PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATIONS OF PLANTS FROM THE FAMILIES EUPHORBIACEAE AND THYMELAEACEAE

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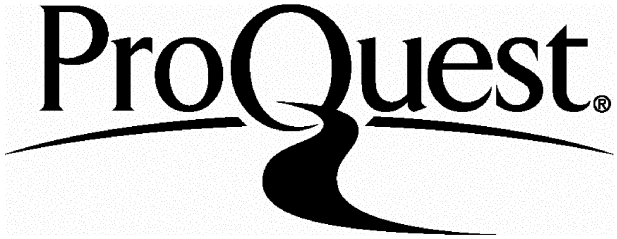

ABSTRACT

Extracts of forty samples from the plant families Euphorbiaceae and Thymelaeaceae were screened for the presence of biologically active diterpene esters. Plant extracts from *Gnidia kraussianus*, *Lasiosiphone kraussianus*, *Thymelaea hirsuta* and *Daphne blagayana* (Thymelaeaceae) as well as extracts from *Sapium indicum* (Euphorbiaceae) induced intense inflammation.

Crude extracts and methanol fractions from the Thymelaeaceae species inhibited TPA-induced differentiation of HL-60 cells. Ether fractions induced platelet aggregation of the human blood at concentrations of 10-50 μg/ml.

Biologically active diterpene esters were isolated and identified. These compounds were purified by multistage fractionation procedures. These involved liquid-liquid partition, centrifugal liquid chromatography, column chromatography, preparative adsor ption and partition liquid chromatography. Final purification was carried out using HPLC.

A series of one and two dimension NMR experiments were carried out to facilitate the structure elucidation of the isolated diterpenes. Mass spectrometry and infrared were used to confirm the chemical structures. Both thin layer and normal phase HPLC proved to be valuable in analysing and purifying small quantities of daphnanes and tiglianes.

Thymelaeatoxin-A and resiniferonol-9,13,14-orthophenyl acetate were isolated from the twigs and leaves of *Thymelaea hirsuta*. The intense irritant effect of *Daphne blagayana* was partially due to the presence of mezerein.

The novel sapintoxin-E, 12-O- (2-methylanminobenzoyl)-4,20-dideoxy phorbol-13-acetate, in addition to sapintoxin-A and sapintoxin-D were isolated from the latex of *Sapium indicum*. The twigs and leaves of *Sapium insigne* yielded the semi-synthetic 12-O-(2-methyl aminobenzoyl)-4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate.

The established technique of irritancy testing was used to assess the potency of selected diterpenes. Compared to tiglianes irritancy induced by daphnanes was rapid and

short lasting. Epidermal hyperplasia induced by mezerein was comparable to that induced by TPA.

In vitro, the isolated compounds were evaluated for their platelet aggregating activity and for their ability to induce differentiation of HL-60 cells. At doses of 1nM and greater, TPA was the most potent diterpene ester in both *in vivo* and *in vitro* assays.

For the detection of differentiation of HL-60 cells, a rapid and effective microdilution *in vitro* assay was developed. This technique was based on quantitation of absorbence of protein-bound eosin dye using a single beam minireader. Irritant daphnanes and tiglianes induced the HL-60 cells to differentiate at doses of 20 nM and greater. Differentiation effect of TPA obtained from the new assay was closely related to the established methods.

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List of Abbreviations

AD_{50}	Dose inducing 50% platelet aggregation
ADP	Adenosine diphosphate
BP	Benzopyrene
c-AMP	Cyclic adenosine monophosphate
$CDCl_3$	Deuterated chloroform
CIMS	Chemical ionization mass spectrometry
CLC	Centrifugal liquid chromatography
COSY	Correlated spectroscopy
D.	*Daphne*
2,5-DHB	2,5-Dihydroxybenzoic acid
D_2O	Deuterium oxide
^{13}C-NMR	Carbon nuclear magnetic resonance
2,5-DHB	
DCC	Droplet counter current
DEPT	Distortionless enhancement by polarization transfer
DNA	Deoxyribose nucleic acid
DOPP	12-Deoxyphorbol-13-phenylacetate
DOPPA	12-Deoxyphorbol-13-phenylacetate-20-acetate
E.	*Euphorbia*
EIMS	Electron impact mass spectrometry
FABMS	Fast atom bombardment mass spectrometry
G.	*Gnidia*
^{1}H-NMR	Proton nuclear magnetic resonance
HPLC	High performance liquid chromatography
ID_{50}	Dose inducing irritancy in 50% of the test animals
Ins-1,4,5-P_3	Inositol-1,4,5-triphosphate
IR	Infrared
M/Z	Mass/charge
M^+	Molecular ion

MEZ	Mezerein = 12-O-(5`-phenyl-2,4-pentadienoate)-5,12,dihydroxy-6α,7α- epoxyresiniferonol-9,13,14-orthobenzoate
mM	Millimolar
MNOBA	3-Nitrobenzyl alcohol
MS	Mass spectrometry
nM	Nanomolar
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser and exchange spectroscopy
Ns	Nucleotides
OD	Optical density
PDA	Phorbol-13,20-diacetate
PDBA	Phorbol-12,20-dibenzyl-13-acetate
PDBu	Phorbol-12,13-dibutyrate
PDE	Phosphodiesterase
PDEH	Phorbol-12,13-diester-12-hydrase
PH	Phorbol
PHDA	Phorbol-12,20-diacetate
PKC	Protein kinase C
pM	Picomolar
PMA	Phorbol-12-myristate-13-acetate
ppm	Parts per million
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PTA	Phorbol-12,13,20-triacetate
PTB	Phorbol-12,13-20-tributyrate
PtdIns-4,5-P_2	Phosphatidyl inositol-4,5-biphosphate
PTLC	Preparative thin layer chromatography
RLCC	Rotational counter current
RNA	Ribonucleic acid
ROPA	Resiniferonol-9,13,14-orthophenylacetate
RP-HPLC	Reversed phase high performance liquid chromatography

RX	Resiniferatoxin = resiniferonol-9,13,14-orthophenylacetate-20-O-(m-methoxy-m`-hydroxy-phenyl acetate)
S.	*Sapium*
SAP-A	Sapintoxin-A = 12-O-(2-methylaminobenzoyl)-4-deoxyphorbol-13-acetate
SAP-AC	4α-Sapintoxin-B-5,20-diacetate = 12-O-(2-methylaminobenzoyl)-4α-5-hydroxyphorbol-5,13,20-triacetate
SAP-C	Sapintoxin-C = 12-O-(2-methylaminobenzoyl)-4,20-dideoxy-5-hydroxyphorbol-13-acetate
SAP-D	Sapintoxin-D = 12-O-(2-methylaminobenzoyl)-phorbol-13-acetate
SAP-E	Sapintoxin-E = 12-O-(2-methylaminobenzoyl)-4,20-dideoxyphorbol-20-methyl-13-acetate
SD	Standard diviation
t-Peak	Time at which maximum irritant effect was evident
THY-A	Thymelaeatoxin-A = 12-O-(cinnamoyl)-5,12-dihydroxy-6α,7α-epoxy-resiniferonol-9,13,14-orthobenzoate
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TPA	12-O-Tetradecanoylphorbol-13-acetate
UV	Ultraviolet
µM	Micromolar

CHAPTER ONE

INTRODUCTION

How near are we to finding a cure for cancer? It is a question scientists in the field of cancer research are frequently asked. It also illustrates the common misconception that cancer is a single disease caused by a single factor. The term cancer covers a wide range of diseases affecting any organ of the body. It is characterised by uncontrolled cell divisions forming abnormal masses. Scientists believe that the majority of human cancers may have been caused by exposure to more than one carcinogenic risk factor. However, it is more likely that a number of human cancers are due to exposure to a small quantity of a carcinogen and repeated exposure to a tumour promoter. While a carcinogen is a substance which causes cancer, a tumour promoter is a substance which is not carcinogenic but one which accelerates cancer growth. Several compounds are known to be tumour promoting agents e.g. phenol, tween and anthralin. However, phorbol esters are the most potent and the most widely studied agents.

Phorbol esters is a term generally used to describe a group of highly toxic plant constituents. These compounds occur naturally in the plant families Euphorbiaceae and Thymelaeaceae in the form of polyhydroxylated diterpene esters. These families are the only reported natural sources for such compounds.

Cultivation of these plant families in home gardens, occupational exposure of employees, consumption of food products derived from a plant source as well as the use of these plants in alternative medicine have all exposed human to these naturally occurring tumour promoters.

1.1 Botanical Considerations

The complexity of relationship between the families Euphorbiaceae and Thymelaeaceae raised considerable debate among taxonomists. Phylogenic, phytochemical and embryologic studies concluded that each is a well defined family (Webster 1994, Borris *et al* 1988, Kapil and Bahtnager 1994, Seigler 1994).

1.1.1 The Plant Family Euphorbiaceae

The plant family Euphorbiaceae, commonly known as the spurge family, is the sixth largest family of flowering plants. It consists of 300 genera and almost 7000 species. Members of this family are widely distributed throughout the world in the form of desert succulents, trees or less commonly herbaceous types. Many species contain irritant or piscicidal substances (Trease and Evans 1978).

The morphological diversity of the members of the Euphorbiaceae family created classification difficulties for both botanists and taxonomists. According to the revised classification by Webster, this family is subdivided into five subfamilies; Phyllanthoideae, Oldfieldioideae, Acalyphoideae, Crotonoideae and Euphorbioideae (Webster 1975, 1987).

Phorbol esters have been isolated from different genera including *Aleurites, Balliospermum, Croton, Euphorbia, Excoecaria, Hippomane, Hura, Jatropha, Micrandra, Sapium, Ostodes, Stillingia and Synadeium* (Evans and Schmidt 1980, Kinghorn 1985). These genera are distributed in the subfamilies Crotonideae and Euphorbiodeae. The subfamilies Phyllanthoideae and Acalyphoideae were reported to be sources of irritants. However, their reported irritant effect was to be less dramatic than that associated with Crotonoideae and Euphorbioideae. Whilst the subfamily Oldfieldioideae appears to be devoid of such activity, further phytochemical research of the family Euphorbiaceae would provide useful data for further chemosystematic consideration.

1.1.2 The Plant Family Thymelaeaceae

The family Thymelaeaceae, or daphne family, consists of 90 genera and 500 species (Trease and Evans 1978). Members of this family are mainly mostly temperate and tropical shrubs. Some are poisonous and contain vesicant resins.

This family has been divided into the subfamilies Gonystyloideae, Aquilarioideae, Gilgiodaphnoideae and Thymelaeoideae. Phytochemical investigations of the genera *Gnidia, Pimelea, Daphne and Thymelaea* revealed that the toxic phorbol esters are widely distributed in the subfamily Thymelaeoideae (Evans and Taylor 1983, Schmidt

1986a). Further, *Gnidia* species yielded a number of anti-leukaemic compounds (Kasai *et al* 1981, Kupchan 1975 and 1976). In Britain, this family is represented by two species of *Daphne*, *D. laureola* and *D. mezereum* (Rendle 1938).

Taxonomists have proposed that the families Euphorbiaceae and Thymelaeaceae are closely related because chemosystematic studies suggested that their toxic constituents are derived from the common precursor, (-)-casbene (Adolf and Hecker 1977, Schmidt 1986b). Phytochemical studies revealed that the toxic diterpenes are only distributed in these two plant families.

1.1.3 The Use of Plant Families Euphorbiaceae and Thymelaeaceae in Traditional Medicine

For many centuries the curing ability of plants from these families has been recognised by many ancient civilisations. For example, seeds of the castor oil plant dating back to 4000 BC have been found in ancient Egyptian tombs but it is thought that its usage dates even further (Bianchini and Corbetta 1977).

Traditional Chinese herbalists have used many species of these two families in the treatment of different ailments. Several *Daphne* species (family Thymelaeaceae) are used in Chinese herbal medicine for circulatory system diseases, analgesic, anti-inflammatory, expectorant, antimalarial, abortifacient, anti-leukaemic and also to eradicate skin infections (Huang 1993). Plants from the genera *Euphorbia* (family Euphorbiaceae) are used as cathartic, purgative, diuretic, cough remedy, anti-asthmatic, spasmolytic and dysentery and as gastrointestinal infections treatment. The juice of the plant *Mercurialis annua* (family Euphorbiaceae) is used to remove warts (Bianchini and Corbetta 1977).

In India and Pakistan, the traditional and medicinal system commonly known as Unani as prescribed by the traditional healer known as Hakim, is the primary source of medical treatment. This system have originated in Greece and was influenced by Arab and other medicine. The materia medica of Unani contains few plants from the families Euphorbiaceae and Thymelaeaceae. The whole plant of *Chrozophora prostrata* (family Euphorbiaceae) is used for the treatment of leprosy while the fruit of *Mallotus*

phillippensis is used as cathartic and anthelmintic. Seeds of *Ricinus communis* and juice of some *Euphorbia* species are used as purgatives. The resin of *Aquilaria agallocha* (family Thymelaeaceae) is used as stimulant (Trease and Evans 1983).

In Africa and Middle America several species of the genera *Acalypha* are used to treat eye conditions, chest pains, leprosy, rheumatic pain, gastrointestinal infections and are also used as snake-bite remedy. The bark, seeds and oil of different species of *Croton* are used as a purgative, a malaria remedy and in syphilis treatment. The latex of *Euphorbia*, which is a characteristic of this genera, is used to treat warts, gonorrhoea and dysentery (Oliver-Bever 1986, Watt and Breyer 1962, Morton 1981). In addition, the oil is used as purgative and tumour treatment while the root or the powdered leaf of many *Euphorbia* species are used for fever, toothache and diabetes treatment. Wounds and tuberculosis are treated using either the juice or the sap of *Jatropha* species. Plants from other genera including *Phyllanthus, Sapium and Alchornia* are the source of many remedies used as mouth washes, emetics and dressing for abscesses (Watt and Breyer 1962, Morton 1981, Oliver-Bever 1986).

Plants of the family Thymelaeaceae, have also been used as the main sources of herbal remedies in India, Middle America and Africa. Infusions of plants from *Gnidia* are used to relieve asthma, cough and influenza while smoke of the burning root is used to treat eye diseases, fever and bad dreams. The flowers of these plants are used as a purgative and abortifacient. Remedies for toothache, backache, sore throat, snake bite, muscle and bone complaints are prepared using either the root or the whole herb of *Lasiosiphon* species (Watt and Breyer 1962, Oliver-Bever 1986, Morton 1981).

In Europe, Croton oil was used as purgative and counter-irritant to the skin. It was not until 1949 that this oil was considered to be toxic for humans and was eventually removed from the British Pharmaceutical Codex. The British Herbal Pharmacopoeia includes monographs for herbal remedies based on plants from the family Euphorbiaceae. These include the use of the entire herb of *Euphorbia pilulifera* and *E. hirta* as anti-asthmatic, expectorant and spasmolytic. In addition, the root of *Stillingia sylvatica*, known as Queen's delight, is used as astringent, cathartic, expectorant, spasmolytic and as dermatological agent (British Herbal Pharmacopoeia part I 1976 and part II 1979).

Several members of Euphorbiaceae and Thymelaeaceae are used in homeopathy, health foods, herbal and other plant based medicines. Whilst the medicinal value of plants has been appreciated for thousands of years, studies to evaluate their therapeutic and toxic properties should not be overlooked.

1.1.4 The Dietary Value of the Euphorbiaceae

Over the centuries the widely used cassava and tapioca have been produced from plants of the family Euphorbiaceae. Next to the sweet potato the cassava, which is the flour produced from the roots of *Manihot esculenta* Crantz., is the most important root type crop in the tropics. Cassava bread has a high nutrition value and replaces wheat bread in India, West Africa and Middle America. It contains 77-88% starch and less than 1% protein. Tapioca is a commercial product consumed throughout the world (Rizk 1987 and Morton 1981). In Middle America the new shoots and young leaves of *Acalypha wilkesiana*, locally known as red hedge, are cooked and eaten. Locally known as chickweed, *Chamaesyce hirta and Euphorbia hirta* L., are used as nutrition during famine in India (Morton 1981).

Noor honey contains the nectar of *Euphorbia* species particularly *Euphorbia ingens*, *E. Coopri* and *E. Virosa*. It causes a burning sensation in both mouth and throat (Watt and Breyer 1962). The dried bark of *Croton eluteria* Benn. is used as flavouring in liquors (Rizk 1987). The nuts of *Cindoscolus maregravii* Phol. as well as their oil expressed are used for cooking (Morton 1981).

1.1.5 The Economic Value of the Family Euphorbiaceae

Several species of this family provide valuable raw materials for a wide range of industries including oil, rubber, textile, food, cosmetics, construction and paints. In the construction industry, the strong and durable timber produced from *Androstachys johnsonii* Prain., *Croton schiedeans*, commonly known as wild cinnamon, *Oldfeldia africana* Benth. and Hook., and *Uapaca* species is used in structural work.

Encouraged by its beauty, the European furniture makers use *Sapium reticulation* wood to replace sandal wood. Sandbox tree, botanical name *Hura crepitans*, is another source of timber for furniture making. The soft wood of *Alchornia latifolia* and *Aeramnus*

lucidus which is known as oyster wood, is utilized for producing small deluxe articles. High quality paper is produced from a mixture of fibre obtained from castor oil tree and bamboo (Watt and Breyer 1962, Morton 1981, Rizk 1987).

Castor oil is produced by expressing the seeds of *Ricinus communis* L. In addition to its medicinal use as purgative, it has been used in the manufacturing of soap, paints and varnishes. Utilizing its water resistant quality, castor oil is also used for coating fabrics and protective coverings for aeroplanes. Furthermore, it is used as plasticizer in liquors and brake fluid when mixed with alcohol. The cake, which is the waste product of castor oil production, is used as a fertilizer (Morton 1981, Watt and Breyer 1962). Tung oil, often called Chinawood oil and obtained from *Aleurites fordii* Hems. and *Aleurites montana* is of great value in the manufacturing of paints, varnishes and for preserving and water proofing purposes. In parts of China, it is also used as lamp oil.

Oil expressed from the seeds of *Jatropha curcas*, commonly known as purge nut, is used for soap and candle making, adulteration of olive oil and for illumination and lubrication purposes while the cake is used as fertilizer and cattle feed (Watt and Breyer 1962). Vegetable tallows and fats are expressed from the seeds of *Stillingia sebifera* and the Chinese tallow tree of *Sapium sebifera*. These tallows are used in the manufacturing of soaps and candles. As drying oil, it was concluded that the seed oils of *Euphorbia heterophylla* L. and *E. marginta* Pursh. are equal or superior to the linseed oil (Rizk 1987).

Rubber is a valuable product produced by several plants of the family Euphorbiaceae. Throughout the world the Hevea or Para rubber tree, botanical name *Hevea brasiliensis*, is the major source of rubber produced. Elastica or India rubber is prepared from the latex or milk of *Hevea brasilensis*. Ceara or manicobe rubber is obtained from *Monihot glaziovii* Muell-Arg. but in caura rubber of Venezuela it is obtained from species of the genera *Micandra*. Species of the genera *Cnidoscolus* yield chilte rubber. Many *Euphorbia* species have been reported as rubber sources e.g. the latices of *Euphorbia gregaria* Marl., *E. mauritanica*, *E. helioscopia* L., *E. tirucalli* commonly known as rubber hedge of Rhodesia, *E. triangularis* Desf. and *E. abyssinica*. However the quality of rubber obtained is considered inferior to Hevea and others (Watt

and Breyer 1962).

Research on several latex-rich species revealed that many species of the family Euphorbiaceae may be considered as liquid fuel sources. When analysed, *Euphorbia lathyris* was among the most useful of all species analysed. Other such species include *Euphorbia tirucalli* L. (common name Pencil tree) and *Alchornia latifolia* SW. (Morton 1981, Rizk 1987).

In Middle America a plant commonly known as sassafras *botanical name Croton reflexifolius* HBK. is mistaken for another C*roton* species *C. eluteria* which is known as sweetwood bark, both are used in food industry. The former is mainly used in EL Salvador for flavouring alcoholic liquors and the later is used in Bahamas for flavouring soft drinks, ice cream, candy and baked goods (Morton 1981). Many of the succulent species of *Euphorbia* are grown in bee farms for the production of honey which has been reported to be irritant to the mouth and the throat. Phytochemical research revealed that toxic diterpene esters are the main irritant constituents of honey produced from the nectars of *Euphorbia ingens*, *E. cooprei* and *E. virosa* (Sosath *et al* 1988).

1.1.6 Other Uses of the Family Euphorbiaceae

The essential oil of the plant *Chamaesyce thymifolia* Millsp., known as eye bright, is used as mosquito and fly repellent spray. Twigs of the yellow balsam or seaside sage, Latin name *Croton flavens*, are used as household cleaning agents in rural areas of Middle America. In addition, the leaves are burned in the dwelling as disinfectant. The latex of *Euphorbia clavarioides* Boiss. is used to make glue while the resin from *E. triangularis* forms the basic constituent in the making of chewing gums (Watt and Breyer 1962, Morton 1981).

The latex of a considerable number of species of *Euphorbia* is used as an ingredient in arrow and fish poison. Its cohesive property produces irritation in the arrow wound and facilitates the absorption of the poison. Examples of plants used as fish poison are the roots of *Euphorbia neriifolia* L. locally known as cactus hedge, the rhizomes of *E. biglandulosa* Desf. and *E. drupifera* Stapf. Other Euphorbiaceous plants used as fish poison are *Aleurites montana* E.H.Wilson. and *Antidesma venosum*. In parts of Africa

Hippomane mancinella L. is used as an arrow poison to kill animals such as leopards and lions (Rizk 1987, Evans 1986a, Evans 1986b).

In several areas of the world, Euphorbiaceous plants are used as ornamental species in gardens, parks and homes. Examples of these plants are *Jatropha gossypifolia* which is grown in India, *Euphorbia tirucalli*, *E. ingens* and *E. royleama* which are grown in Egypt and parts of tropical Africa. Herbaceous *Euphorbia* varieties, known to be vesicant and irritant to the humans and animals, are used as a protective fence in many areas of Europe, Japan and USA. These varieties are *Euphorbia lathyris*, *E. milli*, *E. myrsinites* and *E. wulfenii* (Der Maderos and Rio 1979, Evans 1986a, Evans 1986b).

1.2 Structural Classification of Phorbol Esters

The toxic diterpenes of the plant families Euphorbiaceae and Thymelaeaceae are based upon the hydrocarbons, tigliane, daphnane and ingenane shown in figure 1.1, pp.34. The term phorbol esters, or phorbols, is loosely used by biologists to describe the family of these toxic diterpene esters which is correctly referred to as the tigliane diterpenes . The carbon skeleton of a diterpene is made up of four isoprene units which are usually joined together in a head-to-tail fashion to form a hydrocarbon of twenty carbon atoms.

1.2.1 The Tigliane Diterpenes

In 1923, phorbol was the first compound of this series to be isolated from *Croton tiglium* (Hecker 1971a). Phorbol is considered to be the parent alcohol of the tigliane esters (Figure 1.1, pp. 34) which occur naturally in the esterified from.

The tigliane nucleus is tetracyclic in nature. It consists of a five membered ring-A which is trans-linked to a seven-membered ring-B, a six-membered ring-C which is linked to a cyclopropane ring-D in the cis configuration. In addition, the parent alcohol features six oxygen functions located at C3 in ring-A in the form of an unsaturated ketone, a primary hydroxyl group at C20 of ring-B, a tertiary hydroxyl at C4 on the junction of rings A and B and at C9 on the junction of rings B and C and a glycol at C12 and C13.

In plants, tigliane derivatives exist naturally as esters of the fully hydroxylated

polyol, phorbol, or as esters of various deoxy phorbol alcohols (Evans and Taylor 1983, Evans and Soper 1978). Derivatives of phorbol, 4-deoxyphorbol and 12-deoxyphorbol are commonly isolated from the plant family Euphorbiaceae.

Phorbols have been isolated from plants in three distinct esterified forms. The first group of compounds, known as the 'A' series, is the 12,13-diesters which exhibits a long chain or a high molecular weight acyl function at C12 of the nucleus. The second group of compounds, known as the 'B' series, is the 12,13 diesters with the long chain acyl function at C13 of the nucleus. The third group is characterized by the presence of an acyl group at the C20 primary alcoholic function of phorbol, this group of compounds is known as cryptic compounds because *in vivo* it lacks the well known pro-inflammatory and tumour promoting activities of the 12,13-diesters (Evans 1986a).

TPA, 12-*O*-tetradecanoyl phorbol-13-acetate often called PMA (phorbol-12-myristate-13-acetate), is the first and the most potent tumour-promoting agent isolated from the oil of *Croton tiglium* (Hecker 1968).

1.2.2 The Daphnane Diterpenes

The daphnane diterpenes are a group of naturally occurring tricyclic compounds. They are of a similar structure to the tigliane with the exception that cyclopropane ring-D in the tigliane type opens up to form an unsaturated isopropenyl side chain at C13 in the daphnane nucleus (Figure 1.1, pp. 34). Although the polyol diterpene esters have been found in members of the families Euphorbiaceae and Thymelaeaceae, they appear to be more widely distributed in the latter family. This provides evidence for a close relationship between these two families.

The daphnane type diterpenes occur naturally as series of esters, predominantly orthoesters. They are divided into daphentoxin, 12-hydroxydaphentoxin, resiniferonol and 1-alkyl daphnanes. Daphentoxin was the first identified member of a series of related daphnane orthoesters. It was isolated from mezereon bark, the commercially available bark of *Daphne mezereum* (a member of the family Thymelaeaceae) (Evans and Soper 1978). Mezerein is the best known derivative of the 12-hydroxydaphentoxin orthoester. It was first isolated from the seeds of *Daphne mezereum*. Known as gniditrin and

gnidicin, *Ginidia* species of the Thymelaeaceae yielded three anti-leukaemic esters of this type (Kupchan *et al* 1975). Both the daphentoxin and 12-hydroxy daphentoxin esters possess an epoxide moiety at C6-C7 of the ring-B.

The third of the daphnane series is the resiniferonol type. The epoxide moiety at C6 and C7 of ring-B in the former groups is replaced by a double bond. Furthermore, the secondary hydroxy group at C5 is absent. This type of compounds is specifically found in the genus *Euphorbia* (Evans and Soper 1978). Resiniferatoxin was the first compound of this group to be identified. It was isolated from *Euphorbia unispina*, *Euphorbia resinifera* and *Euphorbia poisonii* of the family Euphorbiaceae (Hegenhahn *et al* 1975, Evans and Schmidt 1976).

The fourth and the final daphnane type is the 1-alkyl daphnane esters. Derivatives of these compounds have similar structure to daphentoxin with two exceptions. First, the olifenic bond between C1 and C2 in daphentoxin is absent in this group. The second is the presence of a fourth macro cyclic ring produced as a result of a carbon atom from the aliphatic orthoester chain being attached to C1 of ring-A. In addition, a benzoate ester may replace the keto function at C3 (Kupchan *et al* 1976, Schmidt 1986a). Literature search revealed that all derivatives of this type of daphnanes were isolated from the family Thymelaeaceae. They have not been detected in the Euphorbiaceae to date.

1.2.3 The Ingenane Diterpenes

The ingenanes are a group of tetracyclic diterpenes found exclusively in the family Euphorbiaceae and particularly in the species *Euphorbia* and *Elaeophorbia* (Evans and Taylor 1983). In 1968, ingenol was the first member of this group to be isolated form *Euphorbia lathyris* by Hecker's group (Hecker 1986). This parent diterpene has many common structural features with phorbol (Figure 1.1, pp. 34). The difference between them however is that ring-C is seven-membered and C8 being linked to C10 by means of a keto bridge. In addition, the keto group at C3 in the tiglianes and the daphnanes have been replaced by a secondary hydroxyl group. Furthermore, the C12 and C13 hydroxyl groups are missing in ingenol.

In plants, ingenane esters were reported to occur naturally as derivatives of the

following nuclei: ingenol, 5-deoxyingenol, 20-deoxyingenol, 16-hydroxy-20-deoxyingenol, 13-hydroxyingenol, 16-hydroxyingenol and 13,19-dihydroxyingenol (Schmidt 1986b). However, since the ingenanes proved to be technically the most difficult to separate from one another few reported their successful isolation and characterization. This suggests that a greater variety of structural types may yet be found.

Despite the apparent structural dissimilarity between the ingenanes and both the tiglianes and daphnanes, their biological activities are notably similar, reflecting their presumed biosynthetic relationship.

1.3 Biological Activities of the Diterpene Esters

The irritant properties of croton oil led Berenblum to detect its tumorigenesis effect on mouse skin. The isolation and structural elucidation of the irritant constituents of the genus *Croton* of the family Euphorbiaceae initiated intensive chemical and biological studies into many other species of this family as a well as the closely related Thymelaeaceae. The isolated tigliane type diterpenes were responsible for eliciting a wide range of responses in different *in vivo* and *in vitro* test assays.

Biological effects of these diterpene esters include inflammation of mammalian skin (Evans and Schmidt 1980b), tumour promotion (Bohrman 1983), stimulation of degranulation in neutrophils (Blumberg 1980, Blumberg 1981), cell proliferation and epidermal hyperplasia (Marks and Furstenberger 1984), lymphocyte mitogenesis (MacDonald 1994), activation of blood platelets (Zucker *et al* 1974) and induction of Epstein-Barr virus early antigen (Ito *et al* 1984).

At biochemical levels, phorbol esters were shown to affect the membrane associated cascade systems. In terms of inflammation and tumour promotion, the most important effects are the stimulation of arachidonic release and prostaglandins synthesis (Billah 1981), induction of ornithine decarboxylase *in vitro* (Yuspa *et al* 1976) and *in vivo* (O'Brein 1976) and activation of phospholipid synthesis (Balmain and Hecker 1974). Phorbol esters were also reported to stimulate RNA, DNA and protein synthesis (Baird *et al* 1971).

Studies of tumour promoting and platelet activating phorbol esters provided

valuable information on the transmission of their effects inside the cells. These studies provided considerable evidence that the phorbol esters and the related diterpenes function, at least partly, through persistent stimulation of the calcium-phospholipid activated protein kinase known as protein kinase C (PKC) (Nishizuka 1984). This protein appears to embody specific phorbol ester receptors that have been characterized in many species and cell types (Niedel *et al* 1983).

The plant derived phorbol esters appear to function by substituting diacyl glycerol, the endogenous compound in animal cells which normally activates PKC (Castagna *et al*1982). Evidence for the involvement of this enzyme in phorbol-induced inflammation, tumour promotion and cell differentiation was based on *in vitro* investigations of PKC activation by phorbol esters (Ryves *et al* 1991, Hasler *et al* 1992). Diacyl glycerol is the product of phosphatidyl inositol hydrolysis (Figure 1.2, pp. 35). It is an important mechanism through which the effects of many neurotransmitters and hormones are transmitted (Aitken 1987). On binding to its membrane, the agonist stimulates the activity of phosphatidyl inositol-4,5-biphosphate (Ptd Ins-4,5-P_2) via phosphodiesterase (PDE) or phospholipase C and a guanine nucleotide binding protein (Ns). Products of this interaction include inositol-1,4,5-triphosphate (Ins-1,4,5-P_3) which releases calcium from intracellular stores and 1,2-diacylglycerol which activates protein kinase C (PKC). Phosphatidyl inositol hydrolysis is summarized in Figure 1.2, pp.35. Phorbol esters act as agonists of PKC inducing activation for longer duration than diacylglycerol (Aitken 1987).

1.3.1 Inflammation

inflammation is the acute effect on humans caused by exposure to plants of the families Euphorbiaceae and Thymelaeaceae containing phorbol esters and related diterpenes. This response is commonly used to assess the pro-inflammatory activity of phorbol esters and phorbol esters containing plants on mouse skin (Schmidt and Evans 1979). This is a direct pro-inflammatory response characterised by the classical symptoms of heat, redness, swelling, pain and the thickening of the tissues (Youlten 1984). Exposure to these compounds results in severe reddening and blistering of the skin and oedema (Evans 1986b, Ambasata *et al* 1986). Skin burning induced by the *Euphorbia* latex may develop into pustules progressing to dry flaky skin and necrosis of the tissues

(Schmidt and Evans 1980a). Contamination of the eyes with the latex of these plants induces keratoconjunctivitis that can lead to blindness (Evans and Edwards 1987).

The presence of different forms of protein kinase C (Parker *et al* 1986, Nishizuka 1988) could explain the different structural requirements for irritant activity of tigliane, daphnane and ingenane esters (Figure 1.3, pp. 36). The parent alcohols phorbol, 12-deoxyphorbol, 4-deoxyphorbol, resiniferonol and ingenol all are inactive as pro-inflammatory agents. The degree of irritant activity depends on the lipophilicity of the acyl functions. Short-chain acyl functions on the molecule are associated with a very rapid onset of the response. However, long-chain acyl functions with higher lipophilicity may be involved in a later but longer duration of action (Hergenhahn *et al* 1982, Hecker 1978). Skin inflammation induced by phorbol esters appeared not to be due to direct effect upon skin microvasculature (Williams *et al* 1981) but was more likely to be the result of an indirect action involving the release of endogenous mediators of inflammation (Williamson and Evans 1981). Evidence for the possible mechanism of action is yet to be confirmed.

1.3.2 Skin Hyperplasia and Tumour Promotion

Skin carcinogenesis is a complex biological process known to occur in a two-stage process. Rous and co-workers reported the enhancing effect of irritation of the tumour promotion process (Marks and Furstenberger 1990). Evidence for chemical promotion or co-carcinogenesis was first provided by Berenblum. He reported that when croton oil (weak or non-carcinogenic) is applied to mouse skin, alternately with small doses of benzo pyrene (BP), it induced a larger number of skin tumours than BP alone. Subsequently, applications of a single subcarcinogenic dose of BP followed by multiple applications of croton oil induced a large number of skin tumours (Boutwell 1984). However, skin tumours failed to materialize following multiple application of croton oil alone (Slaga 1984).

The characteristic three stages of carcinogenesis, commonly known as Berenblum experiments, are illustrated in Figure 1.4, pp. 37. Skin tumours can be induced by sequential application of a subthreshold dose of a carcinogen (initiation stage) followed by repetitive treatment with a non carcinogenic promoter (promotion stage). The

initiation stage only requires a single application of a carcinogen but it is an essentially irreversible step. The promotion phase is initially reversible but becomes irreversible later (Peto 1984). Most initiators appear to be mutagens that can damage the DNA whereby proliferation of the initiated cell is at its minimum, stimulation and normal differentiation is prevented. Tumour promoters are membrane-active compounds requiring a certain frequency of application in order to induce tumours (Trosko *et al* 1984).

Phorbol esters are the most potent of the mouse skin tumour promoters. Treatment of mouse skin with tumour promoting phorbol ester results in profound and acute biological effects. Within a few hours of application, local tissue reactions such as oedema and erythema are evident. These are characteristics of inflammation. Leukocytes infiltrate the dermis (Diamond *et al* 1980) and although there is a temporary block in DNA synthesis, proliferation of cells of the basal layer of epidermis begins within 24 hours (Mastro 1982). Cell division, as well as hypertrophy of superficial cells above the basal cell layer, lead to a dramatic increase in the size of the epidermal layer, increase in keratinization and cell death in the upper epidermal layers (Balmain 1978).

Sustained hyperplasia combined with inflammatory reaction of the skin are critical conditions of skin tumour development (Troskie *et al* 1984). Hyperplasia may be considered as the result of an imbalance between the rates of cell gain and cell loss (Marks and Furstenberger 1993).

Although all known tumour promoters stimulate epidermal hyperplasia, many compounds that induce epidermal proliferation are not tumour promoters. Therefore, hyperplastic transformation has to be distinguished from another response of the skin to external stimuli. Experiments have shown that balanced hyperproliferation induced by mild stimuli is neither accompanied by inflammatory reactions nor by the symptoms of keratinocyte activation. In addition, tumour-promoting phorbol esters induce several morphological and biological changes in mammalian skin (Klein-Szanto 1984) as in 1.1, pp. 38.

Several short term assays have been proposed for the detection of tumour promoters. Such assays include induction of ornithine decarboxylase, activation of

Epstein-Barr virus in human lymphoblastoid cells, alteration of surface-attachment properties of tumour cells and inhibition of intracellular communication (Trosko *et al* 1984). The skin is the most suitable model for investigating carcinogenesis. Mice are generally more sensitive to skin carcinogenesis than rats and hamsters (Slaga 1984). Skin hyperplasia can be induced by a single application of the tumour-promoting phorbol esters. Most of plant phorbol esters are isolated in quantities insufficient for *in vivo* promotion protocols. Therefore, induction of skin hyperplasia on the back skin of mice is commonly used as a rapid preliminary *in vivo* screen for tumour promoters.

Investigations of the mouse skin irritant response and its relation to the initiation-promotion activity of the diterpene esters on the back skin indicated that the degree of biological activity depends on the lipophilicity of the acyl substituents. The short chain acyl derivatives, including benzoyl and phenyl acetate derivatives of several parent diterpenes, are weak promoters. In contrast, long chain analogues are highly active esters. Conjugated acyloxy moieties generally increase irritant activity and simultaneously decrease the promoting activity. As well as the free hydroxyl group at C20, trans-linking of ring-A and ring-B are essential for tumour promotion (Hergenhahn *et al* 1982, Hecker 1978). Despite the high irritant activity of 12-deoxy tigliane and daphnane types, they are inactive tumour promoters. The 4-hydroxy tigliane derivatives are more active tumour promoters than the 4-deoxy analogues (Brooks *et al* 1989).

Epidemiological studies suggested that these plant-derived compounds may play an important role in human cancers (Hecker 1981). The initiation-promotion phenomenon observed in the laboratory appears to be closely related to the human early and late carcinogenesis stages. However, until the role of promotion in animal carcinogenesis is clearly defined, it will be difficult to assess its relevance to human malignancy.

1.3.3 Platelet Aggregation

Structural similarities between TPA and prostaglandins have initiated many studies on the effects of phorbol esters on platelet aggregation. Zucker and his team were the first to demonstrate that TPA would induce a dose-dependent aggregation of human

blood platelets (Zucker *et al* 1974).

The TPA's aggregation of platelets was studied in great detail to clarify the action of drugs on aggregation. It was found that TPA's potent and rapid effects on platelets are not identical to those produced by drugs or adenosine diphosphate (ADP) (Mastro 1982). The most dramatic TPA's structural effects on platelets include vacuole formation within storage granules and dilation of the open canicular system of platelets (White and Estensen 1974). Chemical analysis confirmed that TPA-induced aggregation is associated with partial release of granule contents such as serotonin, adenine nucleotides, 5-hydroxy tryptamine and lactic dehydrogenase (Sano *et al* 1983, Ensten and White 1974).

Aggregation was assayed in an aggregometer which detects changes in light transmission in either stirred or unstirred samples. These changes were found to be a function of time and TPA concentration. Estensen and White reported a linear relationship between the concentration of TPA and the increase of transmitted light (Blumberg 1980). Submicrogram doses of TPA induced two-stage aggregation of human platelets (Westwick *et al* 1980).

A range of biologically active diterpene esters induced a dose-dependent aggregatory response similar to the TPA's (Williamson *et al* 1980, Westwick *et al* 1980). Tigliane derivatives were more active aggregating agents than daphnane ortho esters (Williamson *et al* 1980). The structural requirements for diterpene esters-stimulated platelet aggregation did not correlate directly with inflammation (Evans and Edwards 1987). This is due to the additional requirement of a primary hydroxyl group at C20 of the nucleus for the aggregatory response which is also essential for tumour-promoting activity (Williamson *et al* 1981).

TPA-induced activation of human platelets was reported to be associated with activation of Ca^{+2} -dependent protein kinase (Naka *et al* 1983) identified as PKC (Sano *et al* 1983). *In vivo* the ability of different diterpene esters to induce platelet aggregation correlated very well with their ability to activate different PKC isozymes (Brooks *et al* 1990, Baldassare *et al* 1992).

1.3.4 Cell Differentiation

The addition of TPA to a variety of cell types in culture led to the discovery of a very large number of TPA effects on the cells. First reports indicated that TPA prevented terminal differentiation of Friend-Murine erythroleukaemia cell (Yamasaki *et al* 1981), normal chicken embryo cells (Blumberg 1981), mouse neuroblastoma (Mastro 1982) and hamster epidermal cells (O'Brein and Diomond 1979). Studies of other cell cultures indicated that TPA could induce terminal differentiation and block 8-997/cell division leading to a less transformed state (Rovera *et al* 1980, Yamasaki 1984). Examples of TPA-induced effects in cell culture are shown in Table 1.2, pp. 39.

The characteristics of human promyelocytic leukaemia cells (HL-60 cells) derived from patients with acute promyelocytic leukaemia proved invaluable in a variety of different *in vitro* studies. Contrary to other human myeloid leukaemia cells, the HL-60 cells continuously proliferate in suspension culture, for example RPMI-1640 medium, provided that it is supplemented with a transferrin and insulin. Most leukaemia cells when cultured in a liquid suspension undergo a limited number of cell divisions prior to growth arrest and cell death (Collins 1987).

Various agents induced HL-60 cells to differentiate into four general types of cells, granulocyte, monocytes, macrophage-like cells and eosinophils. TPA and related diterpene esters stimulate the HL-60 cells to differentiate into and acquire many properties of macrophage-like cells (Rovera 1979a, Rovera 1979b, Huberman and Callaham 1979). The process of TPA-induced differentiation of HL-60 cells into macrophage-like cells is rapid but lacks new DNA synthesis and multiple cell divisions that are normally associated with the transition from promyelocytic to a more differentiated myeloid cell (Rovera *et al* 1980). Significant macrophage-like differentiation of HL-60 cells occurs within 20 minutes of exposure to TPA (Rovera *et al* 1979a).

Macroscopic studies revealed that TPA-treated cells exhibit intense adherence to plastic with prominent pseudopodia formation and marked adherence of the cells to each

other. Other types of promoters such as Phenobarbital and saccharin do not have this effect (Rovera *et al* 1979b). This rapid and unique response provides an excellent model system for studying the physiological role of tumour promoters in cell differentiation. Protein kinase C (PKC) is the major intracellular receptor for the tumour promoting diterpene esters (Herbert 1993). Several PKC isotypes have been recognized in HL-60 cells (Kikkawa *et al* 1989, Ryves *et al* 1994). Observations suggest that activation of PKC can not by itself account for the macrophage-like differentiation (Ryves *et al* 1991). The macro cyclic lactone bryostatin binds to and activates PKC in HL-60 cells but actually inhibits TPA-induced HL-60 differentiation in a dose-dependent manner (Kraft *et al* 1986). The nature of the additional TPA action remains to be defined.

Diterpene esters are capable of inducing differentiation of HL-60 cells at nanomolar concentrations. The relative potencies of the diterpenes correlate well with their ability to activate PKC *in vivo* (Aitken *et al* 1987). However, structural requirements for induction of differentiation appear to be more specific than those for irritancy but less specific than tumour-promotion. The primary hydroxyl at C20 is necessary for such activity. In contrast, the C12 or C13 side chain increased the activity but it is not an absolute requirement as it is for tumour-promoting ability.

Although the naturally occurring phorbol esters are best known for their irritant and tumour promoting actions, they exhibit anti-leukaemic activity. Hartwell documented over 100 species in the Euphorbiaceae that are used in folk medicine to treat various forms of cancer (Hartwell 1969). A number of daphnane and tigliane types demonstrated activity against P-388 lymphocytic leukaemia cells *in vivo* (Cassady and Suffness 1980, Borris and Cordell 1984, Kupchan *et al* 1976). Ingenol derivatives isolated from *Euphorbia lathyris*, *Euphorbia esula* and croton oil exhibited anti-tumour and anti-leukaemic activities (Itokawa *et al* 1989, Kupchan *et al* 1976, Harborn and Baxter 1993). The structural similarity between anti-leukaemic and tumour-promoting diterpene esters throws doubts on their use in chemotherapy. Further research could establish the reasons for this contradiction.

