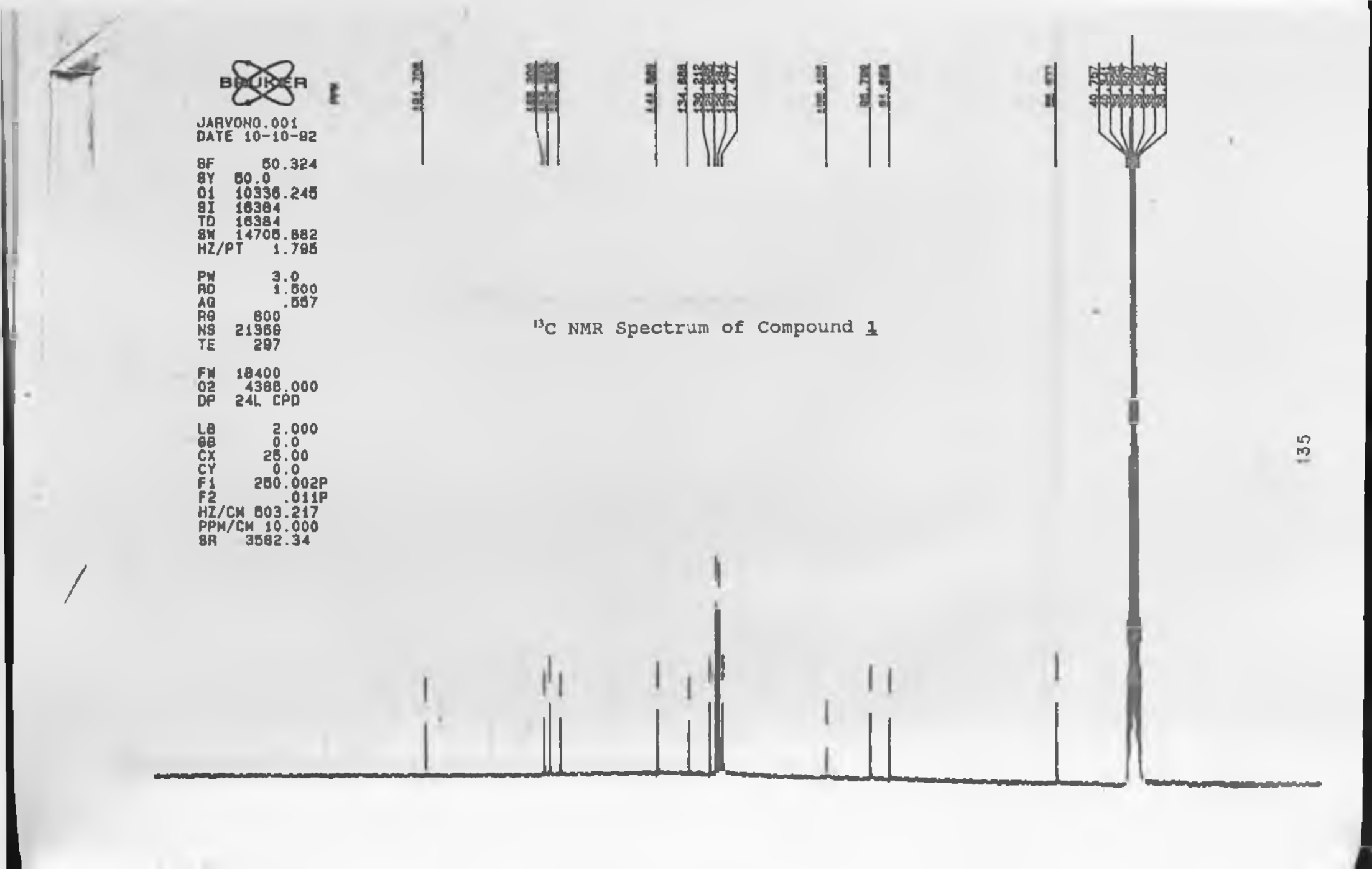


^{1}H NMR Spectrum of Compound **44**



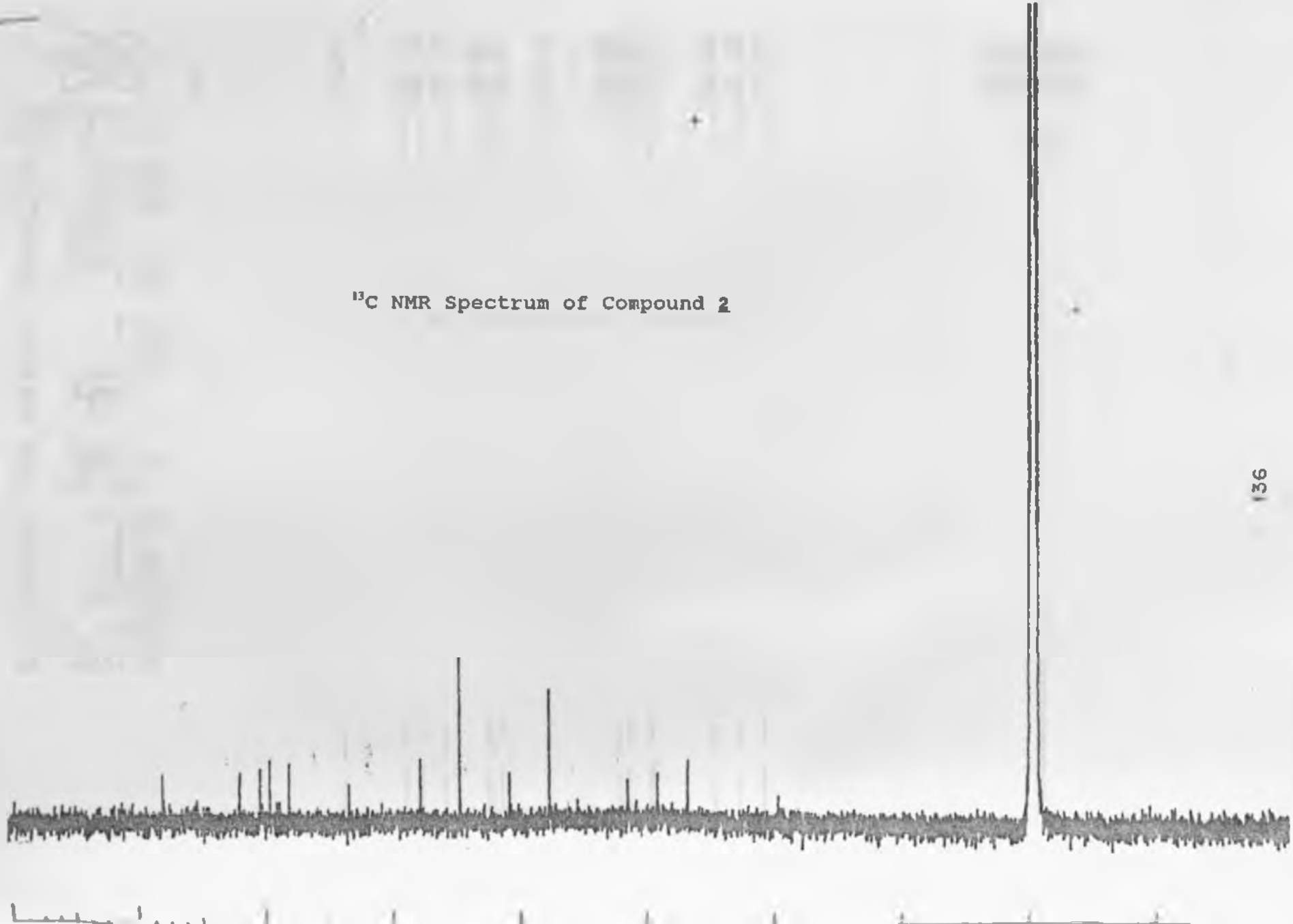
^{1}H NMR Spectrum of Compound **45**





^{13}C NMR Spectrum of Compound 1

^{13}C NMR Spectrum of Compound **2**



BRUKER

PPM

JARVWANG.001
DATE 10-10-92

SF	100.614
SFO	100.620
O1	4376.848
SI	32768
TD	32768
SW	29411.765
HZ/PT	1.795
PW	2.0
RD	1.500
AQ	.557
RG	1600
NS	20000
TE	297
FW	36800
O2	8429.000
DP	18L CPD
LB	2.000
GB	0.0
CX	25.00
CY	0.0
F1	249.999P
F2	-.002P
HZ/CM	1.006E3
PPM/CM	10.000
SA	-5604.00

175.830
163.873
160.717
156.132
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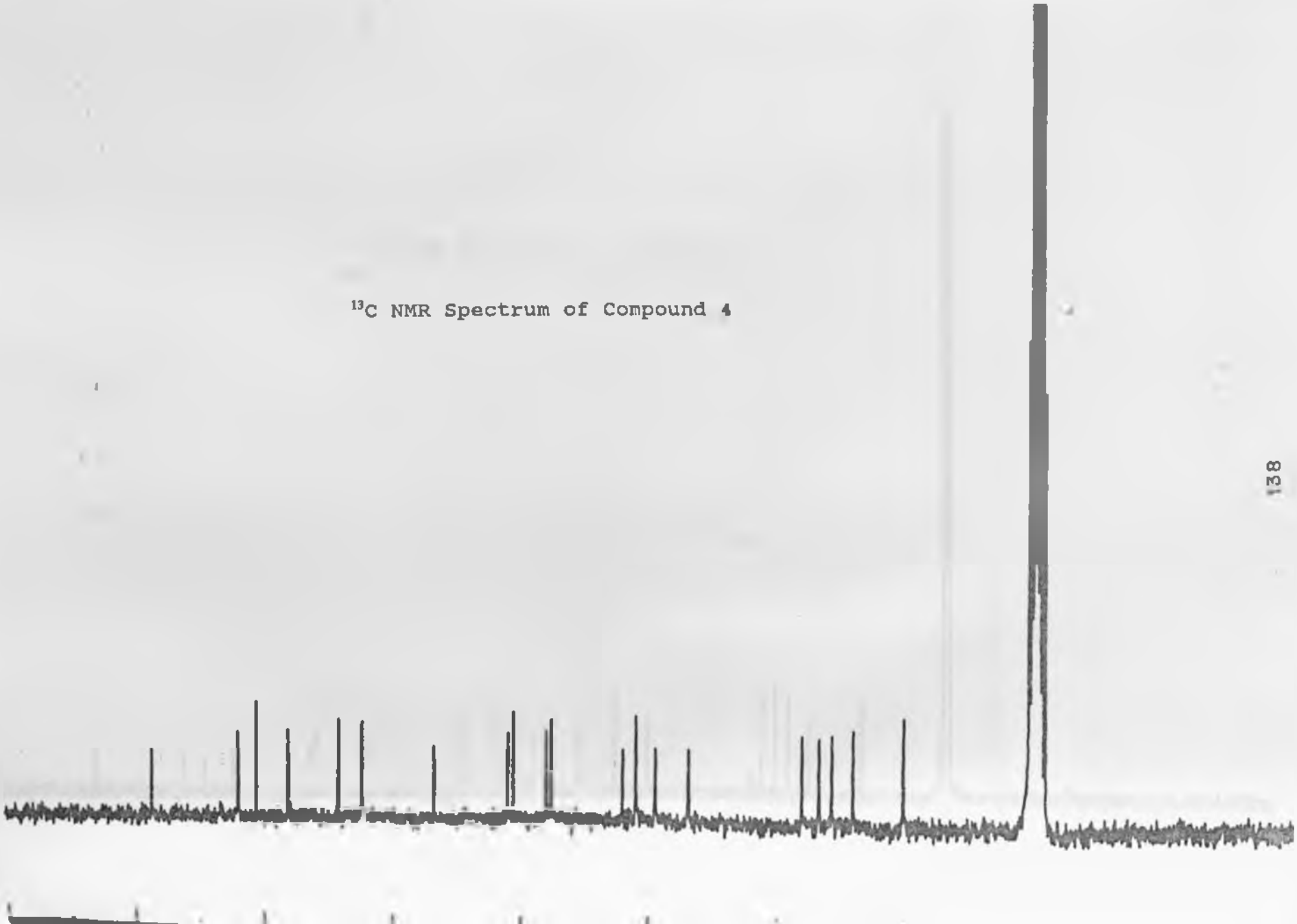
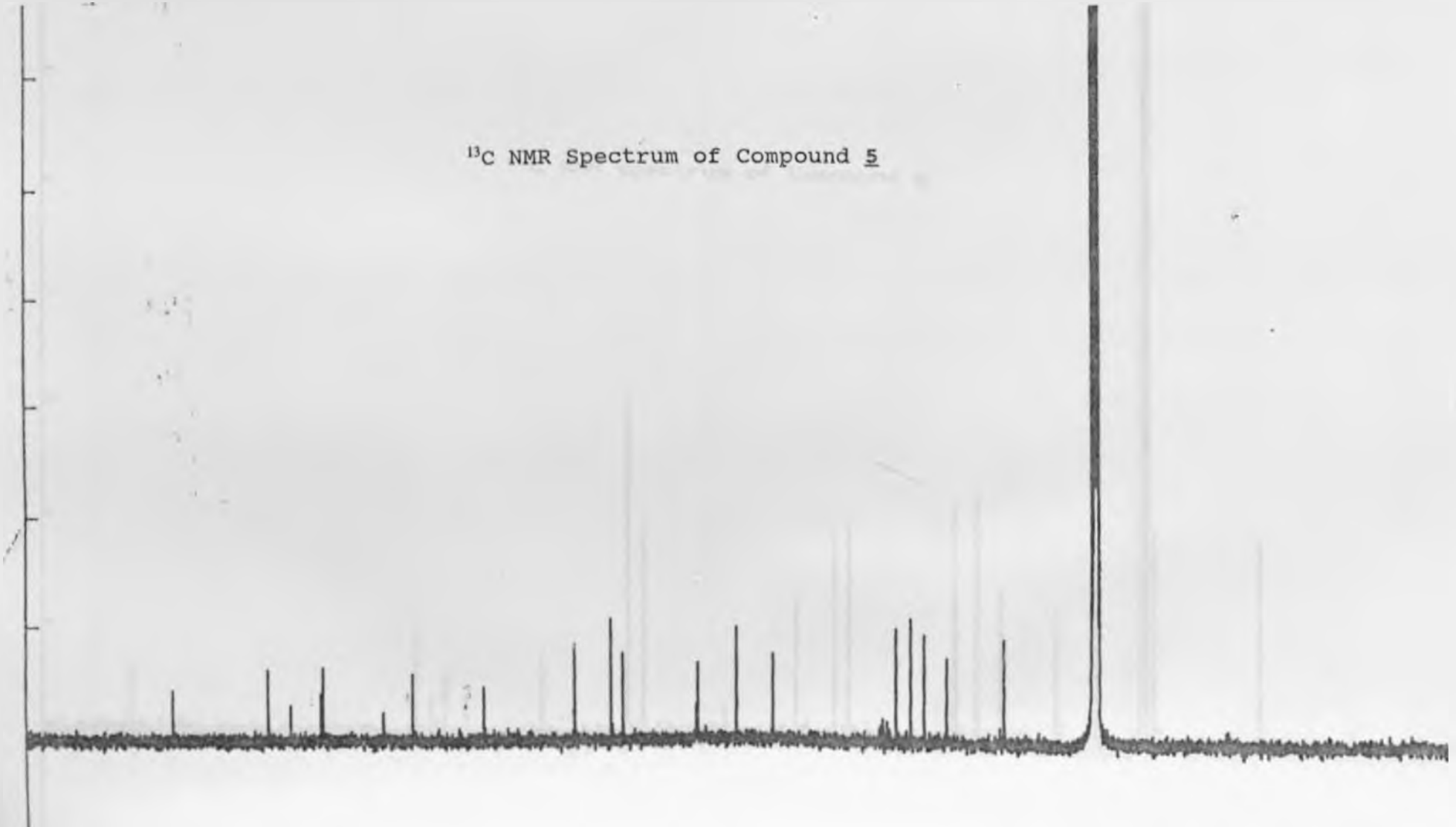
145.050
135.720
121.960
119.975
115.597
115.065
103.014
98.177
93.345
40.128
39.921
39.712
39.502
39.293
39.087
38.877