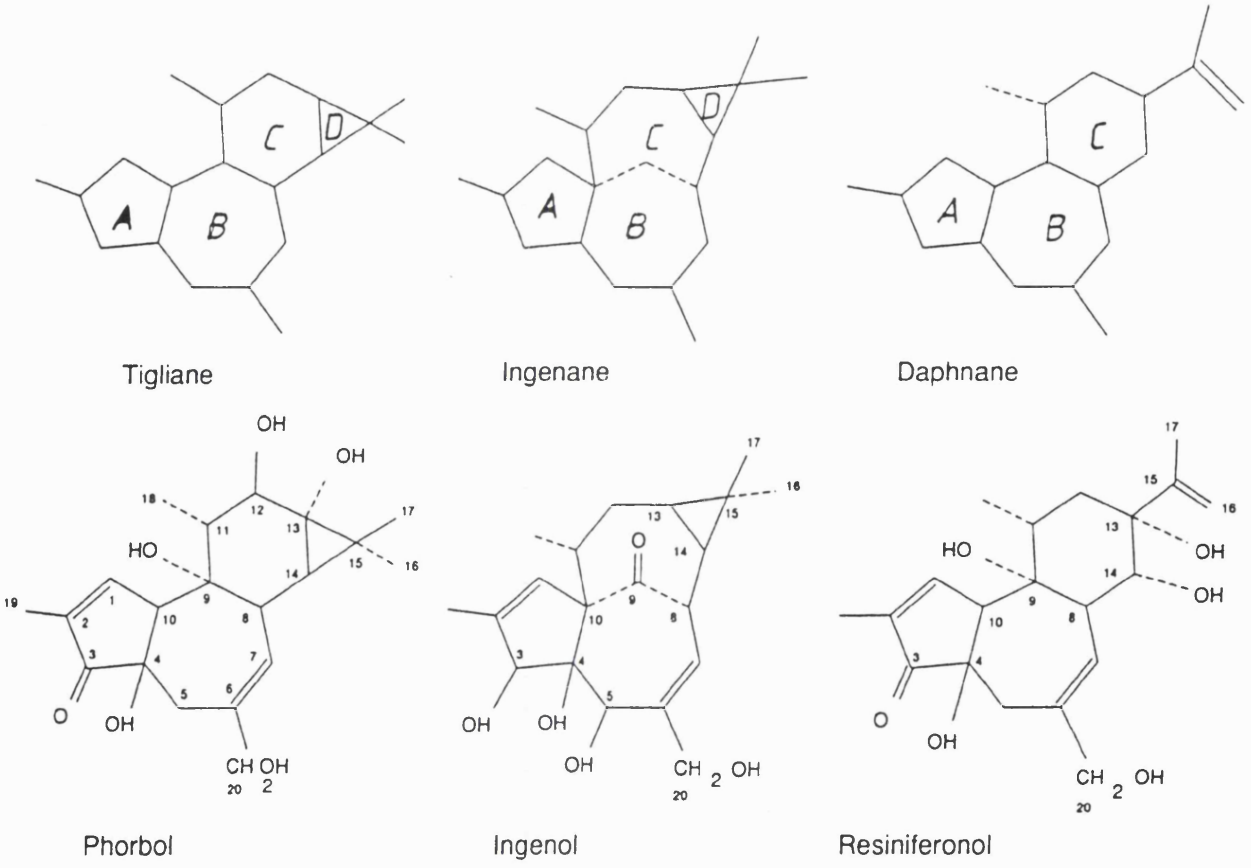


Figure 1.1 Diterpene Parent Hydrocarbons Tigliane, Ingenane and Daphnane of the Biologically Active Diterpene Esters of the Euphorbiaceae and Thymelaeaceae

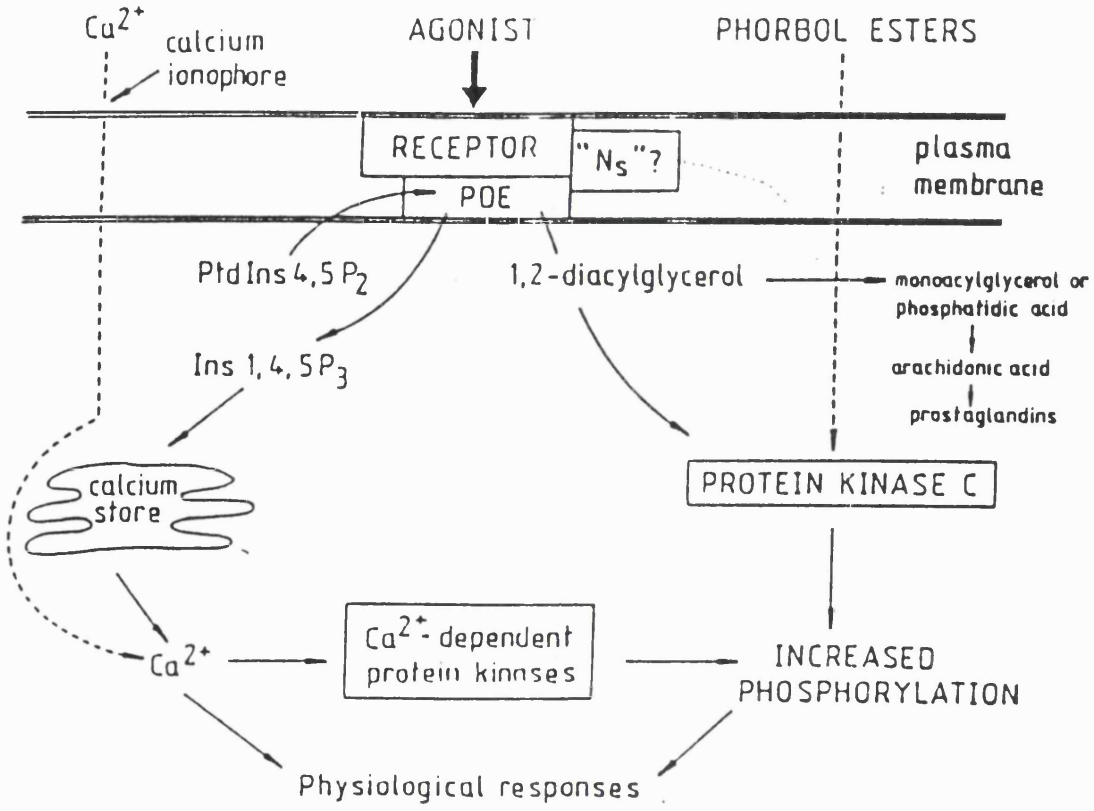


Figure 1.2 The Phosphatidyl Inositol Cycle

The agonist binds to its membrane receptor, stimulates the hydrolysis of phosphatidyl inositol-4,5-biphosphate (PtIns-4,5-P_2) via phosphodiesterase (PDE) or phospholipase C and a guanine nucleotide binding protein (Ns).
Products formed are inositol-1,4,5-triphosphate (Ins-1,4,5-P_3) which releases calcium ions from internal cytosolic stores and 1,2-diacylglycerol which activates protein kinase C (PKC).

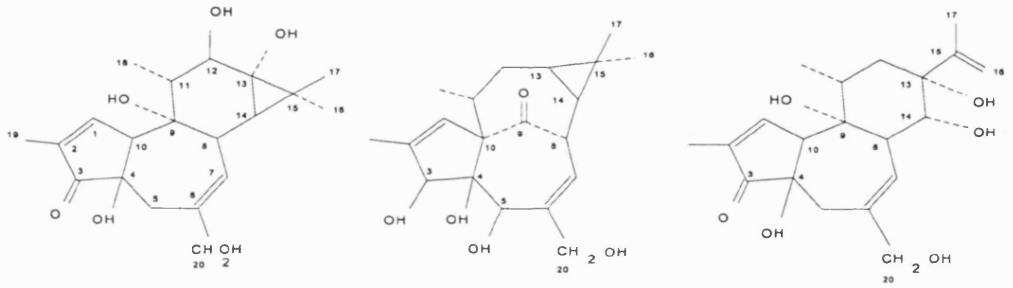


Figure 1 .3 Structure-Activity Relationship in Irritant Diterpene Esters

Structural features essential for irritant activity:

1. The configuration of the link between ring-A and ring-B must be trans
2. The presence of an ortho ester at C9, C13 and C14 in daphnane type
3. An acyl or acetate group at C12 or C13
4. Double bond between C1 and C2

Structural features not necessary for irritant activity

1. A hydroxy or acyl group at C12
2. Free hydroxyl at C20 only in daphnane type
3. A tertiary hydroxyl at C4
4. Cyclopropane ring-D
5. Double bond between C6 and C7
6. Methyl group at C16

Structural features increase irritant activity

1. Increased unsaturation and the length of C12 or C14 acyl residue
2. Aromatic residue at C12 or C13
3. A tertiary hydroxyl group at C4
4. Long-chain substituent at C13

Structural features decreased irritant activity

1. Replacement of the keto group at C3 with a hydroxyl group or acetate
2. Replacement of the double bond between C6 and C7 with epoxide group
3. Elimination of the hydroxy group at C4
4. Replacement of the primary alcohol function at C20 with an acetyl

References:

Hecker 1978, Evans and Soper 1978, Evans *et al* 1990, Ellis 1987, Evans and Edwards 1987, Hergenhan 1982, Adolf *et al* 1982.

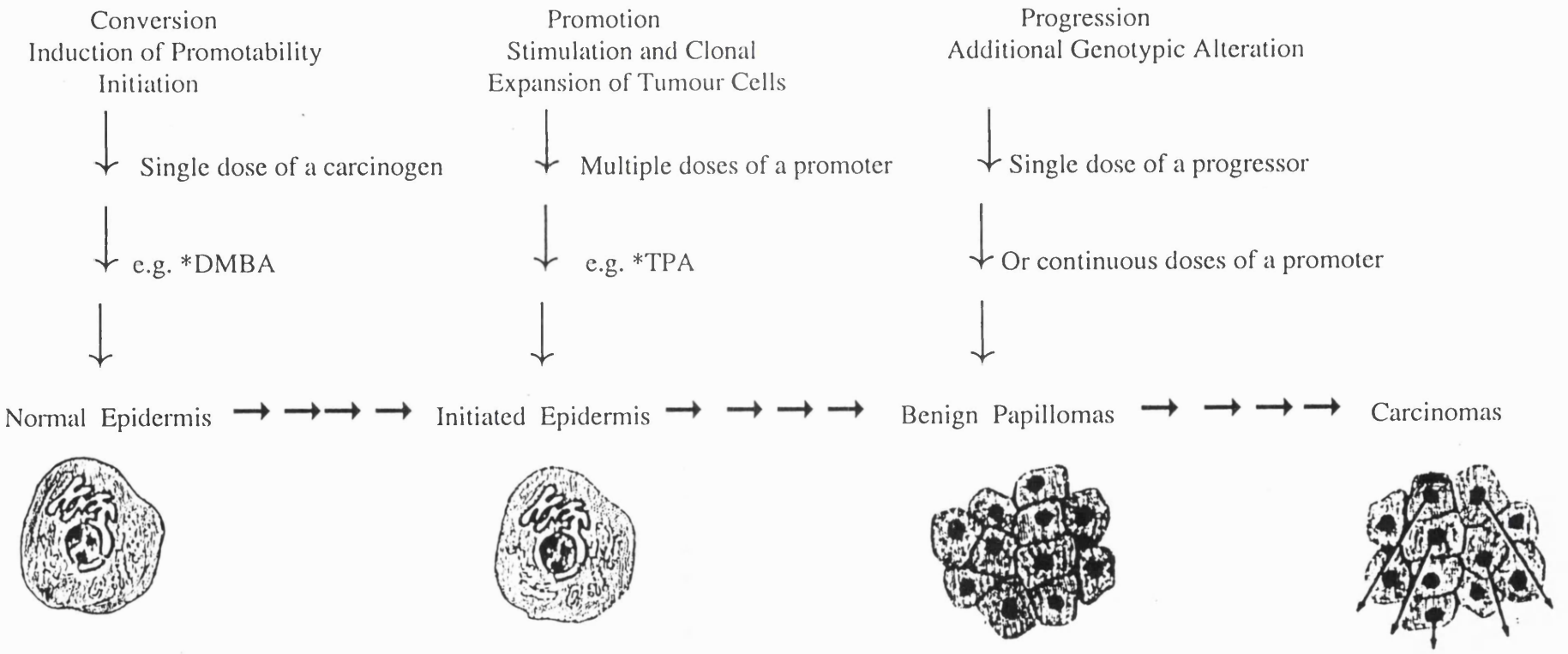


Figure 1.4 The Three Biologically Defined Stages of Carcinogenesis in Mouse Epidermis

*DMBA = 9,10-dimethyl-1,2-benzanthracene *TPA = 12-O-tetradecanoylphorbol-13-acetate

Table 1.1 Morphological and Biochemical Responses of Mouse Skin to Phorbol Esters:

1. Induction of inflammation and hyperplasia
2. Induction of dark basal cells
3. Increase in DNA and RNA synthesis
4. Initial increase followed by a decrease in keratinization
5. Increase in phospholipid synthesis
6. Increase in histone synthesis and phosphorylation
7. Increase in ornithine decarboxylase activity
8. Induction of embryonic proteases in adult mouse skin
9. Increase in prostaglandins synthesis
10. An increase in c-AMP phosphodiesterase activity

References:
- Bohrman J. 1982
- Mastro A.M. 1982
- Diamond L.A. *et al* 1980

Effect	Cell System	Reference
Prevents cell fusion, stimulates cell division	Primary chicken myogenic cells	Mastro 1982
Blocks morphological differentiation	Mouse neuroblastoma	Mastro 1982
Induce differentiation	PC-12 phenochromocytoma neuronal cells	Mastro 1982
Formation of new phenotypes	JB-6 Mouse epidermal cells	Bohrman 1982
Morphologically altered colonies and increased cellular proliferation in soft agar and tumorigenicity when injected into mice	Human foreskin cells	Bohrman 1982
Synthesis of new proteins, increased pre-neoplastic changes	Mouse epidermal cells (SENCAR)	Bohrman 1982
Prevents morphological differentiation	Hamster epidermal cells, mouse cells	Yuspa *et al* 1976
Inhibit differentiation to muscle cells	C3H/1OT1/2 mouse fibroblasts	Bohrman 1982
Blocks spontaneous and induced differentiation	Murine Friend virus leukemia cells	Yamasaki *et al* 1977
Stimulates appearance of macrophage-like and induced differentiation	Hl-60 human promyelocytic leukemia cells	Rovera *et a*l 1980
Stimulates differentiation	Human chronic lymphocytic leukemia cells	Mastro 1982
Stimulates production of T-cell markers	MOLT-3 human lymphoblastic cell line	Mastro 1982

Table 1.2 Some Effects of TPA on Cells in Culture

OBJECTIVES OF RESEARCH

The use of natural products as alternative or complementary medicine has increased dramatically over the past years. Almost 100 species of the families Euphorbiaceae and Thymelaeaceae are used in folk medicine which many herbal remedies are based upon. In addition, many species of these families, especially the Euphorbiaceae, are widely used as ornamental plants. The release of the irritant and tumour-promoting diterpene esters into the soil affecting the growth of other plants have been documented (Ito *et al* 1984).

This research has been designed to investigate the biological activities of selected species of the families Euphorbiaceae and Thymelaeaceae. The primary aim of this study was to evaluate the pro-inflammatory activity of plant extracts from forty species of these families, isolate the irritant constituents and identify their chemical structures. It was anticipated that the effect of storage on the pro-inflammatory activity of plant extracts and pure tigliane and daphnane derivatives would be carried out. This necessitated the study of the chromatographic behaviour of the tigliane and daphnane derivatives to establish a reproducible system in order to analyse and detect these diterpene esters.

An additional aim was to carry out short-term *in vivo* pro-inflammatory and hyperplastic effects of the crude extracts and the isolated compounds to correlate them with the *in vitro* platelet aggregation and HL-60 cell differentiation.

A further objective was to design an alternative *in vitro* assay to replace the current *in vivo* tests to detect the presence of toxic diterpene esters in plant extracts using HL-60 cells. The purpose was to compare the results of this assay with those of the *in vivo* and the *in vitro* tests carried out earlier.

CHAPTER TWO

Biological Screening of Selected Species of the Families Euphorbiaceae and Thymelaeaceae

2.1 Introduction

Although the etiology of many malignant diseases remain unknown, studies of the geographical distribution of their occurrence give some leads to the possible influence of certain environmental or social factors. There is however considerable evidence that environmental factors are important in the pathogenesis of cancers (Hecker 1978, Evans 1986b). Scientists have realised that the majority of human cancers could be the result of exposure to more than one carcinogenic risk factor (cancer producing substance). It is more likely that a number of human tumours are due to exposure to small quantities of a carcinogen and a tumour-promoter, a substance which enhances tumour production. This process is known as syn-carcinogenesis. The diterpene esters of the plant families Euphorbiaceae and Thymelaeaceae, collectively known as phorbol esters, remain the most potent tumour-promoting agents known (Evans 1986b, Culvenor and Jago 1979).

Inflammation is the acute effect of the exposure to these plants. This response is a characteristic of all tumour-promoting diterpene esters. However, not all pro-inflammatory diterpenes are tumour promoters.

Tumour-promotion is a complex multistage process. One of the main problems associated with this type of investigation is the lack of rapid and valid *in vitro* tests for tumour-promotion. To assess tumour-promoting activity, present available techniques used largely depend on the biological activities of the pure forms of phorbol esters isolated from these plant families. These techniques include the induction of hyperplasia in mammalian skin following a single application of tested compounds, tumour-production following initiation with a carcinogen, induction of erythema on mammalian skin, induction of Epestien Barr virus, induction of terminal differentiation of human promyelocytic leukaemia cells (known as HL-60 cells), inhibitory effect on terminal differentiation of Friend Virus transformed cells, enhancement of mutagenesis in bacteria and the ability to induce aggregation of platelets from human blood (Mastro 1982, Evans and Soper 1978).

2.2 Botanical Classification of Plant Species and Their Use

Approximately 40 plant samples were screened for their pro-inflammatory activity, their ability to induce differentiation of HL-60 cells (promyelocytic leukemia cells), inhibition of differentiation of TPA treated HL-60 cells and aggregation of platelets in human blood (Table 2.1, pp. 44). Whilst these samples represented different genera from the families Euphorbiaceae and Thymelaeaceae, only those plant extracts possessing pro-inflammatory activity were tested for induction of epidermal hyperplasia in mammalian skin.

Of the widely published toxic hazards, associated with the majority of plants from these families, their tumour-promoting and irritant effects are the more prominent (for reviews refer to Kinghorn 1979, Evans and Taylor 1983). Clinically, the inflammatory condition is the most serious and immediate toxic syndrome induced by contact with these plants. *Phylanthus* is one of the largest genera in the Euphorbiaceae. Interest in this genus has been stimulated by the wide use of its different species in folk medicine. Traditionally, for example, the Indians use this plant to treat jaundice, viral infections and skin diseases (Unader *et al* 1995, Watt and Breyer-Brandwijk 1962).

The genus Euphorbia is comprised of approximately one thousand species (Webster 1995 & 1987). A large number of these are potentially toxic to humans. Species of this genus are capable of inducing erosion of the corneal epithelium which results in decreasing visual acuity. Oral poisoning was reported in the form of severe irritation and inflammation of oral membranes and the digestive system (Der Marderosian and Roia 1979). Despite the well recognised toxicity of the *Euphorbia* species, many are widely used in folk medicine. Traditional herbalists use many species of this genus in cancer treatment (Hartwell 1969). Table 2.1, pp. 44 shows traditional medicinal uses of selected *Euphorbia* species.

In 1990 the board of the 101st Congress on Unconventional Cancer Treatments discussed one of the treatments offered by clinics in United States and Mexico. This tumour treatment used *Stillingia* roots (Queens's roots botanical name *Stillingia sylvatica* from the family Euphorbiaceae) in several herbal combinations of plants. This protocol is known as Hoxsey's treatment (Gibbons 1990).

Plant species screened in this study were referred to by Grieve for their medicinal and toxic effects (Grieve 1994). *Mercurialis annua*, a member of the Euphorbiaceae commonly known as Garden, French or annual mercury, is used in France as purgative and consumed in Germany by both humans and cattle. In addition, the juice, leaves and seeds of annual mercury are used to treat warts, tumours and swellings (Hartwell 1969).

Sapium indicum, commonly known as sapium, is known to be irritant and poisonous. However, young leaves are eaten as condiment (Grieve 1994). Whilst the bark for example is used as decoction to treat uterine cancer in Cuba (Hartwell 1969), the entire herb is recommended for wart removal and as a diuretic (Bianchini and Corbetta 1977).

Species of the genus *Jatropha* are known to be toxic and irritant. The oil of *Jatropha curcas,* known as physic nut, is frequently used as a remedy for paralysis, eye and skin diseases and as purgative all over West Africa (Watt and Breyer-Brandwijk 1962, Oliver-Bever 1986). In Indonesia and some Arab countries leaves are used to treat tumours (Morton 1981, Hartwell 1969). The use of *Jatropha gossypifolia* in folk medicine and tea infusions in Central America may be related to the high incidence rates of oesophageal cancer especially in Curacao (Morton 1981, Adolf *et al* 1984, Hacker 1987). In India, boils, eczema and rashes are treated with repeated application of the leaves of this plant. which is locally known as red fig nut (Oliver-Bever 1986).

Alchornia species, which was investigated in this study, *Alchornia indica* and *A. cordifolia* are all used to treat tumours by traditional healers in India, Mexico and Guatemala (Hartwell 1969). In tropical Africa, the roots of the latter species are used as antiprotozoal (Oliver-Bever 1986). Traditional Indian healers use root extracts of *A. indica* as purgative and emetic while its leaves are used as anthelmintics (Watt and Breyer-Brandwijk 1962). In addition, Indonesians used species of *Exoecaria* to treat tumours on both hands and feet (Hatrwell 1969).

Species of the family Thymelaeaceae, commonly known as the daphne family, are widely used in traditional medicine particularly in Africa. Some species of the genus *Daphne* are also used in Chinese herbal medicine as abortifacient, analgesic, diuretic and

laxative (Huang 1993). In Southern and Eastern Africa, *Gnidia* species are used as blood purifier and for tooth ache and eye diseases treatment. In Europe infusions prepared from the roots are used to treat asthma (Watt and Breyer-Brandwijk 1962, Cordell *et al* 1988). Leaves of the *Lasiosiphon kraussianus* are applied to burns, wounds, bruises and used as a snake bite remedy. Local inhabitants in Africa reported the dried plant to be very toxic. It is used as arrow poison and fish poison (Cordell 1988, Oliver-Bever 1986, Watt and Breyer-Brandwijk 1962). The Egyptian *Thymelaea hirsuta*, known to the herbalists as methnane, was mentioned by Al-Baytar, the well known physician and botanist in the twelfth century, to be irritant and toxic despite being used as a purgative after dilution of the concoction with honey and as expectorant (Al Baytar 1992).

Table 2.1 Traditional Use of Selected *Euphorbia* Species

Plant Species	Common Name	Organ	Medicinal Use	Reference
E. amygdaloides	Wood spurge	juice	treatment of warts and corns	Grieve 1994
			treatment of cancer	Hartwell 1969
E. esula	Leafy spurge of Europe	leaves	used by homoeopaths to treat Cholera,	Grieve 1994
E. peplus		leaves	diarrhoea &	Huang 1993
E. helioscopia	Sun spurge	leaves	dysentery	
			diuretic	Watt *et al* 1962
			purgative, treatment of gonorrhoea	Hartwell 1969
		juice	treatment of cancer (Europe, India & Africa)	Grieve 1994
E. lathyris	Caper spurge	seeds	purgative	Hartwell 1969
			treatment of warts and cancer	

2.3 Materials and Methods

2.3.1 Plant Material

Plant material used in this study was collected from Africa, Asia and Europe. Some species were collected from Egypt, Nigeria, Pakistan, India, Portugal, Britain and the Former Yugoslavia. The remaining samples were obtained from the department's herbarium.

According to the classification proposed by Webster 1975, 1987 and 1994, plant species of the family Euphorbiaceae represent 21 species of 9 genera from 4 subfamilies as shown in Table 2.2, pp. 60. Plant species from the family Thymelaeaceae represent three species, *kraussianus, hirsuta and blagayana*, of four genera, *Gnidia, Lasiosiphon, Thymelaea* and *Daphne,* from the subfamily Thymelaeoideae.

2.3.2 Extraction of Plant Material

Plant material was finely chopped using an electric mill. It was then exhaustively extracted by cold maceration in redistilled acetone (supplied by BDH) for a period of two weeks. The macerate was shaken daily, filtered twice a week with fresh acetone added after each filtration. The plant debris was removed by final filtration on day fourteen and the acetone was evaporated under vacuum at 35°C using Büchi rotavaporator to yield the crude extract which was then stored under nitrogen at -20°C.

To obtain ether extracts the dry crude extracts were dissolved in 40% aqueous methanol (supplied by BDH) using sonication to ensure complete and homogenous solubility. Sterols and lipids were extracted three times from the aqueous methanol by means of liquid-liquid partition with hexane (supplied by BDH). The methanolic phase was adjusted to 30% using 0.9% sodium chloride solution (supplied by Sigma UK) to prevent formation of emulsion during liquid partition with ether. Diterpenes were then extracted with equal volumes of redistilled ether, until the ether layer became colourless. The ether extract was then washed three times with 0.25% sodium carbonate solution (supplied by BDH) to remove any excess chlorophyll and other plant pigments. The ether extract was then washed with 5% sodium chloride to remove any residual sodium carbonate. The ether fraction was finally dried over anhydrous sodium sulphate (supplied by BDH). Ether was evaporated under vacuum at 35°C flushed with nitrogen and stored

in the freezer at -20°C until required. Hexane fractions were dried over anhydrous sodium sulphate and stored under the same conditions used for both crude and ether extracts. The aqueous methanol fractions were dried under vacuum and later redissolved in water and freeze dried. They were then stored as previously mentioned. Figure 2.1, pp. 61, summarizes the extraction procedure.

Crude extracts, ether, hexane and methanol fractions were tested for their ability to induce inflammation on the mice-ear, for their induction of HL-60 cells differentiation and for their inhibition to differentiation of TPA-treated HL-60 cells. Those extracts which proved to possess pro-inflammatory activity were tested for their ability to induce hyperplasia on mammalian skin. In addition those extracts were tested for their ability to induce platelet aggregation in human blood.

2.3.3 Mouse- ear Erythema Test

The use of the mouse-ear erythema assay for extracts of plant species of the families Euphorbiaceae and Thymelaeaceae provided rapid means for the identification of species which are potentially hazardous in terms of human cancer. This technique was used throughout the fractionation of plant extracts as a biological-guide to isolate the toxic constituents. This test was also used for screening extracts for pro-inflammatory effect. Four CD-1 female mice (supplied by Charls-River) were housed together on woodsaw dust as bedding. They were fed standard food and water and were kept at a temperature of 35°C in 41% humid atmosphere.

Preparation of solutions

Solutions for the test were prepared by dissolving an accurately weighed (using Sartorius balance) 5mg of each extract into 1ml of redistilled acetone (final concentration 25μg/5μl). Subsequent dilution of 1mg/1ml was prepared to give a concentration of 5μg /5μl.

Procedure

Five micro litres aliquots of the solutions were applied to the inner surface of the right ear of each mouse using a 5μl Drummond Microcap. The left ear was used as a negative control. Extracts were tested at doses of 5, 20 and 100µg/ear. The acetone was left to dry by allowing a few seconds between each application.

The Assay Procedure

The ears were examined after 15 minutes, then for 2 hours at 15 minute intervals and subsequently for 72 hours at an hourly interval. 20μl of acetone were applied to the ears of one group of mice as a negative control. Positive control group received o.5μg TPA (supplied by Sigma UK).

Using negative (-) and positive (+) response redness of ears was assessed on all or non basis. Negative response was recorded for any ear showing no difference from the negative control (the left ear). Positive (+) response corresponded to vasodilatation of the major blood vessels of the treated ear, (++) corresponded to vasodilation and remarked redness of the ear between the blood vessels while (+++) corresponded to complete redness of the ear accompanied by oedema. The same procedure was repeated three times to ensure reproducibility of the assay. No mouse was treated more than once. After each experiment mice were disposed off by the animal house personnel using the authorised Home Office schedule-1 procedure.

2.3.4 Platelet Aggregation Test

Blood Collection

Fifty millilitres of venous blood from healthy male donors, who denied taking any medication for the previous 2 weeks, were collected into a 60 ml plastic sterile syringe (supplied by Becton Dickinson) which contains 5ml citrate buffer. The final concentration of the buffer was 10% i.e. 1 part buffer to 9 parts of blood. The blood was incubated at room temperature for fifteen minutes.

Preparation of Citrate Buffer

The citrate buffer contains citric acid 90 millimole (189.13 mg), trisodium citrate 70 millimole (294.10 mg) and glucose 100 millimole (180.2 mg). All were dissolved in 10 ml Millipore calcium-free water (supplied by BDH).

Preparation of PPP and PRP

Blood was centrifuged at room temperature at a speed of 200 gravity for a period of twenty minutes. This was carried out in Falcon tubes containing equal amounts of blood placed at opposite sides of the centrifuge to ensure equal balance. As a result the

blood sample was separated into a yellow upper layer, a middle buffy layer and a lower deep red layer. The supernatant was transferred into a clean Falcon tubes and re-centrifuged at 280 gravity for 10 minutes to remove any residual red and white blood cells. The resultant supernatant was used as the platelet rich plasma (PRP).

Platelet poor plasma (PPP) was obtained from the dark red layer re-spun for 20 minutes at 2700 gravity. The supernatant was transferred into a clean Falcon tube. To avoid any contamination the whole procedure was carried out in a laminar flow cabinet

Adjustment of The Aggregometer

A Payton dual channel Aggregometer was adjusted for 0% light transmission using PRP. PPP was used to adjust the machine for 100% light transmission by following the manual instructions. The aggregometer was switched on, then the heater and the recorder. The temperature knob was adjusted to 37°C and allowed to warm up for 20 minutes. The module range was adjusted to 4. The revolutions of the machine were also adjusted to 900 per minute. 0% transmission was adjusted using the output knob while the 100% transmission was adjusted using the zero knob. The recorder input was turned off to protect the instrument when changing cuvettes.

Platelets Aggregation Procedure

A volume of 500μl of PRP was placed in the cuvette together with a metal stirrer. The PRP was then incubated in the channel for 2 minutes prior to its use in the assay to allow the platelets to warm up to 37°C. Solutions of plant extracts were prepared in redistilled acetone to give final concentrations of 5mg/ml and 1mg/1ml.

10μM (micro molar) of TPA (12-O-tetradecanoylphorbol-13-acetate) was used as a positive aggregation response. Extracts were tested at 10, 50 and 200μg/ml doses. Since higher concntrations were found to induce platelet aggregation, acetone concentrations was never more than 10% during the assay. Samples were tested for platelet aggregation at 4 and 8 minute intervals. Aggregation response was taken as a percentage compared to the standard effect produced by PRP as 0% and PPP as 100%.

2.3.5 HL-60 Cell Differentiation Test

Cell Source and Routine Maintenance

HL-60 cells were grown in RPMI 1640 medium (supplied by ICN International)

and supplemented by 15% heat inactivated foetal calf serum, 2% glutamine and 5% of gentamycin (all supplied by Gibco, UK). The cells were placed in 250 cm^3 plastic tissue culture flasks and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were maintained at varying concentrations between 0.1 and 2 x 10^6 cell/ml by twice a week passage of cultures. Under these conditions, the doubling time of cells was found to be forty eight to fifty five hours. The cell line HL-60 was donated by Professor P. Parker of ICRF London.

Preparation of Test Extracts

Plant extracts were dissolved in acetone to obtain final concentrations of 100μg/5μl for crude extracts and 20μg/5μl for ether, hexane and methanol extracts.

The Assay Procedure

On day one cell cultures were diluted in fresh media to give a final concentration of 1x10^5 cells/ml counted on a two channel haemocytometer. Using a Gilson pipette, a 665μl aliquot of cell suspension was dispensed into 2ml sterile epindroph tube. 35μl of the plant extracts were added to obtain a final concentrations of 200μg/200μl of crude extracts and 40μg/200μl for other fractions. Two fold serial dilutions were carried out in 200μl of cell suspension and then placed in sterile 96-well micro titre plates (supplied by Linbro, Flow Labs Inc). Final concentrations achieved for crude extracts were 500-7.5μg/1ml while those for other fractions were 100-0.75μg/ml.

A total of six negative control wells, containing only cell suspension, and 6 positive control standard wells, containing 10μM of TPA and cell suspension were simultaneously used. 10μl acetone were added to 6 wells to eliminate any effect of acetone on the cells. In addition, control wells with media were used in each plate.

Plates were covered with lids and incubated for forty eight hours under the same conditions as previously mentioned. Experiments were conducted in triplicate wells and were repeated three times to confirm reproducibility. After incubation, macrophage-like differentiation of HL-60 cell cultures was assessed by observation of cell morphology and adhesion. Initially, microscopic examination was conducted on cell cultures to identify cell clumping, adherence and appearance of pseudopodia by gentle pipetting of culture in each well.

Macrophage-like patterns were confirmed using Giemsa stain (supplied by Sigma UK). Giemsa stain procedure was performed by placing an air dried culture film in undiluted Giemsa stain for three minutes. It was then placed in distilled water for four minutes, rinsed with water, air dried and examined under the microscope. The nucleus was stained dark blue to violet and the background cytoplasm was pale blue. Viability of cells was assessed by Trypan blue exclusion test. This was conducted by transferring 200μl of cell suspension mixed thoroughly and allowed to stand for 10 minutes. 30 μl of Trypan blue cell suspension was transferred to a haemacytometer. Stained blue, non viable cells were counted. Viability of healthy culture was never less than 90-95%.

2.3.6 Induction of Hyperplasia on CD-1 Mouse Skin

Preparation of Plant Extract Solutions

Crude extracts were dissolved in acetone to a concentration of 100μg/200μl (0.5mg/1ml). Ether fraction solutions were prepared to give a final concentration of 20μg/200μl (0.1mg/1ml).

Animals

Ten weeks old CD-1 female mice were climatized in the animal house. The four mice in each cage were provided with standard food and drink. The dorsal surface was shaved using a hand held clipper. Two millilitres Imac ® paste diluted with 10ml water was applied to the mice's back to remove any traces of hair. Five minutes later, the area was washed and dried throughly.

The Assay Procedure

Plant extracts were applied to the 1cm^3 prepared area in 200μl acetone. As a positive control, one group of four mice received 20nM TPA. Another group received 200μl of acetone as a negative control. The mice were sacrificed after 48 hours and their skin was removed and placed in 10% formaldehyde solution. The skin was then sent for staining and sectioning. Paraffin sections (5-6μm) were stained with haemoxylin and eosin and photographed at 400 x magnification.

2.4 Results And Discussion

The present study evaluated thirty four species from the family Euphorbiaceae and six samples from the family Thymelaeaceae in four short term *in vivo* and *in vitro*

biological assays. In order to detect a range of classical biological effects induced by tumour promoting phorbol esters.The tests used were inflammation, differentiation of HL-60 cells, platelets aggregation and skin hyperplasia. The aim was to provide a scientific background for assessment of the possible risk factors of the promoter type associated with human exposure to plant material. Biological tests were based on the fact that tumour-promoters, of the phorbol esters type, induce a range of effects including irritation of the mouse ear, adhesion and induction of differentiation of HL-60 cells, induction of platelet aggregation and epidermal hyperplasia in mouse-skin (Evans and Schmidt 1978, Brooks *et al* 1989, Moore 1983).

Pro-inflammatory activity:

Different plant extracts that induce inflammation on the mouse ear was the first test used to detect the presence of the irritant diterpenes in those plant extracts. This procedure was originally devised by Hecker in 1971 (Hecker 1971a) and was later modified by Kinghorn in order to screen species of the genus *Euphorbia* for irritant activity (Kinghorn and Evans 1975). The test was finally refined by Evans and Schmidt for testing comparative irritancy of tigliane and daphnane esters (Evans and Schmidt 1979).

Pro-inflammatory activity was recorded on an all or none basis. This means that the irritant effect was recorded as positive only if inflammation was induced in all animals within the test group. Table 2.3, pp. 63, lists the time at which maximum irritant effect was evident (t-peak) and the duration of both the maximum inflammatory effect and the duration of the irritant effect.

It was evident that at the same dose levels ether fractions were more active than their corresponding crude extracts. This distinction of activity is due to the fact that the diterpene phorbol esters were concentrated in the ether fraction during the partition procedure. In addition, the irritant effect was found to be dose dependent. This means the degree and the persistence of inflammatory effect was directly proportional to the applied dose. Moreover, the onset of irritant effect was inversely proportional to the dose. For example, within five hours of application of 5µg dose per ear the crude extract of the roots of *Gnidia kraussianus* induced vasodilatation of the major blood vessels in the ear.

The same effect was induced within 4 hours when 20µg dose was applied to the ear and within 30 minutes in the case of 100µg/ear. The maximum inflammatory effect induced by the 5µg dose was reached after 18 hours in the forms of vasodilatation of the blood vessels and redness of the ear. This effect, however, disappeared completely after five hours. In comparison, the time for peak inflammatory effect induced by 100µg/ear in the form of vasodilatation, redness and oedema was nine hours. Whilst the effect persisted for more than 96 hours, the onset of irritancy for the ether fraction was 2 hours for 5µg/ear, 1 hour for 20µg/ear and 15 minutes for 100µg/ear. Comparing this data to that of the crude extract, it is clear that the onset of action is shorter for the ether fraction.

Inflammatory activity was detected throughout the roots, stems and leaves of *Gnidia kraussianus*. Extracts from the roots were the most active of all parts of this species. This suggested that the toxic diterpene esters were either abundant in the organ or they are of different nature compared to those in the stems or the leaves. A review of the phytochemical literature on this genus revealed that interest drawn to other *Gnidia* species was due to the isolation of series of antilaeukemic daphnane type from *Gnidia lamprantha, G. subcordata* (Kupchan *et al* 1975 and 1976). A decade later, the research for antineoplastics from natural source led to the isolation of a number of diterpene esters from the roots of *G. kraussianus* Meisner. These were of similar structures to those previously reported by Kupchan and co-workers (Borris and Cordell 1984). Despite the fact that extracts of the roots of this species were found to be highly toxic to P-388 lymphocytic leukaemia cells, neither the extracts nor the pure compounds were tested for irritant activity.

The crude extract and the ether fraction of the *Lasiosiphon kraussianus* herb, a closely related species to *G. kraussianus* Meisner. were found to be more irritant. The effect was more persistent than the extracts from stems and leaves of *G. kraussianus*. An applied dose of 5µg/ear of crude extract induced vasodilatation within ten hours. The vasodilatation progressed to redness of the whole ear which persisted for up to thirty hours. The pro-inflammatory activity of this species could be attributed to a group of compounds based on 12-hydroxydaphentoxin nucleus which is known to be irritant as reported by Evans and Taylor (1983). This type of compounds was isolated from

Lasiosiphon burchelli Meisner. leaves (Coetzer and Pieterse 1974, Schmidt 1986a).

Daphne species were reported to be poisonous and toxic to both humans and animals (Forsyth 1968, Clapham *et al* 1968b). These properties were confirmed following the isolation of irritant constituents such as mezerein, daphentoxin and other daphentoxin derivatives from *Daphne mezereum* L. and *D. genkwa* (Stout *et al* 1970, Ronlan and Wickberg 1970, Kasai *et al* 1981).

Crude extract of the twigs and leaves of *Daphne blagayana* induced severe inflammatory effect on the mouse ear at all applied dose levels (Table 2.3, pp. 63). After twenty four hours from exposure to 5µg/ear, the maximum effect induced by this dose was redness and vasodilatation. This effect decreased gradually but completely disappeared eighteen hours later.

The Egyptian plant *Thymelaea hirsuta* was found to be very irritant to the mouse skin. Vasodilatation of the major blood vessels in the ear was visible within 8 hours following application of 5µg of the crude extract. The same dose of the ether fraction induced the same effect within only one hour. This effect progressed to strongly visible erythema of the ear within six hours. By increasing the dose of the ether fraction to 100µg/ear severe inflammation was induced in 1 hour. This was characterized by redness and oedema of the ear. However, unlike ether fractions of the other species from the Thymelaeaceae, the effect of the maximum applied dose of 100µg/ear *Thymelaea hirsuta* only lasted for forty eight hours.

Plant material from the Thymelaeaceae species was re-extracted 1 and 2 years after the initial extraction. Although it remained irritant to the mouse skin at 20 and 100µg/ear it was not at 5µg indicating the presence of reasonably stable biologically active constituents.

The screening procedure included thirty four different samples from the family Euphorbiaceae from which the first phorbol ester was isolated by Hecker in 1971. The majority of the plant material belong to the genus *Euphorbia*. When using doses of 20, 100 or 200µg/ear both crude extracts and ether fractions failed to induce any sign of

inflammatory effect in the mouse ear erythema test.

Seeds of *Euphorbia lathyris*, *E. helioscopia*, *E. peplus* and *E. myrsinites* were amongst the tested species. Extracts of plant material and latices of these species were reported to induce inflammation to the mouse skin at doses between 3 and 100µg/ear within 4 to 24 hours (Evans and Kinghorn 1977, 1975). Crude extracts of *Euphorbia exigua*, *E. dulcis* and the latex of *E. virgata* were reported to be irritant to mammalian skin (Schmidt and Evans 1980a, Itokawa 1989, Rizk *et al* 1985 and Upadyay *et al* 1980 and 1981). Interpretation from both this research and literature reports have been based upon the age and part of plant material under investigation.

Whilst extracts of the seeds were used in this study, previous studies used extracts from the latex, aerial part or even the whole plant which were found to contain the irritant phorbol esters. Furthermore, the majority of plant material for the current screening was collected at different periods between 1966 and 1982 and stored under the ideal cold, dark and dry conditions.

The irritant constituents isolated from different species of the genus *Euphorbia* were based on the tigliane derivatives, 12-hydroxyphorbol, and on the ingenane type ingenol (Evans and Kinghorn 1974b, Evans 1986a). Although the parent alcohols, phorbol and ingenol, were devoid of irritant activity (Evans and Soper 1979, Evans and Schmidt 1979a) esterified derivatives of 1-hydroxyphorbol and 5-deoxyingenol were highly irritant to mammalian skin. However, it was recently reported that irritant activity of plant material containing ingenane and tigliane types could be lost during storage and in some occasions during handling due to auto-oxidative destruction (Karalai and Hecker 1994).

Despite the extreme care taken in handling the frozen unripe fruits of *Elaephorbia drupifera* Stapf., a closely related genus to *Euphorbia* (Webster 1987), neither the crude nor the ether fraction was found to be irritant to the mouse ear. Repeatedly, ingenol derivatives were reported to be the major irritants from the latex of this plant with an ID_{50} of 7µg/ear (Evans and Kinghorn 1977, Kinghorn and Evans 1975).

In Thailand and other countries in South East Asia and Africa *Excoecaria* species

are well known for their extreme skin irritant and caustic properties especially residing in their latices (Karalai and Hecker 1994). In this study, extracts of the seeds of *Excoecaria crenulata* showed no irritant effect on the mouse ear. Phytochemical investigations of other *Excoecaria* species reported the isolation of cryptic irritants, diterpene esters devoid of irritant activity (Wiriyachitra *et al* 1985). It was also reported that irritant activity disappeared during handling of the latex by oxidative process of the *Excoecaria* factors (Karalai and Hecker 1994).

As pro-inflammatory agents extracts from the twigs and leaves of *Sapium insigne* were inactive. However, extracts from the frozen latex of *Sapium indicum* induced severe inflammation at 5µg/ear. Taylor reported the isolation of unstable irritant 4-deoxyphorbol aldehyde derivatives from the latter species (Taylor *et al* 1981) as well as 4-deoxy-5-hydroxyphorbol derivatives, irritant at 0.01µg/ear (Evans *et al* 1981a). Two years later, several irritant phorbol derivatives were isolated from *Sapium insigne* (Taylor *et al* 1983). Phorbol esters are known to be sensitive to light, moisture, acids, alkali and even oxygen (Evans 1986a). The 4-deoxyphorbol series of ester,the major irritant constituents from the genus *Sapium*, are not only chemically unstable but also convert to biologically inactive 4-α-deoxy epimers on prolonged storage (Evans and Taylor 1983, Evans 1986b).

Extracts of the seeds of the annual mercury plant, *Mercurialis annua* L., produced no inflammatory effect on the mouse ear for doses up to 200µg/ear. All parts of this plant are known to be poisonous. Toxicity was reported to be lost with age and destroyed by drying and boiling (North 1967).

Extracts from *Dalechampia roezliana* Dell Arg., *Alchornia cordifolia, Acaylpha indica* and *Phyllanthus muellerianus* were found to be non-irritant to the mouse skin. These findings agree with the phorbol-12,13-dibutyrate (PDBU) binding assay which concluded that these four genera have no phorbol-like activity (Butler *et al* 1989). Moreover, a review of the phytochemical literature did not provide any evidence for the presence of toxic diterpene esters in any of these genera (Adesida 1972, Evans 1986b, Mukherjee 1967).

At doses up to 100µg/ear, extracts from the genus *Jatropha* were inactive as pro-

inflammatory agents. Phytochemical investigations of this genus concluded that the 12-deoxy-16-hydroxyphorbol derivatives are the main irritant constituents of its seed's oil (Adolf et al 1984). Samples of different organs of *Jatropha gossypifolia* and *J. glandilifero* were collected from Nigeria in 1967 and 1974 and from India in 1982. The lack or irritancy in these samples suggest that the 12-deoxyphorbol esters may have decomposed during storage.

Methanol fractions, sodium chloride and sodium carbonate washes of all species of both families were found to be inactive in the mouse-ear erythema test. However, hexane fractions of the Thymelaeaceous plants were found to be irritant to the mouse ear suggesting the presence of lipophilic irritants.

Induction of HL-60 cell differentiation:

Irritant tumour-promoters induce aggregation and differentiation of HL-60 promyelocytic leukaemia cells (Rovera *et al* 1979, Moore 1983, Huberman and Callaham 1979). Serial dilutions of plant extracts were incubated with HL-60 cells for 48 hours. Plant extracts were also tested for their ability to inhibit differentiation induced by TPA, which is a compound known to induce differentiation at nanomolar concentrations. Results are shown in Table 2.4, pp. 64.

Extracts from different parts of *Gnidia kraussianus* were able to induce differentiation of cells into macrophage-like cells. This was at the low concentration of 7.8µg/ml in the case of crude extracts and at 0.78µg/ml for ether fractions. However, when extracts were added to TPA-treated cells, an inhibitor effect on cell differentiation was detected only at high concentrations between 75 and 500 µg/ml for both ether fractions and crude extracts (Table 2.5, pp. 65). Notably, methanol fractions of the Thymelaeaceae showed inhibitory activity on TPA-treated cells but were devoid of differentiating properties on TPA-free cells. The crude extract and the ether fraction of *Lasiosiphon kraussianus* induced cell differentiation of HL-60 cells. It was noted that addition of ether fraction to TPA-treated cells prevented differentiation at all concentrations between 125 and 0.78 µg/ml. In the case of the crude extract inhibitory effect on cell differentiation was detected only at high concentrations of 31-500µg/ml.

Ether fractions of *Daphne blagayana* and *Thymelaea hirsuta* induced cell differentiation even at the lowest concentration of 0.78µg/ml. For the former species no inhibitory effect was evident on TPA-induced differentiation except at concentrations of 125 and 500µg/ml. Neither the crude extracts nor the ether fractions of the Euphorbiaceous species were able to induce cell differentiation.