^{13}C NMR Spectrum of Compound 3

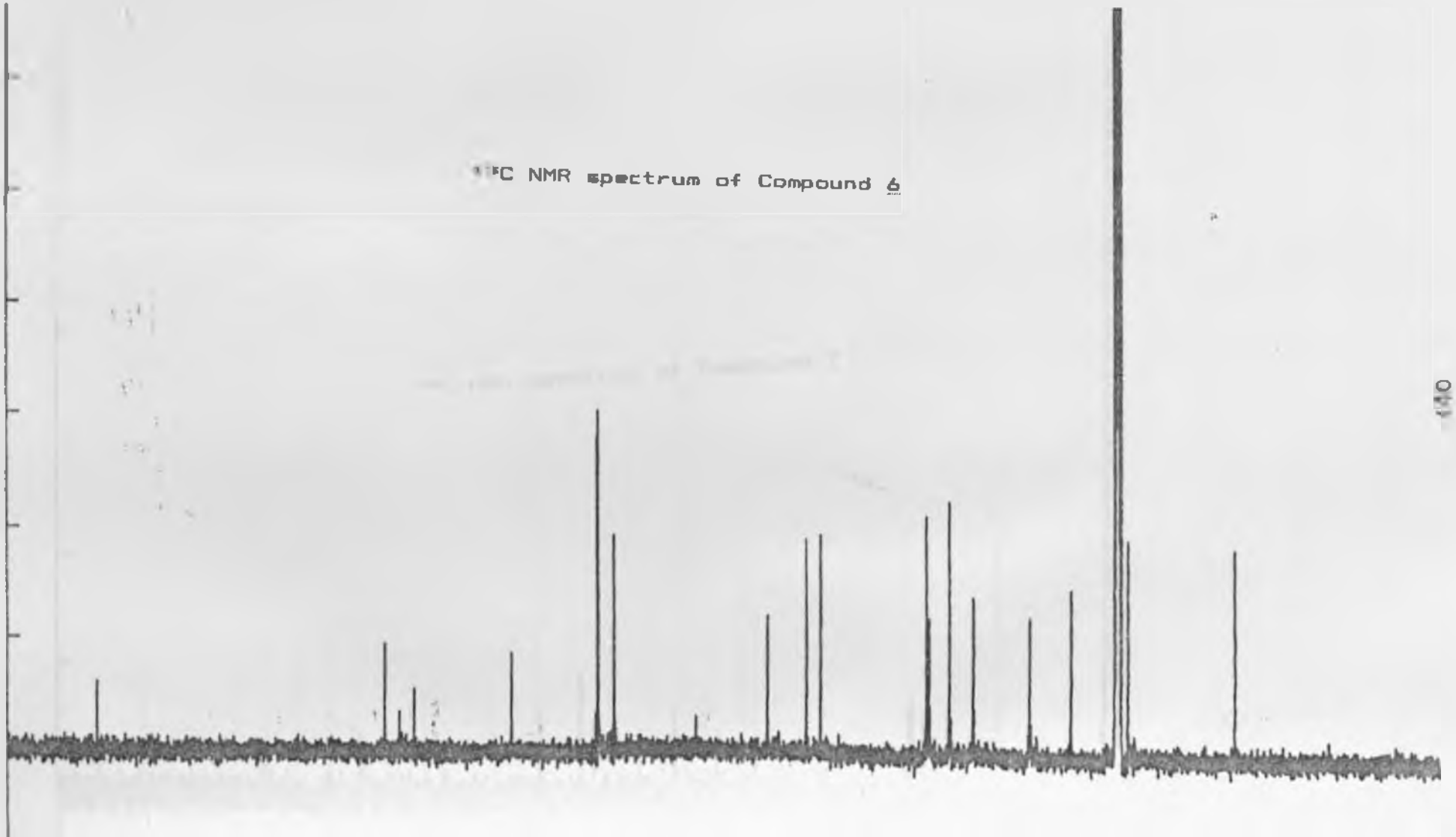
^{13}C NMR Spectrum of Compound 4

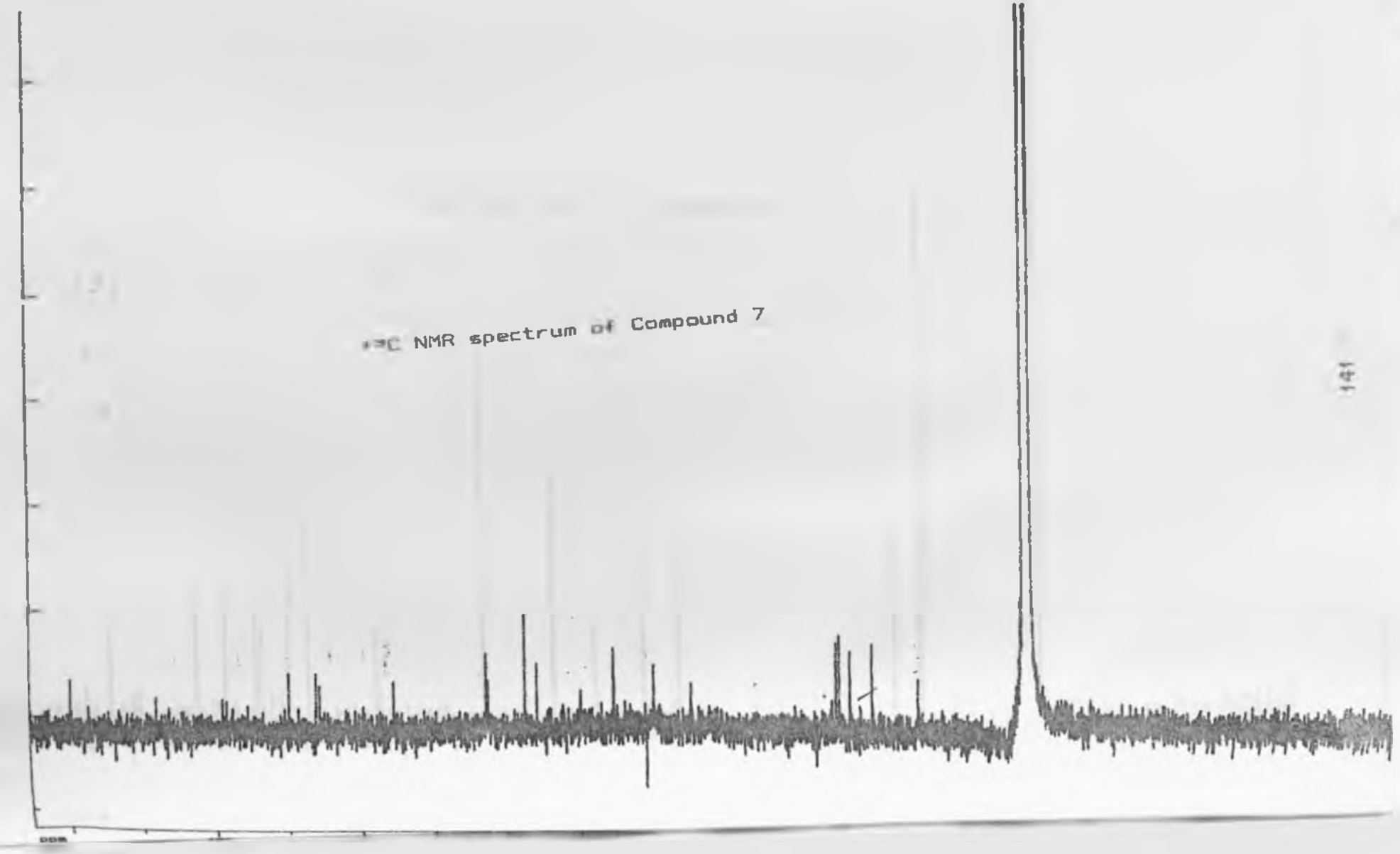


^{13}C NMR Spectrum of Compound 5



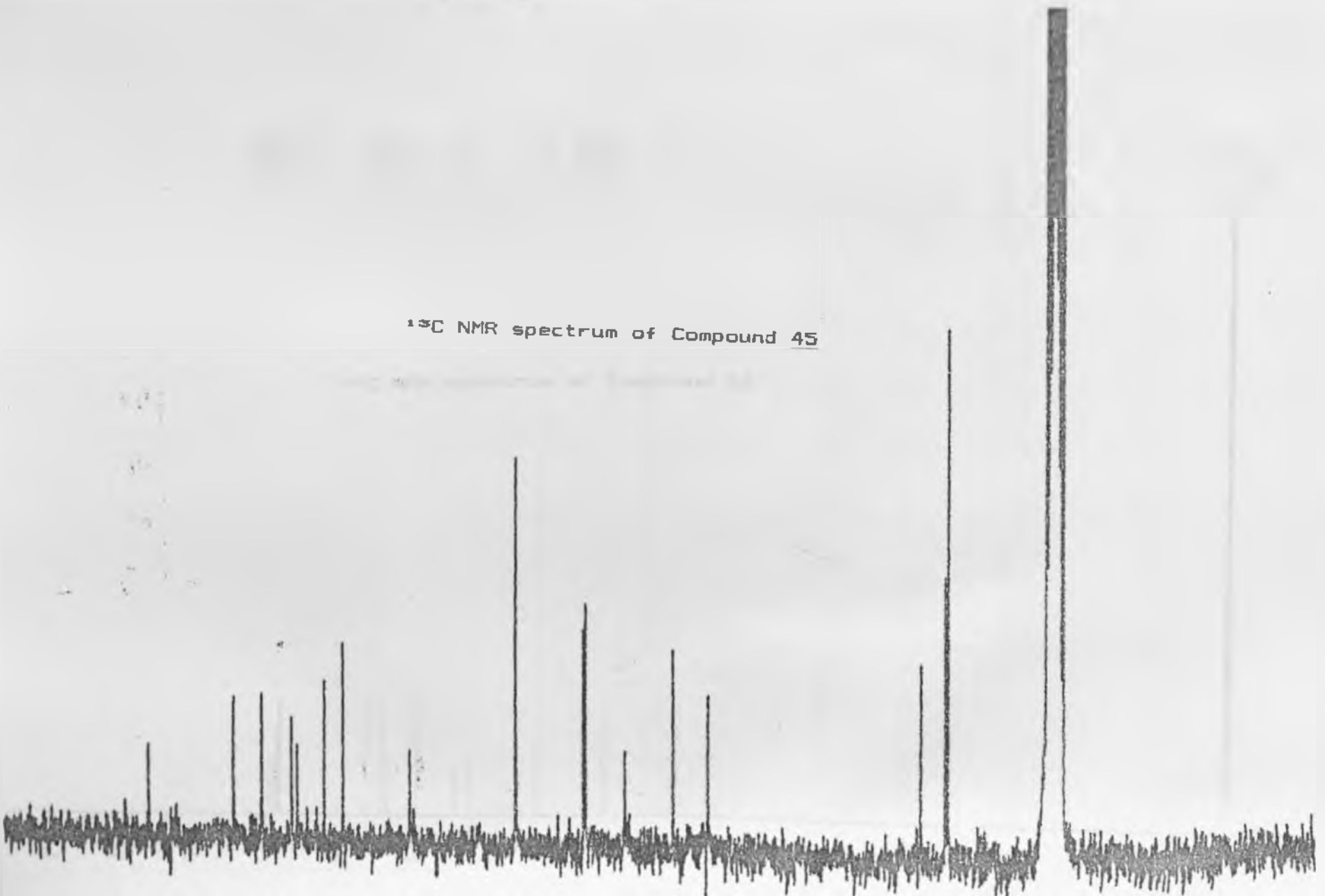