Platelet aggregation:

In addition to the pro-inflammatory and tumour-promoting activities, phorbol esters would induce a dose dependent aggregation of human blood platelets as demonstrated by Zucker and co-workers (Zucker *et al* 1974). Plant extracts were investigated for their aggregating ability from a range of concentrations over 4, 8 and 12 minutes. Table 2.3, pp. 63, and Table 2.6, pp. 66, show that extracts found to be irritant to mammalian skin were able to induce platelet aggregation at concentrations as low as 5µg/500µl which is the same dose level found to be irritant to the mouse ear

Epidermal hyperplasia:

Epidermal hyperplasia has been linked to the early stages of the tumour promotion process (Marks and Frustenberger 1984). Whilst all tumour-promoters of the diterpene ester type are skin irritants, the contrary does not appear to be the case (Kinghorn 1983). Crude extracts and ether fractions of the Thymelaeaceae were tested for hyperplastic activity at 100 and 20 µg respectively. TPA was used as a posituve control at a dose of 50µg. Extracts from *Gnidia kraussianus* and *Lasiosiphon kraussianus* induced significant hyperplasia of CD-1 mouse epidermis (Figure 2.3 C -H, pp. 68-71). However, extracts from *Daphne blagayana* and *Thymelaea hirsuta* failed to induce the hyperplastic response (Figure 2.3 I, J, K & L, pp. 71 & 72). Although tumour promotion is more than sustained hyperplasia, these observations provided preliminary indications of potential tumour-promoting activity of these plants.

Compared to previously published data, on toxic effects of diterpene esters isolated from the families Euphorbiaceae and Thymelaeaceae (Evans 1986, Evans and Taylor *et al* 1983, Kinghorn 1985), this study showed that extracts of species from the family Thymelaeaceae were able at microgramme levels to induce certain biological effects similar to those induced by pure tumour-promoting diterpene esters. However,

extracts from the family Euphorbiaceae, with the exception of *Sapium indicum*, were found to be inactive in all conducted biological tests, mainly due to inactivation on storage.

According to reports on the irritant activity of Euphorbiaceous species, ingenane and tigliane esters appear to be the major toxic constituents (Evans 1986a, Hecker 1971b, Upaday *et al* 1980, Schmidt 1986b). Daphnane orthoesters were reported to be the major toxic constituents isolated from members of both the families Euphorbiaceae and Thymelaeaceae but appear to be more widely distributed in the latter (Schmidt 1986a, Adolf and Hecker 1977). On prolonged storage, daphnane derivatives were reported to be chemically more stable than ingenane and tigliane types (Evans and Taylor 1983, Evans and Soper 1979). The 4-deoxyphorbol series convert to the biologically inactive 4α- deoxy epimers on prolonged storage (Evans and Taylor 1983). Ingenane and highly unsaturated daphnane esters were subjected to auto-oxidation during handling or storing (Karalai and Hecker 1994).

Biological data obtained from this study demonstrated that irritant and toxic properties of the Euphorbials are lost during drying and on prolonged storage. In contrast, tests conducted two years after collecting and storing Thymelaeaceous plants confirmed that toxic constituents, more likely to be daphnane type, are stable and can remain so for many months at room temperature and even after extraction. In addition, when stored under nitrogen at sub-zero temperatures for over a five year period, biological activities of the 4-deoxyphorbol esters from *Sapium indicum* were unaffected. These findings are consistent with previous reports on the stability of phorbol derivatives (Brooks *et al* 1989).

Different parts of the species *Gnidia kraussianus* showed that toxic constituents are widely distributed within this plant. The ability of the extracts to induce platelet aggregation and differentiation correlate well with their capabilities to induce mouse skin inflammation but not necessarily epidermal hyperplasia as in the case of *Daphne blagayana* and *Thymelaea hirsuta* (Table 2.3, pp. 63 and Figure 2.3, pp. 67-72).

Thymelaea's crude extract and ether fraction were unable to induce hyperplasia on the mouse skin. Brooks and co workers have investigated the tumour promoting and

hyperplastic properties of thymelaeatoxin isolated from *Thymelaea hirsuta*. The compound was found to have the ability to induce epidermal hyperplasia at 10µg and to be a second stage tumour-promoting agent in CD-1 female mouse skin (Brooks *et al* 1989). The lack of hyperplastic activity of both the crude extract and the ether fraction could be attributed to many factors. The dose applied to the skin may not have had an adequate biologically effective quantity of the compound. Other compounds, e.g. steroids, in the extract may have interfered with the compound at the receptor level. The latter explanation could apply to the platelets aggregatory response to high concentrations of some plant extracts. Table 2.5, pp.65, shows that 50µg/ml of *Daphne blagayana* crude extract and ether fraction did not induce any effect over eight minutes period, while 10µg/ml induced 70% platelets aggregation. High concentration of plant extracts may contain enough quantities of steroids or other compounds which interfere or block phorbol esters-induced platelet aggregation

Studies on human skin revealed that there is a correlation between results from animal experiments and toxicological effects on humans (Kinghorn 1985). The systematic toxicity of TPA (12-O-tetradecanoylphorbol-13-acetate) following its application to mouse skin was reported by Loomis 1980. He indicated that TPA was absorbed through the skin and translocated to the liver inducing ornithine decarboxylase which is one of the biological effects induced by tumour promoting phorbol esters. Progressive circulatory disease in cattle and mice following chronic exposure to the genus *Pimelea*, family Thymelaeaceae, was reported in Australia (Kelly and Seawright 1978). Other reports have pointed out that co-carcinogenic diterpenoids of the tigliane, daphnane and ingenane derivatives may occur in herbal teas, plant drugs, household and garden ornamental plants and in honey collected from bees fed on nectar from Euphorbiaceous plants (Hecker 1981 & 1987, Der Madersian 1979, Ito 1984).

To what extent do *in vivo* and *in vitro* tests on the mouse skin correlate with toxicity in humans remains the subject of cancer and toxicological research. However, the results of experimental investigations of different co-carcinogens in several target tissues have to be seriously considered.

Table 2.2 Botanical Classification of Plant Species Used in Biological Screening

Subfamily	Genus	Species	Organ	Source / Date
A: Family *Euphorbiaceae				
1.Cortonoideae	*Jatropha*	*curcas*	twigs & leaves (2 samples)	Nigeria /1974
		glandilifero	fruits	India /1982
		gossypiifolia	twigs & leaves	India /1967
			seeds	India / 1974
2. Euphorbioideae	*Excoecaria*	*crenulata*	seeds	Nigeria /1974
	Sapium	*insigne*	twigs & leaves	SOP Herbarium
		indicum	latex	Pakistan / 1980
	Euphorbia	*viregata*	seeds	Bangladish/1967
		terracina L.	seeds	Portugal /1966
		pterococca Brot	seeds	Portugal /1966
		vapoa studii	seeds	Portugal /1966
		peplus L. (petty spurge)	seeds	Portugal /1966
		dulcis L.	seeds	SOP Herbarium
		amygdaloides (Wood spurge)	seeds	SOP Herbarium
		exigua (Dwarf spurge)	seeds	Portugal /1966
		helioscopia	seeds	Portugal /1966
		lathyris	seeds	Portugal /1966
		myrisinites L.	seeds	Portugal /1966
			seeds	Portugal /1966
	Elaeophorbia	*drupifera* Stapf.	fruits (frozen)	Nigeria /1993
3. Phylanthoideae	*Phyllanthus*	*muellerianus*	twigs & leaves	Nigeria /1974
			roots (2 samples)	Nigeria /1974
	Alchornia	*cordifolia* (Schum &Thonn)	fruits	Nigeria /1974
4. Acaylphoideae			fruits/ stems (frozen)	Nigeria /1993
			fruits	Nigeria /1974
	Acalypha	*indica*	roots	Pakistan /1982
			roots (ether extract)	Pakistan /1982
			leaves (ether extract)	Pakistan /1982
	Dalechampia	*roezliana* Dell. Arg.	leaves	India /1967
			seeds	SOP Herbarium
B: Family **Thymelaeaceae				
Thymelaeoideae	*Thymelaea*	*hirsuta*	herb	Egypt /1991
	Daphne	*blagayana*	herb	Yugoslavia/1993
	Gnidia	*kraussianus*	roots	Nigeria /1993
			leaves	Nigeria /1993
			stems	Nigeria /1993
	Lasiosiphon	*kraussianus*	herb	Nigeria /1993

*Classified according to Webster 1975 & 1987 SOP = The School of Pharmacy
**Classified according to Hutchinson 1967

Figure 2.1 Extraction and Partition of Plant Material

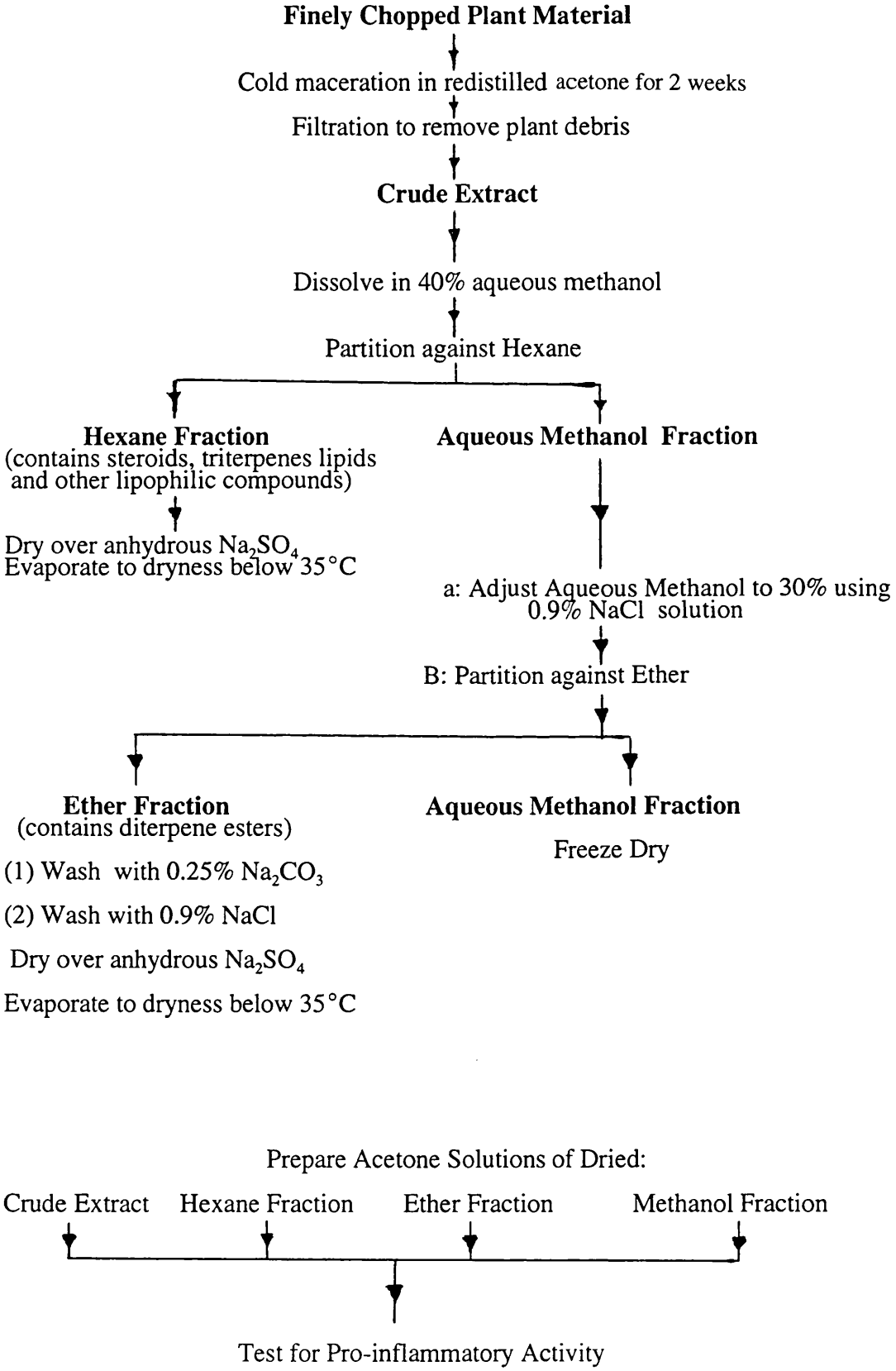


Figure 2.2 A Photograph of the twigs, leaves and flower of *Daphne blagayana*

Species	Organ	Extract	Time to onset of Irritancy (hr)			Degree & time to peak irritancy (hr)			Duration of peak irritancy (hr)			Duration of irritancy (hr)		
			5µg/ear	20µg/ear	100µg/ear	5µg/ ear	20µg/ ear	100µg/ear	5µg/ear	20µg/ear	100µg/ear	5µg/ear	20µg/ear	100µg/ear
Thymelaea hirsuta	herb	crude	8	6	2	8 +	6 +	3 ++	30	36	7	30	36	46
		ether	1	0.5	0.25	6 ++	3 ++	1 +++	6	3	1	23	46	47
		hexane	NI	3	2	NI	3 +	3 ++	NI	1	4	NI	4	21
		methanol	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Gnidia kraussianus	roots	crude	5	4	0.5	18 ++	15 +++	9 +++	2	3	9	23	68	>96
		ether	2	1	0.25	7 ++	7 ++	3 +++	5	17	45	46	77	>93
		hexane	3	3	1	7 ++	7 ++	9 +++	5	7	2	33	43	69
		methanol	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Gnidia kraussianus	stems	crude	21	2	0.5	21 +	21 ++	5 +++	4	3	4	4	28	69
		ether	4	4	0.25	4 +	7 ++	5 +++	3	8	15	41	45	69
		hexane	3	2	2	4 ++	3 ++	5 +++	10	7	2	21	34	46
		methanol	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Gnidia kraussianus	leaves	crude	18	4	1	18 ++	18 +++	10 +++	2	62	14	6	34	47
		ether	6	4	1	7 ++	7 ++	5 ++	3	19	43	42	71	79
		hexane	22	7	5	22 +	22 ++	20 ++	30	2	5	52	68	70
		methanol	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Lasiosiphon kraussianus	herb	crude	10	8	0.75	18 ++	18 +++	10 +++	6	6	38	30	47	>95
		ether	2	1	0.25	10 ++	4 +++	4 +++	10	24	25	46	58	>95
		hexane	NI	1	1	NI	4 +++	4 +++	NI	5	10	NI	71	>95
		methanol	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Daphne blagayana	herb	crude	22	' 16	1	24 ++	21 +++	16 +++	5	27	32	26	56	>95
		ether	3	3	0.5	7 ++	7 +++	5 +++	2	2	23	51	69	>95
		hexane	NI	5	5	NI	6 ++	8 +++	NI	2	5	NI	43	67
		methanol	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Sapium indicum	latex	ether	28	12	5	33 +	16 ++	10 ++	5	3	6	38	49	55

(+) if vasodilatation of the major blood vessels in the ear was observed
(++) if vasodilatation of the blood vessels and redness of the ear in between was observed
(+++) if vasodilatation, redness of the entire ear and oedema was observed (NI) if no sign of irritancy was observed

Table 2.3 Irritant Activity of Plant Extracts

Species	Extract	Concentrations µg /200µl (µg/ml)										
		100	50	25	12.5	6.25	3.13	1.56	1.25	0.63	0.31	0.16
		500	**250**	**125**	**62.5**	**31.2**	**15.6**	**7.81**	**6.25**	**3.13**	**1.6**	**0.78**
Daphne blagayana herb	crude	ND	ND	ND	D	D	D	D	NT	NT	NT	NT
	ether	ND	ND	D	D	D	D	D	D	D	D	D
	hexane	D	D	D	D	D	D	D	D	NT	NT	NT
	methanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Gnidia kraussianus leaves	crude	ND	ND	D	D	D	D	D	NT	NT	NT	NT
	ether	ND	ND	ND	D	D	D	D	D	D	D	D
	hexane	ND	ND	D	D	D	D	D	D	D	D	D
	methanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Gnidia kraussianus Roots	crude	ND	D	D	D	D	D	D	D	NT	NT	NT
	ether	DE	DE	DE	D	D	D	D	D	D	D	D
	hexane	ND	ND	D	D	D	D	D	D	D	D	D
	methanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Gnidia kraussianus stems	crude	ND	ND	ND	ND	ND	ND	ND	NT	NT	NT	NT
	ether	ND	ND	ND	D	D	D	D	D	D	D	D
	hexane	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	methanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lasiosiphon kraussianus herb	crude	ND	ND	D	D	D	D	D	ND	ND	ND	ND
	ether	DE	DE	ND	ND	D	D	D	D	D	D	D
	hexane	D	D	D	D	ND	ND	ND	ND	ND	ND	ND
	methanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Thymelaea hirsuta herb	crude	ND	ND	D	D	D	ND	ND	ND	ND	ND	ND
	ether	ND	D	D	D	D	D	D	D	D	D	D
	hexane	ND	ND	D	D	D	D	D	D	D	D	D
	methanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Results of three experiments conducted in triplets
D= Differentiated cells NT= Not tested
ND= Non differentiated cells DE= Dead cells detected with Trypan blue exclusion test
Differentiation was detected under the microscope for cell clumping, adherence to the wells and macrophage-like patterns
Giemsa stain was used to confirm the presence of macrophage-like cells. Dead cells were detected with trypan blue exclusion test.

Table 2.4 Differentiation of HL-60 Cells Induced by Extracts from the Family Thymelaeaceae

Species	Extract	Concentrations µg /200µl (µg/ml)										
		100	50	25	12.5	6.25	3.13	1.56	1.25	0.63	0.31	0.16
		500	**250**	**125**	**62.5**	**31.2**	**15.6**	**7.81**	**6.25**	**3.13**	**1.6**	**0.78**
Daphne blagayana herb	crude	ND	ND	ND	D	D	D	D	D	NT	NT	NT
	ether	ND	ND	ND	ND	D	D	D	D	D	D	D
	hexane	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	methanol	D	D	D	D	ND	ND	ND	ND	ND	ND	ND
Gnidia kraussianus leaves	crude	ND	ND	D	D	D	D	D	D	NT	NT	NT
	ether	ND	ND	ND	ND	ND	ND	ND	D	D	D	D
	hexane	ND	ND	ND	ND	ND	ND	ND	ND	D	D	D
	methanol	ND	ND	ND	ND	ND	D	D	D	D	D	D
Gnidia kraussianus roots	crude	ND	ND	ND	ND	ND	ND	ND	ND	NT	NT	NT
	ether	ND	ND	ND	ND	ND	D	D	D	D	D	D
	hexane	ND	ND	ND	ND	ND	D	D	D	D	D	D
	methanol	ND	ND	ND	D	D	D	D	D	D	D	D
Gnidia kraussianus stems	crude	ND	ND	D	D	D	D	D	D	D	D	D
	ether	ND	ND	ND	ND	ND	ND	D	D	D	D	D
	hexane	ND	ND	ND	ND	ND	D	D	D	D	D	D
	methanol	ND	ND	ND	ND	ND	ND	ND	D	D	D	D
Lasiosiphon kraussianus herb	crude	ND	ND	ND	ND	ND	D	D	D	NT	NT	NT
	ether	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	hexane	DE	DE	DE	DE	D	D	D	D	D	D	D
	methanol	ND	ND	ND	ND	D	D	D	D	D	D	D
Thymelaea hirsuta herb	crude	ND	ND	D	D	D	D	D	D	NT	NT	NT
	ether	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	hexane	ND	ND	ND	ND	ND	ND	D	D	D	D	D
	methanol	ND	ND	ND	ND	ND	D	D	D	D	D	D

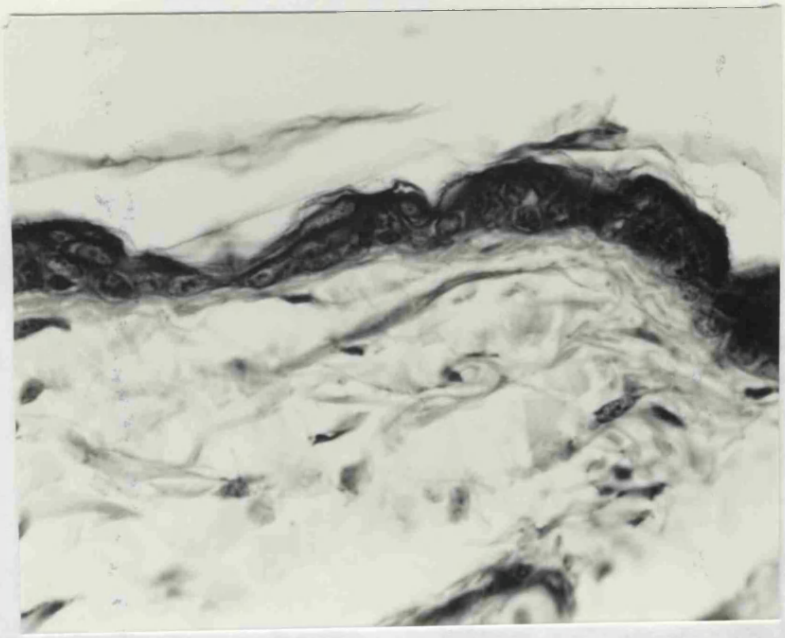
*Results of three experiments conducted in triplets
D= Differentiated cells NT= Not tested
ND=Non differentiated cells DE= Dead cells detected with trypan blue exclusion test
Differentiated cells were detected under the microscope for cell clumping, adherence to the wells and macrophage-like differentiation
Macrophage-like patterns was confirmed using Giemsa stain. Dead cells were detected with trypan blue exclusion test.

Table 2.5 The Effect of Extracts from Thymelaeaceae on TPA-Treated Cells

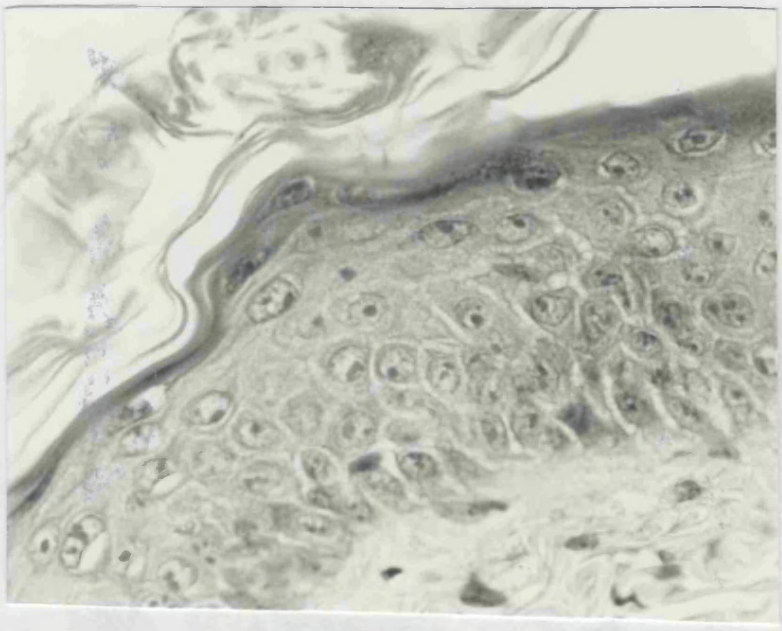
Table 2.6. Platelets Aggregation Induced by Crude Extracts and Ether Fractions from the Family Tymelaeaceae

Species	Fraction / Extract	Concentration (µg/ml)	% Platelets Aggregation			SD +/-
			4Min	8Min	12Min	
Thymelaea hirsuta herb	crude	10	0	0	0	0
		50	34.7	60	60	4.5
	ether	10	59	75	75	1.4
		50	29.8	67.5	67.5	2.25
Gnidia kraussianus roots	crude	10	37.3	70	70	4.6
		50	0	0	0	0
	ether	10	30	60	60	3.6
		50	0	0	0	0
Gnidia kraussianus stems	crude	10	42	70	70.3	3
		50	0	0	0	0
	ether	10	42	60	60	6.65
		50	0	0	0	0
Gnidia kraussianus leaves	crude	50	0	0	0	0
		200	18.3	30	30	7.6
	ether	10	0	0	0	0
		50	21	40	40	1.73
Lasiosiphon kraussianus herb	crude	10	30.67	60	60	1.22
		50	0	0	0	0
	ether	10	27.6	65	65	3.27
		50	0	0	0	0
Daphne blagayana herb	crude	10	16	50	70	6.5
		50	0	0	0	0
	ether	10	27.3	67.5	67.5	2.25
		50	0	0	0	0

SD= Standard Deviation calculated by Probit Analysis Programme



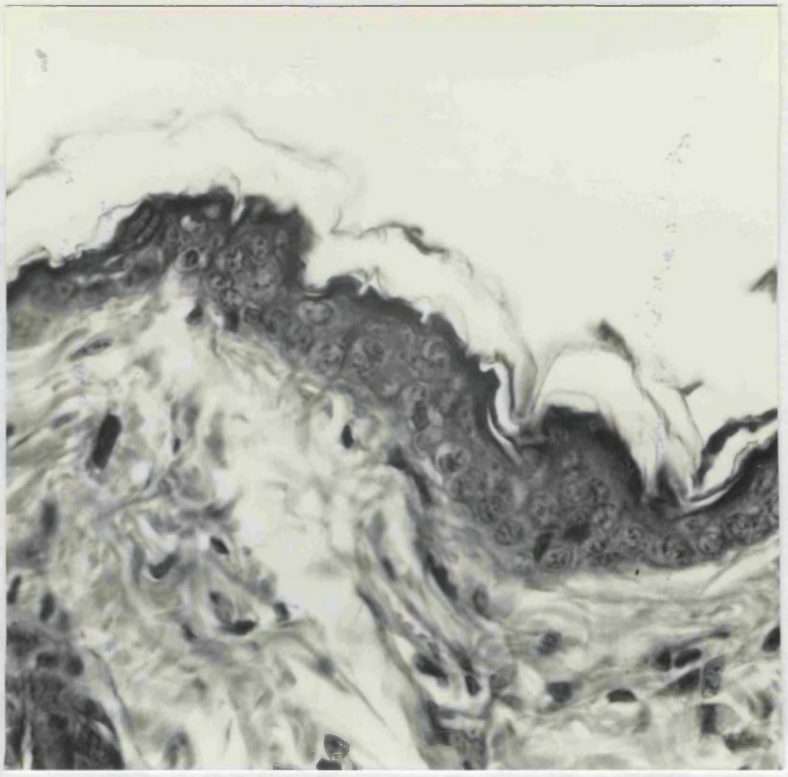
A: Mouse skin 48 hours after 200µl acetone (Negative Control)



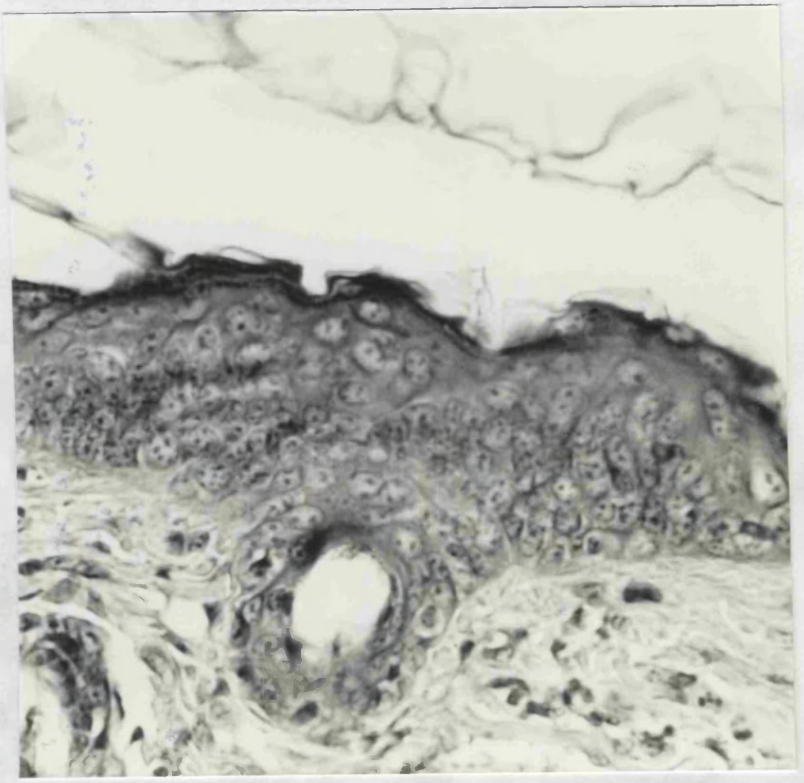
B: Marked hyperplasia and increased keratinization induced by 50nM of TPA (Positive Control)

Figure 2.3 Histological Appearance of Female CD-1 Mice Skin After48 Hours of a single treatment with TPA, Acetone or Plant Extracts

All extracts were applied topically dissolved in 200µl acetone
Stain: Haemotoxylin and Eosin Magnification X 400

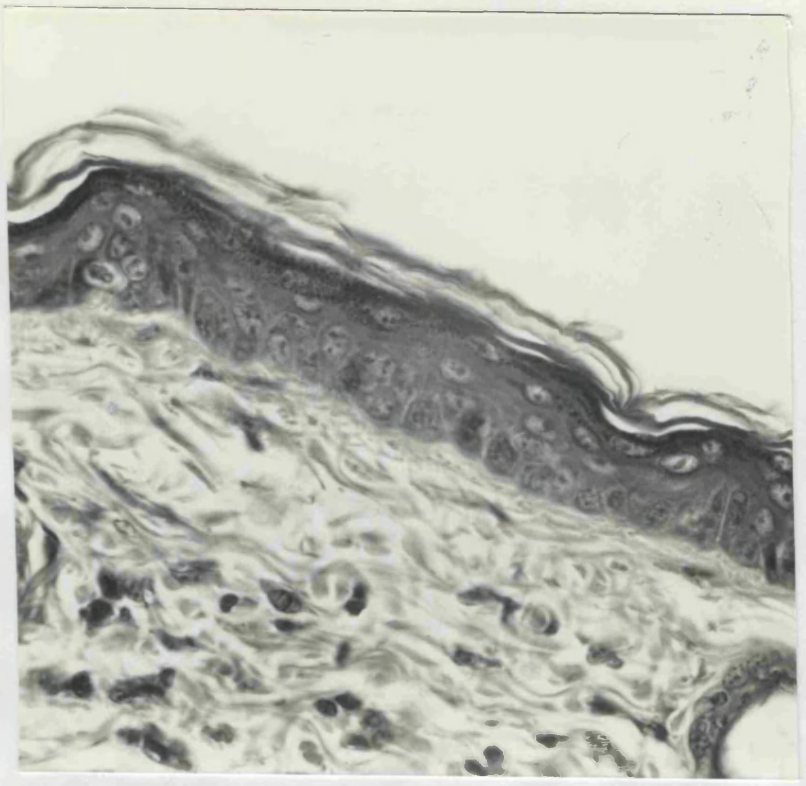


C: Crude Extract 100µg

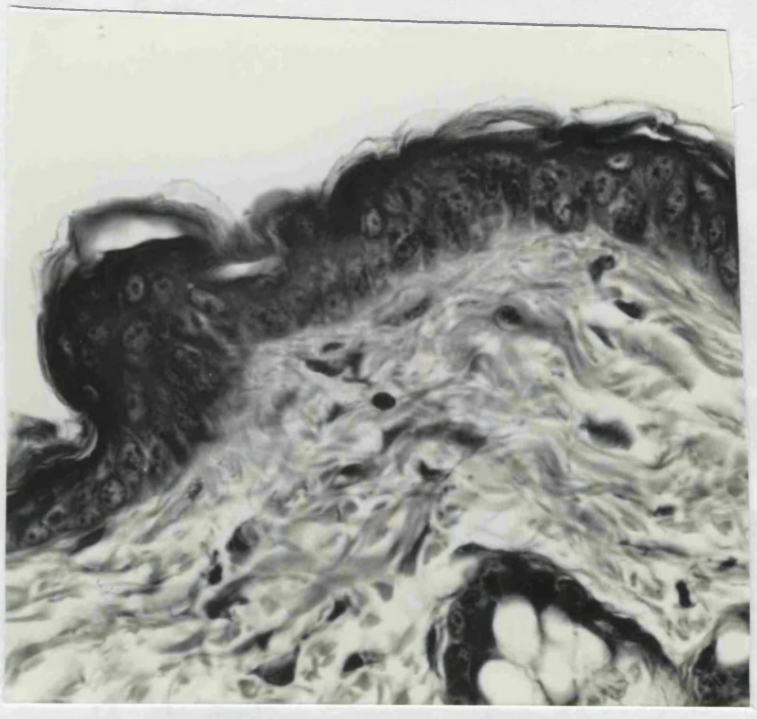


D: Ether fraction 20µg

Figure 2.3 Continued
Increased Keratinization and marked hyperplasia induced by plant extracts from the roots of *Ginidia kraussianus*

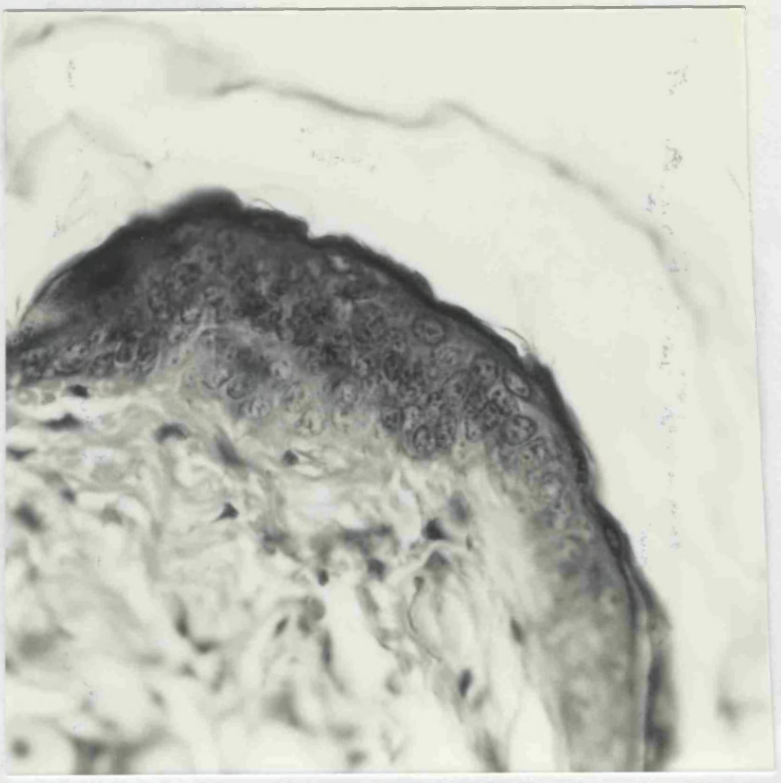


E: Slight hyperplasia induced by 100µg crude extract

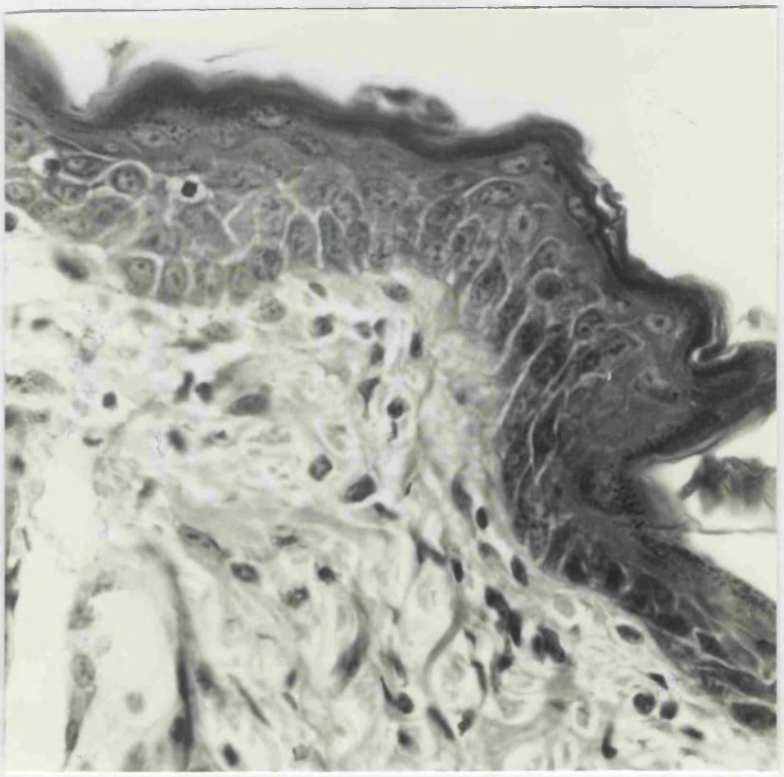


F: Hyperplasia induced by 20µg of ether fraction

Figure 2.3 Continued
Hyperplasia induced by extracts from the stems of *Gnidia kraussianus*

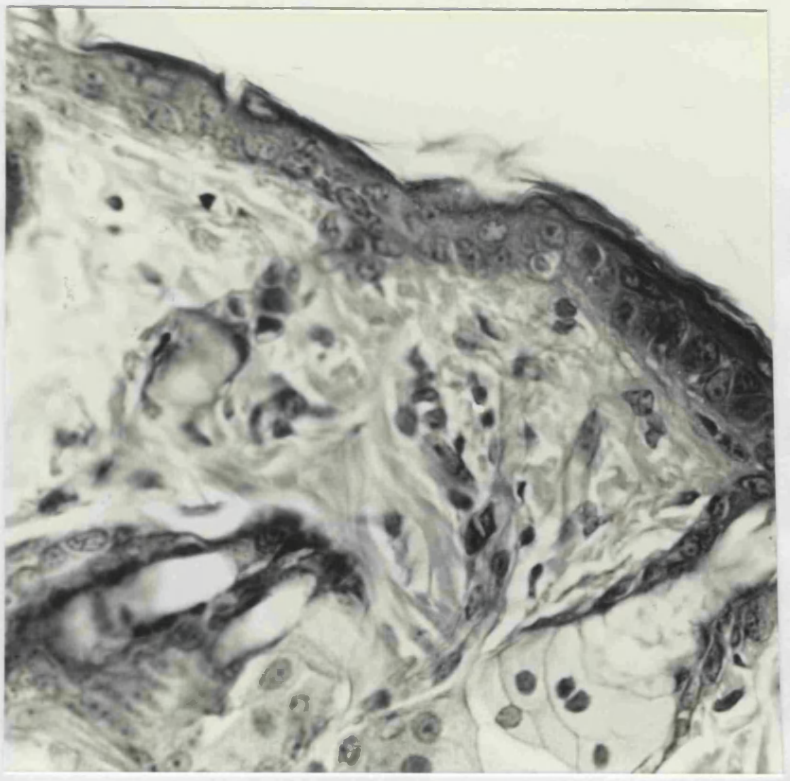


G: Crude extract 100µg

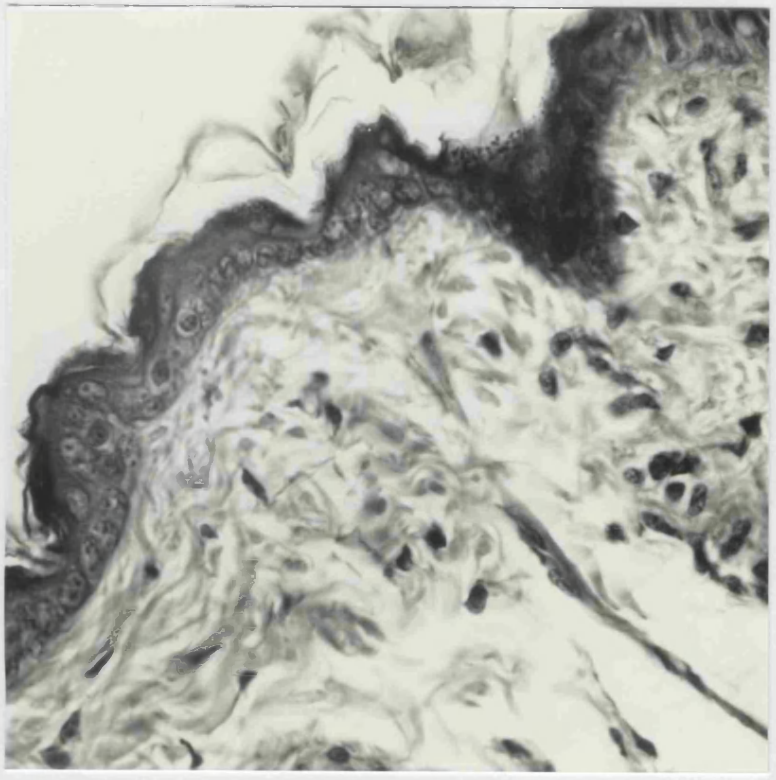


H: Ether fraction 20µg

Figure 2.3 Continued
Marked hyperplasia and increased Keratinization induced by extracts from *Lasiosiphon kraussianus* herb

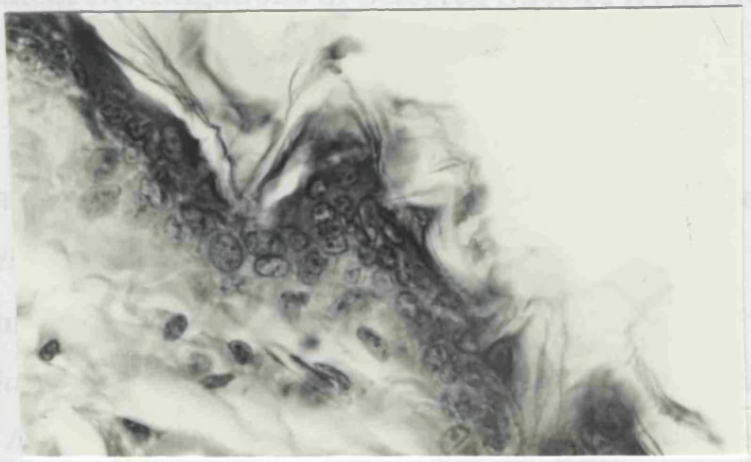


I: Crude extract 100µg

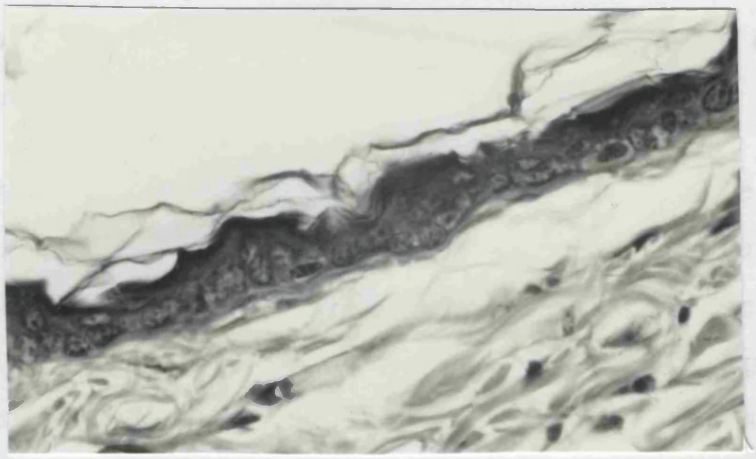


J: Ether fraction 20µg

Figure 2.3 Continued
Normal epidermis (I) and increased keratinization (J) following a single treatment with extracts from *Thymelaea hirsuta* herb



K: Crude extract 100µg



L: Ether fraction 20µg

Figure 2.3 Continued
Increased Keratinization induced by plant extracts from *Daphne blagayana* herb

CHAPTER THREE

Phytochemical Investigations of Selected Species from the families Euphorbiaceae and Thymelaeaceae

3.1 Isolation and Characterization of Daphnane Diterpenes from *Thymelaea hirsuta* L.

3.1.1 Introduction

Thymelaea hirsuta L. is a small and short thymelaeaceous shrub which grows throughout the Arab countries from the western desert of Egypt to Morocco (Rizk *et al* 1984, Boulos 1983). It is the only species of Thymelaeaceae that is indigenous to Egypt (Tackholm 1974). Methnan is the commonly known Arabic name. Other names include metnan and methnan akhdar in addition to the French name passerine (Boulos 1983). Fibres of the bark are tough. They are used for the manufacture of good quality paper (Cordell 1988). The plant is reputed to be toxic to both humans and animals. However, a decoction prepared from the leaves is used as an anthelmintic, powerful hydragogue, cathartic, expectorant and for dandruff treatment (Zohary 1962).

According to the proposed classification by Wagenitz, the tribe Thymelaea belongs to the subfamily Thymelaeoideae to which *Thymelaea hirsuta* L. belongs (Cordell 1988). Previous chemical investigations of this plant revealed the presence of toxic 12-hydroxy daphnane orthoesters (Brooks *et al* 1990). These compounds were reported to induce intense inflammation of the skin, the eyes and the mucous membranes specially around the mouth (Rizk *et al* 1984).

This study provided the opportunity to examine extracts from the Egyptian shrub *Thymelaea hirsuta* L. for a more complete biological evaluation in different *in vivo and in vitro* systems. Furthermore, two daphnane orthoesters were purified from small quantities of frozen extracts with the aid of normal phase HPLC. The structures and stereochemistry of the isolated compounds were elucidated using data obtained from two-dimensional NMR experiments including COSY, NOESY and C-H correlations.

3.1.2 Extraction of Plant Material and Isolation of the Irritant Diterpenes:

Leaves and twigs were collected in the Egyptian western desert in 1988 and 1990. Plant material was air dried and finely chopped using an electric mill. Powdered plant material was exhaustively extracted by cold maceration in acetone at room temperature for one week. Following filtration and removal of plant debris, acetone was removed by reduced pressure distillation below 40°C to yield a green tar-like crude extract which was dissolved in 40% methanol with the aid of gentle heat at 50°C. The methanol solution was partitioned with hexane to remove green pigments and lipids. The aqueous methanol phase was re-extracted three times with diethyl ether. The ether phase was washed with 1% sodium carbonate to remove any remaining pigment followed by 0.5% NaCl solution. Finally, all fractions were dried over anhydrous sodium sulphate and solvents were evaporated by distillation in vacuo at 40°C. All fractions were kept under a blanket of nitrogen at -20°C. Both ether and hexane fractions showed marked inflammation on the mouse ear (Table 2.3, pp. 63).

95 mg of the ether fraction was dissolved in chloroform and absorbed on one gram deactivated silica gel and applied to the top of a 20 cm long and 2.5cm diameter silica flash column. The whole procedure was carried out in a fume cupboard. Elution of the column commenced with hexane followed by a mixture of hexane and acetone at a ratio of 90 hexane to ten acetone. Polarity was increased gradually by increasing the acetone ratio by a factor of ten and reducing the hexane ratio by the same factor. The column was washed with acetone then with methanol to ensure complete elution of the column. 100 fractions, 15 ml each, were collected and grouped into 15 fractions based on their similarities on analytical TLC. The fractions were tested separately for pro-inflammatory activity on the mouse ear. Active fractions were purified further to isolate the irritant constituents. The following daphnanes were isolated and characterized:

3.1.2.1 Isolation and Characterization of Thymelaeatoxin-A, 12-O-(Cinnamoyl)-5,12-Dihydroxy-6α,7α-Epoxy-Resiniferonol-9,13,14-Orthobenzoate or 12-O-(Cinnamoyl)-12-Hydroxydaphentoxin

This compound was the major irritant constituent of fractions eluted with hexane:acetone 80:20 and 70:30. Colour reaction on TLC suggested the presence of

daphnane type diterpene. After spraying with 60% H_2SO_4 and heating at 110°C for 15 minutes, a brown spot was detected in daylight which fluoresced yellow-brown under the UV lamp at 365 nm.

Initially, preparative adsorption TLC was used to purify thymelaeatoxin-A. 10mg of the active fractions were applied to 0.5mm unbuffered plate which was developed twice in hexane:chloroform 15:10. One side of the air dried plate was sprayed with 60% H_2SO_4 and heated at 110°C for 15 minutes until no colour change was observed. A brown zone, having an R_f value of 0.56, was scraped off the plate and eluted with chloroform:acetone 2:1 over a 1x1 cm celite column.

Final purification of this compound was achieved using a semi-preparative HPLC silica column. The column was eluted with chloroform:hexane 90:10, for 10 minutes, at a flow rate of 1ml/minute followed by chloroform:methanol 99:1 at the same flow rate. The major constituent was eluted from the column after 23.5 minutes. Both analytical HPLC and two dimensional TLC showed that the elute was a single component. Evaporation of the solvent under a stream of nitrogen yielded a glassy colourless resin. Thymelaeatoxin-A exhibited the following spectral characteristics:

Mass Spectrum

High resolution EIMS 130°C
Measured Mass 629.2212 Daltons
calculated Mass 628.2303 Daltons corresponding to $C_{36}H_{36}O_{10}$

Table 3.1.1 FAB Mass Spectral Data of Thymelaeatoxin-A:

3.2V, Matrix MNOBA+Na

m/z	%Abundance	Inference
651	17	M+1+Na $C_{36}H_{36}O_{10}Na$
629	35	M+1 $C_{36}H_{36}O_{10}$
481	60	M+1-(R`COO·)
427	82	M+1-(R`COO· +C_4H_6)
381	30	M+1-(R`COO· +R``CO·)
362	17	M+1-(R`COO·+R``CO· +H_2O)
329	65	M+1-(R`COO·+R`CO· +H_2O+CH_3OH)
311	25	M+1-(R`COO·+R`CO·+2H_2O+CH_3OH)
289	100	M+1-(R`COO·+R`CO·+3H_2O+CH_3OH)
258	60	M+1-(R`COO·+R`CO·+3H_2O+CH_3OH+CH_2OH)
149	30	R`COOH+1
122	30	R``COOH
105		C_6H_5CO·

R` = Cinnamoyl R`` = Benzyl

Table 3.1.2 ^{1}H-NMR Spectral Data of Thymelaeatoxin-A:

$CDCl_3$, 400 MHZ, TMS = 0.0000 ppm, Figure 3.1, page 120.

PPM	Integration	Multiplicity	J Hz	Inference
7.7322	2H	m		Aromatic benzoate H3″& H7″
7.6623	1H	d	1	Olefinic H2`
7.6012	1H	m		H1
7.5409	2H	m		Cinnamate H5′ & H 9`
7.3920	6H	m		Cinnamate H 6`,7`,8` Benzoates H 5``,4``,6``
6.4018	1H	d	15.9	Olefinic H3`
5.1828	1H	s		H 12
5.0591	2H	s		H 16
4.9723	1H	d	2.44	H 14
4.2650	1H	d	2.16	H 5
3.9932	2H	d	2.44	H 20
3.9523	1H	m		H10
3.7769	1H	dd	5.4	OH 20 (D_2O exchanged)
3.6858	1H	d	2.48	H 8
3.6550	1H	s		H 7
3.5158	1H	s		OH 4 (D_2O exchanged)
2.5661	1H	q	7.25	H 11
2.3617	1H	m		OH 5 (D_2O exchanged)
1.9082	3H	s		H 17
1.7842	3H	m		H 19
1.4140	3H	d	7.24	H 18

H′= Cinnamate H″= Benzoate

Table 3.1.3 ^{13}C-NMR (DEPT) Spectral Data of Thymelaeatoxin-A:

$CDCl_3$, 100.622 MHZ, TMS=0.00, Figure 3.2, page 121.

PPM	Integration	Inference
209.89	C	C 3
166.29	C	C1`
160.82	CH	C 1
146.44	C olefinic	C2`
143.25	C	C 15
137.88	C	C 2
135.62	C aromatic	C4`
134.41	C	C aromatic
131.10	CH	C aromatic
130.10	CH	C aromatic
129.36	2 CH	C aromatics (cinnamate)
128.70	2 CH aromatics	C 9` & C 5`
128.51	2 CH aromatics	C aromatics (cinnamate)

C′= Cinnamate C″= Benzoate

Table 3.1.3 continued

PPM	Integration	Inference
126.43	2 CH aromatics	C aromatics (benzoate)
118.28	C	C 1″
117.56	CH olefinic	C3`
114.24	CH_2	C 16
84.63	C	C 13
81.22	CH	C 14
79.01	C	C 4
78.92	CH	C 12
72.59	C	C 9
72.54	CH	C 5
65.75	CH_2	C 20
64.85	CH	C 7
60.86	C	C 6
47.89	CH	C 10
44.61	CH	C 11
35.99	CH	C 8
19.32	CH_3	C 17
18.75	CH_3	C 18
10.31	CH_3	C 19

C′= Cinnamate C″= Benzoate

Table 3.1.3 COSY H-H NMR Spectral Data of Thymelaeatoxin-A:
$CDCl_3$, 400MHz, TMS = 0.0000 ppm, Figure 3.3, page 122 &123.

Signals connection (ppm-ppm)	H-H connections
1.4140 - 2.5661	H 18 - H 11
1.7842 - 3.9523	H 19 -H 10
1.7482 - 7.6012	H 19 - H 1
1.9082 - 5.0591	H 17 - H 16
2.3617 - 3.7769	OH 5 - OH 20
2.3617 - 3.9932	OH 5 - 2H 20
3.7769 - 3.9932	OH 20 - 2H 20
3.5158 - 3.9523	OH 4 - H 10
3.6550 - 4.2650	H 7 - αH 5
3.6858 - 4.9723	H 8 - H 14
3.9523 - 7.6012	H 10 - H 1
4.2650 - 3.6550	H 5 - H 7
6.4018 - 7.6623	H 3` - H 2`
7.3920 - 7.7322	H aromatics - H aromatics
7.3920 - 7.5409	H aromatics - H aromatics

Table 3.1.4 NOESY H-H NMR Spectral Data of Thymelaeatoxin-A:
$CDCl_3$, 400MHz, TMS = 0.0000 ppm

Signals connection (ppm-ppm)	H-H connections
1.4140 - 7.5409	H 18 - H aromatics
1.4140 - 5.1828	H 18 - H 12
1.4140 - 2.5661	H 18 - H 11

Table 3.1.4 continued

Signals connection (ppm-ppm)	H-H connections
1.7842 - 7.6012	H 19 - H 1
2.5661 - 3.6858	H 11 - H 8
2.5661 - 5.1828	H 11 - H 12
3.6550 - 4.9723	H 7 - H 14
3.7769 - 4.2650	H 10 - αH 5
3.7523 - 3.9932	OH 20 - 2H 20
5.1828 - 1.9080	H 12 - H 17
5.1828 - 1.4140	H 12 - H 18
5.1828 - 2.5661	H 12 - H 11
6.4018 - 7.5409	H 3′ - H Aromatics (Cinnamate)
7.7322 - 7.3920	H 3″,7″- H 5″,4″,6″(Benzoate)

Table 3.1.5 C-H One bond Correlation NMR Spectral Data of Thymelaeatoxin-A: $CDCl_3$, TMS= 0.00 ppm, Figure 3.4, page 124.

Signals connection (ppm-ppm)	Inference
160.82 - 7.6012	C 1 - H 1
146.44 - 7.6623	C 2` - H 2`
131.10 - 7.3920	C aromatics - H aromatics
130.10 - 7.3920	C aromatics - H aromatics
129.36 - 7.392	2C aromatics - 2H aromatics
128.70 - 7.5409	2C aromatics - 2H aromatics
128.51 - 7.392	2C aromatics - 2H aromatics (cinnamate)
126.43 - 7.7322	2C aromatics - 2 H aromatics (benzoate)
117.56 - 6.4018	C 3` - H 3`
114.24 - 5.0591	C 16 - H 16
81.22 - 4.9723	C 14 - H 14
78.92 - 5.1828	C 12 - H 12
72.54 - 4.2650	C 5 - H 5
65.75 - 3.9932	C 20 - 2H 20
64.85 - 3.6550	C 7 - H 7
47.89 - 3.9523	C 10 - H 10
44.61 - 2.5661	C 11 - H 11
35.99 - 3.6858	C 8 - H 8
19.32 - 1.9082	C17 - 3H 17
18.75 - 1.4140	C 18 - 3H 18
10.31 - 1.7842	C 19 - 3H 19

Table 3.1.6 C-H Long Range Correlation NMR Spectral Data of Thymelaeatoxin-A: $CDCl_3$, TMS=0.0000 ppm, Figure 3.5, page 125.

Signals connection (ppm-ppm)	Inference
137.88 - 1.7842	C 2 - 3H 19
143.25 - 1.9082	C 15 - 3H 17
114.24 - 1.9082	C 16 - 3H 17
118.28 - 4.9723	C 1" - H 14
84.63 - 5.0591	C 13 - 2H 16
72.59 - 7.3920	C 9 - H aromatics (Benzoate)

Table 3.1.6 continued

Signals connection (ppm-ppm)	Inference
64.85 - 3.6858	C 7- H 8
60.86 - 3.9932	C 6 - 2H 20
35.99 - 3.6550	C 8 - H 7
18.75 - 5.1828	C 18 - H 12

UV Spectral Data of Thymelaeatoxin-A: λmax Methanol

205, 214(sh), 223, 279 nm

IR Spectral Data of Thymelaeatoxin-A: Thin film on NaCl disc, $CHCl_3$

3460, 3000m, 1700, 1640, 1580, 1500,1455,1360, 1330, 1260, 1210, 1175, 1090, 1020, 970, 930d, 875, 760 cm^{-1}.

3.1.2.2 Isolation and Characterization of Resiniferonol-9,13,14-Orthophenylacetate:

This compound was isolated from the hexane phase produced during liquid-liquid partition of the crude extract. The irritant effect of the hexane phase detected on the mouse-ear (Table 2.3, pp. 63) was attributed to the presence of this resiniferonol ester.

300mg of the hexane phase was chromatographed over a 20x2.5 cm flash column slurry-packed in cyclohexane. Elution with gradient mixture of cyclohexane:acetone (95:5, 90:10, 85:15, 80;20, 75;25, 60:40, 50:50, 25:75 and 10:90) followed by 100% acetone yielded 135 fractions, 15ml each. Fractions were grouped into 10 based on similarities on TLC plates. Subfractions 4 and 5 eluted with cyclohexane:acetone 80:20 were found to be irritant to the mouse ear. TLC analysis of these fractions revealed the presence of daphnane orthoesters characterized by the detection of a black spot in day light which fluoresced bright orange under 365nm UV-lamp.

Subfractions 4 and 5 were combined and chromatographed over a 1.5x65 cm sephadex LH-20 column packed in methanol. 120 fractions, 10ml each, were collected and grouped according to TLC analysis. The semi-pure fractions were chromatographed on preparative adsorption 1mm thick preparative TLC plate which was developed twice in cyclohexane:dichloroform:methanol at ratios of 45:45:15. A band having an R_f value of 0.36 was scraped off the plate and then extracted with chloroform and acetone over a celite column (1x2cm). Analytical TLC indicated that the elute from the celite column

was not pure enough for NMR analysis. Therefore, the solvent was evaporated under vacuum at 35°C and yielded a yellow semi-solid residue weighing 7mg.

The residue was dissolved in chloroform and then purified on a semi-preparative normal phase HPLC column. 1mg of the chloroform solution was injected into the HPLC column and then eluted with hexane:chloroform 5:95 at a flow rate of 1ml/min. The elute was dried under a stream of nitrogen gas, redissolved in chloroform:methanol and re-injected into the HPLC column eluted with a mixture of chloroform:methanol 99.8:0.2. The pure diterpene eluted at 13.57 min. Analysis of the HPLC-peak and 2-D TLC plate confirmed the presence of a single pure compound having an R_f value 0.16 (chloroform:mehtnol 99.8:0.2). Removal of the solvent under a stream of nitrogen gas offered a chalky white residue. The compound exhibited the following spectral properties:

Mass Spectrum

High resolution FABMS Matrix MNOBA+Na revealed:
Measured mass = 487.2093 Daltons corresponding to $C_{28}H_{32}O_6Na$
Actual mass = 464.2199 Daltons corresponding to $C_{28}H_{32}O_6$

Table 3.1.8 EI Mass Spectal Data of Resiniferonol-9,13,14-Orthophenylacetate: 30eV, 110°C

m/z	%Abundance	Inference
464	2.5	M^+
446	5	M^+-H_2O
428	2.5	$M^+-(2H_2O)$
355	2.5	$M^+-(C_2O_5COO^{\cdot})$
328	35	$M^+-(2H_2O+C_2H_5COO^{\cdot}+C_2H_3)$
310	100	$M^+-(H_2O+C_6O_5CH_2COOH)$
292	20	$M^+-(2H_2O+C_6H_5COOH)$
136	16	$C_6H_5CH_2COOH$
91	60	$C_6H_5CH_2$
83	100	C_6H_{11}
69	20	C_5H_9

Table 3.1.9 ^{1}H-NMR Spectral Data of Resiniferonol-9,13,14-Orthophenylacetate: $CDCl_3$, 400MHz, TMS = 0.0000ppm, Figure 3.6, page 126.

PPM	Integration	Multiplicity	J Hz	Inference
7.4588	1H	t	1.53	H 1
7.3758	2H	d	1.308	H4` & H8`
7.2905	3H	m		3H aromatics
5.8710	1H	bs		H 7
4.7081	2H	d	0.63	2H 16
4.2540	1H	d	2.76	H 14
4.0984	2H	t	13.4	2H 20

Table 3.1.9 continued

PPM	Integration	Multiplicity	J Hz	Inference
3.2095	2H	s		2H 2`
3.1340	1H	t		H 8
3.1060	1H	bs		H 10
2.6000	1H	bs		βH 5
2.5600	1H	m		H 11
2.4153	1H	s		OH 19 (D_2O exchanged)
2.2721	1H	d	18.8	βH 5
2.1590	2H	m		H12
1.8210	3H	q	1.2	3H 19
1.6170	1H	bs		OH 20 (D_2O exchanged)
1.5170	3H	s		3H 17
0.9662	3H	d	7.16	3H 18

The previously unreported ^{13}C-NMR of this compound was also recorded.

Table 3.1.10 ^{13}C-NMR DEPT Spectral Data of Resiniferonol-9,13,14-Orthophenylacetate: $CDCl_3$, 100MHz, TMS=0.00 ppm, Figure 3.7, page 127.

PPM	Integration	Inference
206.00	C	C 3
158.48	CH	C 1
146.32	C	C 15
138.92	C	C 2
136.53	C	C 6
134.99	C	C 3`
130.86	2CH	C 4` & C 8`
127.69	2CH	C 5` & C 7`
126.60	CH	C 6`
125.45	CH	C 7
117.74	C	C 1′
110.72	CH_2	C 16
84.47	C	C 13
81.21	C	C 9
80.76	CH	C 14
73.92	C	C 4
69.34	CH_2	C 20
55.45	CH	C 10
41.00	CH_2	C 2`
39.77	CH_2	C 5
38.92	CH	C 11
35.68	CH_2	C 12
33.04	CH	C 8
19.88	CH_3	C 17
18.81	CH_3	C 18
10.26	CH_3	C 19

Table 3.1.11 COSY H-H NMR Spectral Data of Resiniferonol-9,13,14-Orthophenylacetate: $CDCl_3$, 400MHz, TMS = 0.0000 ppm, Figure 3.8, page 128.

Signals connection (ppm-ppm)	H-H connections
0.9662 - 2.5600	3H 18 - 2H 12
1.5170 - 4.7081	3H 17 - 2H 16
1.8210 - 7.4588	3H 19 - H 1
1.8210 - 3.1060	3H 19 - H 10
2.2721 - 2.6000	αH 5 - βH 5
2.2721 - 5.8710	αH 5 - H 7
2.1590 - 2.5600	H 12 - H 11
2.5600 - 0.9662	H 11- 3H 18
3.1340 - 5.8710	H 8 - H 7
3.1340 - 4.2540	H 8 - H 14
3.1060 - 1.8210	H 10 - 3H 19
3.1060 - 7.4588	H 10 - H 1

Table 3.1.12 NOESY H-H Spectral Data of Resiniferonol-9,13,14-Orthophenylacetate:, $CDCl_3$, 400MHz, TMS=0.0000 ppm, Figure 3.9, page 129.

Signals connection (ppm-ppm)	H-H connection
3.1060 - 2.2721	H 10 - αH 5
3.1060 - 0.9662	H 10 - 3H 18
3.1340 - 2.6000	H 8 - βH 5
2.4153 - 3.1340	OH 4 - H 8
3.1060 - 2.1590	H 10 -H 12
3.1060 - 7.4588	H 10 - H 1
3.2095 - 7.3758	2H 2` - H 4` & H 8`
4.0984 - 5.8710	2H 20 - H 7
4.2540 - 1.5170	H 14 - 3H 17
4.2540 - 3.1340	H 14 - H 8
4.2540 - 5.8710	H 14 - H 7
0.9662 - 7.4588	3H 18 - H 1
2.1590 - 1.5170	H 12- 3H 17

IR Spectral Data of Resiniferonol-9,13,14-Orthophenylacetate: Thin film on NaCl disc, $CHCl_3$
3 680, 3600, 3400, 3040, 2400, 1720, 1520, 1480, 1420, 1200, 1040, 920, 760 cm^{-1}

3.1.3 Discussion

Two biologically active daphnane esters were isolated from *Thymelaea hirsuta* herb. EIMS, FABMS, proton, carbon, proton-proton and proton-carbon NMR techniques were found to be most valuable for the identification and structure elucidation of the individual compounds.

Both of the isolated compounds were daphnane -9.13,14-orthoesters but of a

different nature. One compound was identified as 12-O-(cinnamoyl)-5,12-dihydroxy-6.7-epoxy-resiniferonol-9,13,14-orthobenzoate, commonly known as thymelaeatoxin-A, and is based upon the 5β,12β-dihydroxyresiniferonol-6α,7α-oxide (12-hydroxydaphentoxin) parent nucleus. The second compound was identified as resiniferonol-9,13,14-orthophenylacetate which is based upon the resiniferonol nucleus.

The mass spectrum of thymelaeatoxin-A exhibited a molecular ion at 651m/z, when run by Fast Atom Bombardment (FAB) with MNOBA+Na as a matrix. High resolution and laser mass spectroscopy confirmed that 651m/z corresponded to the molecular ion 628m/z plus one sodium atom (M^++Na). Both resiniferatoxin and thymelaeatoxin-A have a molecular weight of 628 Daltons. However, the two compounds belong to two closely related groups of daphnane diterpenes. Resiniferatoxin is a resiniferonol derivative characterized by both the presence of a double bond between C6 and C7, substitued aromatic ester group at C20 and the absence of the hydroxyl group at C5and C12. In contrast, thymelaeatoxin-A belongs to 5,12-dihydroxy-6α,7α-epoxy resiniferonol substituted with cinnamate group at C12 and has a primary alcohol at C20.

The fragmentation pattern exhibited by the mass spectrum provided the first evidence that the isolated compound was a 12-hydroxydaphentoxin. The fragmentation pathway of the 12-hydroxydaphentoxin derivatives is characterized by the elimination of the ester group at C12 in the form of RCOO· radical or RCOOH group. The orthoester group is eliminated as RCOOH, RCOO· or RCOO· radicals (Evans and Soper 1978). The loss of 147 mass units from the molecular ion 628m/z with the formation of the fragment ion 481m/z suggested the presence of a cinnamate substituent. Subsequent fragmentation yielded the fragment ion 381m/z indicating the presence of a benzoyl group. Although the mass spectrum did not specify the position of the ester groups, studies of C12 substituted daphnane mass spectra indicated that these substituents exhibit characteristic fragment ions corresponding to the elimination of RCOOH and RCOO· associated with the loss of H_2O (Huang *et al* 1985). The summary of the pathway shown in Table 3.1.1, pp. 75, is consistent with observations reported by Huang and co-workers. Fragment ions observed at 149 and 122m/z provided further evidence for the presence of a cinnamate and a benzoyl moieties in the molecule. Elimination of 31 mass units from the base peak ion at 289 m/z with the formation of fragment ion at 258m/z is typical of the 6,7-epoxy

group as previously reported by Schmidt (1986a).

The ^{1}H-NMR spectra of daphnane diterpenes exhibit characteristic signals for the isopropylene group at C13 (Evans and Taylor 1983). In thymelaeatoxin-A ^{1}H-NMR spectrum, the two protons singlet at 5.059 ppm and the three protons singlet at 19.08ppm corresponded to two protons on C16 and three protons on C17 respectively. Two-dimensional COSY H-H NMR spectrum exhibited a cross-peak connection between signals at 5.059 and 1.908ppm thereby confirming the presence of the isopropylene side chain at C13.

Daphnane esters dominantly occur in the orthoester form with an oxygen bridge linking carbon 9,13 and 14. Therefore, the proton at C14 has a diagnostic signal in the proton NMR spectrum (Schmidt 1986). In the spectrum of thymelaeatoxin-A, the proton on C14 exhibited a doublet at 4.26ppm. COSY and NOESY spectra exhibited a cross-peak connecting the doublet at 3.6858 ppm to that at 4.26ppm, confirmed that the earlier signal corresponded to the proton at C-8. Each doublets has a coupling constant J=2.4 Hz suggesting that both protons could be in either the α or the β configuration. Molecular modelling using Sybyl software suggested that both H8 and H14 are in the β-configuration to the orthoester. The one H singlet at 5.18ppm was assigned to H12 indicating a substituted C12 and replacing the 2H multiplet at 2.36ppm in daphentoxin. Configuration of protons and functional groups was assigned according to data from the NOESY spectrum. Table 3.1.13, pp. 84, lists proton-proton connectivity of thymelaeatoxin-A.

Table 3.1.13 Proton-Proton Connectivity of Thymelaeatoxin-A

COSY Connections (PPM-PPM)	H-H Correlation
1.4140-2.5661	H18-H11
1.7842-3.9523	H19-H10
3.5158-3.9523	OH4-H10
4.2650-3.6550	H5-H7
3.9932-3.7769	H20-OH20
2.3617-3.9932	OH5-H20
7.6012-1.7842	H1-H19
3.6858-4.972	H8-H14
6.4018-7.6623	H3'-H2' (trans connection)

Olefinic protons on C-2' and C-3' were assigned according to data from Varian NMR Spectra (Varian 1962). Proton-proton connectivity and coupling constants of 15.98 and 16.02 Hz provided conclusive evidence of trans configuration of H3' and H2'.

IR signals in the fingerprint region of 900-700 cm^{-1} indicated the presence of olefinic and aromatic protons in the structure. These findings supported the signals of aromatic protons in ^{1}H-NMR between 7.73 and 7.39 ppm and the chemical shifts in ^{13}C-NMR between 146 and 126 ppm. However, the complexity of these signals did not allow specific assignment to neither cinnamate nor ortho benzoate aromatic protons.

Resiniferonol-9,13,14-orthophenylacetate was isolated from the hexane phase of *Thymelaea hirsuta*. Electron impact mass spectrum of this compound exhibited a fragmentation pathway typical of resiniferonol derivatives. Low voltage EI technique at 30eV exhibited a very weak molecular ion at 464 m/z at 2.5% relative abundance. High resolution FABMS with MNOBA+Na as a matrix provided a base peak ion of 487 m/z corresponded to $C_{23}H_{32}O_6Na$ confirming that 464 m/z is the molecular ion of the isolated compound.

Fragment ions exhibited at 446 and 310 m/z are consistent with the elimination of water and phenyl acetate groups respectively. This pattern is a characteristic of resiniferonol orthoester which has a double bond between C6 and C7. In this type of compounds the free hydroxyl group at C20 is eliminated as a molecule of water as compared to elimination of 31 mass units of methanol in the case of 6,7-epoxy resiniferonol derivatives (Evans and Taylor 1983). Elimination of the orthoester group $C_6H_5CH_2COOH$ was evident in the spectrum with the base peak ion at 310 m/z. In the low mass region the fragment ion 136m/z indicated the presence of a phenyl acetic acid moiety $C_6H_5CH_2COOH$. Further fragmentation into $C_6H_5CH^{\cdot}_2$ and C_6H_{11} radicals was proposed by the presence of fragment ions 91 and 83 m/z respectively (Table 3.1.8, pp. 80). The mass spectrum was devoid of further fragment ions for a second ester group. Proton NMR and carbon NMR exhibited signals for a 12-deoxydaphnane orthoester. The isopropylene group at C13 was presented by the doublet of 2H on C16 at 4.7ppm and the singlet of 3H at C17 at 1.57ppm in proton NMR. The olefinic proton on C7 was shifted

down field to 5.8710 ppm in contrast to the upfield position in the 6,7-epoxide derivatives at 3.6550ppm as in the case of thymelaeatoxin-A. The diagnostic proton on C-14 which resonated at 4.25ppm confirmed the presence of an orthoester group. A NOESY experiment demonstrated that it coupled with 1H on C8 (Table 3.1.12, pp. 82).

The two protons on C12 occurred as a multiplet at 2.1590 ppm connected to three protons on C17 and 1H on C11 as shown in the COSY H-H NMR spectrum (Figure 3.8, pp. 128). The two protons on C5 resonated as a doublet at 2.2721 ppm and as a broad singlet at 2.6000 ppm. The large coupling constant of 18.8Hz was consistent with the correlation between alpha and beta oriented protons. In addition, the two signals had a very strong COSY cross peak but without NOESY H-H connections. The olefinic proton on C7 showed a strong NOESY connection with protons on C14 and C8 suggesting a beta orientation.

Assignment of signals in the ^{1}H-NMR spectrum was confirmed from the H-H bond connections in the COSY spectrum summarized in Table 3.1.11, pp. 82. Molecular modelling on Sybyl software and data from the NOESY experiment (Table 3.1.12, pp. 82) supported the proposed configuration of protons in the structure and the connection of the methylene group with aromatic protons of the orthoester.

The ^{13}C-NMR spectrum for resiniferonol-9,13,14-orthophenylacetate orthoester was not reported previously. Signals were assigned by comparing them to previously reported daphnane spectra. The infra red spectrum provided supporting evidence for the presence of an aromatic substituent and alkene group in the finger print region but had no specific information regarding structural elucidation.

Resiniferonol-9,13,14-orthophenylacetate was isolated as a semi-synthetic compound of acid-catalysed transesterification of resiniferatoxin (Evans and Schmidt 1979b, Schmidt and Evans 1978). It was isolated as a natural product from *Euphorbia poissonii*, family Euphorbiaceae (Gordge 1992). In contrast, this study is the first to report the same compound being isolated from *Thymelaea hirsuta*, a member of the family Thymelaeaceae. The occurrence of the same daphnane derivative in both families provides further support to the proposed taxonomic relationship between them. It is also

possible that it is a degradation product of 12-hydroxy daphentoxins. Elimination of substituents on C12 on long storage or during chromatographic experiments was previously reported by Brooks (1989).

This investigation of extracts from *Thymelaea hirsuta has* shown that inflammatory activity is retained after several years of storage under sub zero temperatures. However, the loss of the acyl substituents from the irritant diterpenes and/or intra-structural rearrangement should not be overshadowed. Both isolated compounds were found to be active in a range of *in vitro* and *in vivo* biological systems (Chapter 4 & 5).

3.2 Isolation and Structural Elucidation of the Irritant 12-Hydroxy Daphentoxin Ester from the Twigs and Leaves of *Daphne blagayana*

3.2.1 Introduction

Since the eleventh century, species from the genus *Daphne* have been recognised as virulent poisons (Stout *et al* 1970). Human poisoning and fatalities, particularly among children, were commonly identified as a results of ingestion of the berries (Forsyth 1968, Lampe 1984).

Daphne blagayana is an ever green dwarf shrub originally recognised by Count Blagay in Slovania during 1837 and was described by Freyer (Freyer 1838). Its creamy-white flowers are very fragrant making it very attractive as a garden ornament. This species is native of Romania, Bulgaria, Albania, North Greece and former Yugoslavia. It has been cultivated in UK since 1875 (Brickell and Mathew 1976).

Daphne blagayana is a member of the family Thymelaeaceae, subfamily Thymelaeoideae, tribe Daphne (Borris, Blasko and Cordell 1988). In describing the anatomy of this species Cortesi presumed, on the basis of homogeneity of the genus *Daphne*, that its bark has properties analogous to those of *D. gnidium*, *D. mezereum* and *D. laureola* (Cortesi 1941).

No information on the poisonous or medicinal properties of *D. blagayana* has been found in the literature. However, anatomical similarities to other *Daphne* species proposed similar medicinal uses and toxicological effects. Although most of the *Daphne*

species are known to be irritant and caustic to the skin and mucous membranes, medicinal uses have been reported throughout Europe, China and Arab countries. In Europe, *Daphne* was used as a stimulant, diuretic, antiviral, tonic, purgative and abortifacient (North 1967). Other uses include an antidote for snakebite, anticancer and treatment for skin diseases; rheumatism and malaria (Grieve 1931, Borris, Blaski and Cordell 1988, Boulos 1983). The ancient Chinese pharmacopoeia recommended *Daphne* to treat circulatory disorders as an anti-inflammatory and antibacterial agent. Recent uses include antileukaemic and anti-cancer of the nasopharynx (Huang 1993).

All parts of the plants of this genus are poisonous particularly the seeds, the bark and the berries. Ingestion of the berries results in severe, often fatal, poisoning. Skin contact produces blisters and severe redness of the area (Forysth 1968, Lampe 1984). Mezerein and other daphentoxin derivatives were found to be the major toxic substances in *D. mezereum* (Ronla´n and Wickberg 1970). Irritant 12-hydroxydaphentoxins were only isolated from other *Daphne* species (Lee 1981, Stout *et al* 1970).

Extracts from the twigs and leaves of *D. blagayana* were found to be active in a range of *in vivo* and *in vitro* biological systems (Chapter 2). Despite the striking toxicity of this plant, no chemical investigations have been carried out before this research. Therefore, plant extracts were subjected to chemical studies in an attempt to identify the toxic principles of this species.

3.2.2 Extraction and Fractionation of Plant Material

Six hundred grams of the twigs and leaves of *Daphne blagayana* were collected in Yugoslavia and authenticated by Mr Gardner of the Royal Botanic Gardens Kew, London. The plant material was extracted and fractionated as previously described in Chapter 2. The ether fraction was dried over anhydrous sodium sulphate and the solvent was evaporated under pressure and below 40°C temperature to produce 400mg of the green residue.

Further fractionation of the resin was achieved by gradient elution centrifugal liquid chromatography (CLC) using 2mm thick silica gel plate. The plate was eluted with 100ml of cyclohexane, 100ml cyclohexane:ether 2:1, 200ml ethyl acetate:acetone 8:2,

200ml acetone:methanol 8:12 and was finally washed with 200ml methanol. Fractions were collected and later grouped according to similarities on TLC plates. Each fraction was tested for pro-inflammatory activity using the mouse ear assay (Table 2.3, pp. 63). Active fractions were purified further to isolate the irritant constituents. The daphnane ester in the following section was isolated and characterised.

3.2.3 Purification And Structural Elucidation Of Mezerein: 12-O -(5′-Phenyl-2′,4′-Pentadienoate)-5β,12β-Dihydroxy-6α,7α-Epoxyresiniferonol-9,13,14-Orthobenzoate, or 12-O-(5′-Phenyl-2′,4′-Pentadienoate)-12-Hydroxydaphentoxin

This compound was found to be the major toxic diterpene from *Daphne blagayana* twigs and leaves. It was present in three CLC fractions and was initially purified by charcoal column in order to remove most of the green pigment. The column was eluted with chloroform, chloroform:methanol 1:4, chloroform:ethanol 1:1, ethanol and methanol. The compound eluted with chloroform:ethanol 1:4 together with some oily contaminant. The solvent was evaporated and the residue was chromatographed on sephadex LH-20 column eluted with chloroform:methanol 3:7 to yield a white-yellow residue which was found to be irritant to the mouse ear.

The residue was chromatographed on a preparative adsorption plate buffered at PH 7 and developed three times in chloroform:acetone 8:2. The plate was dried, sprayed at one edge with 60% H_2SO_4 and heated at 110°C for 15 minutes. Examination of the plate in day light revealed a grey zone which fluoresced yellow under 365 nm UV light with an R_f value of 0.2.

Final purification of this zone was achieved using semi-preparative normal phase HPLC column eluted with chloroform:hexane 90:10 at a flow rate of 1ml/min. The major constituent eluted at 31.95 minutes. The elute was re-injected into the column, eluted with chloroform:methanol 99.8:0.2 and yielded the pure compound at R_t of 22.62 minutes.

The white amorphous crystals exhibited the following spectral characteristics:

Mass Spectrum

High resolution FAB mass spectrum exhibited:
Measured mass of 677 Daltons corresponding to $C_{38}H_{38}O_{10}Na$
Actual mass of 653.879 Daltons corresponding to $C_{38}H_{38}O_{10}$

Table 3.2.1 FAB Mass Spectral Data of Mezerein: Matrix MNOBA+Na 188mV

m/z	Abundance	Inference
677	50	$M^+ + Na$
649	50	M^+-(Na+5H)
323	100	M^+-(R`COO` + R``COOH+Na+2H_2O)
175	95	$C_6H_5(CH)_4COOH$

Table 3.2.2 ^{1}H-NMR Data of Mezerein:

$CDCl_3$, 400MHz, TMS = 0.0000 ppm, Figure 3.11, page 131.

PPM	Integration	Multiplicity	J= Hz	Inference
7.7280	2H	m		H 3″, H 7″
7.5940	1H	m		H 1
7.4750	2H	m		H 11′, H 7′
7.4428	1H	m		Olefinic C2`
7.3750	2H	m		H 6″, H 4″
7.3451	2H	m		H 8′, H 10′
7.3410	1H	m		H 4′
7.3253	1H	m		H 3′
6.9245	1H	m		H 9′
6.8527	1H	m		H 5″
5.9600	1H	s		H 5′
5.1480	1H	s		H 12
5.0484	2H	s		H 16
4.9500	1H	d	2.37	H 14
4.2725	1H	s		H 5
3.9660	2H	m		2H 20
3.8780	1H	m		H 10
3.7940	1H	m		OH 20 (D_2O exchanged)
3.6670	1H	d	2.559	H 8
3.6560	1H	s		H 7
3.5080	1H	s		OH 4 (D_2O exchanged)
2.5390	1H	q	7.32	H 11
2.3340	1H	m		OH 5 (D_2O exchanged)
1.8970	3H	s		3H 17
1.7845	3H	m		3H 19
1.4008	3H	d	7.23	3H 18

H′= Phenyl pentadienoate H″= Benzoate

The previously unreported ^{13}C NMR spectrum of mezerein was also recorded

Table 3.2.3 ^{13}C-NMR DEPT Spectral Data of Mezerein:

100MHz, $CDCl_3$, TMS= 0.00 ppm, Figure 3.12, page 132.

PPM	Integration	Inference
209.49	C	C 3
165.87	C	C 1`
160.43	CH	C1
145.77	CH	C 2`
142.87	C	C 15
141.43	CH	C 9′

Table 3.2.3 continued

PPM	Integration	Inference
136.96	C	C 2
135.80	C	C 2``
135.20	C	C 6`
129.68	CH	C 3′
129.31	CH	C 4`
128.88	2CH	C 8` &C 10`
128.10	2CH	C 4″ & C 6″
127.28	2CH	C 11′ & C7`
126.03	2CH	C 7″& C 3″
125.85	CH	C 5″
120.19	CH	C 5′
117.86	C	C 1``
113.80	CH_2	C 16
84.23	C	C 13
80.83	CH	C 14
78.62	C	C 4
78.45	CH	C 12
72.20	C	C 9
72.12	CH	C 5
65.21	CH_2	C 20
64.38	CH	C 7
60.48	C	C 6
47.51	CH	C 10
44.20	CH	C 11
35.58	CH	C 8
18.93	CH_3	C 17
18.32	CH_3	C 18
9.91	CH_3	C 19

C′= Phenyl pentadienoate C″= Benzoate

Table 3.2.4 COSY H-H NMR Spectral Data of Mezerein:

$CDCl_3$, 400MHz, TMS = 0.0000 ppm, Figure 3.13, page 133.

Signals connection (ppm-ppm)	H-H connections
1.4008 - 2.5390	3H 18 -H11
1.7845 - 3.8180	3H 19 - H 10
1.7845 - 7.5940	3H 19 - H 1
3.8780 - 7.5940	H10 - H1
1.8970 - 5.0484	3H 17 - 2H 16
3.9660 - 3.7940	2H 20 - OH 20
3.6670 - 4.9500	H 8 - H 14
3.6560 - 4.2725	H 7 - H 5
5.9600 - 7.3451	H 9` - H 8`& H10`
7.3750 - 68527	H4"&H6"-H5"
7.3410 - 7.4074	H4`-H2`
7.3253 - 7.4074	H3`-H2`
7.4722 - 7.3451	H7`& H11`-H8`&H10`

Table 3.2.5 NOESY H-H NMR Spectral Data of Mezerein: $CDCl_3$, 400MHz, TMS = 0.0000ppm, Figure 3.14, page 134.

Signals connection (ppm-ppm)	H-H connections
1.4008 - 5.1480	3H 18 -H 12
1.4008 - 7.5940	3H 18 - H 1
1.8970 - 7.7280	3H 17 - H 1
1.8970 - 4.9500	3H 17 - H 14
1.8970 - 5.1480	3H 17 - H 12
1.8970 - 5.0484	3H 17 - 2H 16
2.5390 - 5.1480	H 11 - H 12
2.5390 - 3.6560	H 11 - H 7
2.5390 - 1.4008	H 11 - 3H 18
3.6560 - 1.7845	H 7 - 3H 19
3.6560 - 4.9500	H 7 - H 14
3.6560 - 3.9660	H 7 - 2H 20
3.6560 - 3.6670	H 7 - H 8
3.8780 - 3.9660	H 10 - 2H 20
4.2725 - 1.7845	H 5 - 3H 19
4.2725 - 3.9660	H 5 - 2H 20
4.2725 - 3.6670	H 5 - H 8
4.2725 - 3.8780	H 5 - H 10
5.0484 - 5.1480	2H 16 - H 12
5.9600 - 6.9160	H 5′ - H 7′, H 11′
5.9600 - 7.4047	H 5′ - H 8′, H10′
6.9160 - 7.4722	H 7′, H 11′- H 4′, H 5′
7.3451 - 6.9160	H 8′, H 10′- H 7′, H 10′
6.9245 - (7.3253 -7.4722)	H9′- aromatics & olefinics of phenylpentadienoate

Table 3.2.6 C-H One Bond Correlation NMR Spectral Data of Mezerein: $CDCl_3$, TMS = 0.0000ppm, Figure 3.15, page 135.

Signals connection (ppm-ppm)	C-H connections
9.90 - 1.7800	C 19 - 3H 19
18.32 - 1.4008	C 18 - 3H 18
18.93 - 1.8970	C 17 - 3H 17
35.58 - 3.6670	C 8 - H 8
44.21 - 2.5390	C 11 - H 11
47.51 - 3.8780	C 10 - H 10
64.38 - 3.6560	C 7 - H 7
65.22 - 3.9660	C 20 - 2H 20
72.10 - 4.2725	C5 - H 5
78.45 - 5.1480	C 12 - H 12
80.64 - 4.9500	C 14 - H 14
113.80 - 5.0484	C 16 - 2H 16
120.19 - 5.9600	C9` - H 9`
125.85 - 6.8527	C 5`` - H5``
126.03 - 7.7281	C 3″ & C 7″- H 3″& 7″
127.28 - 7.4722	C 11″& C 7″- H 11″& 7″

Table 3.2.6 continued

Signals connection (ppm-ppm)	C-H connections
128.88 - 7.3451	C 8′& C 10′ -H8′ & H 10′
128.10 - 7.3750	C 6`` & C 4`` - H 6`` & H 4``
129.31 - 7.3499	C 4′ - H 4′
129.68 - 7.3410	C 3′ - H 3′
141.43 - 6.9245	C 9″ - H 9″
145.77 - 7.4428	C 2′ - H 2′
160.00 -7.594	C 1 - H 1

Table 3.2.7 C-H Long Range Correlation NMR Spectral Data of Mezerein: $CDCl_3$, TMS = 0.0000ppm, Figure 3.16, page 136.

Signals connection (ppm-ppm)	C-H connections
9.91 - 1.7800	C 19 - 3H 19
18.93 - 5.0484	C 17 - 2H 16
35.58 - 3.6560	C 8 - H 7
60.48 - 4.2725	C 6 - H 5
80.63 - 5.1480	C 14 - H 12
84.23 - 5.0484	C 13 - 2H 16
117.86 - 4.9500	C 1`` - H 14
120.19 - 5.9600	C 2` - H 5`
113.80 - 1.8900	C 16 - 3H 17
136.96 - 1.7800	C 2 - 3 H 19
142.87 - 1.8970	C 15 - 3H 17
209.49 - 7.5940	C 1- H 1

IR Spectral Data of Mezerein: Thin film on NaCl disc, $CHCl_3$

3480, 3010, 1710, 1620, 1320, 1240, 1140, 1080, 1010, 760 and 695 cm^{-1}

3.2.4 Discussion

The presence of daphnane ester in *D. blagayana* was initially detected from the colour reaction of both the irritant fractions and the pure compound on analytical TLC plates. The structure was elucidated by analysing and comparing the spectral data to those previously published.

The ^{1}H-NMR spectrum (Figure 3.11, pp. 131) indicated that the isolated compound is a daphentoxin type hydrocarbon. Of this spectrum, two olefinic protons were exhibited as a singlet at 5.0484ppm and three methylene protons exhibited as a singlet at 1.8970ppm. These signals were assigned as two protons on C16 and three protons on C17 respectively thereby confirming the presence of an isopropyl group at C13 in the daphnane nucleus replacing the two methyl groups of ring-D in the tigliane

type compounds. COSY H-H correlation of the two signals (Table 3.2.4, pp. 91) provided further supporting evidence.

In addition, the signal at approximately 5.8ppm, associated with the olefinic proton on C7 in tiglianes and resiniferonol types, was not evident in the ^{1}H-NMR spectrum of the isolated compound being shifted upfield at 3.6560ppm. This is consistent with the replacement of the doublet bond between C6 and C7 in the former two groups with an epoxy group in the daphentoxin derivatives (Evans and Taylor 1983).

Most of the presently known daphnanes have an orthoester structure characterized by the chemical shift of the proton on C14 (Schmidt 1986a). One- proton doublet at 4.9500ppm in the ^{1}H-NMR spectrum, corresponding to 1H on C14, indicated the presence of an ortho ester group.

The nature and the position of the ester groups were based on the data from the FABMS, H-H, H-C NMR and infra red spectra. FAB mass spectrum, Table 3.2.1, pp.90, exhibited a molecular ion at 677m/z which corresponding to $C_{38}H_{38}O_{10}Na$. It is concluded that the isolated compound has a molecular weight of 654 Daltons. Subsequent fragment ions did not provide much information about the molecule. However, in the lower region of the spectrum the prominent fragment ion at 175m/z indicated the presence of $C_{11}H_{10}O_2$ which was later assigned to the 5-phenyl-2,4-pentadienoate substituent. The IR signals at 695 and 760cm^{-1} indicated the presence of 5-adjacent aromatic CH groups i.e, monosubstituted benzene ring.

The ^{1}H-NMR indicated the presence of two aromatic systems with signals at 7.728, 7.417-7.34ppm which integrated for ten protons. Signals at 6.9245, 5.9600, 7.3410 and 7.3240 ppm were integrated for four olefinic protons. Proton-proton connections of the aromatic and the olefinic protons were revealed in the COSY and NOESY spectra. The olefinic protons correlated only with one aromatic system. The signal of one olefinic proton at 5.96ppm of H5' correlated with one proton multiplet at 6.8527ppm of H 9 and the multiplet of two aromatic protons at 7.3410 ppm of H4' and 7.3240ppm of H3'. The aromatic proton multiplet at 7.3750ppm correlated with one aromatic proton doublet at 7.2810ppm suggesting the presence of an isolated aromatic system. Table 3.2.4, pp. 91

summarizes the correlation between the aromatic and olefinic protons.