^{13}C NMR spectrum of Compound 6

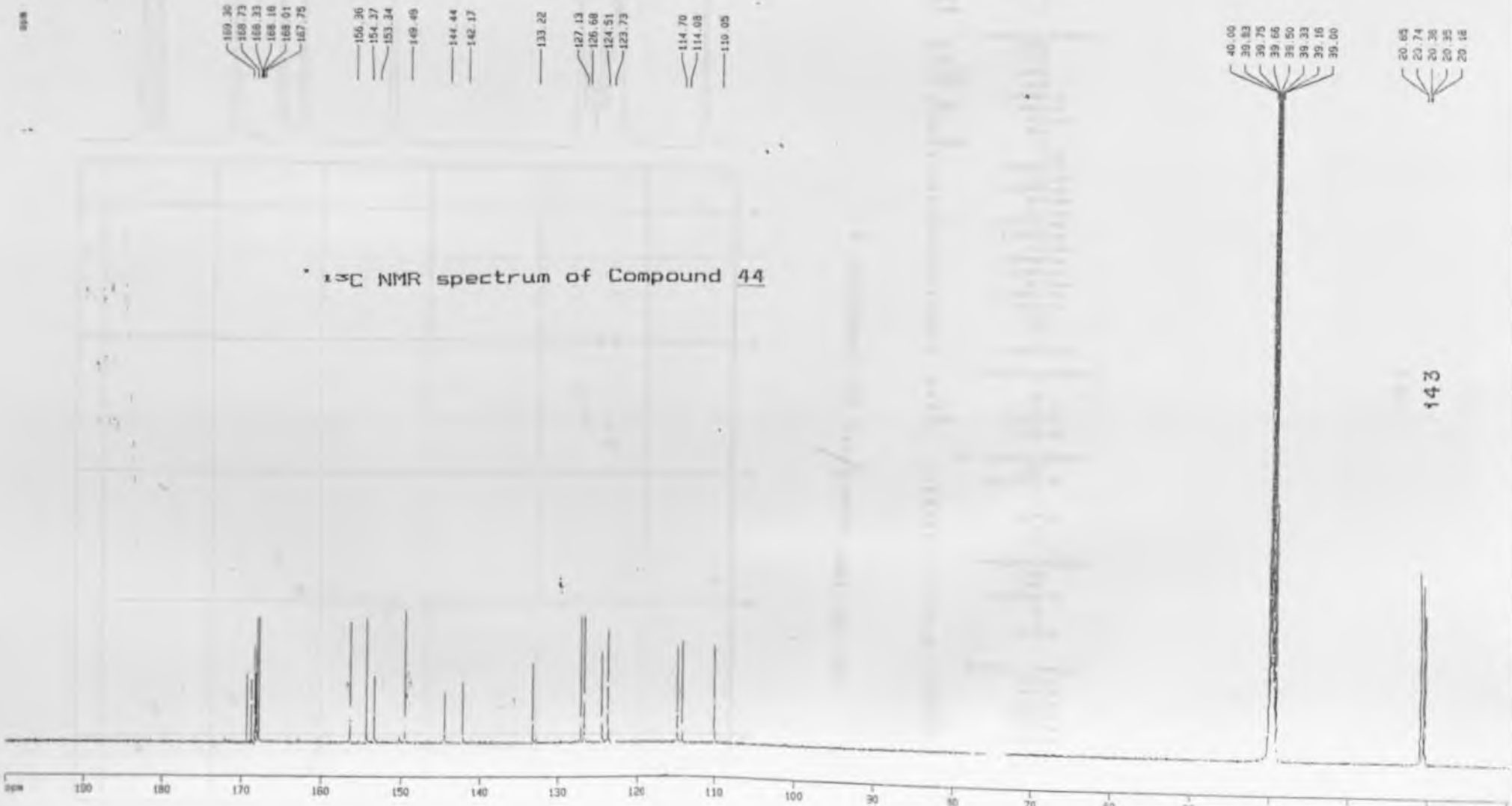




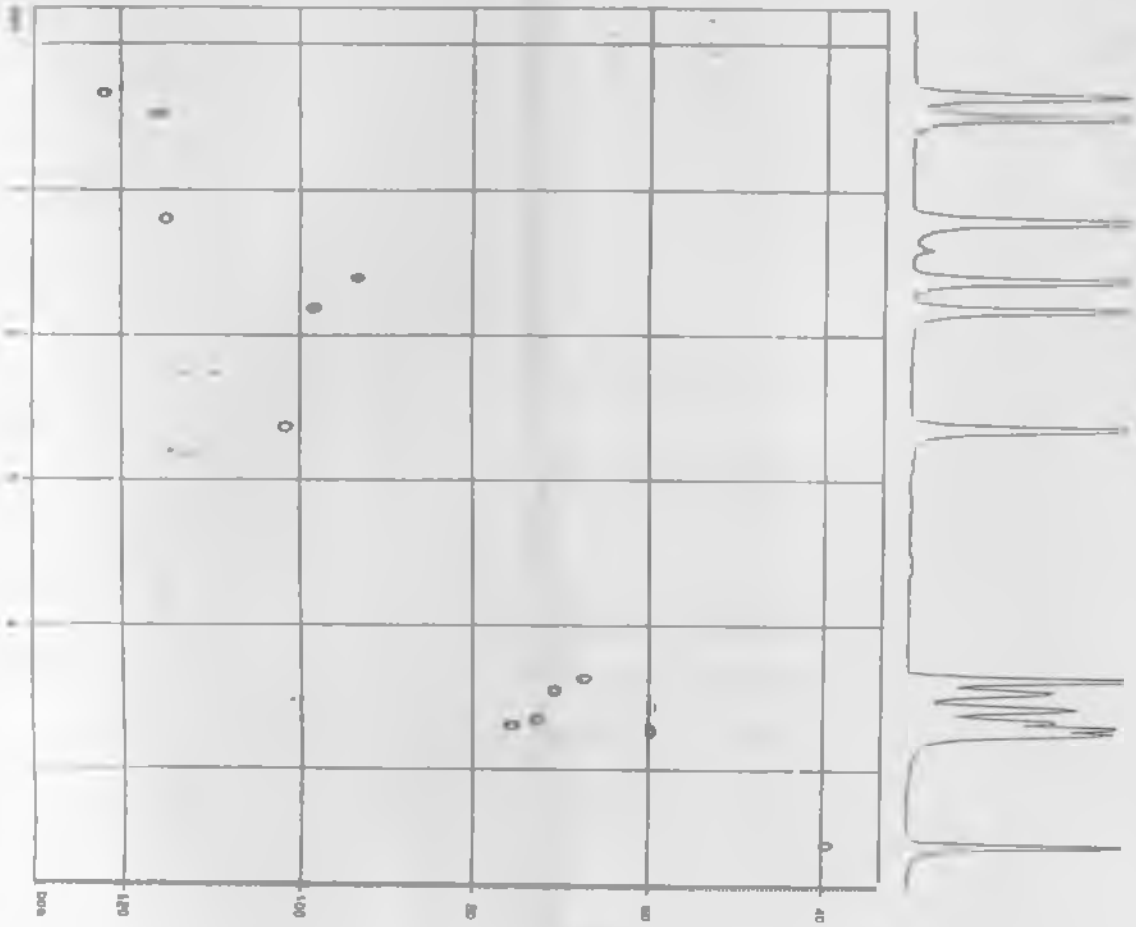
^{13}C NMR spectrum of Compound 7

^{13}C NMR spectrum of Compound 45



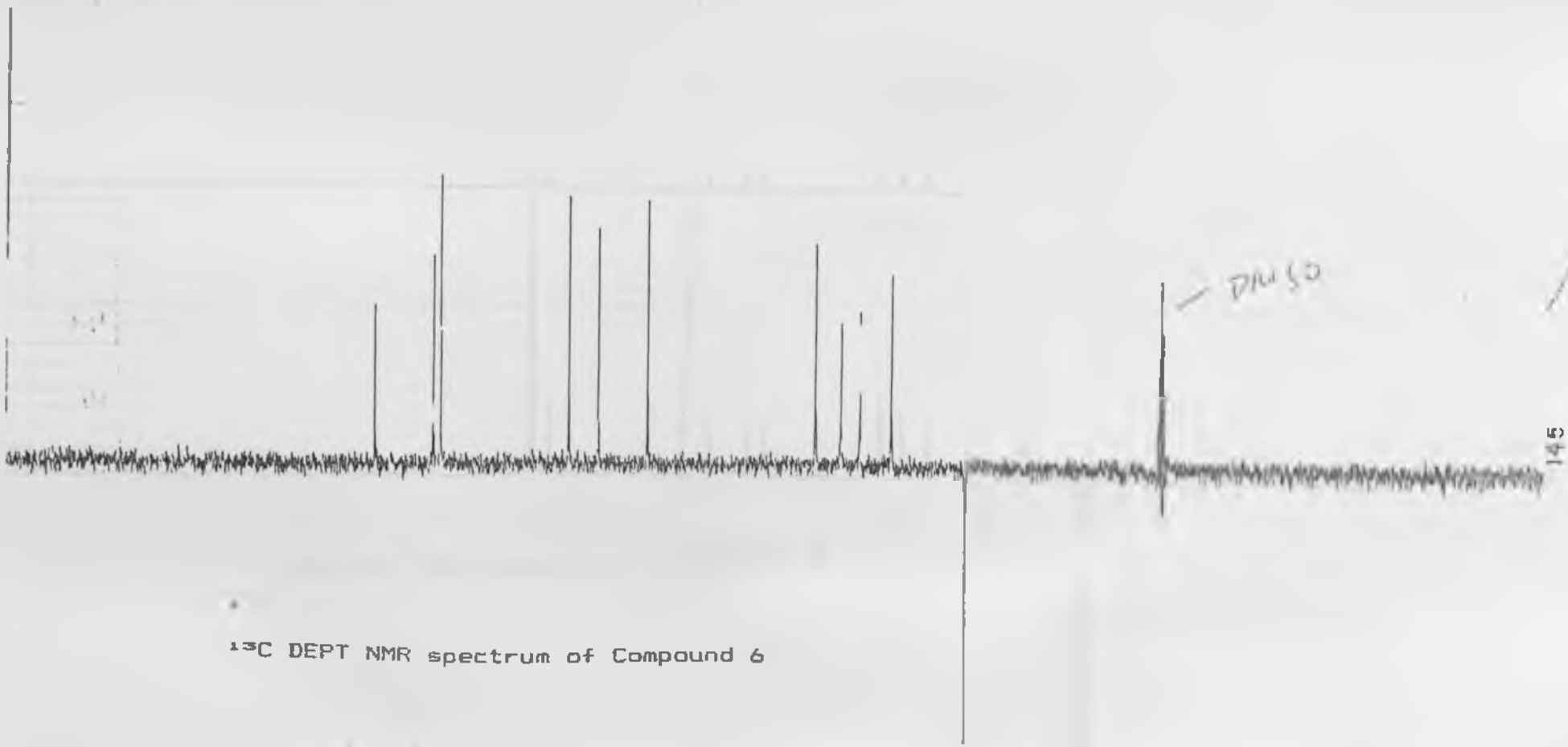