In the infrared the signal at 1010 cm^{-1} indicated the presence of trans-linked olefinic protons. In the proton NMR spectrum the coupling constant of the signal at 5.9600 corresponding to H5', J=15.27 Hz, is typical of trans configuration of the unsaturated hydrocarbons indicating that the olefinic protons on the phenyl pentenoate function on C12 have a trans configuration.

The ^{13}C-NMR spectrum exhibited two signals corresponding to two methylene groups. The signal at 113.8ppm was assigned to methylene group at C16. This was confirmed from its connection to the two protons signal at 5.04ppm in the C-H one bond correlation spectrum. The chemical shift of 65.2ppm corresponded to a CH_2 group attached to a hydroxyl group and was found to be correlated to one proton multiplet at 3.79ppm which exchanged with D_2O. This confirms that the quaternary C6 is connected to a primary alcohol group CH_2OH. The absence of a third methylene group in the DEPT ^{13}C-NMR spectrum suggested substituted C5 and C12 and was supported by the presence of the one proton singlet at 5.14ppm associated with the one proton on C12 and the one proton singlet at 4.27ppm associated with one proton on C5 in the 12,5-dihydroxydaphnane. In contrast, in the case of daphentoxin the two protons on C12 exhibited a two protons multiplet at 2.36ppm (Schmidt 1986). Correlation of the three protons on C17 at 1.8970ppm with the aromatic protons at 7.728ppm in the NOESY spectrum, confirmed the presence of an ester group on C12 and not on C5.

Biological tests indicated that irritant phorbol esters having a tertiary hydroxyl group on C4 exhibit the beta-configuration (Hecker 1968, Hecker and Schmidt 1974). The compound induced severe irritation on the mouse ear at a dose level of 3ng/ear (Chapter 4). Configuration of protons of the isolated hydrocarbon was established on data from coupling constants and NOESY H-H correlation spectrum (Table 3.2.5, pp. 90). The hydroxyl group on C5 correlated with that on C4 indicating that they both have the beta-configuration. X-ray studies of the molecular structure of mezerein concluded that the epoxide group has the α-configuration to the cycloheptane ring-B (Nyborg and Cour 1975). The NOESY spectrum shown here demonstrated a correlation between protons

on C1, C10 and the methyl group at C11 in the α-configuration. Coupling between protons on C8 and C14, J=2.3 Hz, in addition to NOESY connection of both protons to that on C7 suggested the β-configuration for these protons.

Molecular modelling of the isolated compound using the Sybyl software, provided additional support to the structural conformation (Figure 3.17, pp. 137). The cyclohexane ring is in the boat conformation. The cycloheptane ring-B appears to be the base plane of the molecule to which the cyclopentane ring-A and the cyclohexane ring-C are attached. Comparing these findings with those obtained from x-ray diffraction studies by Nyborg and Cour (1975), it can be concluded that the hydroxydaphentoxin derivative isolated from the twigs and leaves of *D. blagayana* is mezerein.

Using a charcoal column to remove chlorophyll pigment from the irritant ether fraction was found to be effective. However, long chain fatty acids eluted together with other compounds. This was first detected by the presence of yellow coloured zone on TLC plates which changed to brown when left exposed to air overnight. ^{1}H-NMR spectrum confirmed the presence of long chain fatty acid contaminants.

Variable ratios of charcoal:silica were examined for maximum adsorption of pigments, minimum contamination and maximum recovery of compounds of interest. Optimization of the column was achieved by comparing eluates behaviour on analytical TLC and ear erythema test. A set of three columns of 0.5x5cm were packed with different charcoal to silica gel ratios, 1:1, 2:1 and 1:2 respectively. Columns were eluted with chloroform, chloroform:methanol and ethanol. Eluates from columns were chromatographed on analytical TLC plates. Solvents were evaporated under nitrogen gas and residues were tested for pro-inflammatory activity. The optimum ratio was found to be two parts of silica gel to one part of acid washed activated charcoal.

This chemical investigation of aerial parts of *D. balagayana* revealed that the irritant property of this species is partially attributed to the presence of mezerein. Minor irritants were detected. However, when isolated these compounds were in such small quantities that rendered them insufficient for NMR spectroscopy. Although ^{1}H-NMR of mezerein was previously reported (Ronla'n and Wickberg 1970), this study is the first to

report full ^{13}C-, NOESY, COSY, one bond C-H correlation and long range C-H correlation NMR spectra. These techniques proved to be invaluable in structural elucidation with no chemical derivation of the compound.

3.3 Isolation and Characterization of Nitrogen-Containing Tigliane Derivatives from Extracts of *Sapium indicum* and *Sapium insigne*

3.3.1 Introduction

The lactiferous genus *Sapium* consists of one hundred and twenty species of shrubs and trees distributed in the tropics and subtropics of both hemispheres. In the revised classification of the family Euphorbiaceae, the genus *Sapium* was retained in the tribe Hippomaneae within the subfamily Euphorbioideae (Webster 1994). Several species of this genus provide excellent rubber and have been exploited for that purpose (Schultes and Raffauf 1990). Evidence of the toxic effects of this genus on humans is contradictory. In Mexico and El Salvador the sap is reputed harmful and poisonous and therefore used for arrow poison. However, in Costa Rica and Panama *Sapium* trees are not reported to be poisonous. The copious latex of *Sapium indicum* and *Sapium insigne* is acrid and vesicant (Mitchell 1979). Despite its well known toxicity, the root bark of *S. indicum* is used by Indian traditional healers as emetic, purgative, hydrophobia and insanity treatments (Ambasta *et al* 1986, Grieve 1994). Fruits are used as fish-poison and leaves as a dye source. Wood from *S. insigne* is durable and is in great demand among furniture manufactures for its beauty (Ambasta *et al* 1986).

Extracts from *Sapium indicum* were previously investigated for piscicidal and pro-inflammatory activities (Taylor *et al* 1981a, b, c, d). The fruits and seeds of this species have yielded two series of biologically active 4-deoxyphorbol esters. Whilst the major toxins were the nitrogen containing sapintoxins the aliphatic analogues occurred in smaller quantities (Taylor *et al* 1982). In addition, this species was reported to contain biologically inactive 4α-deoxy-5-hydroxyphorbol derivatives (Hecker *et al* 1977, Evans *et al* 1981) and aliphatic tigliane diesters were isolated from the closely related species *S. insigne* (Taylor *et al* 1983).

During this study, extracts from the fruits of *S. indicum* and the twigs and leaves

of *S. insigne* were examined for the effect of storage on their biologically active tigliane constituents.

3.3.2 Isolation and Characterization of the Semi-Synthetic, 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate from the Twigs and Leaves of *Sapium insigne*

One hundred and twenty six grams of twigs and leaves of *S. insigne* were extracted with acetone. The crude extract, of 2.6 grams, was partitioned as previously outlined in Chapter 2 to yield 130mg yellow ether residue. The crude extract and the liquid-liquid partition residues were not irritant to the mouse-ear when tested at 5, 20 and 100μg/ear.

The ether residue was dissolved in 1ml chloroform, then applied to 1mm thick silica gel plate and was later developed twice in cyclohexane:ethylacetate (7:3). The blue fluorescent zone having an R_f value of 0.437 was scraped off the plate, extracted with acetone and re-chromatographed on 0.5mm silica gel plate. The plate was developed in chloroform:methanol 7:3. A band having an R_f value of 0.273, was scraped off the plate extracted with acetone:methanol 1:1 four times and dried over anhydrous sodium sulphate. The solvent was evaporated under pressure and the 4mg colourless resin was incubated with 3ml acetic anhydride:pyridine 1:2 for 12 hours in the dark at room temperature. The reaction product was purified on a sephadex LH-20 column eluted with chloroform:methanol 7:3. Recovery of the compound was monitored with a hand-held UV lamp set at 254 nm. When the analytical TLC plate was sprayed with 60% H_2SO_4 and heated at 110°C for 15 minutes, the eluted derivative produced a brown-orange spot in day light with bright blue fluorescence under 254 and 365nm UV light. The structure of the white crystallized compound was determined from the following spectroscopic data.

Mass Spectrum:

FABMS Matrix MNOBA+Na: 646 Daltons having 100% abundance corresponding to $C_{34}H_{41}O_{10}NNa$

Laser MS Matrix 2,5-DHB: confirmed that the mass ion of 647 m/z having 100% abundance corresponding to M^++1+Na.

Table 3.3.1 EI Mass Spectral Data of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydoxyphorbol-5,13,20-Triacetate: 30eV, 110° C

m/z	Abundance %	Lost Ion	Inference
624	2.8	-	M^++1 $C_34H_{41}O_{10}N$
564	3.9	60	M^++1- (CH_3COOH)
504	10.6	60	M^++1- (CH_3COOH+CH_3COOH)
354	21	150	M^++1 - (2xCH_3COOH+R`COOH)
335	10	17	M^++1- (2xCH_3COOH +R`COOH+OH)
311	10	43	M^++1-(2xH_3COOH+R`COOH+OH+CH_3CO·)
294	67	17	M^++1-(2xCH_3COOH+R`COOH+CH_3CO· +2xOH)
151	100		$C_6H_4NHCH_3COOH$
134	75		$C_6H_4NHCH_3CO$·
105	50		C_6H_4CO· + $C_6H_4NH_3$
63	50		Possibly aromatic acetates

R` = $C_6H_4NHCH_3$

Table 3.3.2 ^{1}H-NMR Spectral Data of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-hydroxyphorbol-5,13,20-Triacetate:

$CDCl_3$, 400MHz, TMS = 0.0000 ppm, Figure 3.18, page 138.

PPM	Integration	Multiplicity	J=Hz	Inference
7.8004	1H	dd	1.55	H 4`
7.6743	1H	q (D_2O exchanged)	4.918	NH
7.6309	1H	s		H 1
7.4141	1H	m (d,t,d)	1.4	H 6`
6.6950	1H	d	8.361	H 7`
6.59715	1H	m (d,t,d)	0.7	H5`
6.3905	1H	d	4.749	H 5
5.7759	1H	m		H 7
5.7606	1H	s (D_2O exchanged)		OH 9
5.6825	1H	d	9.65	H 12
4.5215	2H	dd	11.05	2H 20
3.5796	1H	m		H 10
2.9330	3H	d	4.8	CH_3N
2.7061	1H	t	4.72	H 4
2.4188	1H	m		H 8
2.1431	3H	s		3H Acetate C 5
2.0675	3H	s		3H Acetate C13
1.9036	3H	s		3H Acetate C20
1.7333	3H	m		3H19
1.6799	3H	m		H 11
1.3015	3H	s		H 16
1.2546	3H	s		H 17
1.1347	1H	d	5.44	H 14
0.9560	3H	d	6.3	H 18

Table 3.3.3 ^{13}C-NMR DEPT Spectral Data of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate:

$CDCl_3$, 100MHz, TMS = 0.00 ppm, Figure 3.19, page 139.

PPM	Integration	Inference
204.83	C	C3
173.89	C	C 1`
170.71	C	C 13 (Carbonyl)
169.31	C	C 20
167.98	C	C 5
160.68	C	C 1
152.32	C	C 3``
138.08	C	C 2
136.62	C	C 6
135.05	CH	C 5`
134.25	CH	C 7
131.30	CH	C 7`
114.47	CH	C 4`
110.87	CH	C 7`
109.15	C	C 2`
78.62	C	C 9
75.78	CH	C 12
69.81	CH	C 5
65.93	CH_2	C 20
65.31	C	C 13
52.09	CH	C 10
48.84	CH	C 4
42.81	CH	C 11
42.48	CH	C 8
35.95	CH	C 14
29.56	CH_3	CH_3N
25.91	C	C 15
23.60	CH_3	C 17
21.15	CH_3	CH_3 Acetate
20.97	CH_3	CH_3 Acetate
20.45	CH_3	CH_3 Acetate
16.82	CH_3	C 16
15.19	CH_3	C 18
10.18	CH_3	C 19

Table 3.3.4 COSY H-H NMR Spectral Data of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate:

$CDCl_3$, 400MHz, TMS = 0.0000 ppm, Figure 3.20, page 140.

Cross Peaks PPM-PPM	H-H Connection
0.9560 - 1.6799	18 - 11
1.6799 - 5.6825	11 - 12
1.1347 - 2.4188	14 - 8
2.4188 - 5.7759	8 - 7
2.7061 - 3.5796	4 - 10
2.7061 - 6.3905	4 - 5
2.9330 - 7.6743	NH-CH_3N
3.5796 - 1.6799	10 - 11
3.5796 - 7.6309	10 - 1
4.2687 - 4.7742	20 - 20
6.5971 - 7.4141	5` - 6`
6.5971 - 7.8004	5` - 4`
6.6950 - 7.4141	7` - 6`
5.7759 - 2.4188	7 - 8

Table 3.3.5 NOESY H-H NMR Spectral Data of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate:

$CDCl_3$, TMS = 0.0000 ppm, Figure 3.21, page 141

Cross Peaks PPM-PPM	H-H Connection
0.9560 - 5.6825	18 - 12
0.9560 - 7.6309	18 - 1
1.1347 - 5.7759	14 - 7
1.6799 - 7.6743	11-HN
2.4188 - 1.3015	8 - 16
2.4188 - 1.6789	8 - 11
4.2687 - 5.7759	20 - 7
5.6825 - 1.1347	12 - 14
6.3905 - 2.7061	5 - 4
6.6950 - 2.9330	7` - CH_3N
6.6950 - 7.4141	7` - 6`
6.6950 - 7.8004	7` - 4`
5.7759 - 4.5215	7 - 20
5.7606 - 5.6825	OH 9 - 12
1.9036 - 4.5215	Acetate - 20
2.1431 - 6.3905	Acetate - 5

Table 3.3.6 C-H One Bond Correlation NMR Spectral Data of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate:
$CDCl_3$, TMS = 0.00 ppm, Figure 3.22, page 142 -144.

Cross-Peaks PPM-PPM	C-H Connection
10.18 - 1.7333	C 19 - H 19
15.19 - 0.9560	C 18 - H 18
16.82 - 1.3015	C16 - H 16
20.45 - 1.9036	C Acetate - H 5
21.15 - 2.1431	C Acetates - H 13
23.60 - 1.2546	C 17 - H 17
29.56 - 2.9330	CH_3N - 3H(CH_3N)
35.95 - 1.1347	C 14 - H 14
42.81 - 2.4188	C 8 - H 8
48.84 - 2.7061	C 11- H 11
52 .09 - 3.5796	C 10 -H 10
65.93 - 4.5215 (4.77425 & 4.2687)	C 20- 2H 20
69.81 - 6.3905	C 5 - H 5
75.78 - 5.6825	C 7 - H 7
110.87 - 6.6950	C 7` - H 7`
114.47 - 6.59715	C 5` - H 5`
131.30 - 7.8004	C4` - H 4`
134.25 - 5.7759	C 7 - H 7
135.05 - 7.4141	C 6` - H 6`
160.68 - 7.6309	C 1 - H 1

IR Spectral Data of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate: Thin film on NaCl disc, $CHCl_3$
3400, 2925, 1745, 1705, 1690, 1580, 1520, 1430, 1375, 1260, 1230, 1125, 1085, 1050, 975, 750 cm^{-1}.

3.3.3 Isolation of Sapintoxins From The Fruit Oil of *Sapium indicum*

An ether-soluble resin of *Sapium indicum* fruits oil was prepared according to the chart in Figure 2.1, pp. 6, and was stored at -20C for more than five years. 350 ml of this resin was separated by centrifugal liquid chromatography (CLC) using 4 mm-thick silica gel disc and was eluted with the following gradient at a flow rate of 4ml/min: toluene:hexane 3:1 50ml, toluene 100ml, toluene:ethylacetate 9:1, 6:1, 4:1, 3:1, 1:1, 1:3,1:4, 1:6, 1:9 (100ml of each mixture), ethylacetate 100% 150ml and acetone 200ml. 10ml fractions were collected and monitored by a hand held UV lamp set at 254 nm.

Fractions were grouped according to similarities on analytical TLC. Each subfraction was tested for irritant activity on the mouse-ear. Fractions eluted with toluene:ethylacetate

1:6, 1:9 and ethylacetate 100% induced severe inflammation to the mouse ear. These fractions were purified further to isolate the following diterpene esters:

3.3.3.1 Isolation and characterization of Sapintoxin-A, 12-O-(2-Methylaminobenzoyl)-4-Deoxyphorbol-13-Acetate:

This compound was eluted from the CLC plate with toluene:ethylacetate 1:6 which was purified using preparative adsorption chromatography. The TLC plate was developed three times in cyclohexane:ethylacetate 3:7. A blue UV fluorescent band was recovered and extracted from the silica gel with chloroform:acetone mixed at a ratio of 1:1 to yield a lemon yellow residue.

Final purification was achieved on semi-preparative HPLC using hexane:chloroform 1:99 as eluent at a flow rate of 1ml/min followed by chloroform:methanol 99.8:0.2 at the same rate. Eluate having a retention time (R_t) of 15.9minutes was collected. The solvent was evaporated under a stream of nitrogen and the colourless resin was found to be a single component on 2D-TLC. The spectral characteristics of the isolated compound are as follows:

Mass Spectrum
FAB MS MNOBA + Na Matrix:

Measured Mass 546.2464 Daltons

Accurate Mass 546.2468 Daltons corresponding to $C_{30}H_{37}O_7N$ Na

Table 3.3.7 EI-Mass Spectral Data of Sapintoxin-A: 30eV, 110°C

m/z	%Abundance	Lost Ion	Inference
523	30		$M^+ = C_{30}H_{37}O_7N$
445	2	78	$M^+ - (C_6H_6)$
373	55	72	$M^+ - (C_6H_5NHCH_3COO^·)$
355	5	18	$M^+ - (C_6H_5NHCH_3COO^· + H_2O)$
330	10	43	$M^+ - (C_6H_5NHCH_3COO^· + H_2O + CH_3CO^·)$
313	60	17	$M^+ - (C_6H_5NHCH_3COO^· + H_2O + CH_3CO^· + OH)$
295	25	18	$M^+ - (C_6H_5NHCH_3COO^· + H_2O + CH_3CO^· + OH + H_2O)$
151	100		$C_6H_5NHCH_3COOH$
134	90	17	$C_6H_5NHCH_3CO^·$
105	30		$C_6H_5CO^{·+}$

Table 3.3.8 ^{1}H-NMR Spectral Data of Sapintoxin-A:
$CDCl_3$, 400MHz, TMS=0.0000 ppm, Figure 3.24, page 146.

PPM	Integration	Multiplicity	J=Hz	Inference
7.8200	1H	dd	1.5	Aromatic H4'
7.7092	1H	m	-	NH
7.5676	1H	s	-	H1
7.4100	1H	m (d, t, d)	1.5	Aromatic H6'
6.6900	1H	d	8.4	Aromatic H7'
6.5930	1H	t	1.5	Aromatics H5'
5.6589	1H	d	2.14	H12
5.6314	1H	s (D_2O exchanged)		OH9
5.5500	1H	m	-	H7
4.0375	2H	q	13.54	2H20
3.2730	1H	m	-	H10
2.9321	3H	s	-	CH_3N
2.8693	1H	m	-	H4
2.5227	1H	m	-	H11
2.4426	1H	t	5	H8
2.1820	2H	m	-	H5
2.1318	3H	s	-	3H 13 Acetate
1.7239	3H	m	-	3H19
1.6500	1H	bs (D_2O exchanged)		OH20
1.3207	3H	s	-	3H16
1.1960	3H	s	-	3H17
1.1220	1H	d	5.34	H14
0.9590	3H	d	6.415	3H18

The two protons singlet at 7.6932 ppm changed to one proton singlet following addition of a drop of D_2O. The three protons doublet at 2.9286 ppm corresponding to the methyl protons in the CH_3N group collapsed into a singlet upon the addition of D_2O, which is due to its coupling to the exchanged NH proton at 7.6932ppm as shown in the COSY H-H NMR spectrum.

Table 3.3.9 ^{13}C-NMR Spectral Data of Sapintoxin-A:
$CDCl_3$, 100MHz ,TMS =0.00 ppm, Figure 3.25, page 147.

PPM	Integration	Inference
209.72	C	C3
173.79	C	C13 (Carbonyl)
168.05	C	C1′
159.65	CH	C1
152.26	C	C3′
142.08	C	C2

Table 3.3.9 continued

PPM	Integration	Inference
136.47	CH	C6
134.95	CH	C6′
131.32	CH	C4′
126.40	CH	C7
114.46	CH	C5′
110.87	CH	C7′
109.36	C	C2′
77.24	C	C9
76.14	CH	C12
67.14	CH_2	C20
65.43	C	C13
54.12	CH	C10
44.19	CH	C11
42.54	CH	C4
42.07	CH	C8
35.66	CH	C14
29.61	CH_3	CH_3-N
29.58	CH_2	C5
25.70	C	C15
23.71	CH_3	C16
21.17	CH3	CH3 (Acetate)
16.89	CH3	C17
15.11	CH3	C18
10.23	CH3	C19

Table 3.3.10 COSY H-H NMR Spectral Data of Sapintoxin-A:
$CDCl_3$, TMS = 0.0000 ppm, Figure 3.26, page 148.

Cross Peaks PPM-PPM	H-H Connection
0.9590-1.6500	H18 - H9
1.7239-3.2730	H19 - H10
1.7239-7.5676	H19 - H1
2.1820-2.8693	H5 - H4
2.4426-1.1220	H8 - H14
2.4426-5.5500	H8 - H7
2.5227-2.1318	H11 - 3H Acetate
2.1820 - 4.0375	2H5-2H20
6.593-7.8200	H5′ - H4′
6.593-7.4100	H5′ - H6′
6.6900-7.4100	H7′ - H6′

Table 3.3.11 NOESY H-H NMR Spectral Data of Sapintoxin-A:
$CDCl_3$, TMS =0 .0000 ppm, Figure 3.27, page 149.

Cross Peaks PPM-PPM	H-H Connection
1.3207-1.7239	H17-H19
1.3207-7.8200	H17-H4'

Table 3.3.11 continued

Cross Peaks PPM-PPM	H-H Connection
1.1220-5.5500	H14-H7
5.5500-4.0375	H7-2H20
2.1820-4.0375	H5-2H20

Table 3.3.12 C-H One Bond Correlation NMR Spectral Data of Sapintoxin-A: $CDCl_3$, TMS = 0.0000 ppm.

Cross Peaks PPM-PPM	C-H Connection
159.65 - 7.5676	C1-H1
134.95 -7.4100	C6′-H6′
131.32 - 7.8200	C4′-H4′
126.40 - 5.5500	C7-H7
114.46 - 6.5930	C5′-H5′
110.87 - 6.6900	C7′-H7′
76.14 - 5.6589	C12-H12
67.14 - 4.0375	C20-2H20
54.12 - 3.2730	C10-H10
44.19 - 2.5227	C11-H11
42.07 - 2.4426	C8-H8
35.66 - 1.1220	C14-H14
29.07 - 2.9321	$C\underline{H_3}N$-3H (CH_3N)
23.71 - 1.1960	C16-H16
16.89 - 1.3207	C17-H17
15.11 - 0.9590	C18-H18
10.23 - 1.7239	C19-H19

IR Spectral Data of Sapintoxin-A: Thin film on NaCl disc, $CHCl_3$
3400, 3000, 2400, 1700, 1529, 1420, 1110, 1040, 925, 745, 660 cm^{-1}.

3.3.3.2 Isolation and Characterization of the Novel Sapintoxin-E, 12-O-(2-Methylaminobenzoyl)-4,20-Dideoxyphorbol-20-methyl-13-Acetate:

This compound was isolated from CLC fractions eluted with toluene:ethylacetate 1:9 and 100% ethyl acetate. Initial purification was carried out on 0.5mm preparative silica gel TLC plate developed three times in cyclohexane:ethylacetate:methanol 7:3:1. The recovered blue UV fluorescent band was re-chromatographed on a preparative adsorption TLC plate developed twice in chloroform:methanol 90:10. A band having an R_f value of 0.47 was scraped off the plate and then extracted with acetone and chloroform. Semi-preparative normal phase HPLC was used as a final purification step. The HPLC column was eluted with chloroform:methanol 99.8-0.2 at a flow rate of 1ml/min to yield the colourless compound at 16.9 minutes. The isolated diterpene have the following spectroscopic data:

Mass Spectrum

High resolution FAB mass spectrum exhibited:
Measured Mass = 507.6247 Daltons
Actual Mass = 507.6247 Daltons , corresponding to $C_{30}H_{37}O_6N$

Table 3.3.13 EI Mass Spectral Data of Sapintoxin-E: 30eV, 110°C

mz	% Abundance	Lost Ion	Inference
507	2		M^+ corresponding to $C_{30}H_{37}O_6N$
445	2	62	$M^+ - (CH_3COOH+H)$
430	2	15	$M^+ - (CH_3COOH+H+CH_3^{\cdot})$
279	10	151	$M^+ - (CH_3COOH+H+CH_3^{\cdot}+C_6H_4NHCH_3COOH)$
149	65		$C_6H_3NHCH_3COO^{\cdot}$

Table 3.3.14 ^{1}H-NMR Spectral Data of Sapintoxin-E:
$CDCl_3$, 400MHz ,TMS = 0.0000 ppm, Figure 3.28, page 150.

PPM	Integration	Multiplicity	J=Hz	Inference
7.8037	1H	dd	1.5	H4′
7.6932	2H	s	-	NH & H1
7.4055	1H	m	-	H6′
6.6891	1H	d	8.47	H7′
6.5854	1H	t	14.59	H5′
5.6241	1H	d	9.65	H12
5.6060	1H	s	-	OH9
5.3168	1H	m	-	H7
4.8664	1H	bs	-	H4
3.5254	1H	m	-	H10
2.9286	3H	d	4.47	3H ($C\underline{H}_3N$)
2.6254	2H	m	-	2H5
2.3297	1H	bs	-	H8
2.1266	3H	s	-	3H (Acetate)
1.8685	3H	s	-	3H20
1.7493	3H	m	-	3H19
1.6553	1H	m	-	H11
1.5914	3H	s	-	3H16
1.1984	3H	s	-	3H17
1.0792	1H	d	5.58	H14
0.9621	3H	d	6.3	3H18

Following the addition of a drop of D_2O, the two protons broad singlet at 7.6932ppm changed to one proton singlet and the one proton singlet at 5.606ppm disappeared from the spectra. In addition, the three protons doublet at 2.9286ppm collapsed into a singlet which is due to the coupling between the one proton singlet at 7.6932ppm corresponding to the NH and the three protons in CH_3N group respectively.

Table 3.3.15 ^{13}C-NMR Spectral Data of Sapintoxin-E:

$CDCl_3$, 100MHz ,TMS = 0.00 ppm, Figure 3.29, page 151.

PPM	Integration	Inference
208.58	C	C3
173.67	C	C13 (Carbonyl)
168.03	C	C1′
162.73	CH	C1
152.00	C	C3′
140.58	C	C2
138.40	C	C6
134.94	CH	C6′
131.32	CH	C4′
127.34	CH	C7
110.91	CH	C7′
109.00	C	C2′
78.34	C	C9
76.29	CH	C12
71.08	CH	C11
65.54	C	C13
51.44	CH	C10
42.99	CH	C4
42.38	CH	C8
29.72	CH_2	C5
29.29	CH_3	$\underline{C}H_3N$
25.82	C	C5
23.71	CH_3	C16
21.76	CH_3	C20
21.15	CH_3	Acetate at C13
16.95	CH_3	C17
15.30	CH_3	C18
10.13	CH_3	C19

The previously unreported Correlation spectra for this compound were also recorded as listed below:

Table 3.3.16 COSY H-H NMR Spectral Data of Sapintoxin-E:

$CDCl_3$, TMS = 0.0000 ppm, Figure 3.30, page 152.

Cross Peaks PPM-PPM	H-H Connection
6.6891 -7.4055	H7′-H6′
6.5854 -7.8037	H5′-H4′
6.5854 -7.4055	H5′-H6′
5.6241 -1.6553	H9-H11
5.3168 -1.8685	H7-3H20
4.8664-1.8685	H4-3H20
3.5254-7.6932	H12-H1
3.5254-1.7493	H10-3H19

Table 3.3.16 continued

Cross Peaks PPM-PPM	H-H Connection
3.5254-2.6254	H10-2H5
2.9286-7.6923	3H($C\underline{H}_3$N)-HN
2.6254-4.8664	H5-H4
2.6254-3.525	H5-H10
2.3297-5.3168	H8-H7
2.3297-1.0792	H8-H14
1.8685-5.3168	3H20-H7
1.7493-7.6932	H19-H1
1.7493-5.606	3H19-OH9
1.7493-3.5254	3H19-H10
1.1984-2.3297	3H17-H8
0.9621-1.6553	3H18-H11

Table 3.3.17 NOESY H-H NMR Spectral Data of Sapintoxin-E:

$CDCl_3$, TMS = 0.0000 ppm

Cross Peaks PPM-PPM	H-H Connection
7.8037-1.1984	H4′-3H17
7.8037-6.5854	H4′-H5′
7.6932-0.9621	$\underline{H}$N-3H18
7.6932-1.5914	$\underline{H}$N-3H16
7.6932-3.5254	H1-H10
6.6891-2.9286	H7′-CH_3N
6.6891-7.4055	H7′-H6′
5.6241-0.9621	H12-3H18
5.6060-1.0792	OH9-H14
5.3168-1.0792	H7-H14
5.3168-1.8685	H7-3H20
4.8664-1.8685	H4-3H20
4.8664-2.6254	H4-2H5
3.5254-1.6553	H10-H11
3.5254-2.625	H10-H5
3.5254-5.3168	H10-H7
3.5254-7.6932	H10-H1
2.9286-6.6891	CH_3N-H6′
2.6254-1.7493	2H5-3H19
2.6254-2.3297	2H5-H8
2.6254-4.8664	2H5-H4
2.3297-1.5914	H8-3H16
1.8685-4.8664	3H20-H4
1.8685-5.3168	3H20-H7
1.7493-7.6932	H19-H1
1.7493-4.8664	H19-H4
1.6553-0.9621	H11-3H18
1.6553-1.1984	H11-3H17
1.6553-2.3217	H11-H8

IR Spectral Data of Sapintoxin-E: Thin film on NaCl disc, $CHCl_3$

3680, 3640, 3000, 2960, 2200, 1700, 1520, 1489, 1420, 1220, 1050, 930, 880, 760, 660 cm^{-1}.

3.3.3.3 Isolation and Characterization of Sapintoxin-D, 12-O-(2-Methylaminobenzoyl)-Phorbol-13-Acetate:

This compound was the major irritant constituent in the ethylacetate CLC fraction. It was initially purified on adsorption preparative TLC plate developed three times in chloroform:methanol 99:1. A blue UV fluorescent zone having an R_f value of 0.05 was scraped off the plate, extracted with chloroform:acetone 1:1 then dried over anhydrous sodium sulphate and dissolved in chloroform. The chloroformic solution was injected into a semi-preparative normal phase HPLC column and eluted with chloroform:methanol 99.8:0.2 at a flow rate of 1ml/min. Final purification was achieved by repeating the above HPLC procedure using the same parameters. The isolated diterpene has the following spectral characters:

Mass Spectrum

FABMS using MNOBA+Na as a matrix:

Measured Mass = 562.2413 Daltons

Accurate Mass = 562.2417 Daltons corresponding to $C_{30}H_{30}O_8NNa$

Table 3.3.18 EI Mass Spectral Data of Sapintoxin-D: 30eV, 110°C

m/z	%Abundance	Lost Ion	Inference
539	5	-	M^+
389	10	150	M^+- (RCOO·)
329	7.5	60	M^+- (RCOO·+CH_3COOH)
311	7.5	18	M^+- (RCOO·+CH_3COOH+H_2O)
293	7.5	18	M^+- (RCOO·+CH_3COOH+H_2O+H_2O)
263	10	30	M^+- (RCOO·+CH_3COOH+H_2O+H_2O+CH_2O)
151	70	-	RCOOH
149	85	1	RCOO· - H
71	70	-	C_6H_{11}

R = $C_6H_4NHCH_3$

Table 3.3.19 ^{1}H-NMR Spectral Data of Sapintoxin-D:

$CDCl_3$, 400MHz, TMS = 0.0000 ppm, Figure 3.31, page 153.

PPM	Integration	Multiplicity	J=Hz	Inference
7.8509	1H	dd	1.5	H4′
7.7037	1H	m	-	NH
7.6106	1H	s	-	H1

Table 3.3.19 continued

PPM	Integration	Multiplicity	J=Hz	Inference
7.3995	1H	m	-	H6′
6.6856	1H	d	8.38	H7′
6.5958	1H	t	7.73	H5′
5.7100	1H	d	4.7	H7
5.6486	1H	s	-	OH9
5.6224	1H	superimposed on 5.6486ppm		H12
4.0356	2H	q	9.92	2H20
3.3100	1H	m	-	H8
3.2772	1H	m	-	H10
2.9270	3H	d	4.9	3H (CH_3N)
2.6380	1H	s	-	αH5
2.5398	1H	m	-	βH5
2.4382	1H	s	-	OH4
2.2955	1H	m	-	H11
2.1260	3H	s	-	3H (Acetate at C13)
1.7760	3H	m	-	3H19
1.6396	1H	bs	-	OH20
1.3662	3H	s	-	H17
1.2577	3H	s	-	H16
1.1277	1H	d	5.24	H14
0.9277	3H	d	6.43	H18

Addition of a drop of D_2O the one proton multiplet at 7.7037 ppm corresponding to N<u>H</u> disappeared while the three protons doublet at 2.9270 ppm corresponding to$C\underline{H}_3NH$ collapsed to a sharp singlet. In addition, the one proton singlet at 5.6486ppm and at 1.6396ppm each corresponding to a hydroxyl group at C9 and C20 respectively disappeared completely.

Table 3.3.20 ^{13}C-NMR Spectral Data of Sapintoxin-D:
$CDCl_3$, 100MHz, TMS = 0.00 ppm, Figure 3.32, page 154.

PPM	Integration	Inference
208.93	C	C3
173.86	C	Carbonyl at C13
168.23	C	C1′
160.74	CH	C1
140.45	C	C2
152.23	C	C3′
134.81	CH	C6′
132.90	C	C6
131.47	CH	C4′
129.21	CH	C7
114.38	CH	C5′
110.77	CH	C7′
109.63	C	C2′

Table 3.3.20 continued

PPM	Integration	Inference
78.30	C	C9
76.15	CH	C12
73.72	C	C4
68.03	CH_2	C20
65.78	C	C13
56.18	CH	C10
43.21	CH	C11
39.10	CH	C8
38.62	CH_2	C5
36.40	CH	C14
29.57	CH_3	CH_3N
25.57	C	C15
23.83	CH_3	C16
21.20	CH_3	CH_3 (Acetate at C13)
16.97	CH_3	C17
14.45	CH_3	C18
10.13	CH_3	C19

Table 3.3.21 COSY H-H NMR Spectral Data of Sapintoxin-D:
$CDCl_3$, TMS = 0.0000 ppm, Figure 3.33, page 155.

Cross Peaks PPM-PPM	H-H Connection
0.9277 - 2.2955	H18-H11
1.7760 - 3.2772	H19-H10
1.7760 - 7.6106	H19-H1
3.3100 - 5.7100	H8-H7
3.310 0- 1.1277	H8-H14
2.9270 - 7.7037	3H (CH3N)-HN
3.2772 - 1.7760	H10-H19
5.6486 - 2.2955	H12-H11
6.5958 - 7.3995	H5′-H4′
6.5958 - 7.8509	H5′-H6′
6.6856 - 7.3995	H7′-H6′
2.2955 - 0.9277	H11-H18
2.2955 - 5.6486	H11-H12
2.5398 - 5.7100	βH5-H7
2.6380 - 5.7100	αH5 - H7

Table 3.3.22 NOESY-NMR Spectral Data of Sapintoxin-D:
$CDCl_3$,TMS = 0.0000 ppm

Cross Peaks PPM-PPM	H-H Connection
2.2955-3.2772	H11-H10
2.2955-1.3662	H11-H17
3.3100-1.2577	H8-H16
3.3100-2.2955	H8-H11
3.3100-2.4382	H8-OH4

Table 3.3.22 continued

Cross Peaks PPM-PPM	H-H Connection
4.0356-5.7106	2H20-H7
5.6224-0.9277	H12-H18
3.2727-2.5398	H10-βH5
5.7100-1.1277	H7-H14
0.9277-5.6486	H18-OH9
0.9277-7.6106	H18-H1
2.5398-4.0356	βH5-2H20
6.6856-7.3995	H7-H6′
6.5958-7.8509	H5′-H4′
6.5958-7.3995	H5′-H6′
2.5398- 3.2772	αH5-H10

IR Spectral Data of Sapintoxin-D: Thin film on NaCl disc, CHCl3:

3880, 3820, 3400, 3000, 2400, 1700, 1529, 1480, 1420, 1129, 1050, 930, 779, 670 cm^{-1}.

3.3.4 Discussion

Four nitrogen-containing phorbol derivatives have been isolated from the ether fractions of *Sapium insigne* and *Sapium indicum*. The structures of the isolated compounds were elucidated from the data obtained by means of different spectroscopic experiments.

Analytical TLC plates provided evidence for the presence of a group of tigliane type diterpenes termed sapintoxins. After spraying the plates with 60% H_2SO_4 and heating them at 110°C for 15 minutes, spots were detected having brown-yellow colour in day light and blue fluorescence under UV lamp. This colour reaction is almost identical to that previously reported for sapintoxins (Taylor *et al* 1981a).

Compounds isolated during this investigation have a methyl amino benzoate moiety at C12. Evidence for the presence of this side chain was obtained from the fragmentation pathway of the molecular ion in mass spectra. An ion corresponding to the loss of 151 or 149 mass units from the molecular ion was prominent in th EI mass spectra of the four isolated compounds indicating the presence of a methyl aminobenzoate substituent. This was confirmed by the presence of either a base peak (100% abundance) at 151 m/z or at 149 m/z in the lower region of the spectra (Table 3.3.1; pp. 99, Table 3.3.7; pp. 103, Table 3.3.13; pp. 107, Table 3.3.18; pp. 110). The sequential loss of acyl derivative, with the formation of fragment ions 313, 311 m/z and the dehydroxylated fragment ion at 294 m/z exhibited in

the spectra is typical of 4-deoxyphorbol derivatives. It was noted that the molecular ion in the EIMS of the isolated compounds exhibited low relative abundance of 2-5%. The use of FABMS technique with MNOBA+Na as a matrix showed dramatic increase in the relative abundance of the moleular ion plus Na up to 100% in the case of the semi-synthetic sapintoxin from *S. insigne*.

The tigliane nucleus was identified from the ^{1}H- and ^{13}C-NMR spectra. In the ^{1}H-NMR spectra, the signals at approximately 1.88 and 5 ppm for the isopropylene side chain at C13 in daphnanes (Section 3.1 and 3.2) were replaced by signals at 1.2 and 1.3 ppm each corresponding to three protons of a methyl group. In addition, the ^{13}C-NMR spectra exhibited signals at 16 and 23 ppm each corresponding to a methyl group.

Data from the one bond C-H correlation spectra of SAP-A and sapintoxin triacetate demonstrated that the three protons signal at 1.2ppm correlated with the methyl carbon at 23ppm and three protons signal at 1.3ppm correlated with the methyl carbon at 16ppm. The lack of COSY H-H connection of either signals, together with the C-H correlation, provided significant evidence that the former group of signals corresponded to 3H at C-16 and the latter one corresponded to 3H at C17.

The semi-synthetic compound isolated from *S. insigne* is the 4α-deoxy-5-hydroxy phorbol triacetate derivative. In addition to the base peak of 151m/z in the mass spectrum, the ^{1}H-NMR provided supporting evidence for the presence of the N-methyl aminobenzoyl group in the compound. The two doublets at 7.6743 ppm and 2.9330 ppm, having strong COSY connection and a coupling constant of 4.8Hz, are typical of protons at NH and NCH_3 respectively. Further evidence was obtained from the C=O stretching absorbion at 1690 cm^{-1} in the IR spectrum. This is typical for anthranilates as previously reported (Hecker *et al* 1977). The ^{13}C-NMR DEPT spectrum exhibited only one signal at 65.93ppm corresponding to a CH_2 group. Protons of the CH_2 group were represented by signals at 4.7742 and 4.2687 ppm in the ^{1}H-NMR with a coupling constatnt of 11.05Hz and were assigned to 2H on C20 according to one bond C-H, COSY and NOESY connections (Table 3.3.4; pp. 101, Table 3.3.5; pp. 101, Table 3.3.6; pp. 102).

Signals for three acetate groups were also evident in both the carbon and the proton

NMR spectra. Assignment of the ester groups were supported by MS and COSY spectra. This was in agreement with the role (Evans & Soper 1978) that the N-methyl amino benzoyl ester group at C12 was eliminated from the molecule as the aryloxy radical (M^+-150), whereas the acetate at C13 split off as acetic acid (M^+-60) as outlined in Table 3.3.1, pp. 99. This fragmentation pathway is characteristic of the A-series phorbol esters which exhibit a higher molecular weight acyl function at C12. Further evidence was obtained from NOESY connection of the signal of 1H on C11 at 1.6799 ppm with that of NH of the methyl aminobenzoyl at 7.6743 ppm confirming that the higher molecular weight acyl group is attached to C12.

Assignment of the olefinic ^{1}H-NMR signals to their corresponding aromatic protons are based on data (Table 3.3.23, pp. 115) from the values of coupling constants supported by the COSY and NOESY connections.

Table 3.3.23 Aromatic Protons Connections of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-hydroxyphorbol-5,13.20-Triacetate:

^{1}H-NMR ppm	J= Hz	COSY	NOESY	Inference
6.6950 d H7`	8.3	7.4141 H 6`	2.9330 $\underline{CH_3}N$	H7`- H 6` & CH_3N
6.5972 d,t,d H5`	7.3, 0.7	7.4141 H6`	7.4141 H6` 7.8004 H4`	H5`- H6` H5`- H4`
7.8004 dd H4`	1.59	6.5972 H7`	6.5972 H5`	H4`- H7` & H5`
7.4141 d,t,d H6`	1.5	6.6950 H7` 6.5972 H5`	6.6950 H7` 6.5972 H5`	H6`- H7` & H5`

The semi-synthetic compound isolated from *Sapium insigne* was inactive in the standard ear-erythema test at doses up to 20µg/ear. However, it was reported that 4-deoxy-20-hydroxy phorbol derivatives induce inflammation to the mouse-ear (Taylor 1981). The lack of this activity in the acetylated sapintoxin is partly attributed to the absence of a free hydroxyl group at C20. Neverthless, epimerization of the biologically active AB-trans sapintoxins into the inactive cis-analogues together with characterisitic ^{1}H-NMR signals were previously reported (Hecker 1977, Evans and Taylor 1983).

Identification of the 4α-phorbol nucleus was based on the chemical shifts of certain

protons in the ^{1}H-NMR spectrum supported by data from H-H space NOESY correlation in the NOESY spectrum. Examination of the ^{1}H-NMR spectrum of the semi-synthetic sapintoxin, for the presence of the characteristic chemical shifts for the AB-cis epimer, revealed that 1H on C11 exhibited a multiplet at 1.67ppm which is characteristic of the AB-cis phorbol isomer while the proton on C-10 resonated at 3.5796 ppm. Supporting evidence for the H11 assignment was provided by its C-H and COSY connection to the H10 multiplet. Based on NOESY and C-H connections the singlet at 5.7606 ppm was assigned to the proton of the hydroxyl group on C9. The proton on C4 has COSY and NOESY connections with the doublet at 6.3905ppm exhibited by the proton on C5, provided further evidence that the compound under investigation is a 4α-deoxy-5-hydroxyphorbol derivative.

Structural modelling using Sybyl software indicated that H-H correlation between H4 and H5 could not be in the trans analogue. Similar conclusion was achieved from NOESY connection between the α-orientated H8 and H1 providing strong support for the presence of a cis link between ring-A and ring-B. NOESY connections between H12 and the methyl protons on C18 in addition to H10 and the proton of the hydroxyl group on C9 further supported the AB-cis configuration.

Assignments of the proton and the carbon NMR signals for their corresponding atoms were confirmed by the C-H and COSY H-H correlation. Summary of these correlations and their implications on structural elucidation are listed in Table 3.3.4; pp. 101, Table 3.3.5; pp. 101 and Table 3.3.6; pp. 102. From the above data and literature search it has been concluded that the semisynthetic compound from extracts of twigs and leaves of *S. insigne* is the 12-O-(2-methylaminobenzoyl)-4α-deoxy -5-hydroxyphorbol-5,13,20-triacetate. This is the first report of this compound from this plant. The compound was not irritant to the mouse ear at doses up to 20μg/ear. This AB-cis isomer was unable to induce aggregation of human platelets or differentiation of HL-60 cells.

Three nitrogen-containing tigliane diesters were isolated from the fruits oil of *Sapium indicum*. The major compound was a 4-deoxyphorbol derivative, 12-O-(2-methylamino benzoyl)-4-deoxyphorbol-13-acetate, SAP-A (Figure 3.34a, pp. 156). In addition, a minor phorbol diester was isolated from the ether soluble fraction of the oil and was identified as 12-O-(2- methylaminobenzoyl)-phorbol-13-acetate, SAP-D (Figure 3.34b, pp. 156).

Furthermore a new member of the sapintoxin series was also isolated and identified by spectroscopic methods. The structure of the new sapintoxin, labelled sapintoxin-E, was 12-O-(2-methylaminobenzoyl)-4,20-dideoxy phorbol-20-methyl-13-acetate (Figure 3.34c, pp. 156).

The fragmentation pathway in the mass spectra of the isolated compounds indicated that the acyl substituents were methyl amino benzoate and acetate. Assignment of the acyl substituents to C12 and C13 respectively was based on NMR data. A characteristic of the ^{1}H-NMR of sapintoxins is the CH_3N doublet at 2.9ppm which is coupled to the NH signal at 7.7 ppm. The CH_3N doublet changed to a sharp singlet upon the addition of a drop of D_2O while the NH signal completely disappeared. Identification of Sapintoxin-A was based on comparison between its proton NMR and mass spectral data and those in literature (Taylor *et al* 1982, 1981a). The mass ion at 523 m/z suggested the presence of sapintoxin-A or sapintoxin-C. Both compounds have the same parent nucleus 4-deoxyphorbol and the same molecular weights however, substituents on C5 and C20 are different. The presence of $2CH_2$ signals in ^{13}C-NMR DEPT spectrum corresponding to 2H5 and 2H20 suggested that the isolated compound is sapintoxin-A. The ^{1}H-NMR was typical of the phorbol diester sapintoxin-A with a free primary hydroxyl group on C20. Assignment of the carbon signals of the previously unreported ^{13}C-NMR spectrum was guided with the DEPT spectrum and was later confirmed by the C-H one bond correlation NMR spectrum.

The COSY H-H NMR spectrum exhibited strong correlation between the 2H multiplet at 2.1820ppm and the 1H multiplet at 2.8693 ppm. This H-H correlation suggested that the former multiplet corresponds to the 2H on C5 rather than H11 as previously published (Evans *et al* 1981). In addition, the 2H5 multiplet correlates with 2H20 quartet at 4.0375ppm confirming the assignment. In the ^{13}C-NMR spectrum the CH signal at 44.19ppm is connected to 1H multiplet at 2.5227 ppm supporting the assignment of these two signals to C11 and H11 respectively. Signals of the aromatic protons were assigned according to the COSY H-H correlations. Assignment of the aromatic carbons signals was guided with C-H and COSY cross peaks. Analysing the spectroscopic data concluded that the major diterpene in the fruits oil of *S. indicum* is the 12-O-(2-methylaminobenzoyl)-4-deoxyphorbol-13-acetate (sapintoxin-A).

Sapintoxin-D was also isolated from *S. indicum*. The loss of two water molecules and one methine-oxy radical from the molecular ion in the EI mass spectrum (Table 3.3.18, pp. 110) indicated the presence of two hydroxyl groups and one primary alcohol in the structure. The ^{1}H-NMR spectrum of this compound was typical of a phorbol diester with a primary hydroxyl group on C20, a secondary hydroxyl group at C5, an N-methylaminobenzoate at C12 and an acetate moiety at C13 (Taylor *et al* 1981b). This was confirmed by analysing data from the ^{13}C-NMR spectrum. DEPT ^{13}C-NMR indicated that the signal at 68.03 ppm represents C20 primary alcohol function while the signal at 38.62ppm represents C5. In addition, the 2H on C5, resonating at 2.6380ppm and 2.5398ppm have COSY correlation with 1H on C7 at 5.7100ppm.

According to the x-ray diffraction analysis of phorbol, the 3H on C18 are α-orientated (Hecker and Schmidt 1974). The NOESY connection of the 3H on C18 to 1H1, 1H12 and OH9 but not H7 indicated that the latter proton is β-orientated. This was supported by the NOESY connection of H7 to 2H20 and was later confirmed by Sybyl model of the compound. NOESY correlation of H8 to H11 indicated that the former proton is β-oriented. The multiplet at 2.5398ppm represented β-H5 while the singlet at 2.6380ppm represented α-H5. This assignment was supported by NOESY connection to β-2H20 and α-H10 respectively. Spectroscopic data exhibited by this compound confirmed that the second biologically active constituent of *S. indicum* is 12-O-(2-methylaminobenzoyl)-phorbol-13-acetate, sapintoxin-D.

The third compound isolated for the fruit oil was a new 4,20-dideoxyphorbol derivative. The EI mass spectrum exhibited a 2% relative abundance M^{+} ion at m/z 507. The fragmentation pathway was similar to those for sapintoxins. However, the loss of 15 mass units replacing the elimination of 31 mass units suggested that the primary alcohol group at C20 has been replaced with a methyl group. The ^{1}H-NMR spectra was similar to sap-A with the following exceptions. The 2H20 quartet at 4.03756ppm in sapintoxin-A was replaced with 3H singlet at 1.8685ppm. In addition, the new 3H signal has COSY connection to 1H7 and NOESY connection to H7 and H14. The 1H14 was shifted downfield to 4.8664ppm and exhibited COSY correlation with 2H5 at 2.6254ppm. The ^{13}C-NMR spectrum confirmed the presence of an additional methyl group in the DEPT spectrum at 21.76ppm and the absence of 2H20 signal. The only CH2 in the DEPT spectrum at 29.72ppm was assigned to C5.

Based on the presented spectral data the compound was identified as the novel 12-O-(2-methyaminobenzoyl)-4,20-dideoxyphorbol-13-acetate, sapintoxin-E.

Three sapintoxins were isolated from the frozen fruit oil of *Sapium indicum*. Two of them were active in a number of *in vitro* and *in vivo* tests (Chapter 4 and 5). In contrast, 4α-deoxy-5-hydroxyphorbol triacetate has been identified as a semi-synthetic constituent from *S. insigne*. The twigs and leaves of this plant were collected in India in 1980 and were stored in cool, dry and dark conditions. The isolated compound was biologically inactive in a range of *in vivo* and *in vitro* tests (Chapter 4 and 5). The change of configuration of the link between ring A and ring B from the active trans-form to the inactive cis-form was reported to take place during crystallization and as a result of exposure to alkali (Taylor *et al* 1981a, 1981b). This study has demonstrated that biological activity of sapintoxins are retained for more than 5 years when stored at -20°C. Investigation of extracts of two *Sapium* species indicated that storage of extracts at subzero temperature prevents epimerization of sapintoxins to the inactive AB-cis isomers. In addition, the use of two-dimensional NMR experiments for structural elucidation of the isolated compounds provided further evidence for the re-assignment of previously reported spectral data of sapintoxins and identification of a new 4,20-dideoxyphorbol derivative, sapintoxin-E.

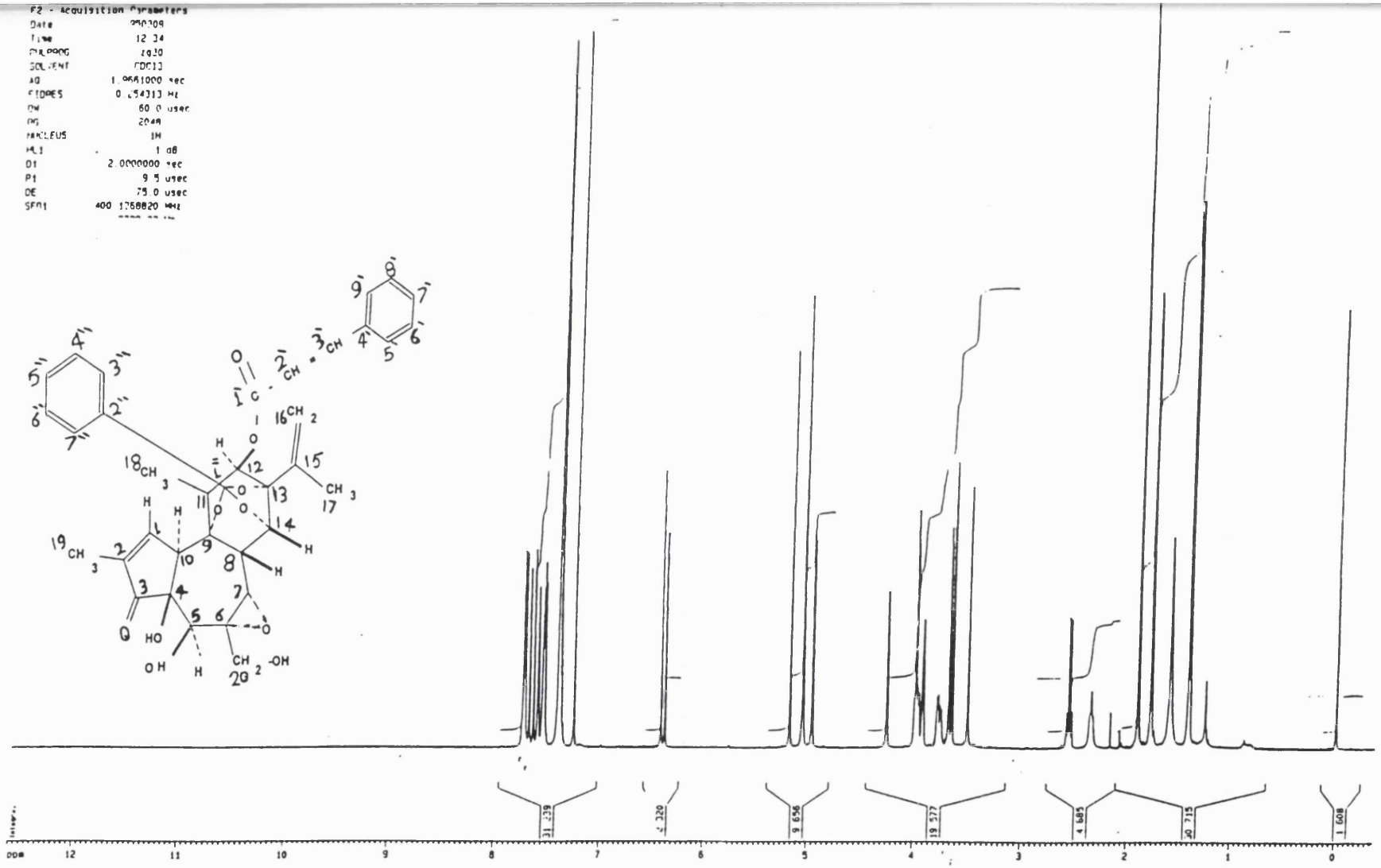


Figure 3.1 ^{1}H-NMR Spectrum (400MHz, $CDCl_3$) of Thymelaeatoxin-A, 12-O-(Cinnamoyl)-5-Hydroxy-6,7-Epoxy-Resiniferonol-9,13,14-Orthobenzoate

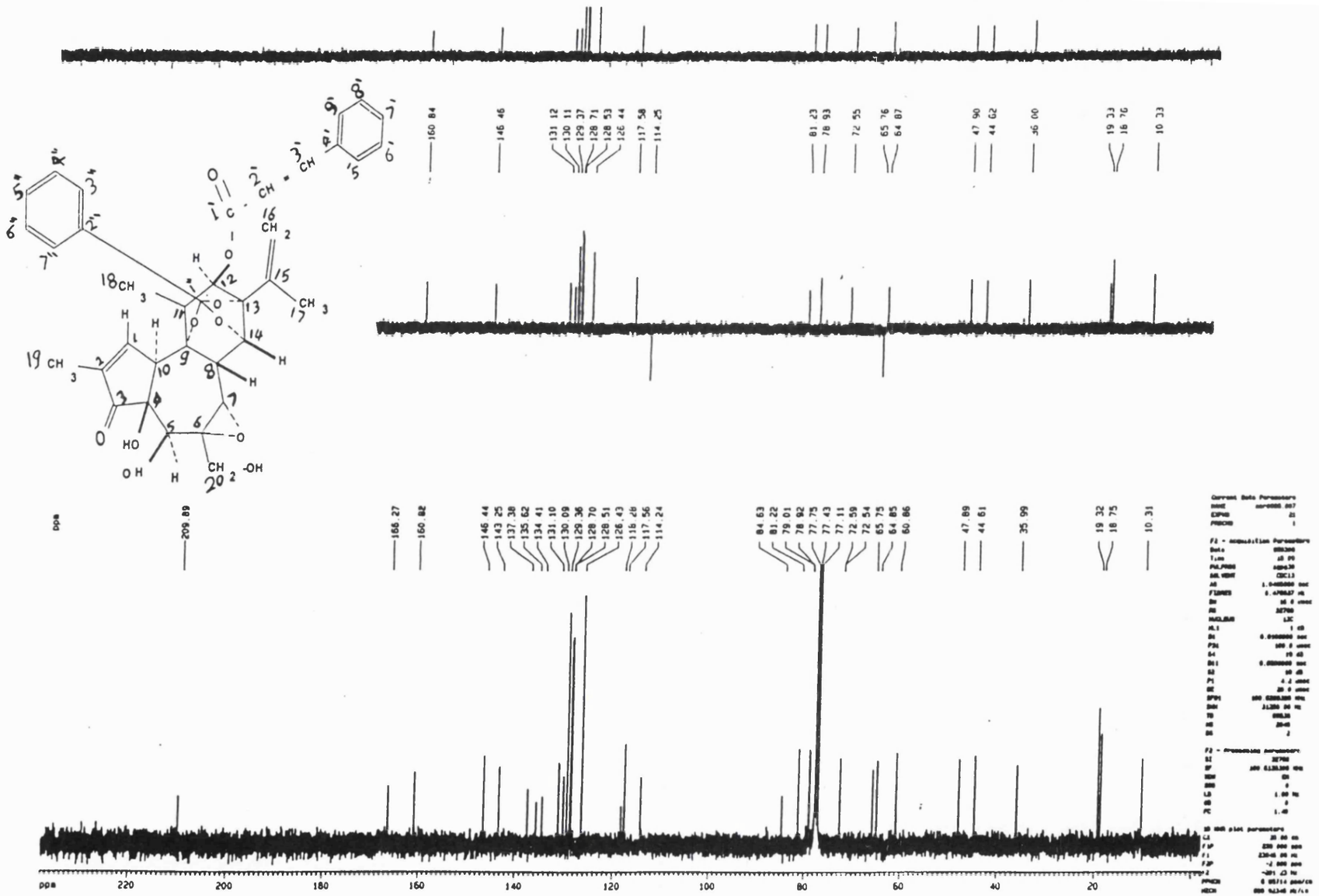


Figure 3.2 ^{13}C NMR Spectrum (100MHz, $CDCl_3$) of Thymelaeatoxin-A, 12-O-(Cinnamoyl)-5-Hydroxy-6α,7α-Epoxy-Resiniferonol-9,13,14-Orthobenzoate

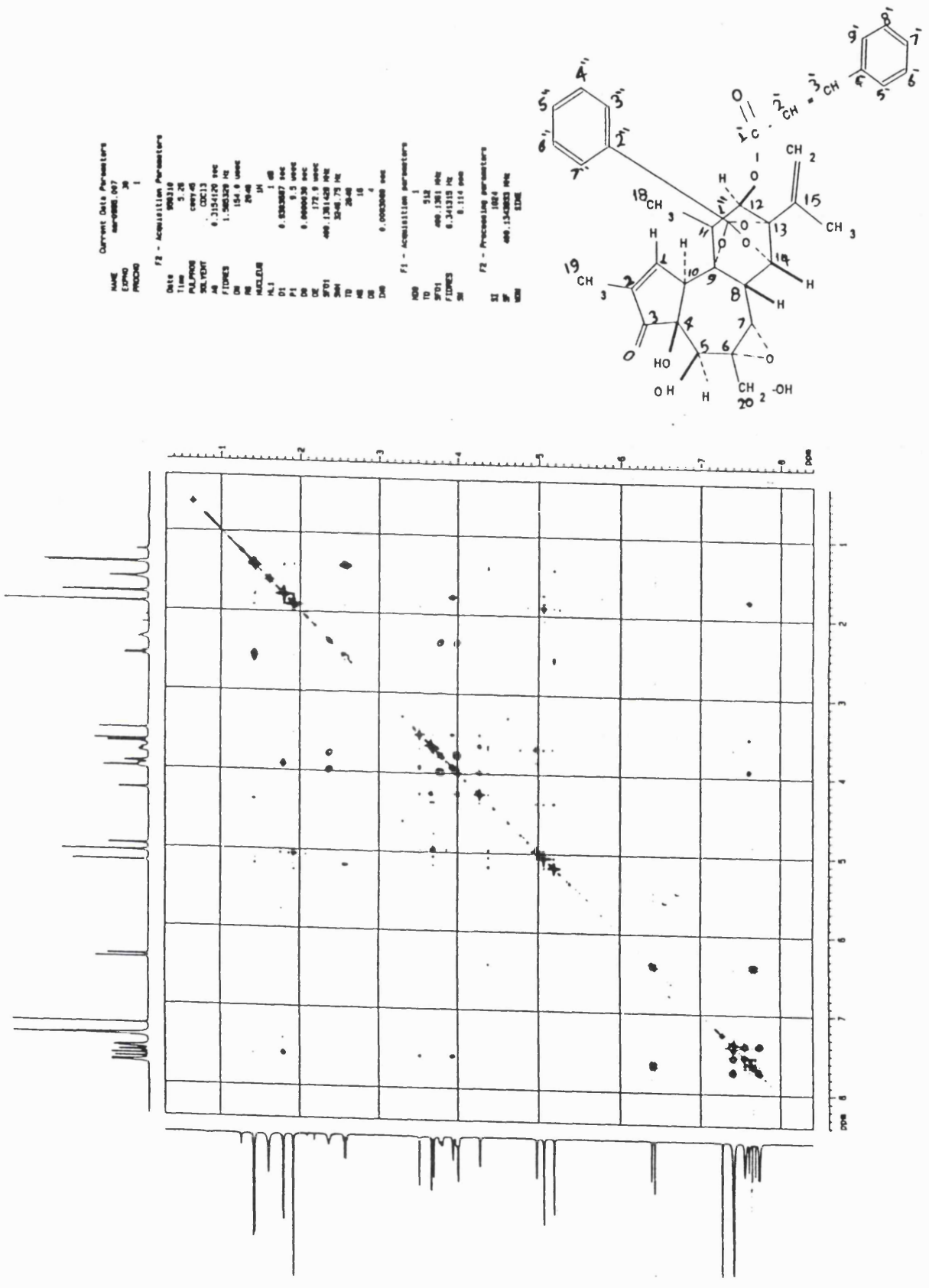


Figure 3.3 COSY H-H NMR Spectrum of Thymelaeatoxin-A, 12-O-(Cinnamoyl)-5-Hydroxy-6α,7α-Epoxy-Resiniferonol-9,13,14-Orthobenzoate

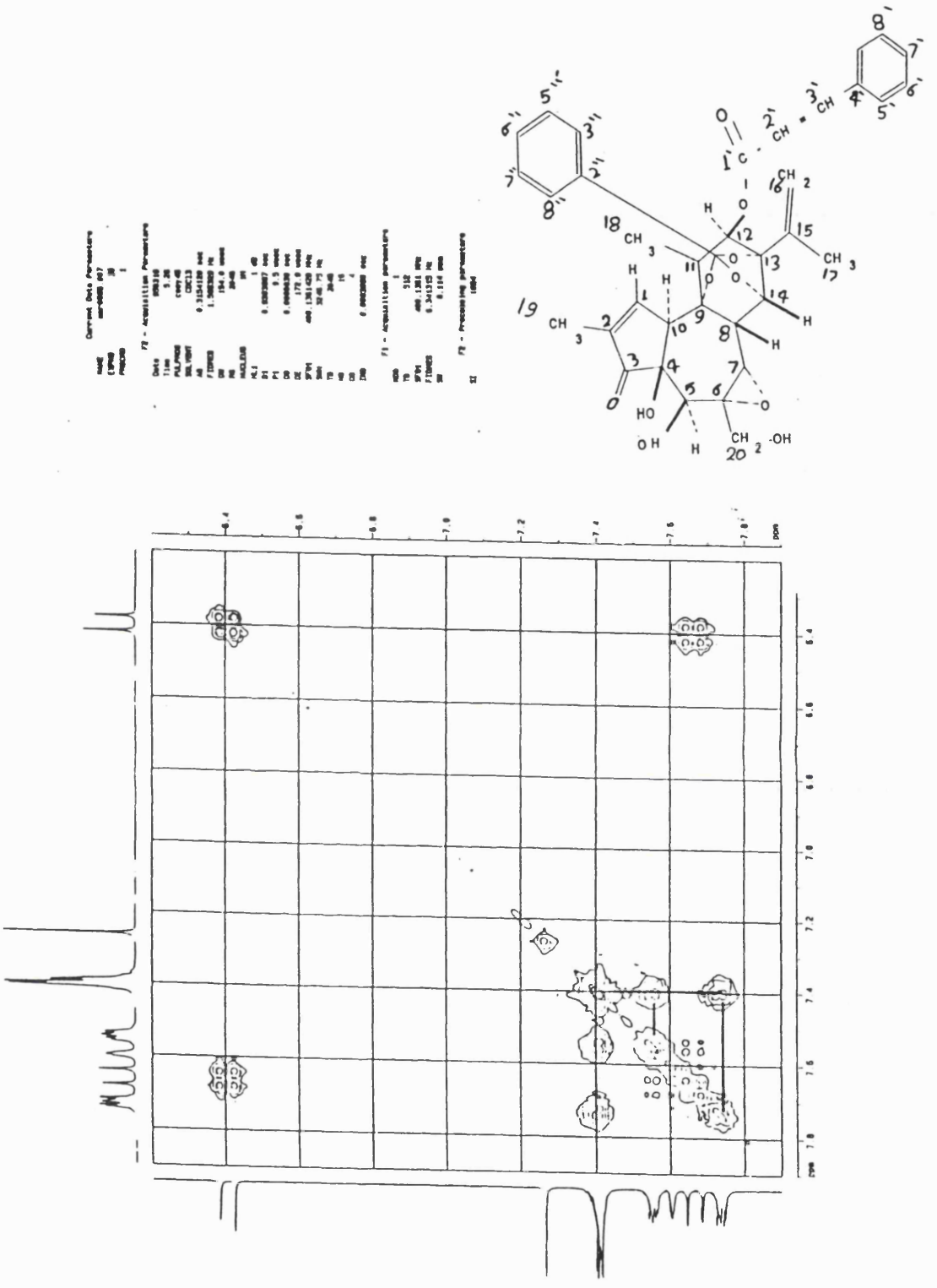


Figure 3.3A COSY H-H NMR Spectrum of Thymelaeatoxin-A, 12-O-(Cinnamoyl)-5-Hydroxy-6α,7α-Epoxy-Resiniferonol-9,13,14-Orthobenzoate showing the aromatic and olefinic proton-proton connections

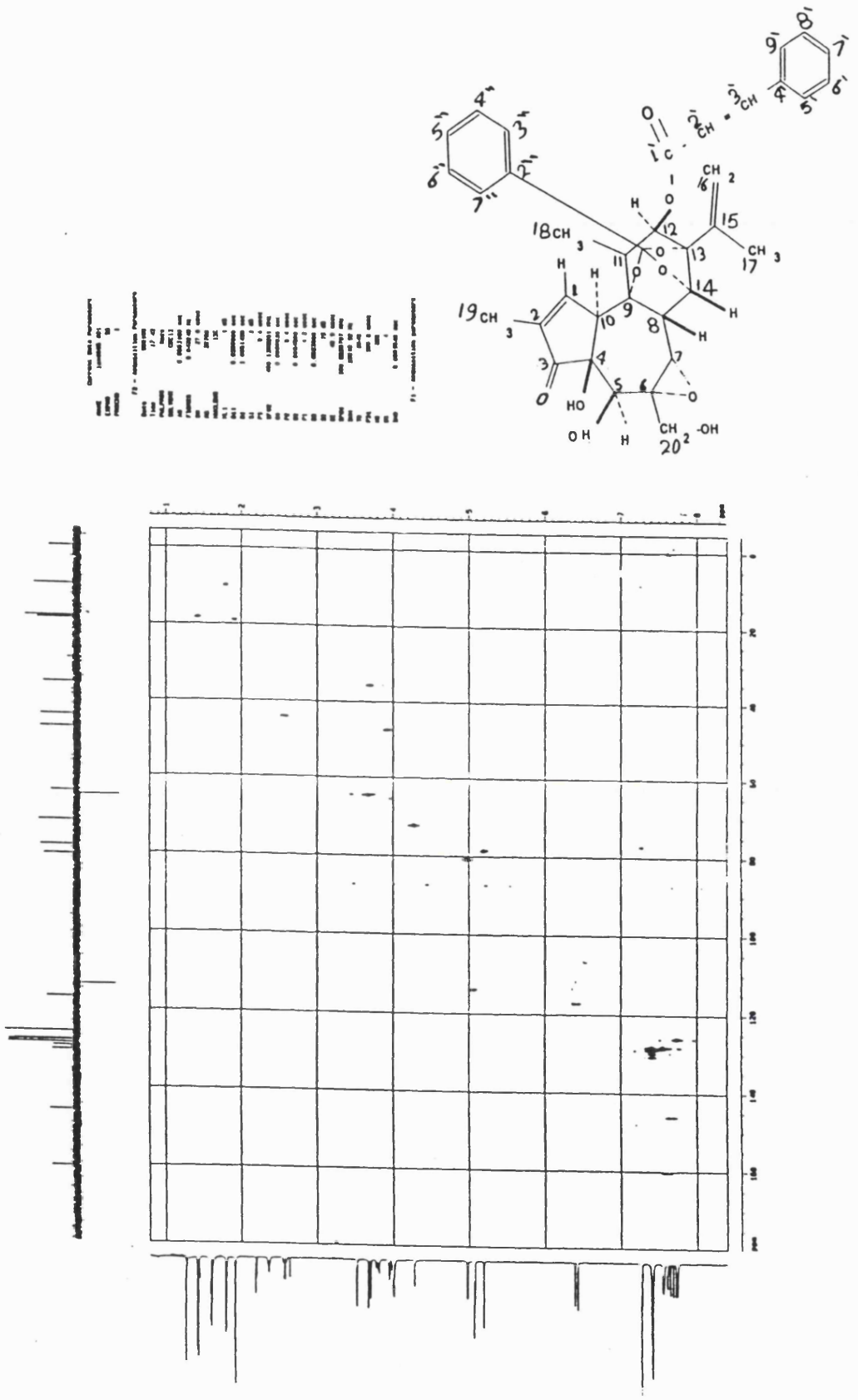


Figure 3.4 C-H One Bond Correlation NMR Spectrum of Thymelaeatoxin-A, 12-O-(Cinnamoyl)-5-Hydroxy-6α,7α-Epoxy-Resiniferonol-9,13,14-Orthobenzoate

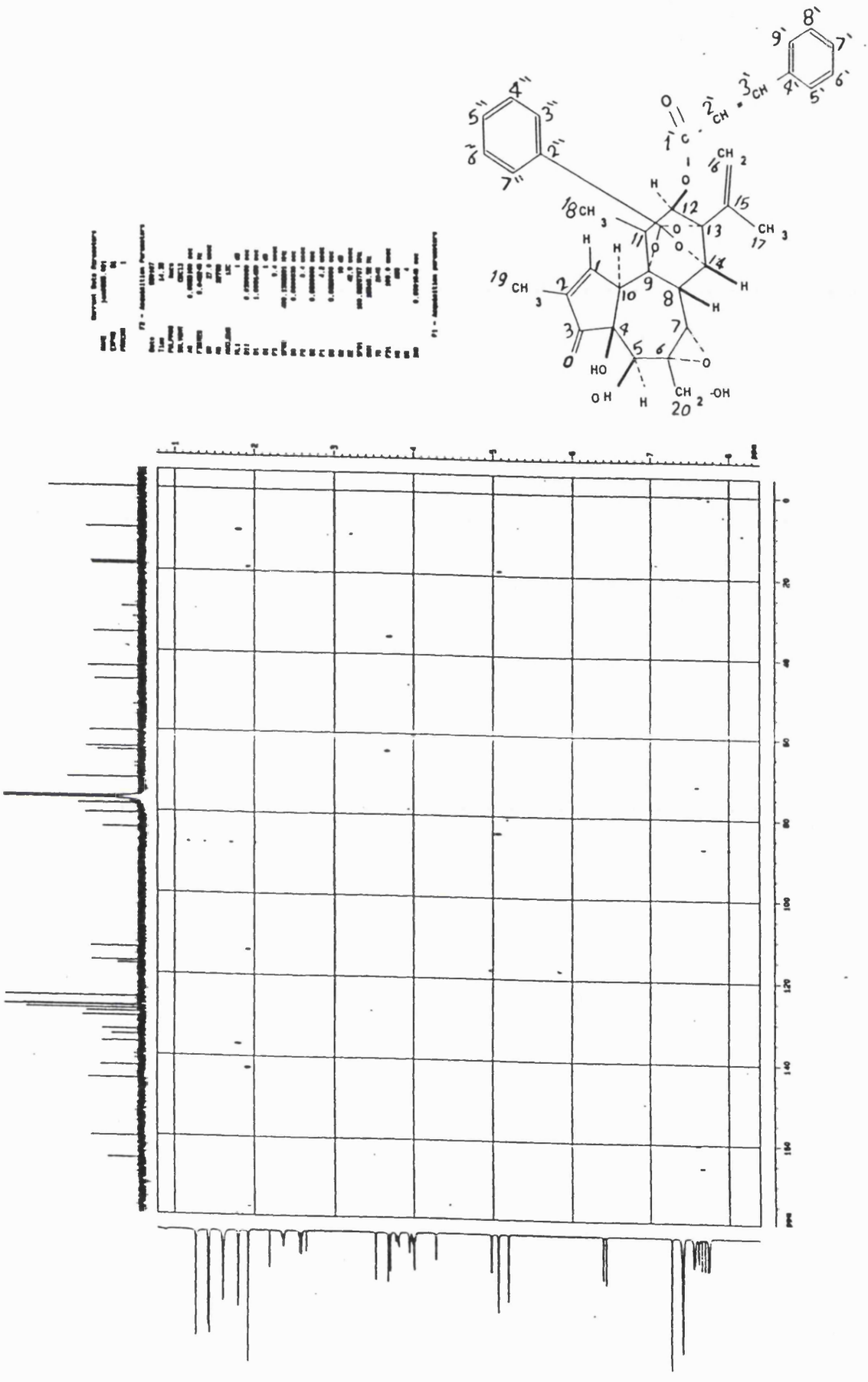


Figure 3.5 C-H Long-Range Correlation NMR Spectrum of Thymelaeatoxin-A, 12-O-(Cinnamoyl)-5-Hydroxy-6α,7α-Epoxy-Resiniferonol-9,13,14-Orthobenzoate

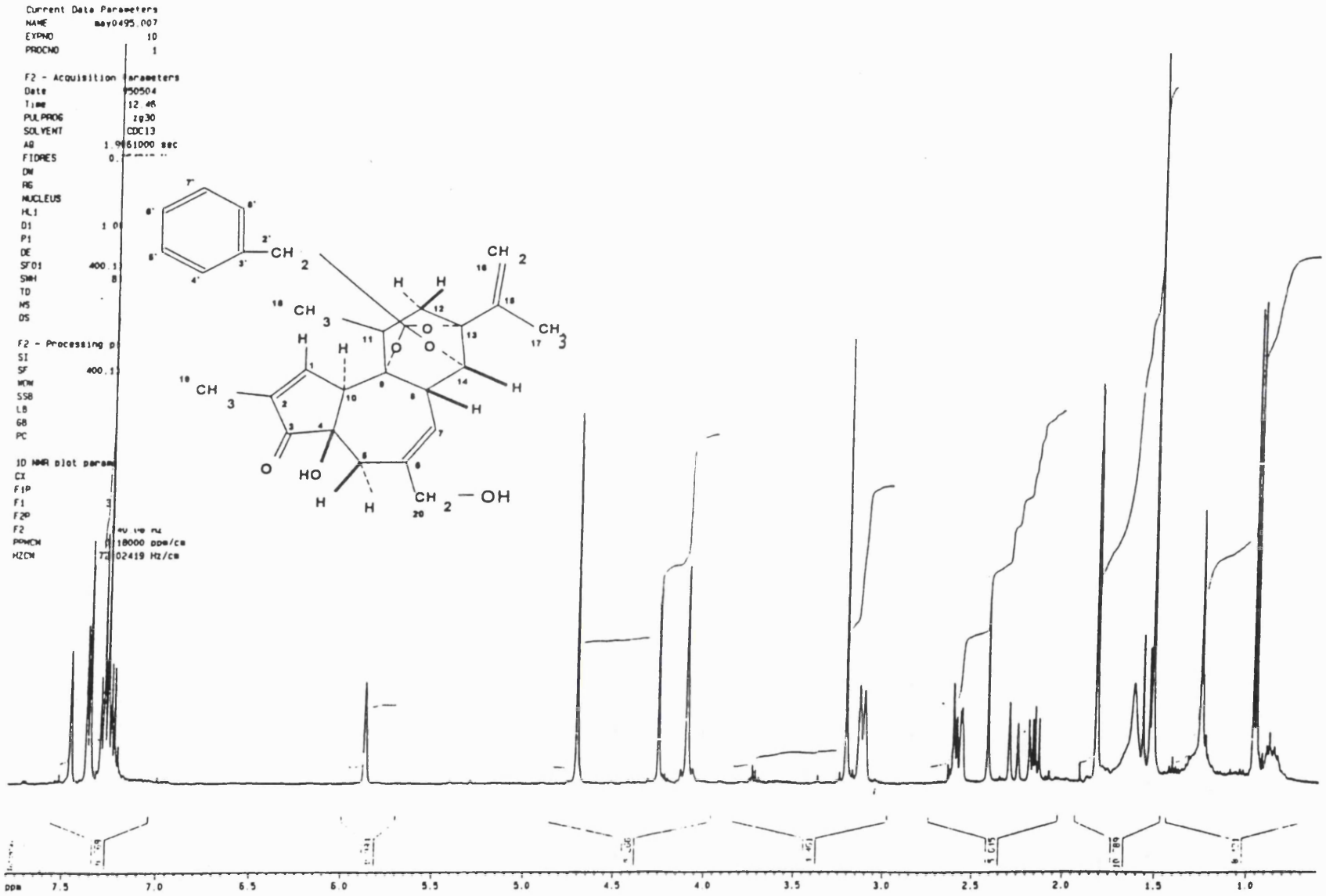


Figure 3.6 ^{1}H NMR Spectrum (400 MHz, $CDCl_3$) of Resiniferonol-9,13,14-Orthophenyl acetate

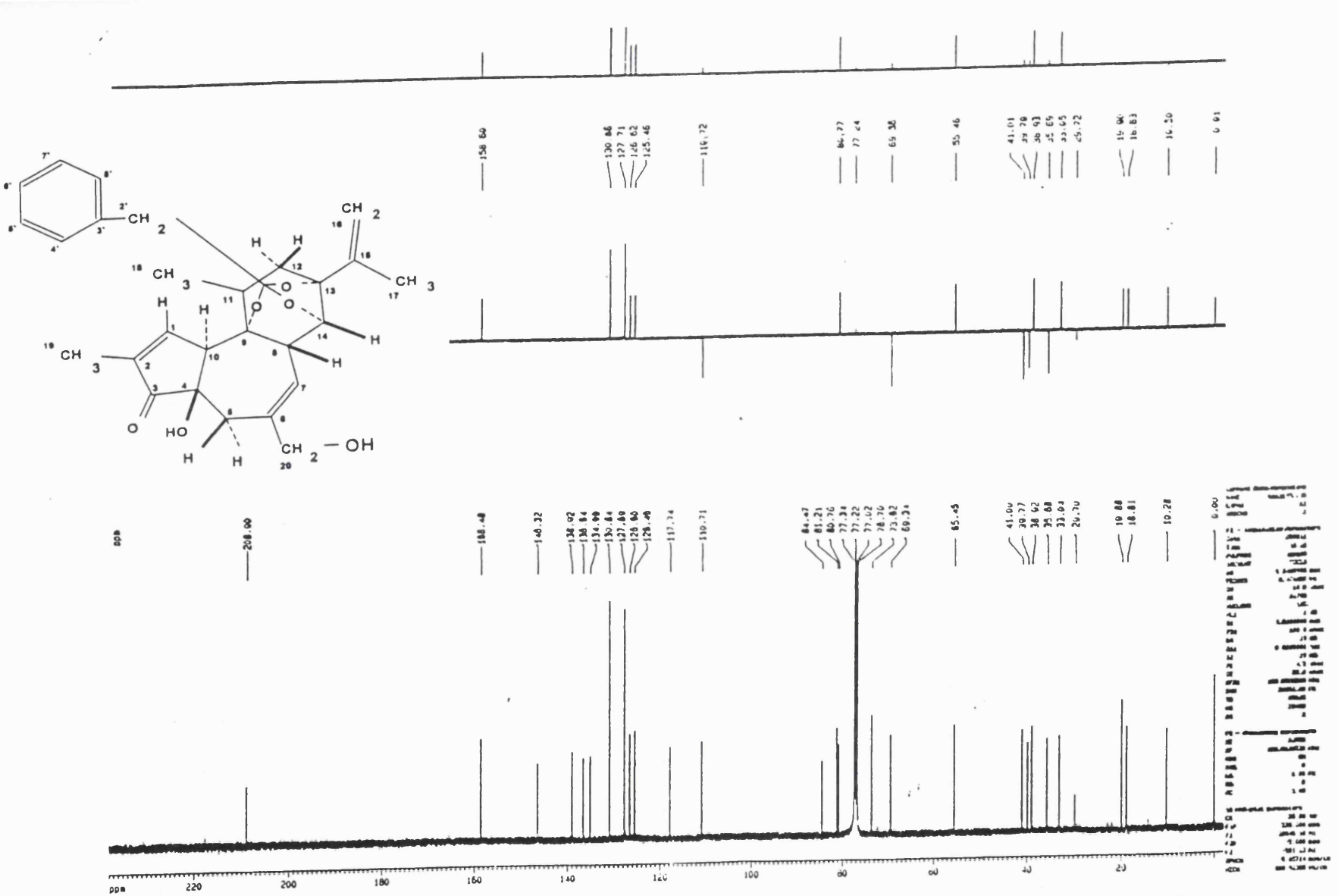


Figure 3.7 ^{13}C NMR Spectrum (100 MHz, $CDCl_3$) of Resiniferonol-9,13,14-Orthophenylacetate

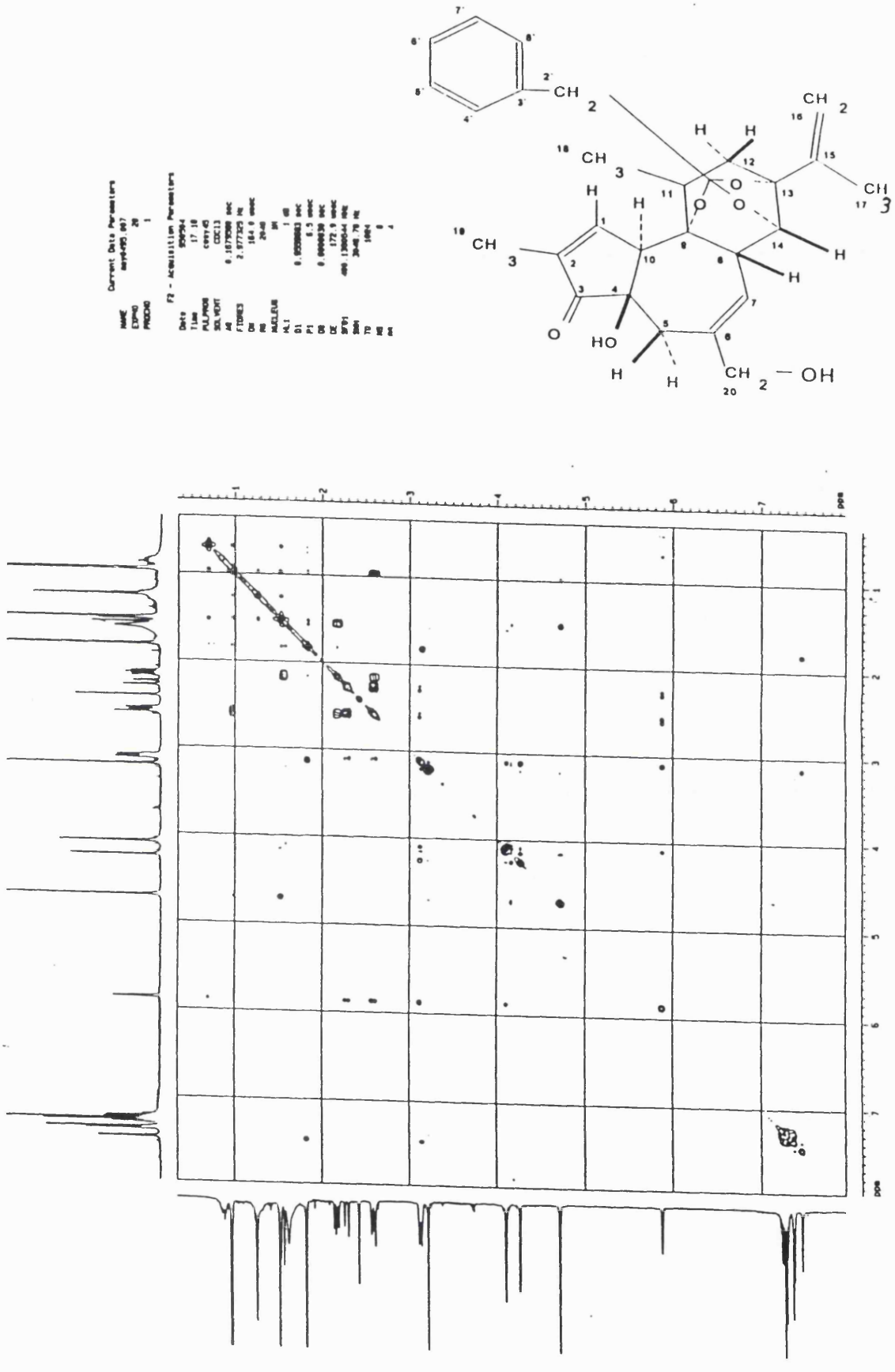


Figure 3.8 COSY H-H NMR Spectrum of Resiniferonol-9,13,14-Orthophenylacetate

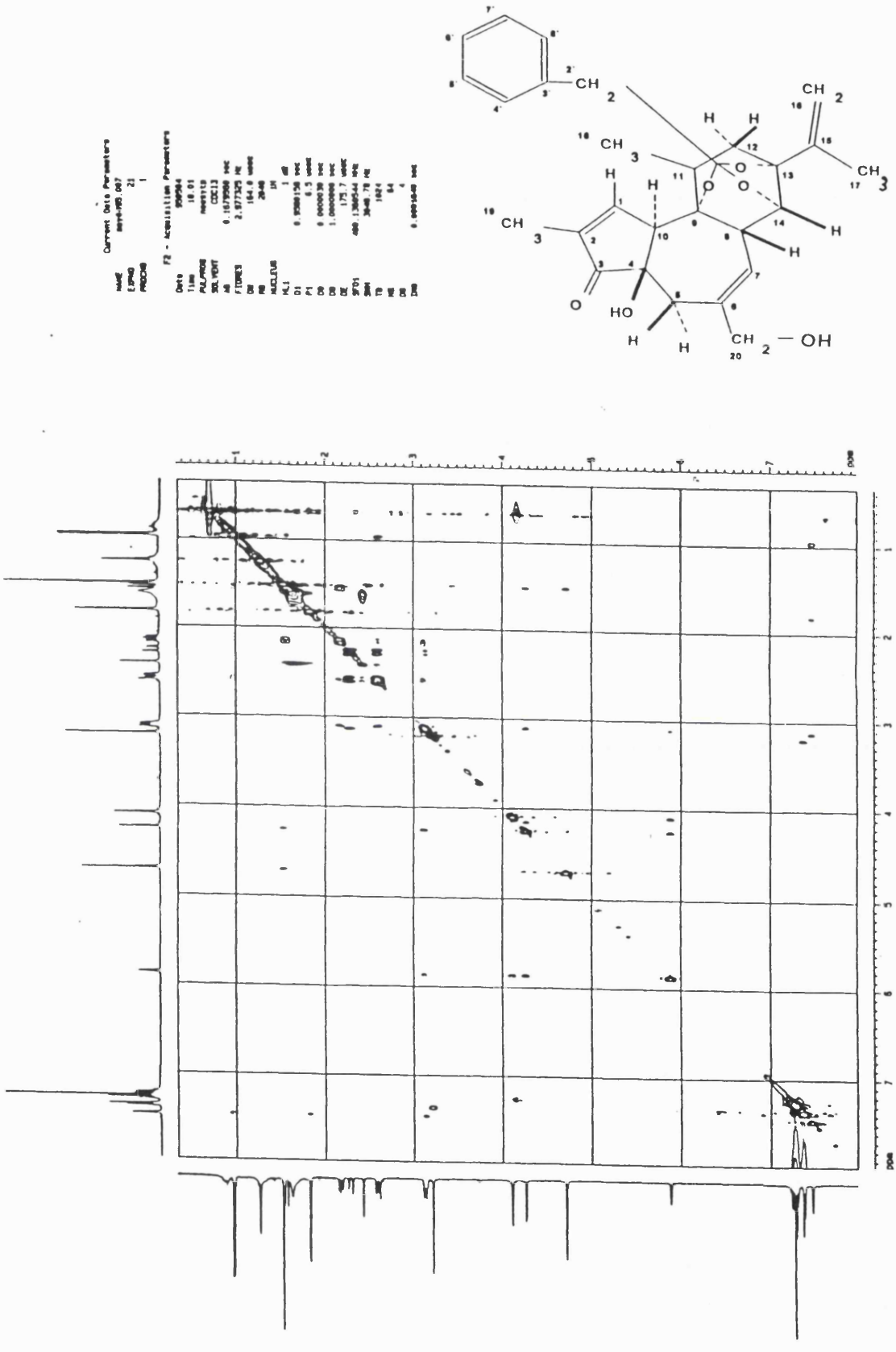


Figure 3.9 NOESY H-H NMR Spectrum of Resiniferonol-9,13,14-Orthophenylacetate

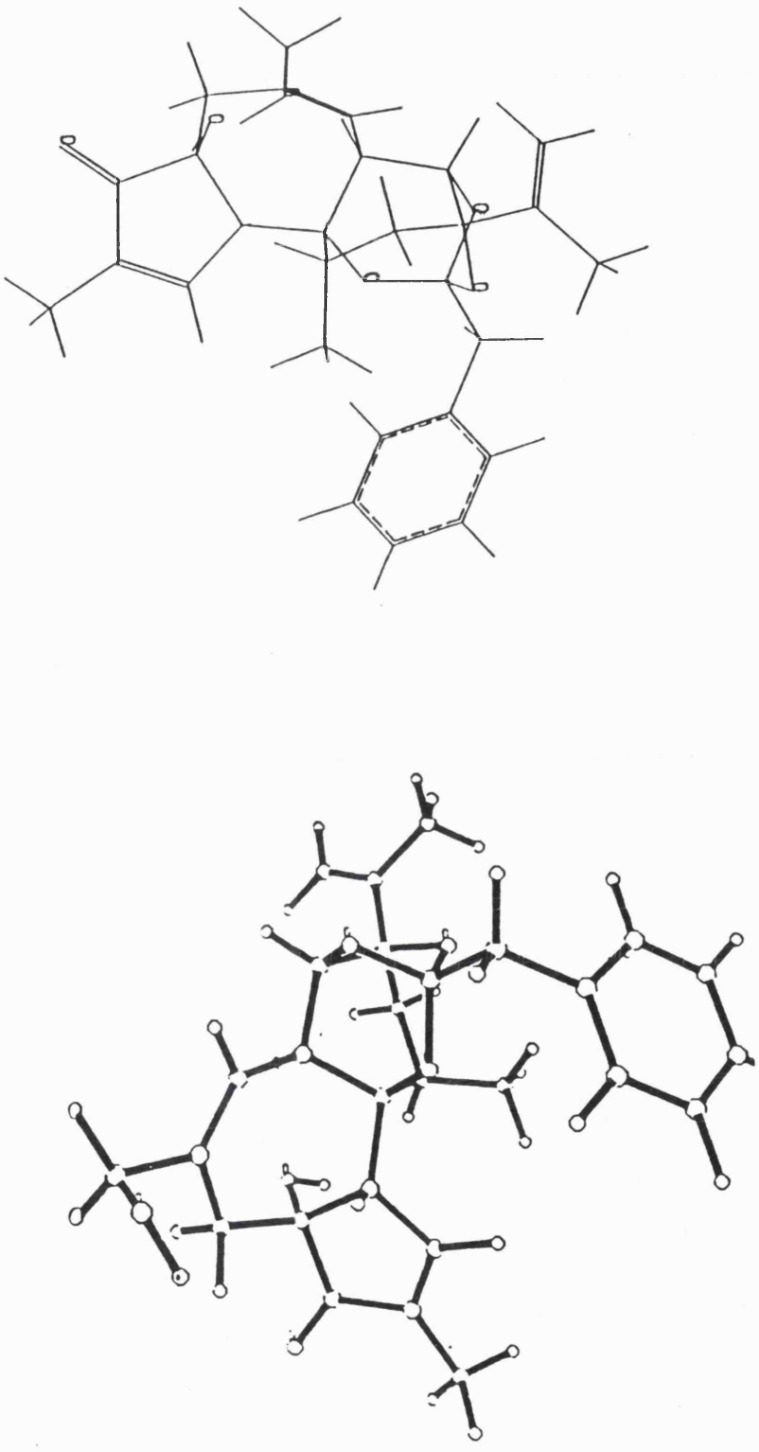


Figure 3.10 A Computer-generated drawing (Sybyl)of the molecular structure of Resiniferonol-9,13,14-orthophenylacetate based on NMR spectral data