^{13}C NMR spectrum of Compound 44

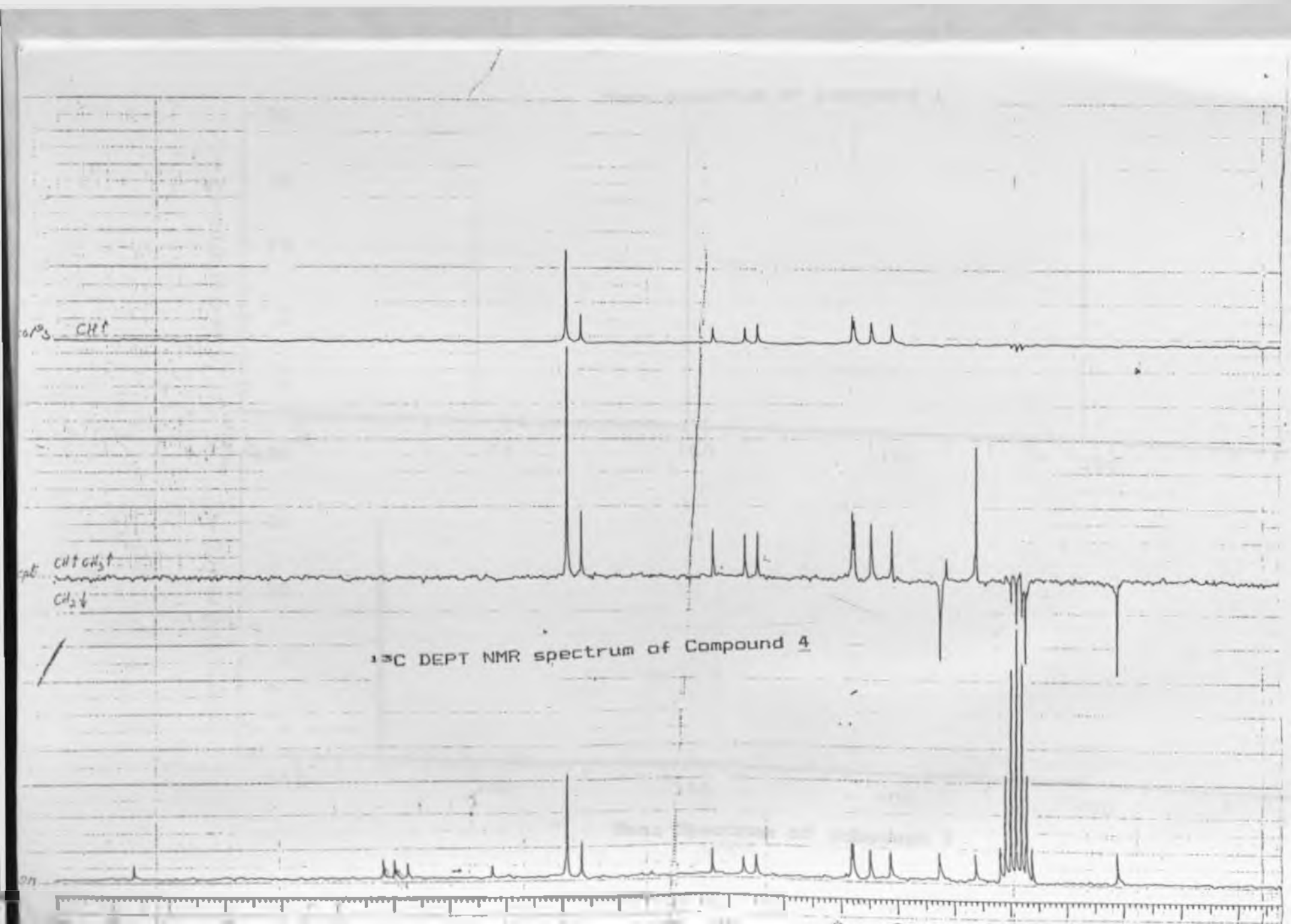


^{1}H-^{13}C NMR spectrum of Compound 4

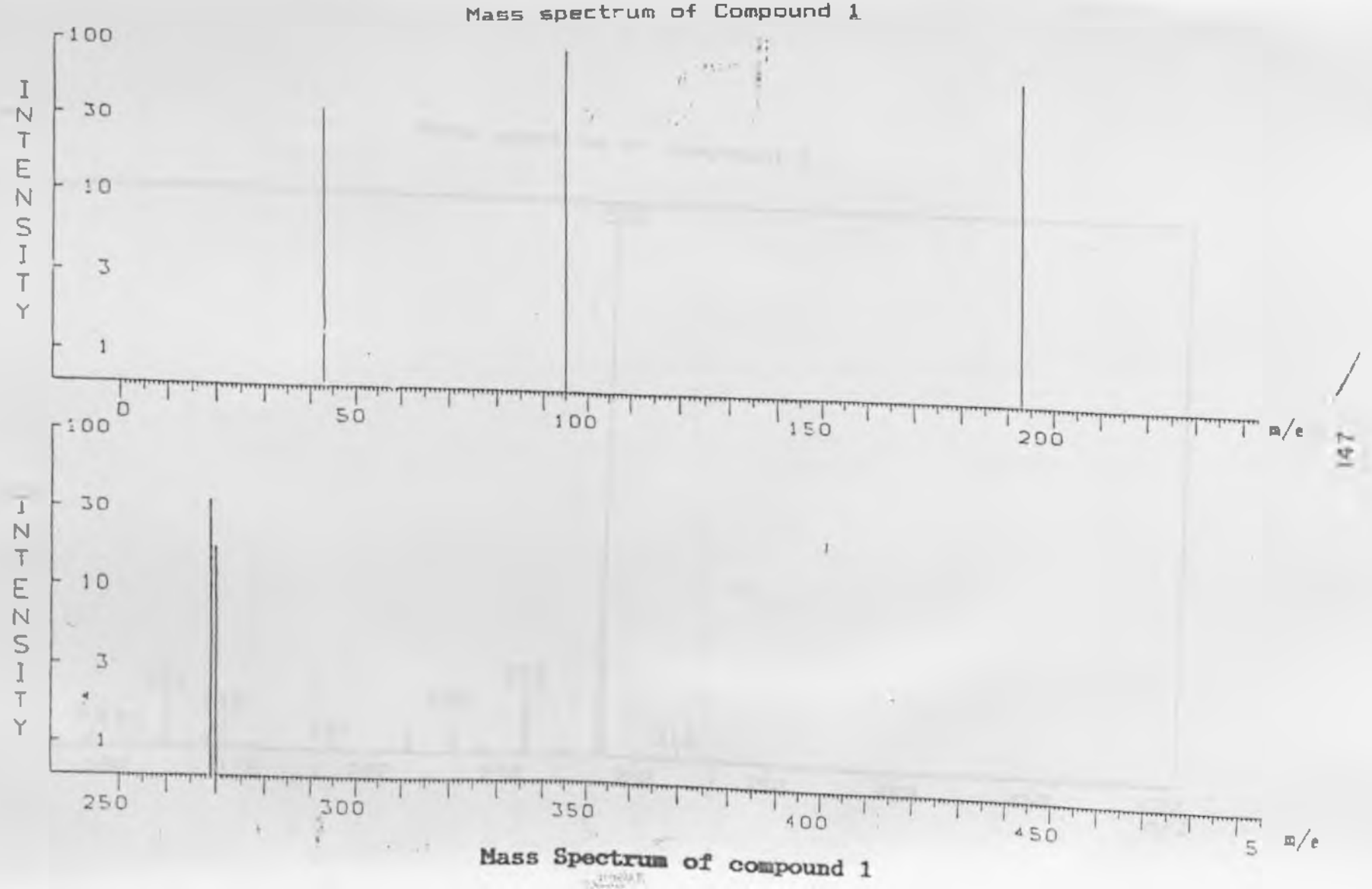
125 MHz



^{13}C DEPT NMR spectrum of Compound 6

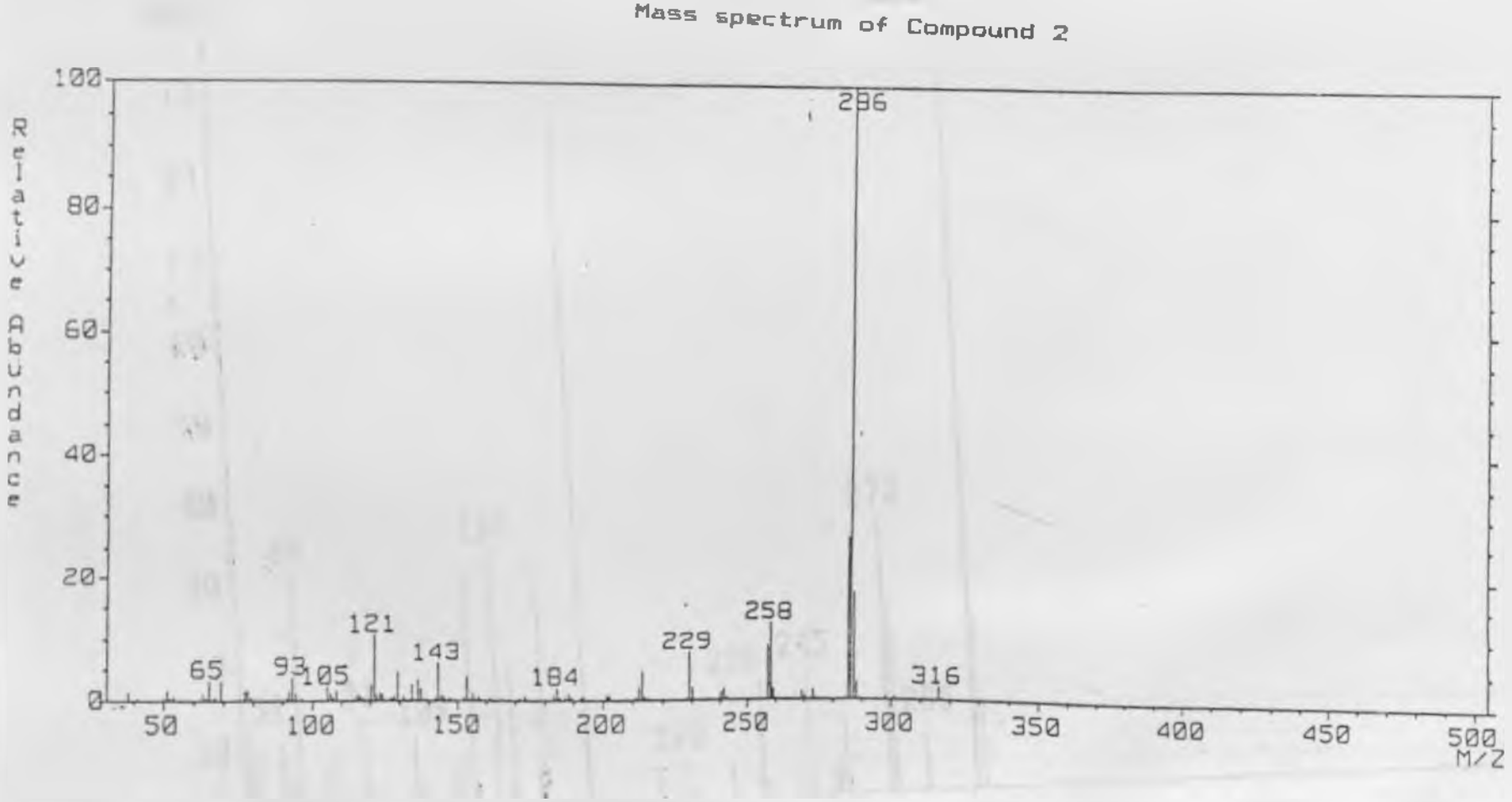