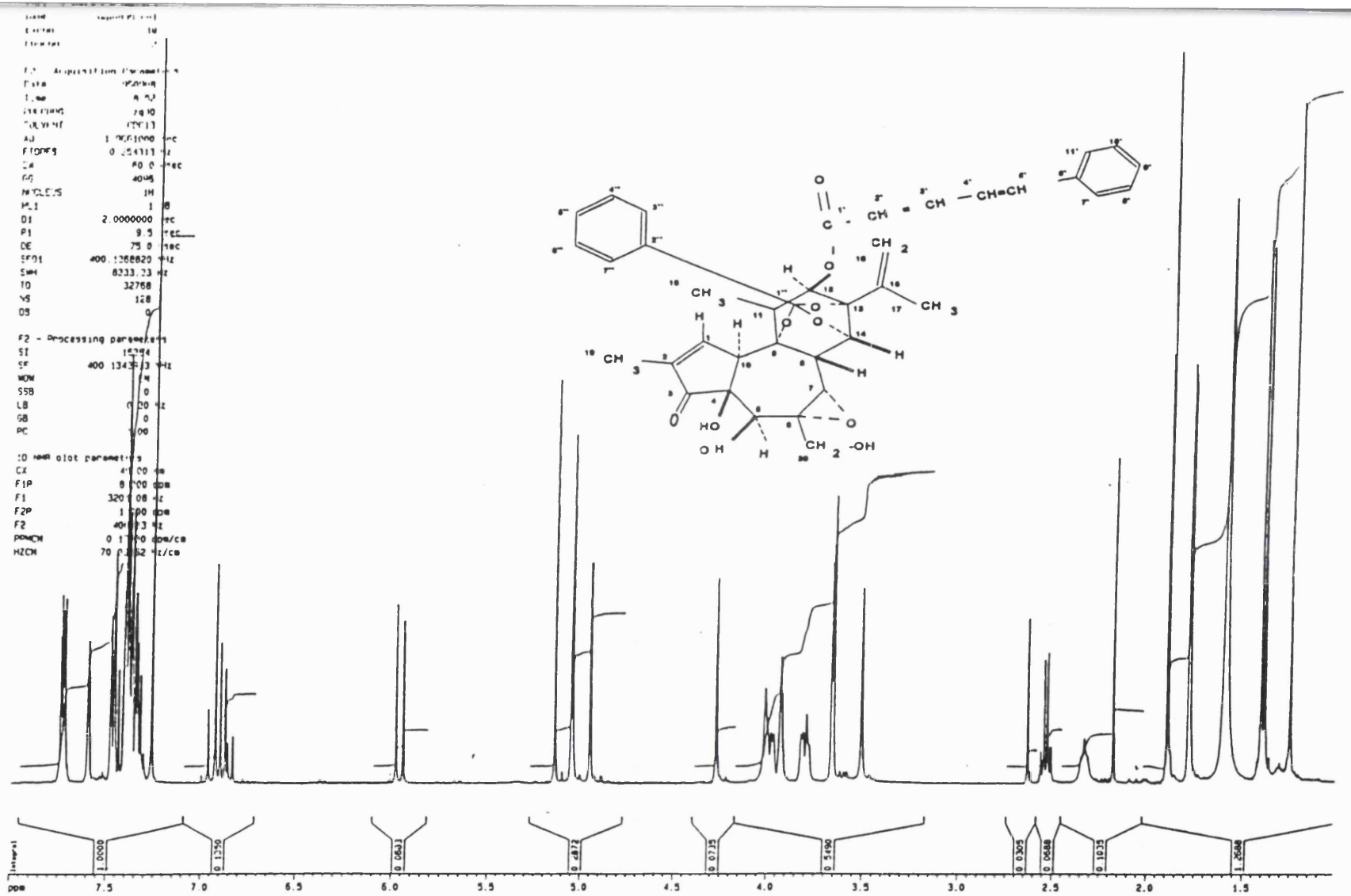


Figure 3.11 ^{1}H NMR Spectrum (400MHz, $CDCl_3$) of Mezerein, 12-O-(5-Phenyl-2,4-Pentadienoate)-5-Hydroxy-6α,7α-Epoxyresiniferonol-9,13,14-Orthobenzoate

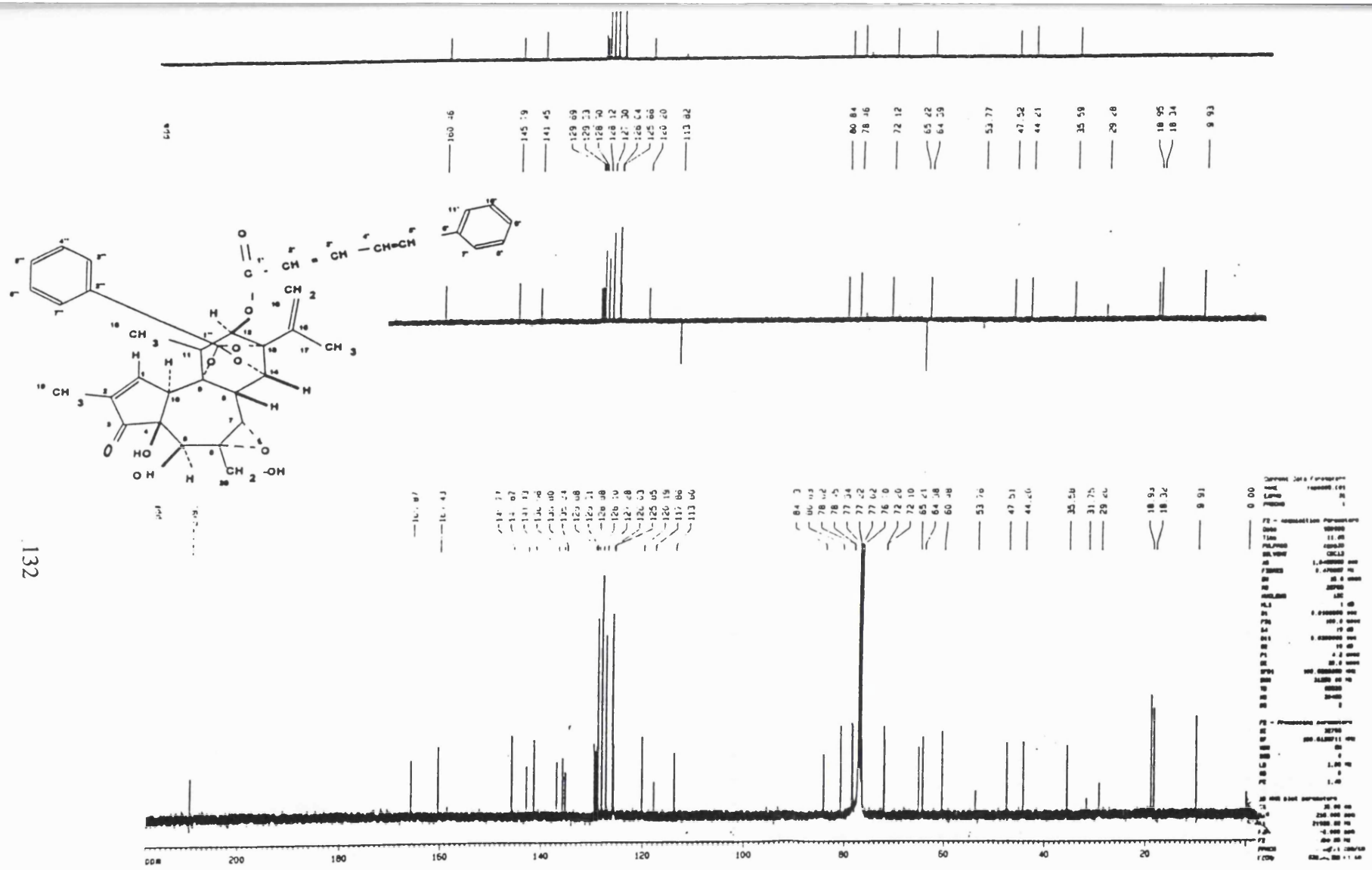


Figure 3.12 ^{13}C NMR Spectrum (100MHz, $CDCl_3$) of Mezerein, 12-O-(5-Phenyl-2,4-Pentadienoate)-5-Hydroxy-6α,7α-Epoxyresiniferonol-9,13,14-Orthobenzoate

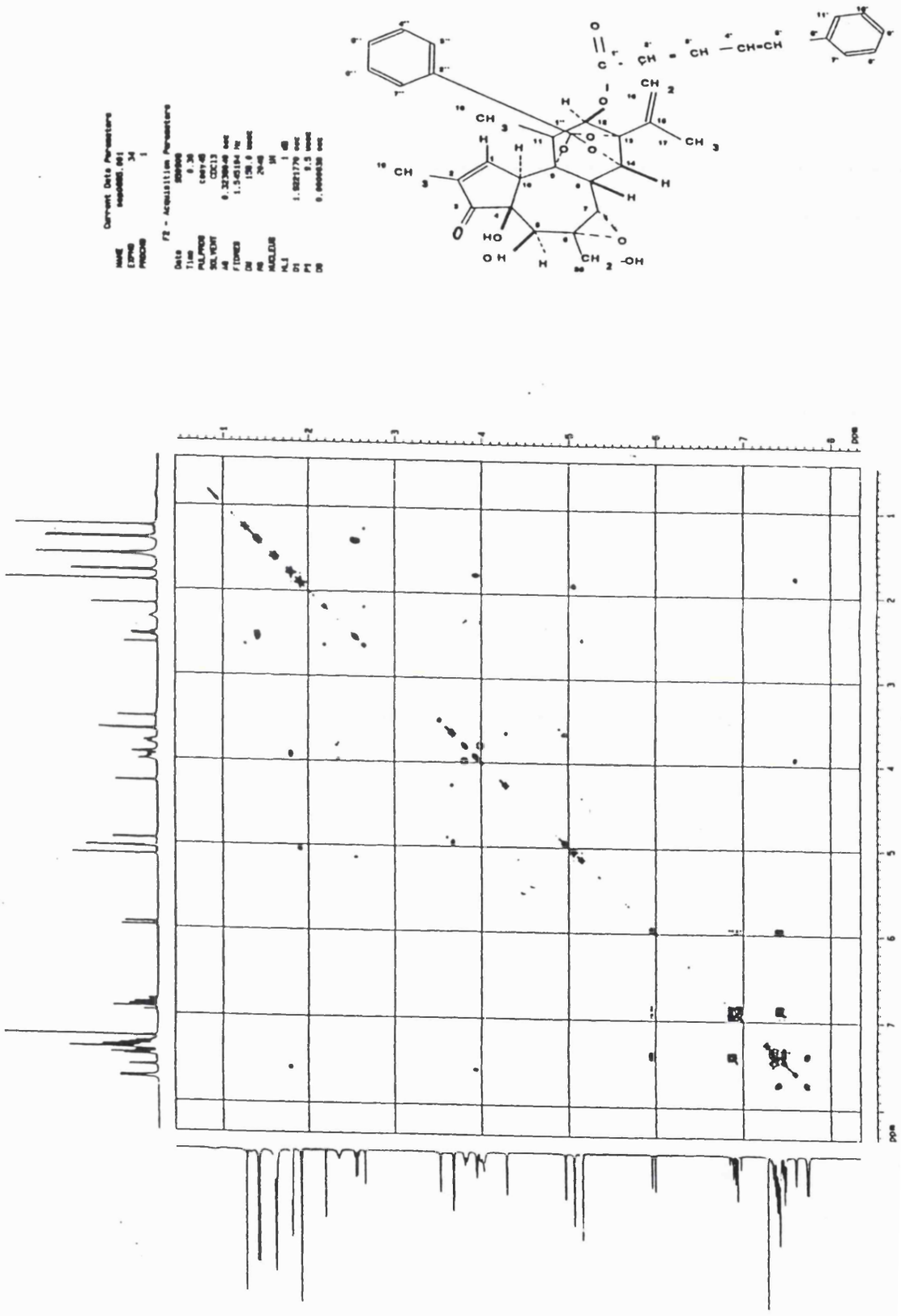


Figure 3.13 COSY H-H NMR Spectrum of Mezerein, 12-O-(5-Phenyl-2,4-Pentadienoate)-5-Hydroxy-6α,7α-Epoxyresiniferonol-9,13,14-Orthobenzoate

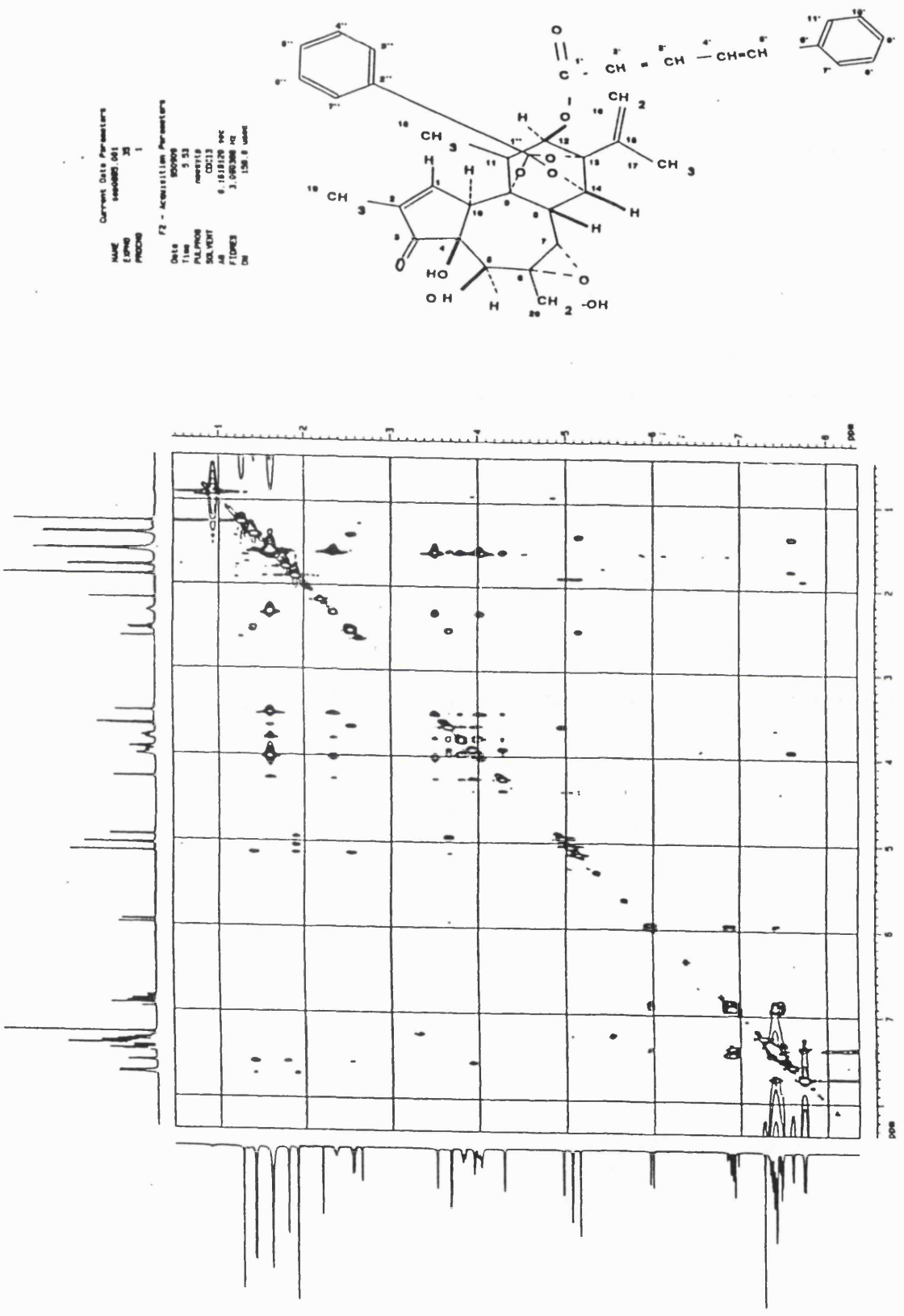


Figure 3.14 NOESY H-H NMR Spectrum of Mezerein, 12-O-(5-Phenyl-2,4-Pentadienoate)-5-Hydroxy-6α,7α-Epoxyresiniferonol-9,13,14-Orthobenzoate

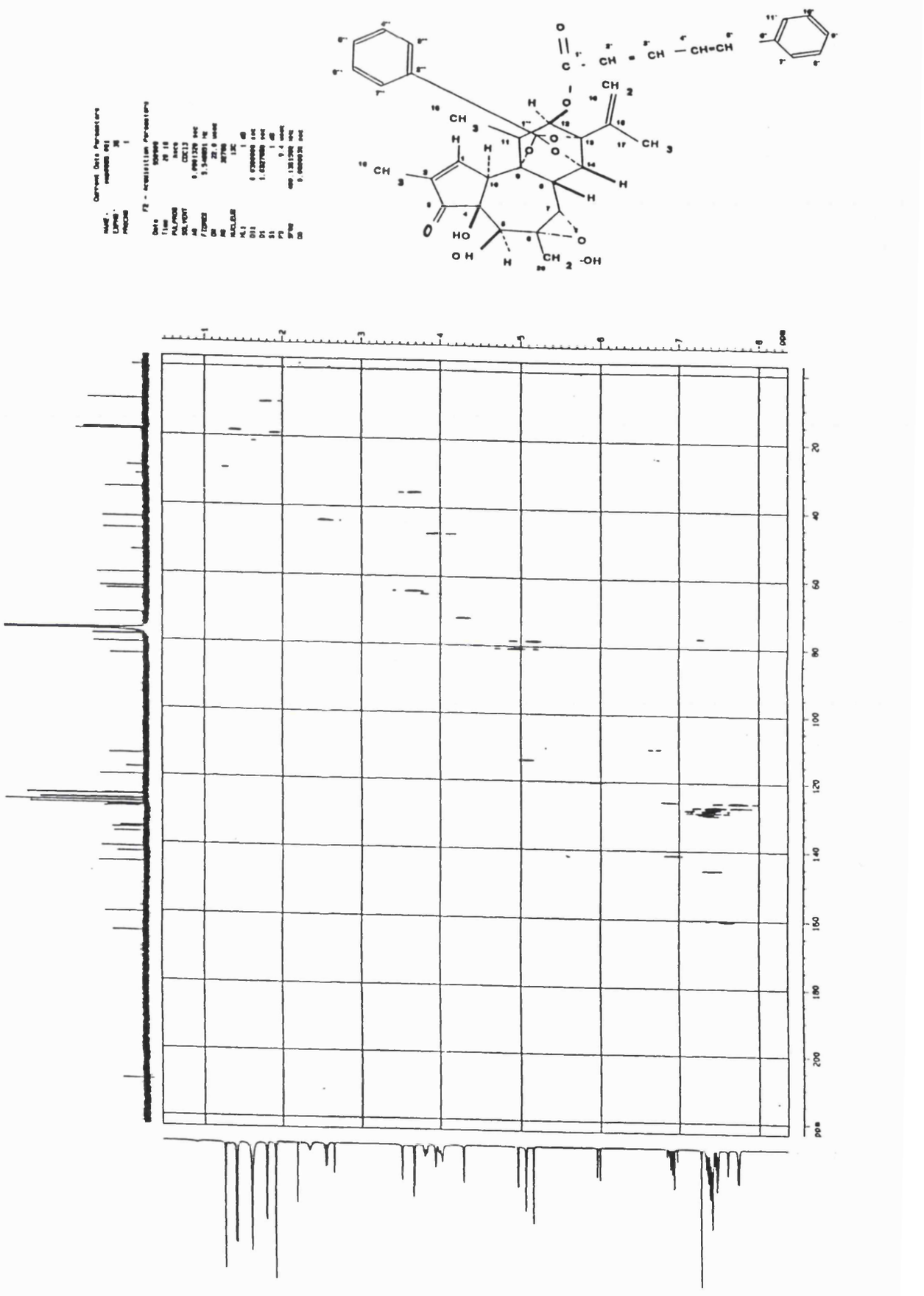


Figure 3.15 C-H One Bond NMR Spectrum of Mezerein, 12-O-(5-Phenyl-2,4-Pentadienoate)-5-Hydroxy-6α,7α-Epoxyresiniferonol-9,13,14-Orthobenzoate

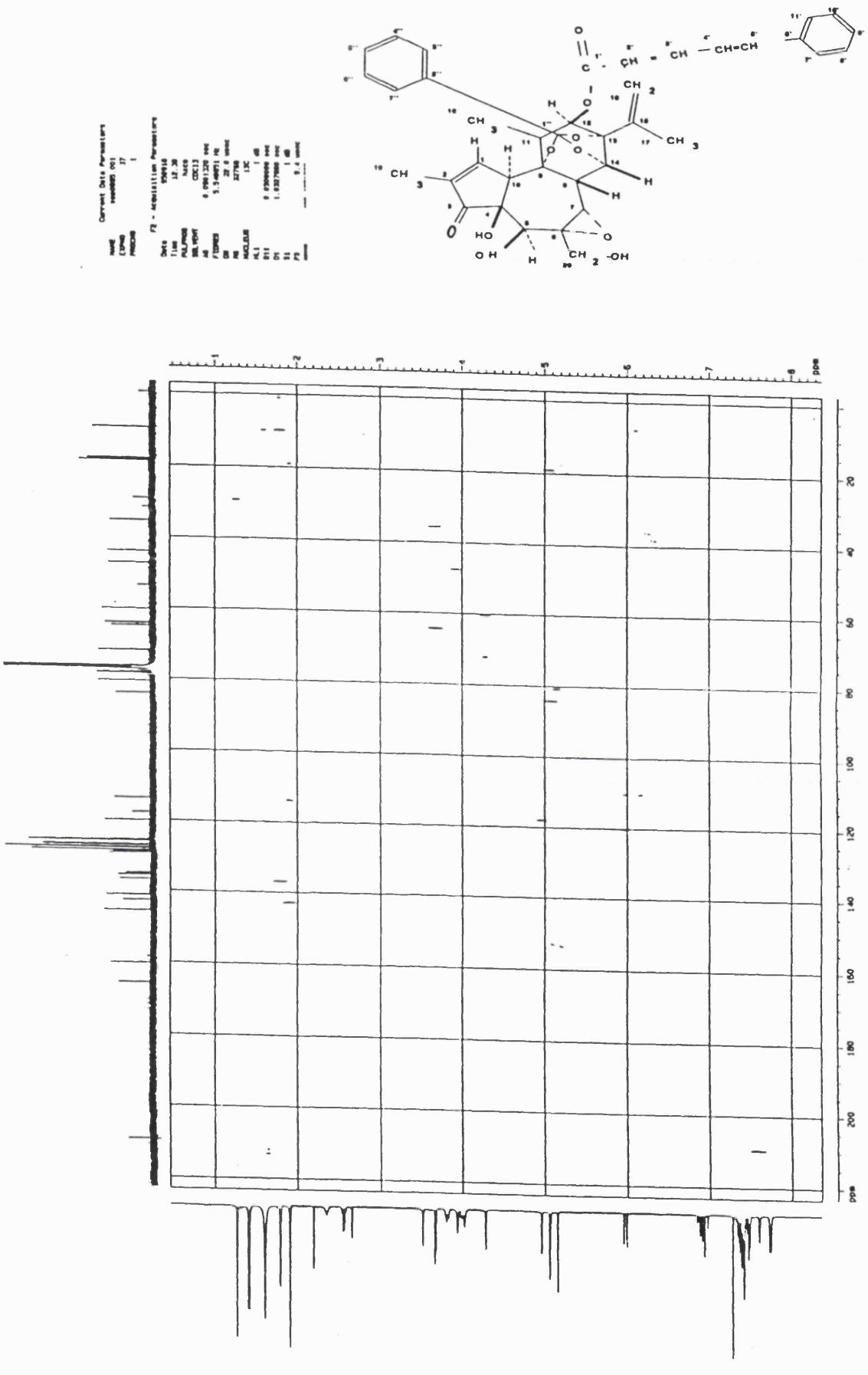
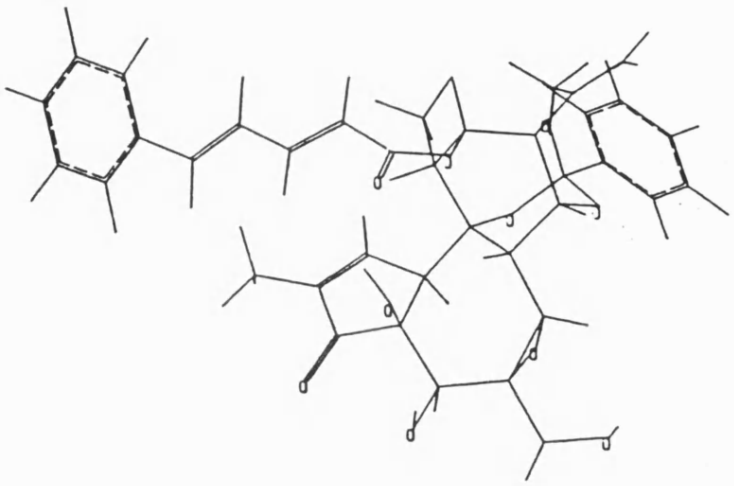
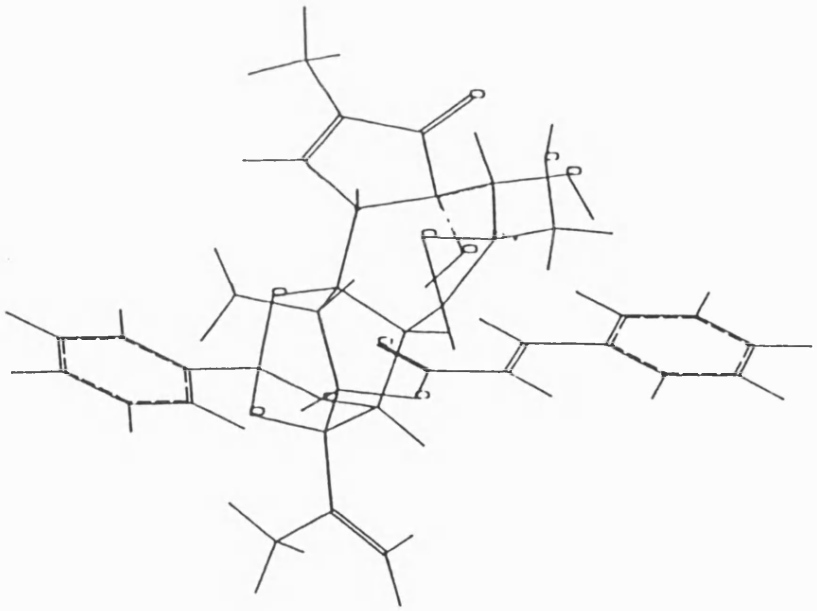


Figure 3.16 C-H Long Range NMR Spectrum of Mezerein, 12-O-(5-Phenyl-2,4-Pentadienoate)-5-Hydroxy-6,7-Epoxyresiniferonol-9,13,14-Orthobenzoate



Molecular structure of Mezerein



Molecular structure of thymelaeatoxin-A

Figure 3.17 A Computer-generated drawing (Sybyl)of the molecular structure of mezerein and thymelaeatoxin-A based on NMR spectral data

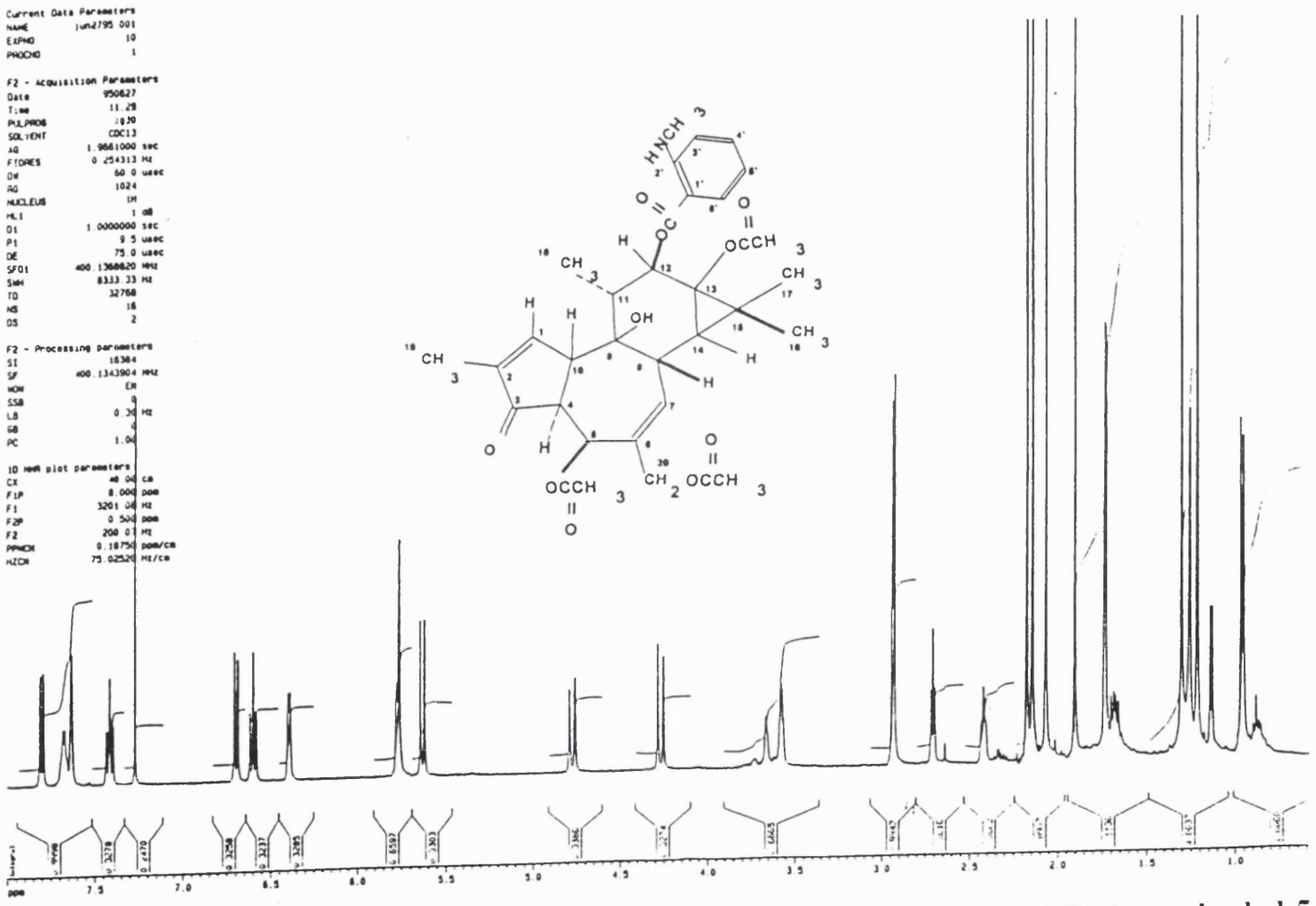


Figure 3.18 ^{1}H NMR Spectrum (400MHz, $CDCl_3$) of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate

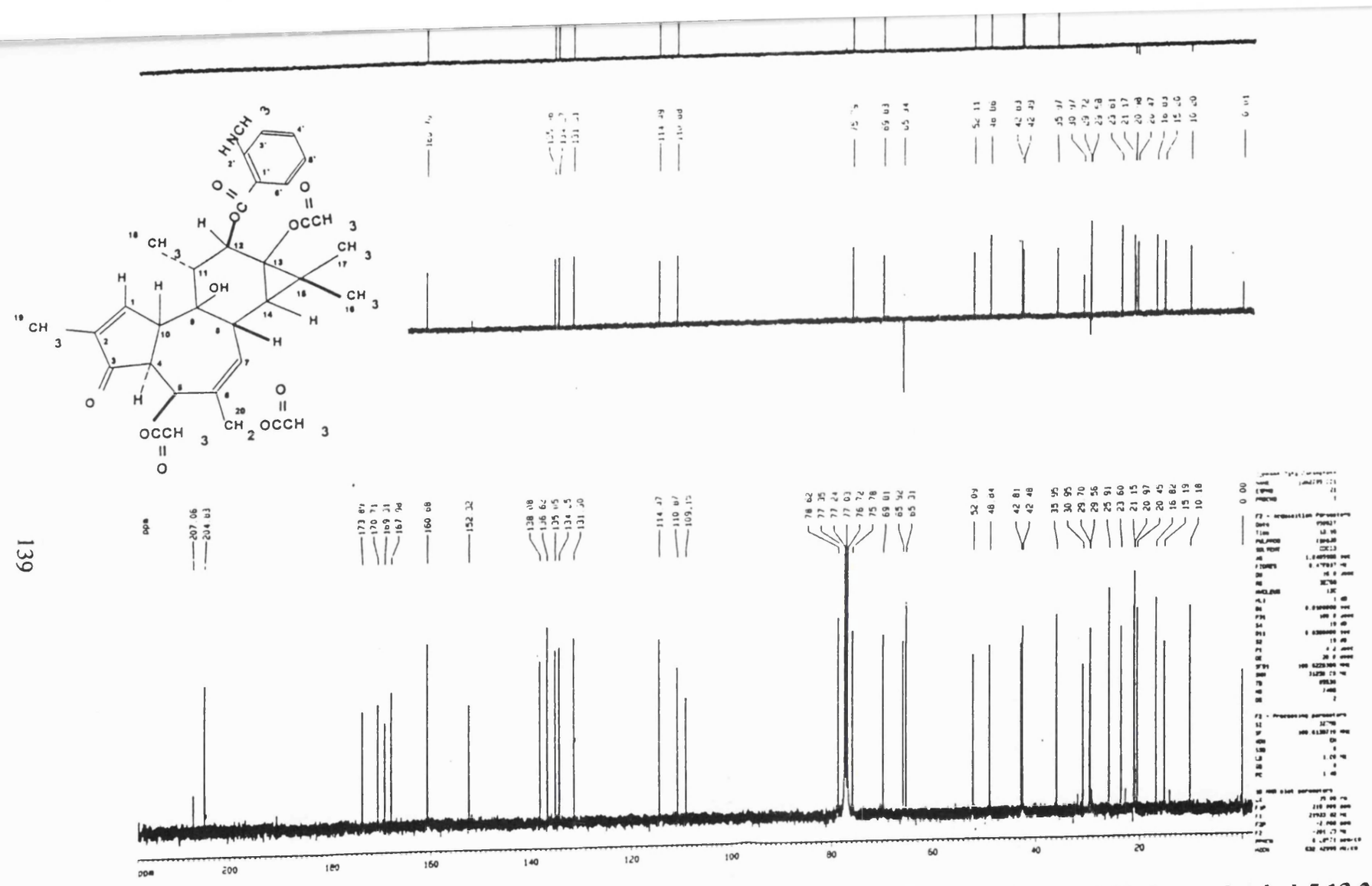


Figure 3.19 ^{13}C NMR Spectrum(100MHz, $CDCl_3$) of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate

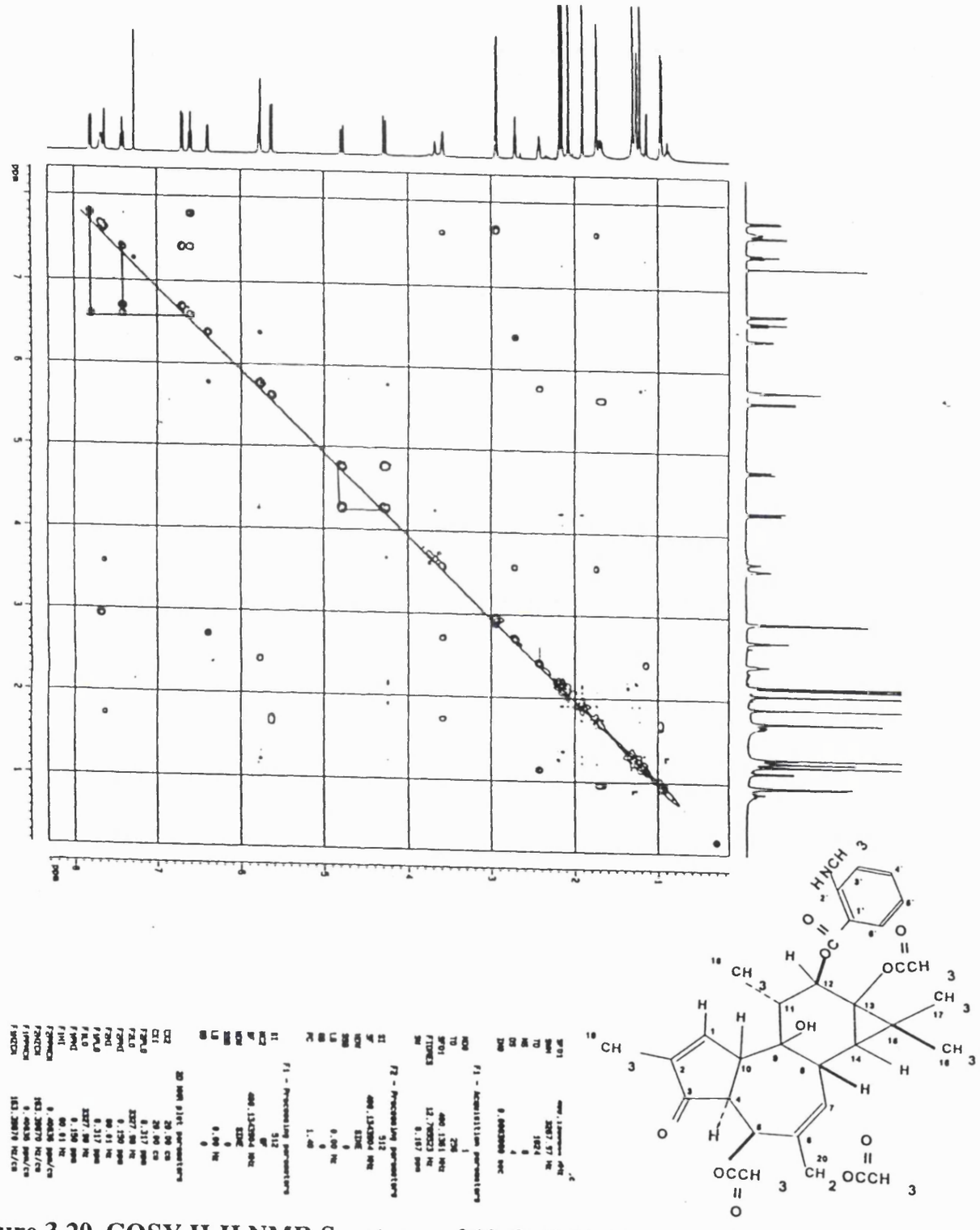


Figure 3.20 COSY H-H NMR Spectrum of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate

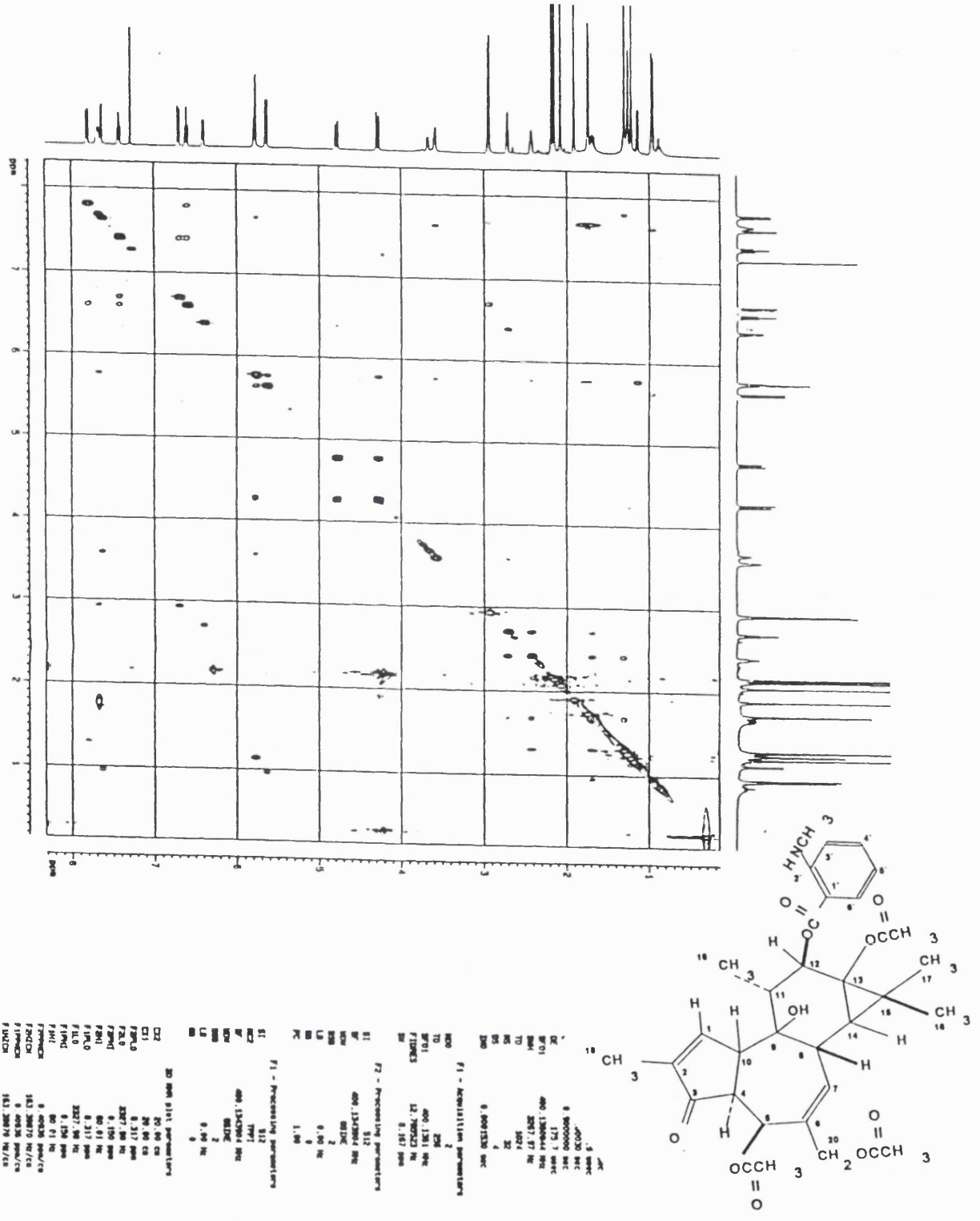


Figure 3.21 NOESY H-H NMR Spectrum of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-Hydroxyphorbol-5,13,20-Triacetate