^{13}C DEPT NMR spectrum of Compound 4

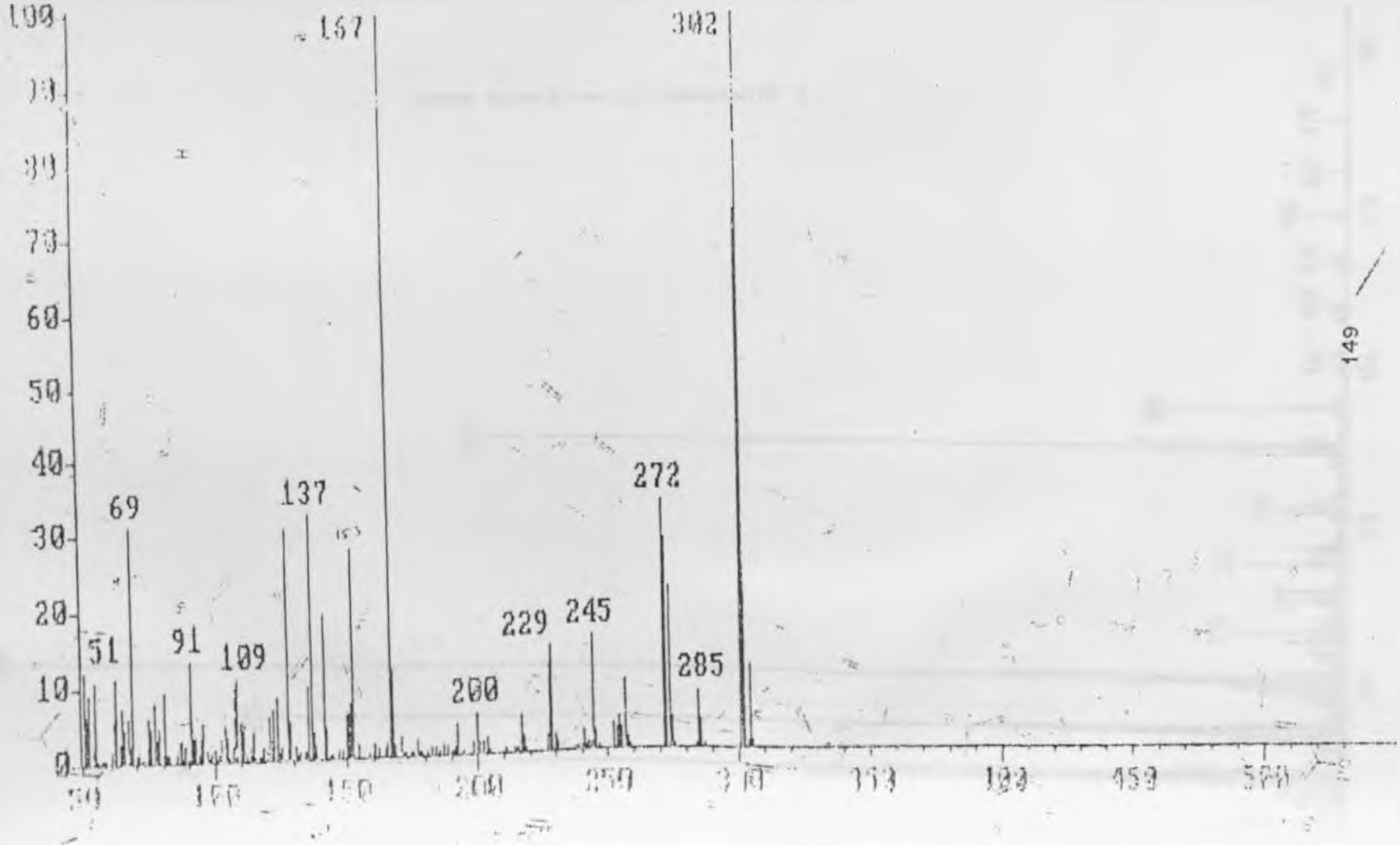


Mass Spectrum of compound 1

147


Mass spectrum of Compound 2
RELATIVE ABUNDANCE
100
80
60
40
20
0
50
100
150
200
250
300
350
400
450
500
M/Z
65
93
105
121
143
184
229
258
286
316

Mass spectrum of Compound 3

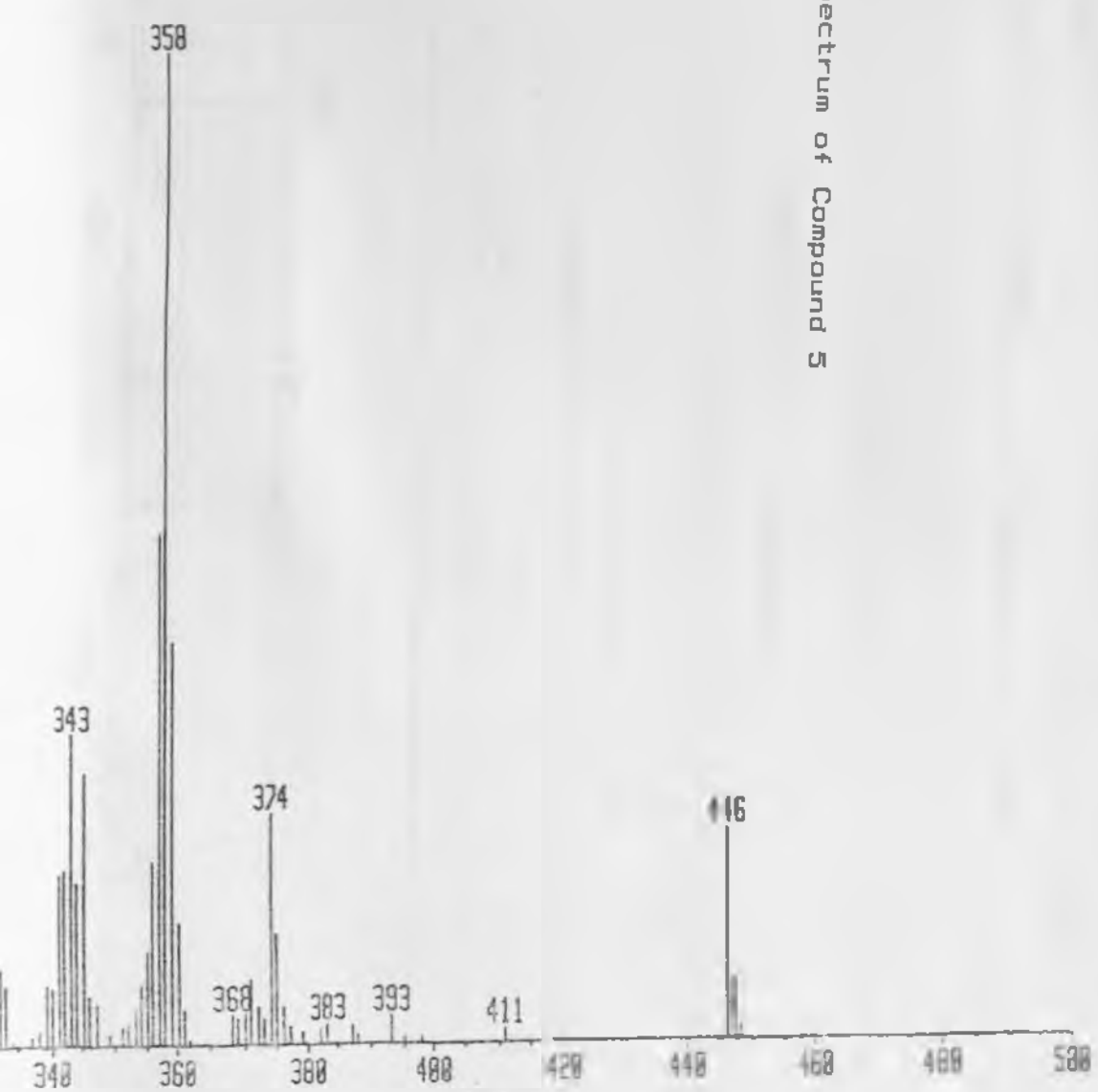


Mass spectrum of Compound 4

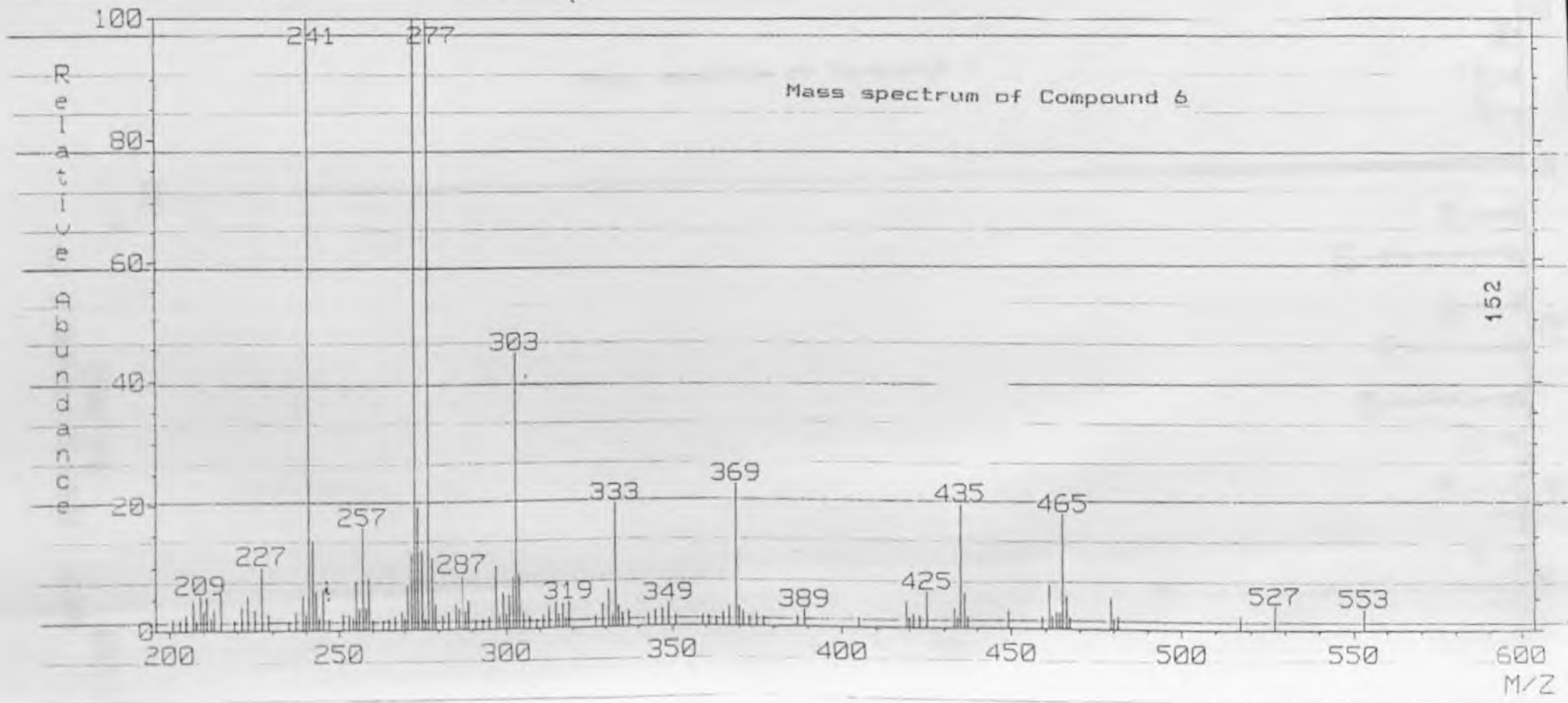
x100 Bgd=15 6-MAY-93 15:11+0:01:04 Cl+
=3452828 Acnt:JARVIS

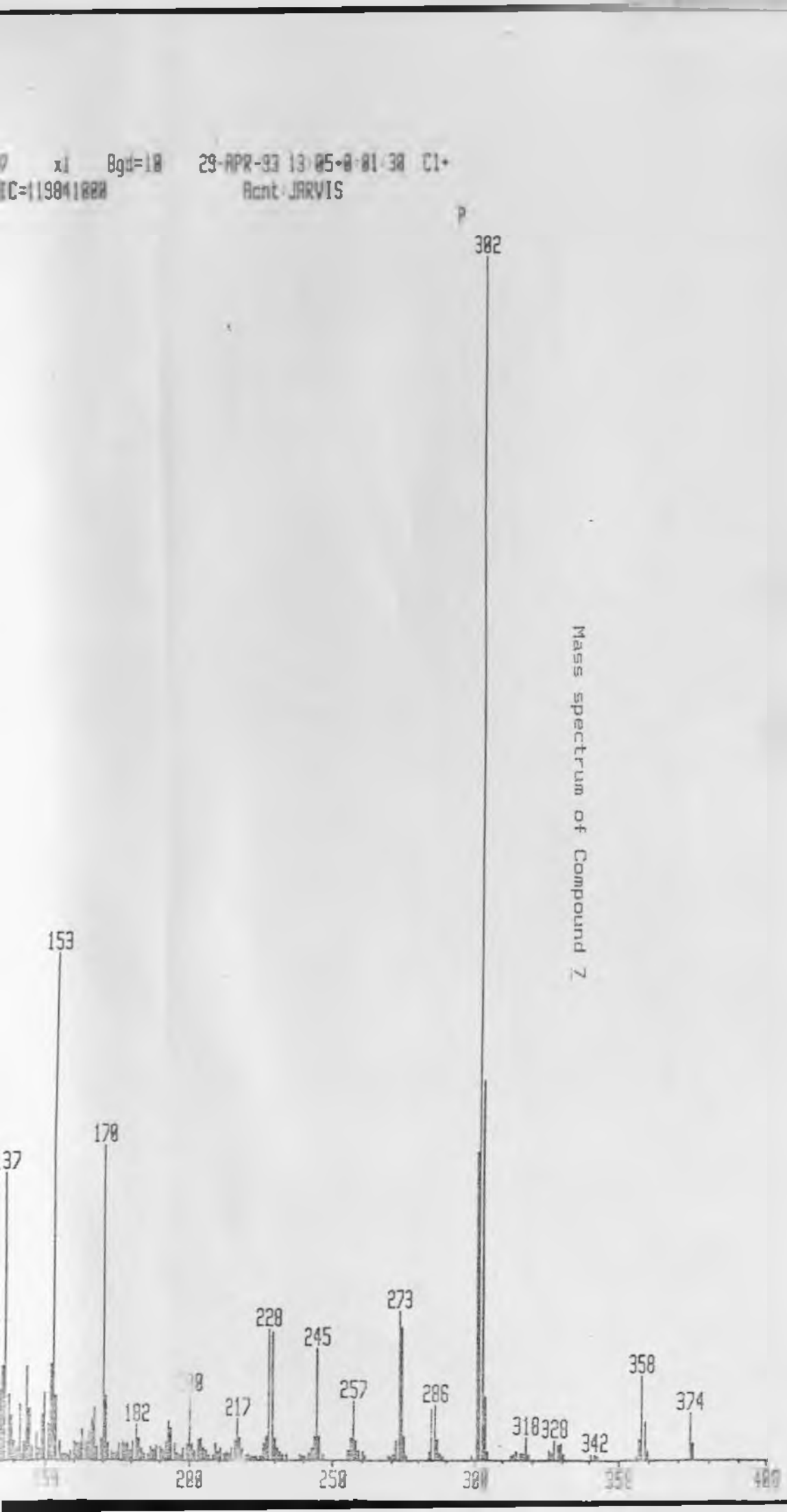
P

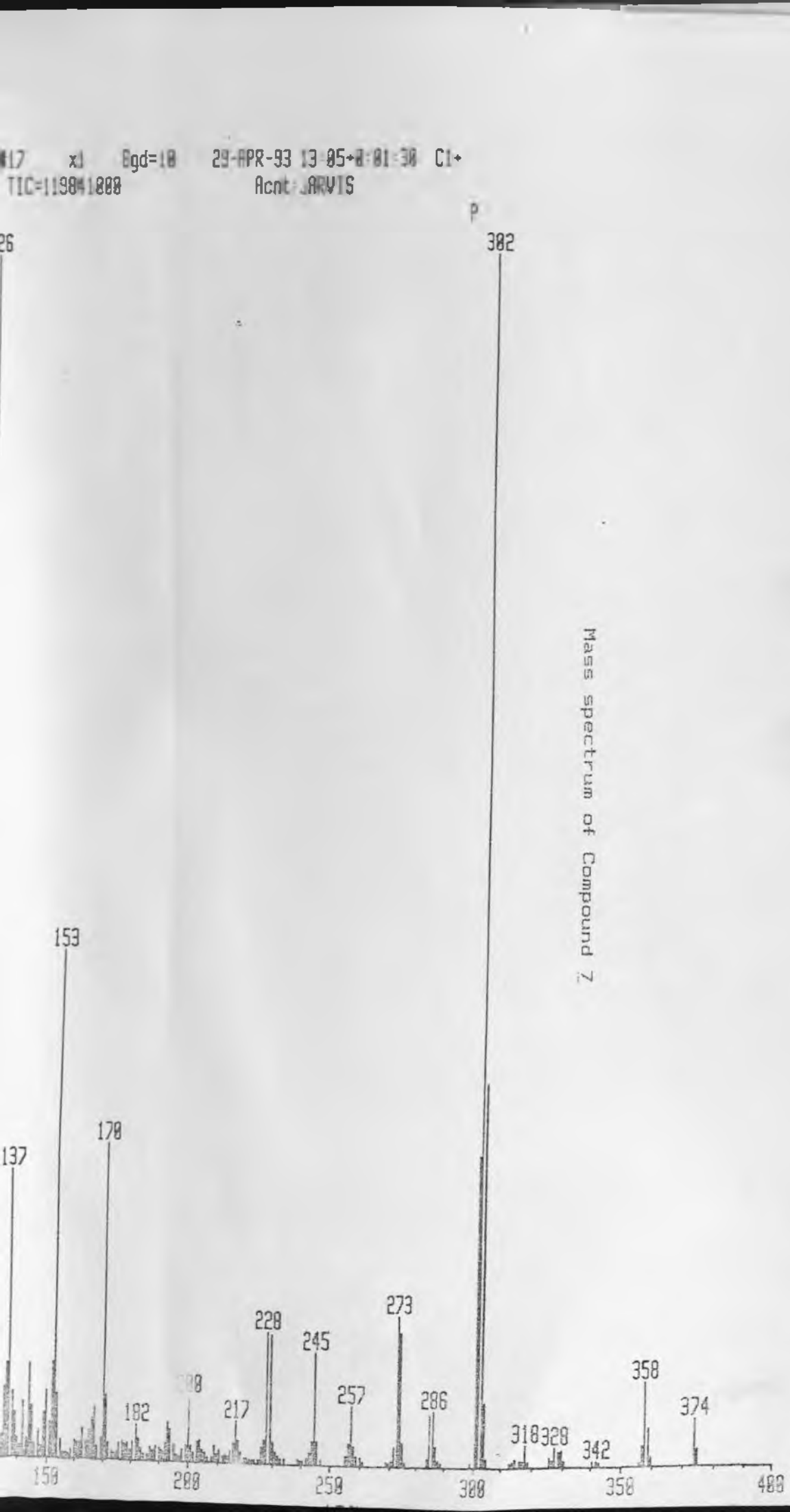
Mass spectrum of Compound 5



MASS SPECTRUM Data File: 92208 20-MAR-93 19:05
Sample:
RT 0'07" FAB(Pos.) GC 1.4c BP: m/z 277.0000, Int. 97.4116 Lv 1.50
Scan# (2)




xl Bgd=18 29-APR-93 13:05-0:01:30 CI+
Acnt:JARVIS
382
153
170
37
228
245
273
358
374
182
217
257
286
318
328
342
200
250
300
350
400
Mass spectrum of Compound 7


#17 x1 Bgd=10 29-APR-93 13:05→0:01:30 C1+
TIC=11984 1000 Acnt: JARVIS
P
382
153
170
137
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273
358
374
182
217
257
286
318
328
342
150
200
250
300
350
400
Mass spectrum of Compound 7