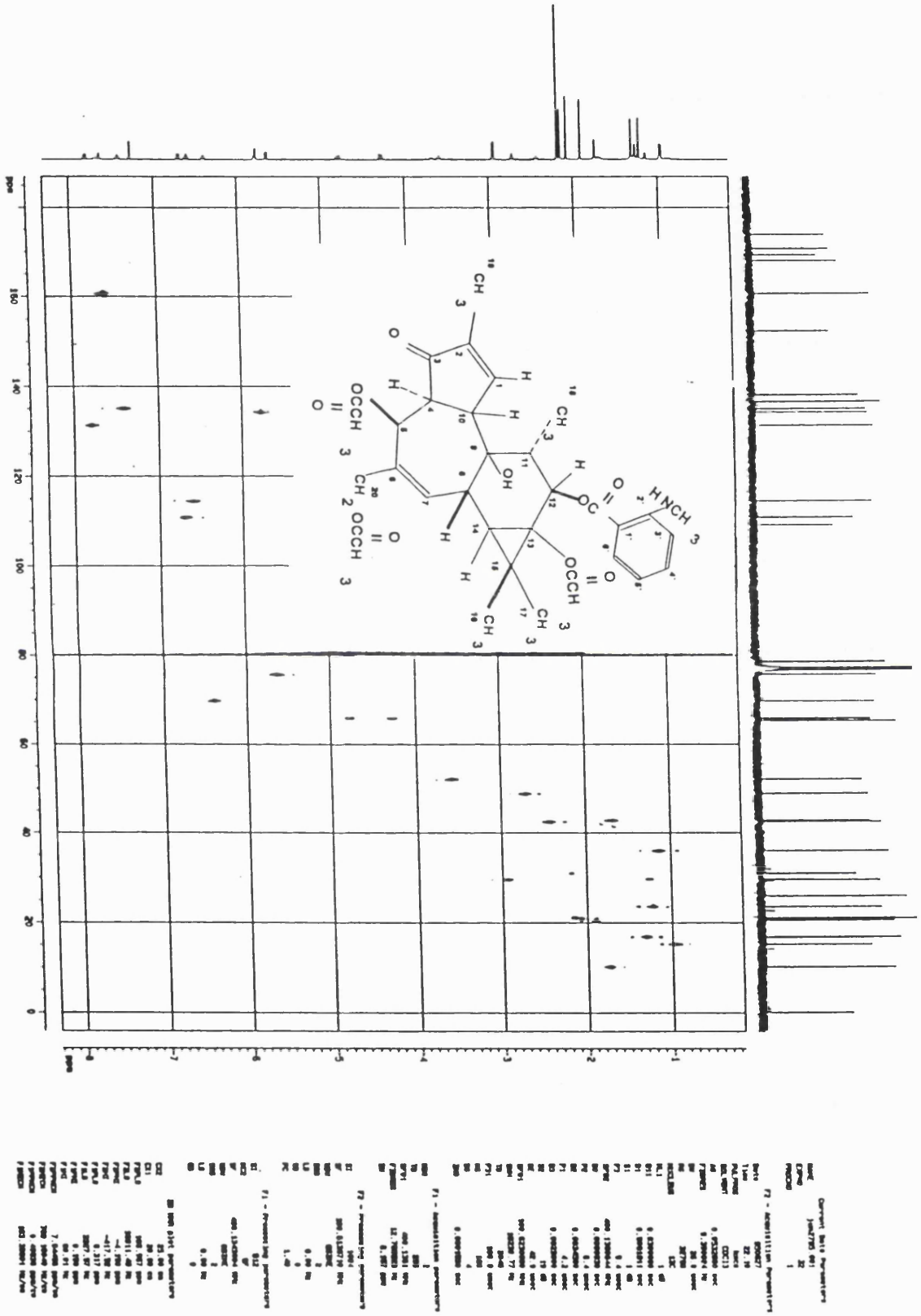


Figure 3.22 C-H One Bond NMR Spectrum of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy--5-Hydroxyphorbol-5,13,20-Triacetate

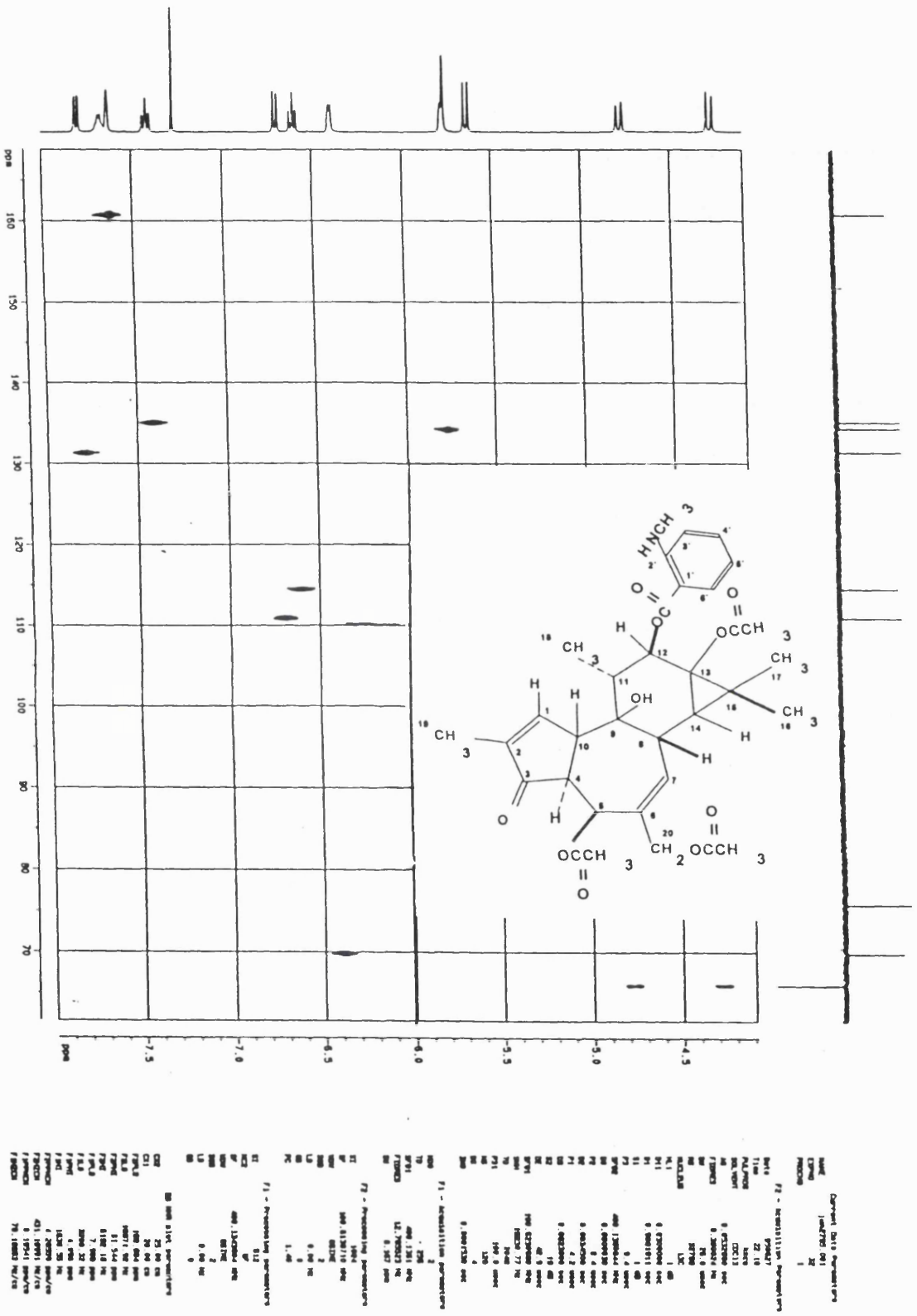


Figure 3.22A C-H One Bond NMR Spectrum of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy--5-Hydroxyphorbol-5,13,20-Triacetate Showing Carbon-Proton Connections in Methine and Methylene groups

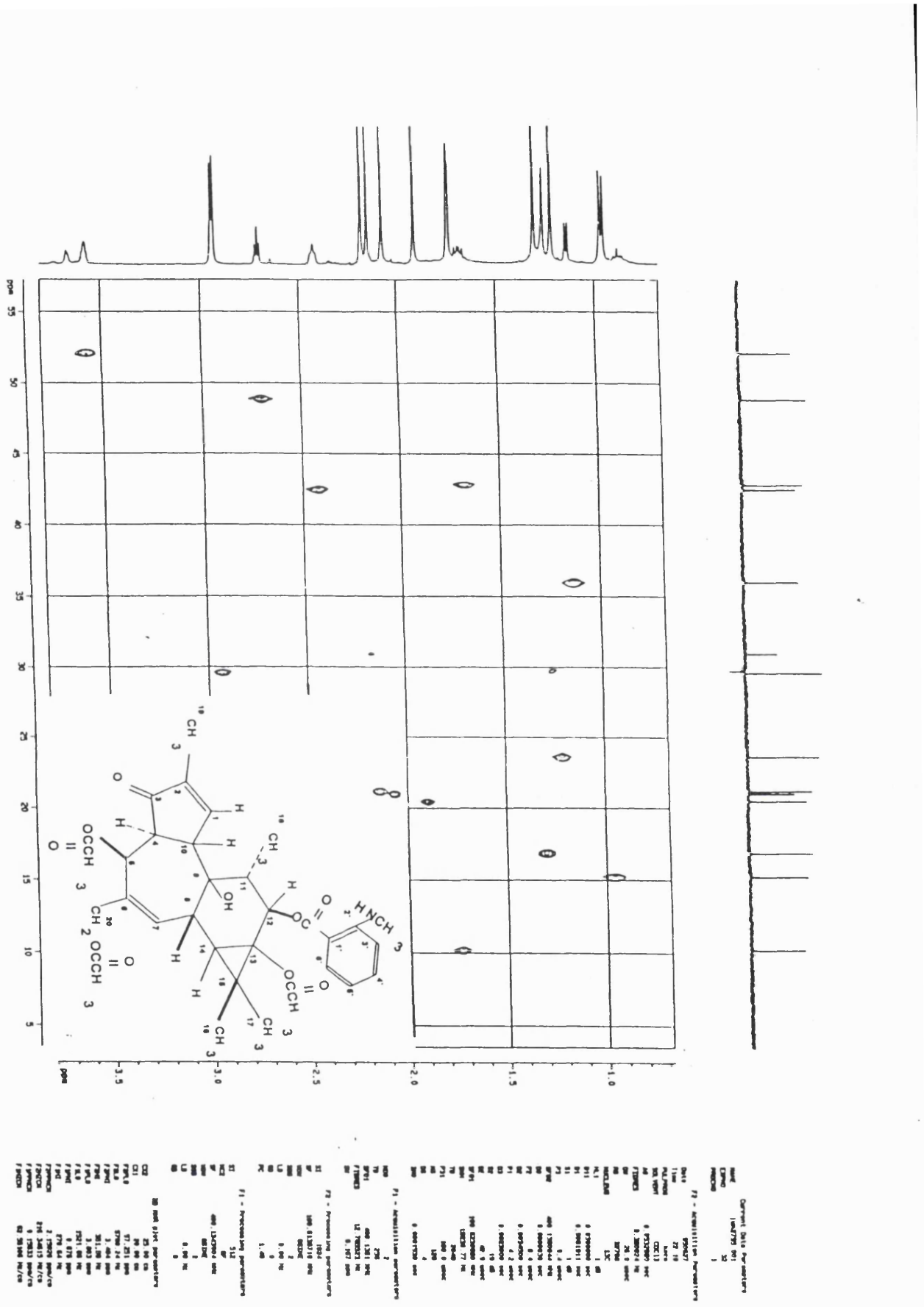


Figure 3.22B C-H One Bond NMR Spectrum of 12-O-(2-Methylaminobenzoyl)-4α-Deoxy--5-Hydroxyphorbol-5,13,20-Triacetate Showing Carbon-Proton Connections in Methyl and Methine groups

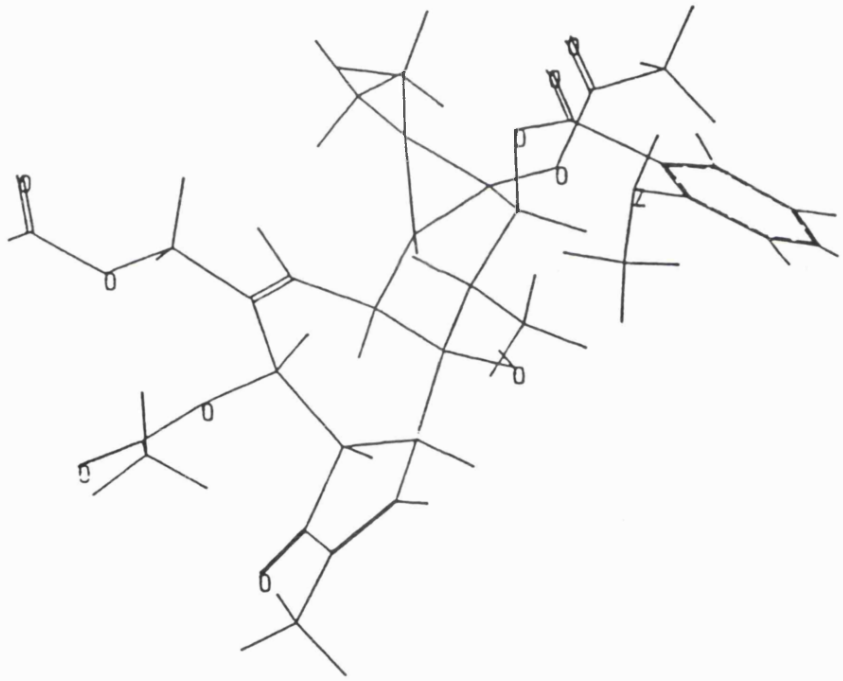


Figure 3.23 A Computer-generated drawing (Sybyl) of the molecular structure of 12-O-(2-Methylaminobenzoyl)4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate based on NMR spectral data

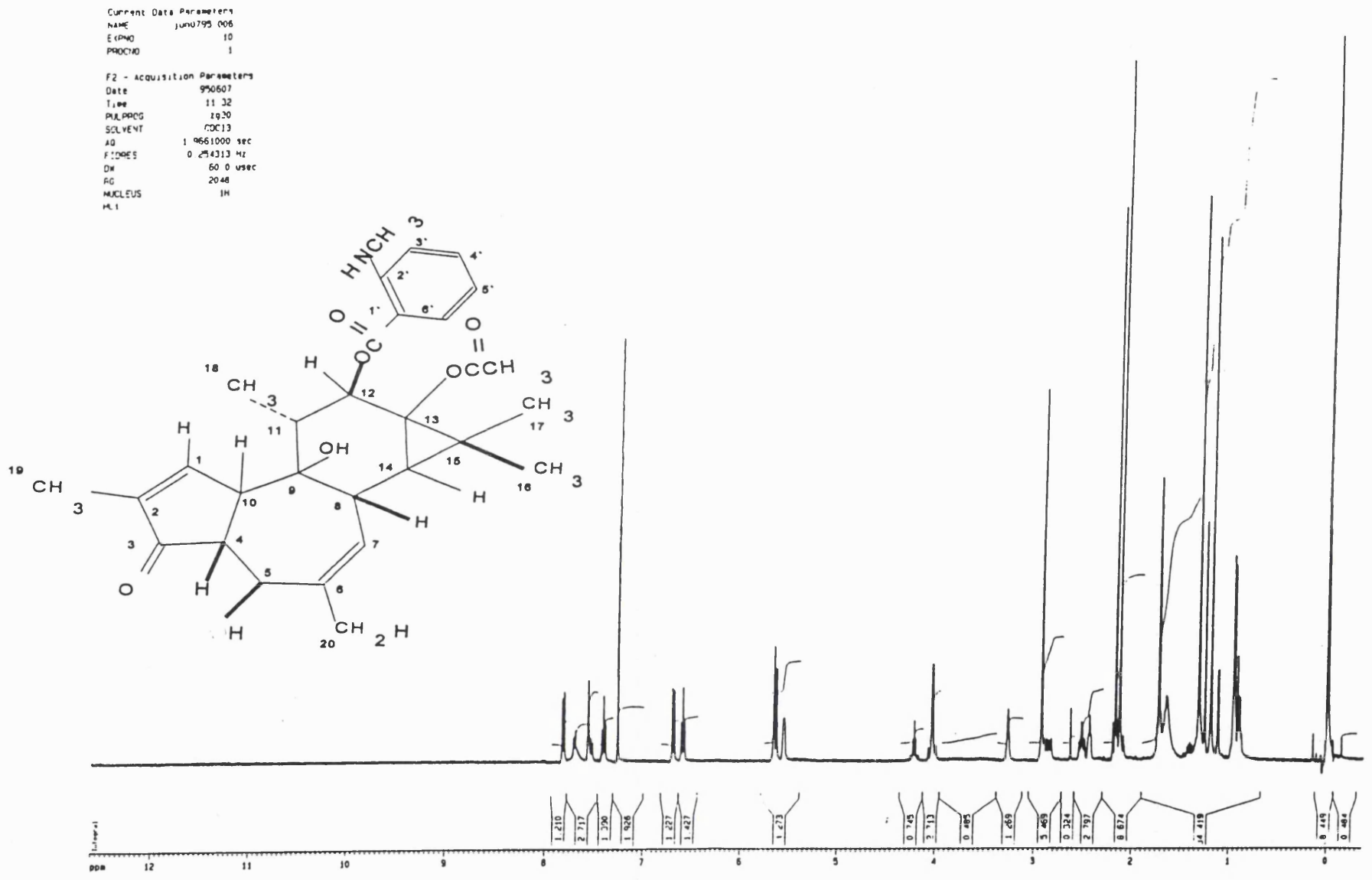


Figure 3.24 ^{1}H NMR Spectrum(400MHz, $CDCl_3$) of Sapintoxin-A, 12-O-(2-Methyl aminobenzoyl)-4-Deoxyphorbol-13-Acetate

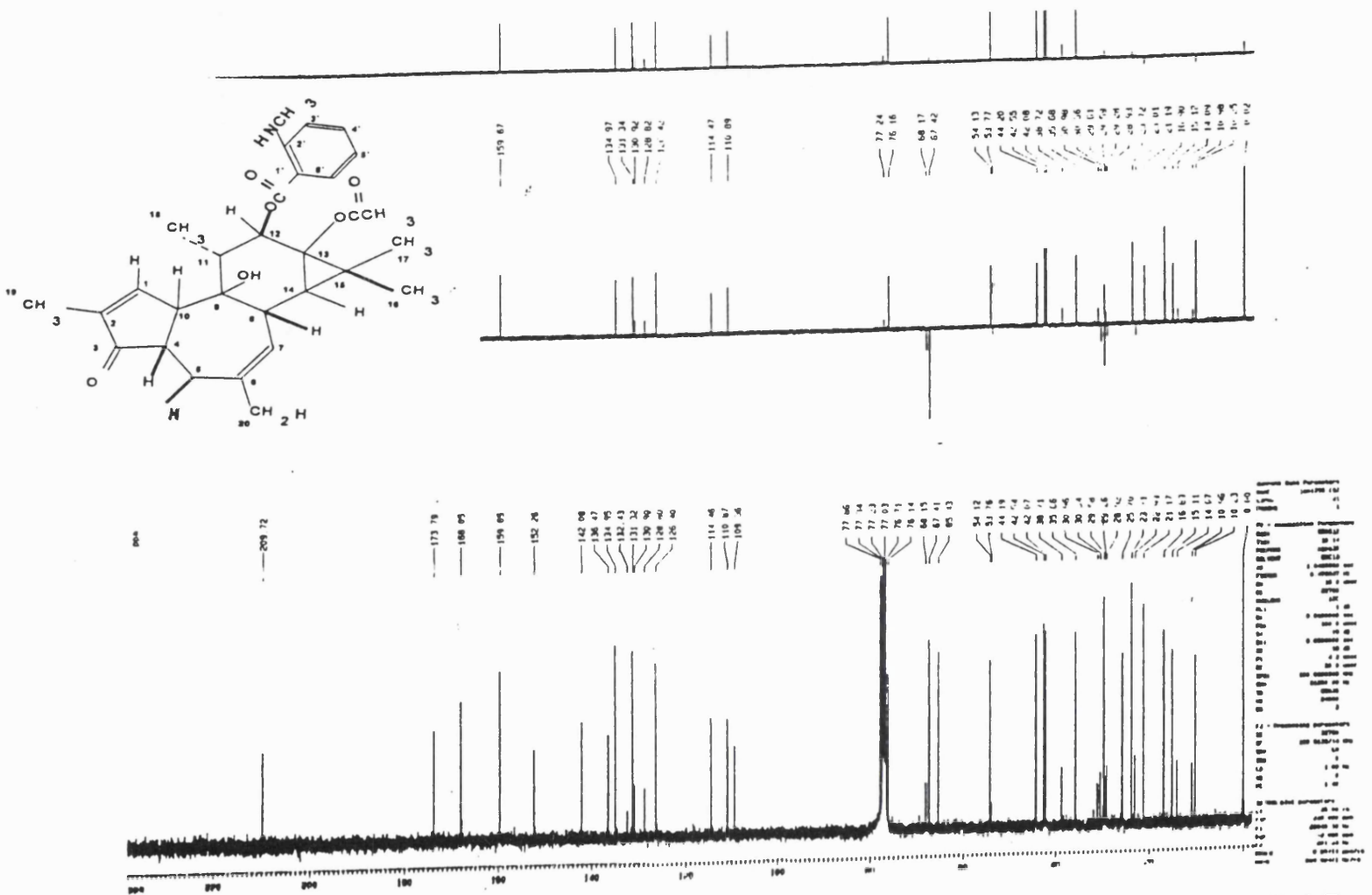


Figure 3.25 ^{13}C NMR Spectrum (100MHz, $CDCl_3$) of Sapintoxin-A, 12-O-(2-Methyl aminobenzoyl)-4-Deoxyphorbol-13-Acetate

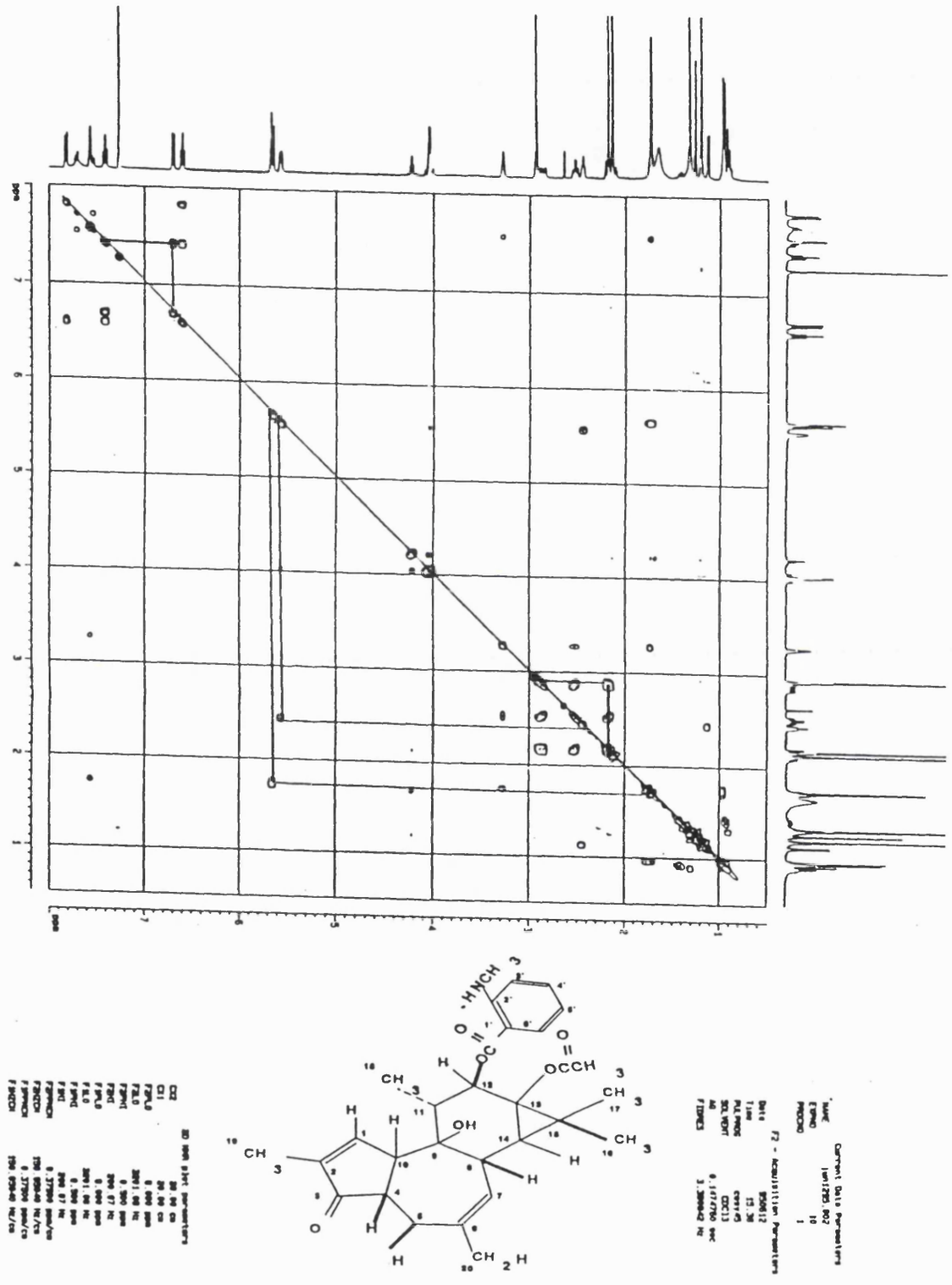


Figure 3.26 COSY H-H NMR Spectrum of Sapintoxin-A, 12-O-(2-Methylaminobenzoyl)-4-Deoxyphorbol-13-Acetate

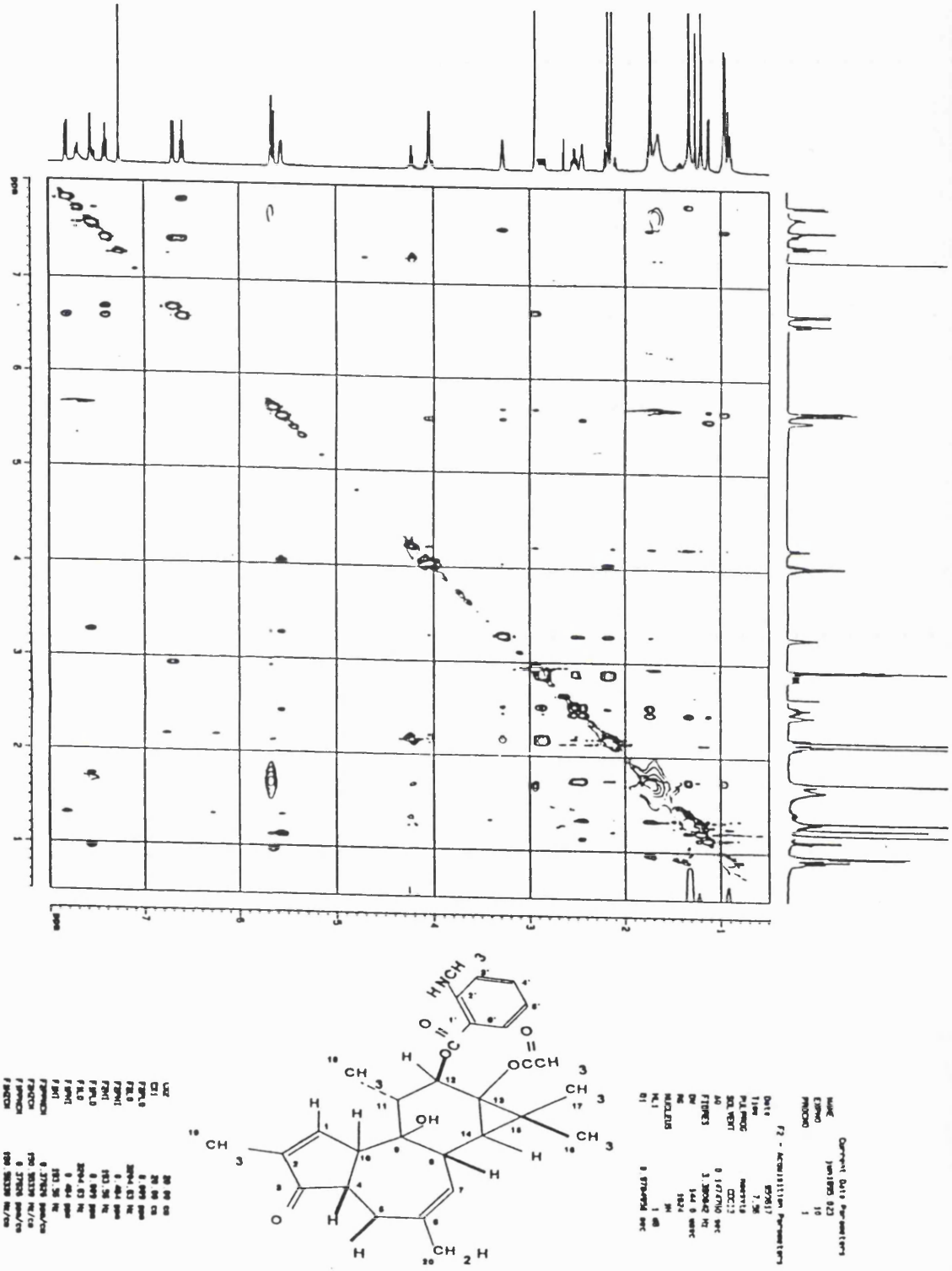


Figure 3.27 NOESY H-H NMR Spectrum of Sapintoxin-A, 12-O-(2-Methylaminobenzoyl)-4-Deoxyphorbol-13-Acetate

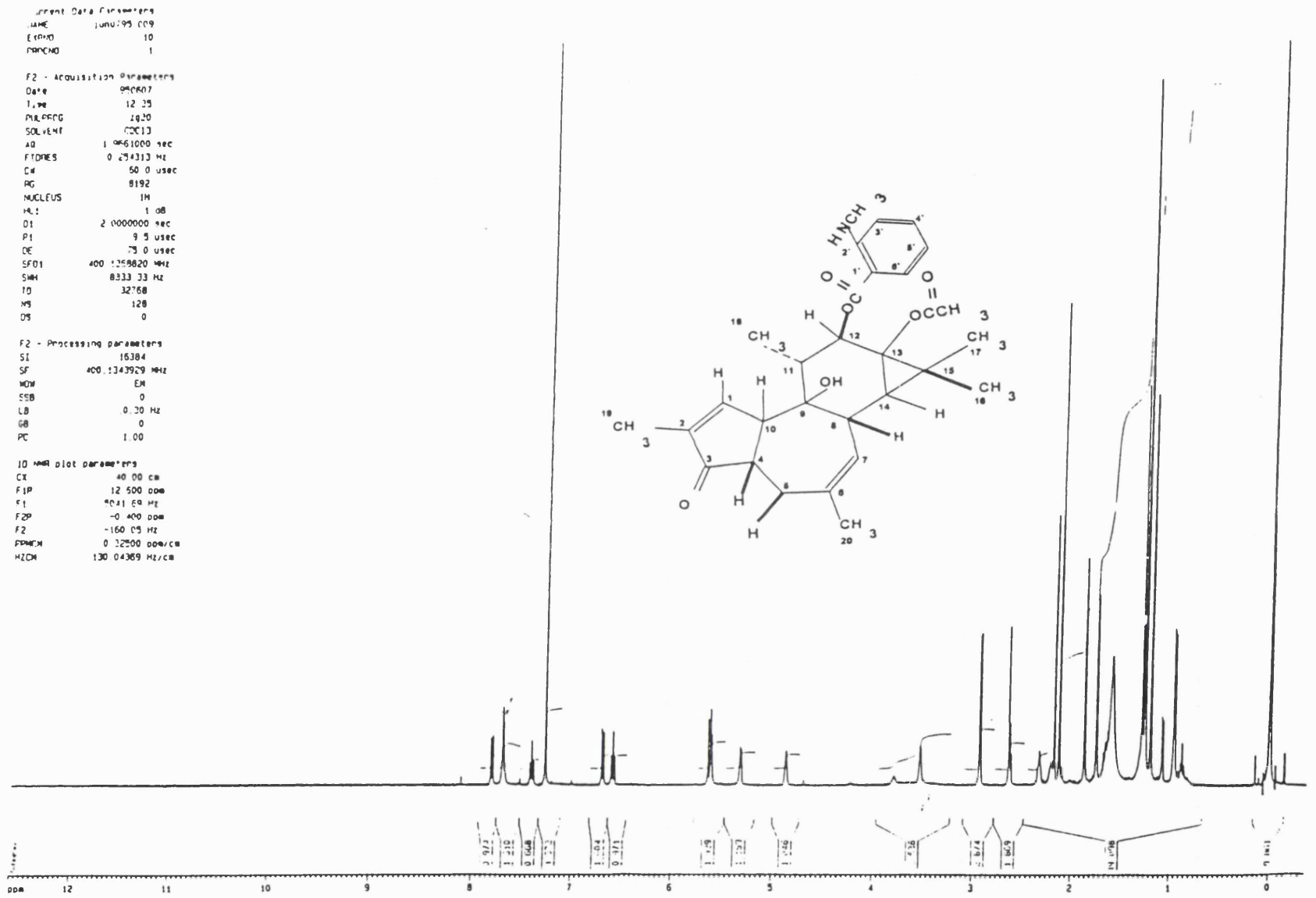


Figure 3.28 ^{1}H NMR Spectrum (400MHz, $CDCl_3$) of Sapintoxin-E, 12-O-(2-Methylaminobenzoyl)-4,20-Dideoxyphorbol-20-methyl-13-Acetate

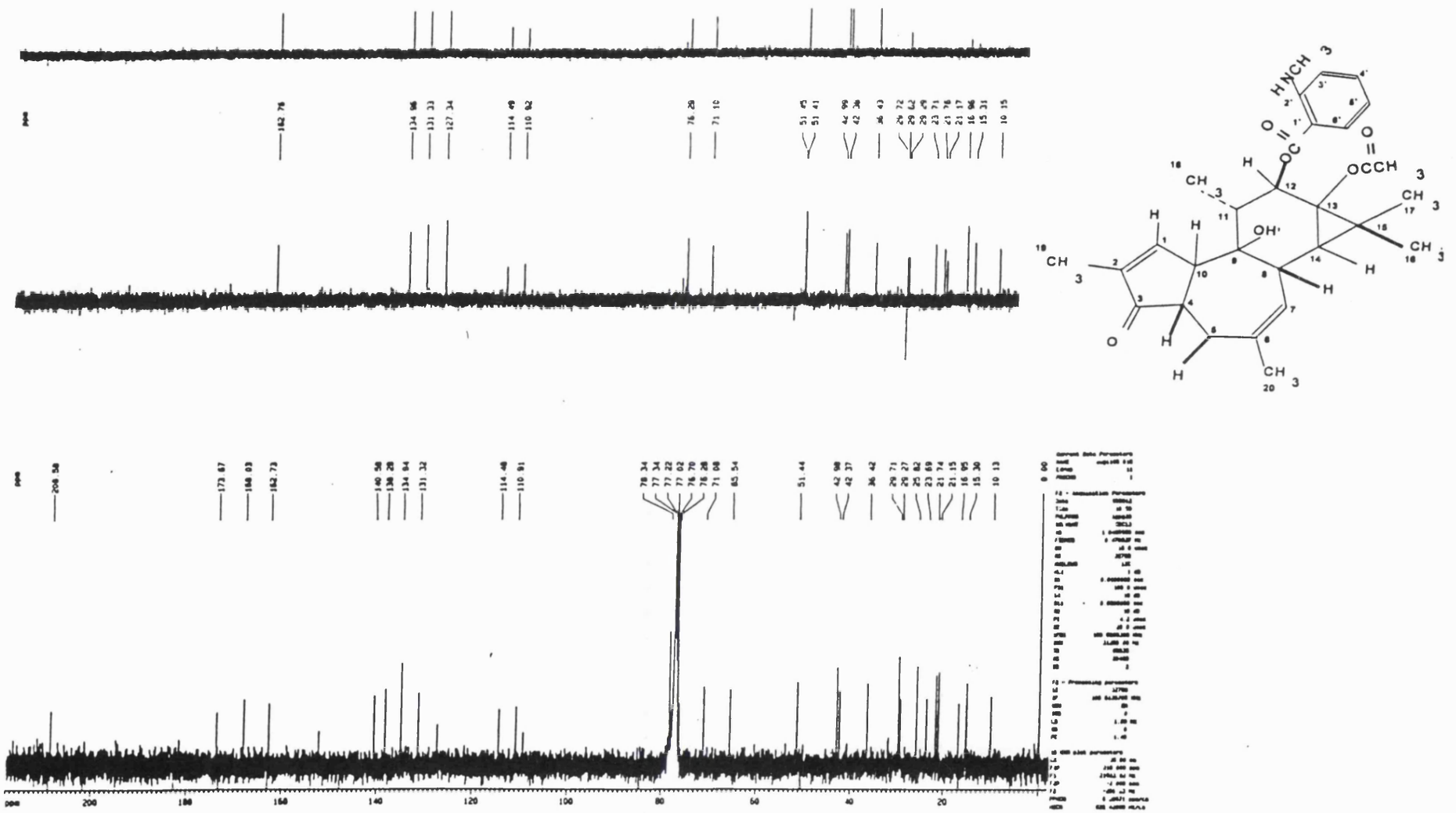


Figure 3.29 ^{13}C NMR Spectrum(100MHz, $CDCl_3$) of Sapintoxin-E, 12-O-(2-Methylaminobenzoyl)-4,20-Dideoxyphorbol-20-methyl-13-Acetate

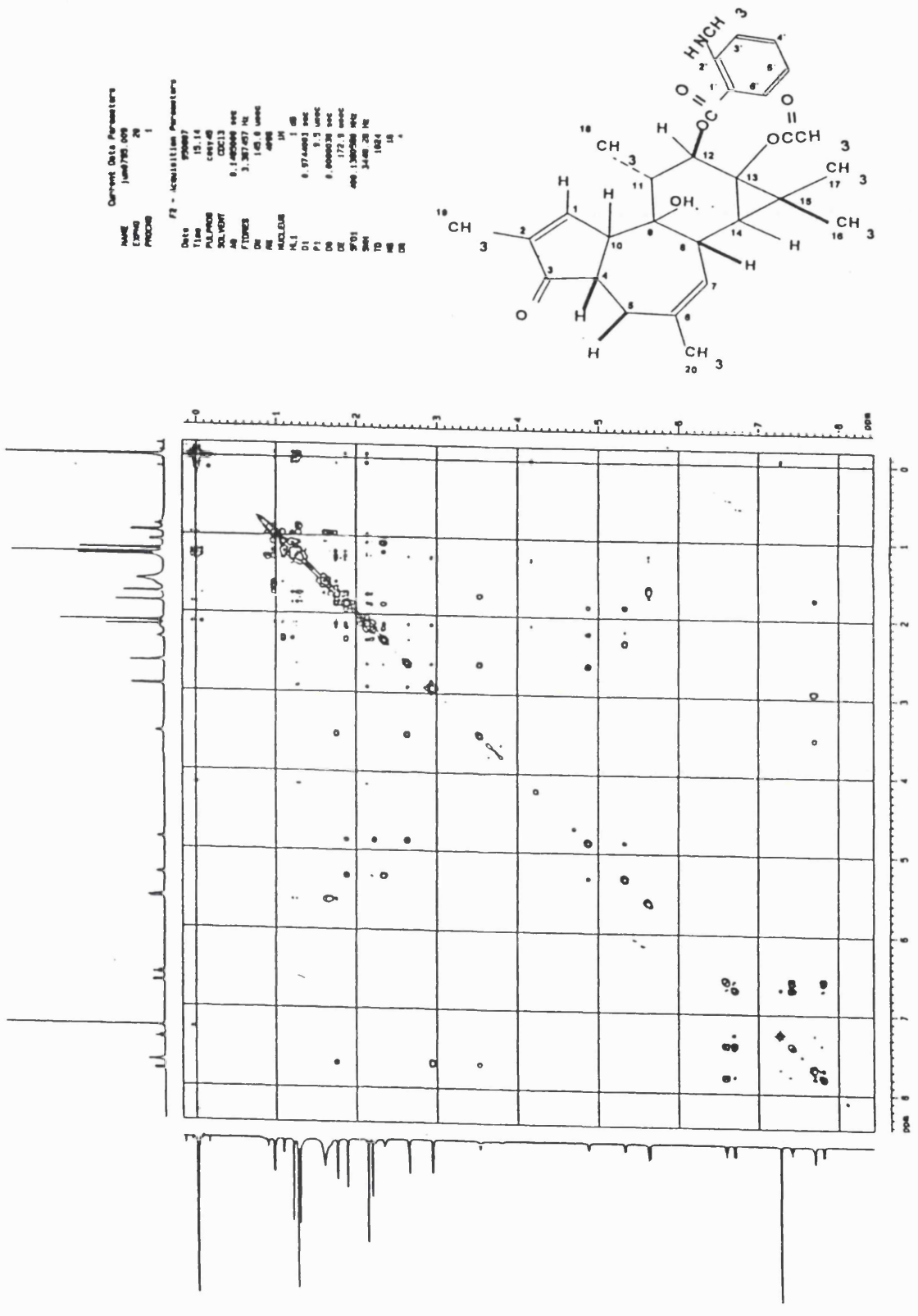


Figure 3.30: COSY H-H NMR Spectrum of Sapintoxin-E, 12-O-(2-Methylaminobenzoyl)-4,20-Dideoxyphorbol-20-methyl-13-Acetate

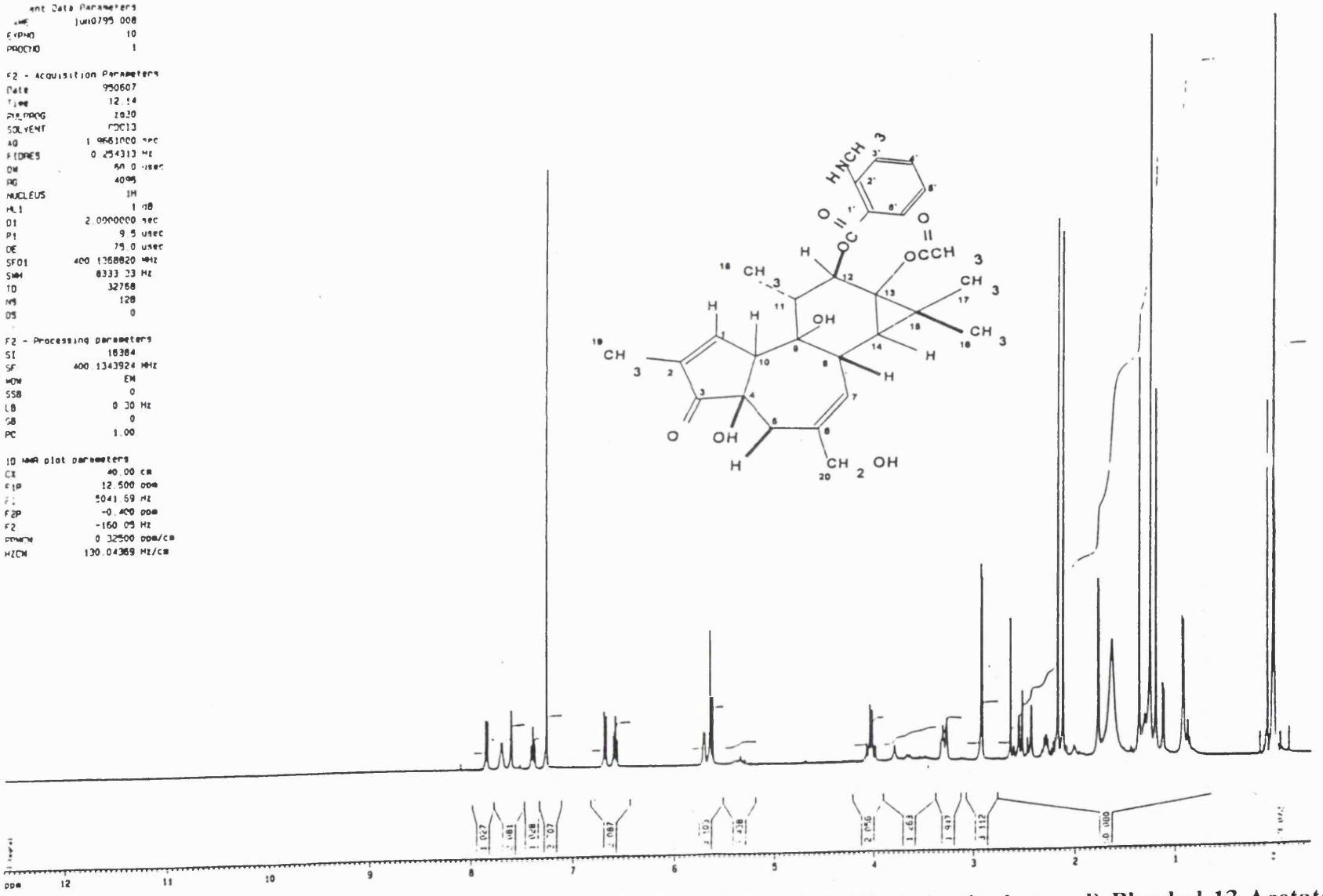


Figure 3.31 ^{1}H NMR Spectrum (400MHz, $CDCl_3$) of Sapintoxin-D, 12-O-(2-Methylaminobenzoyl)-Phorbol-13-Acetate

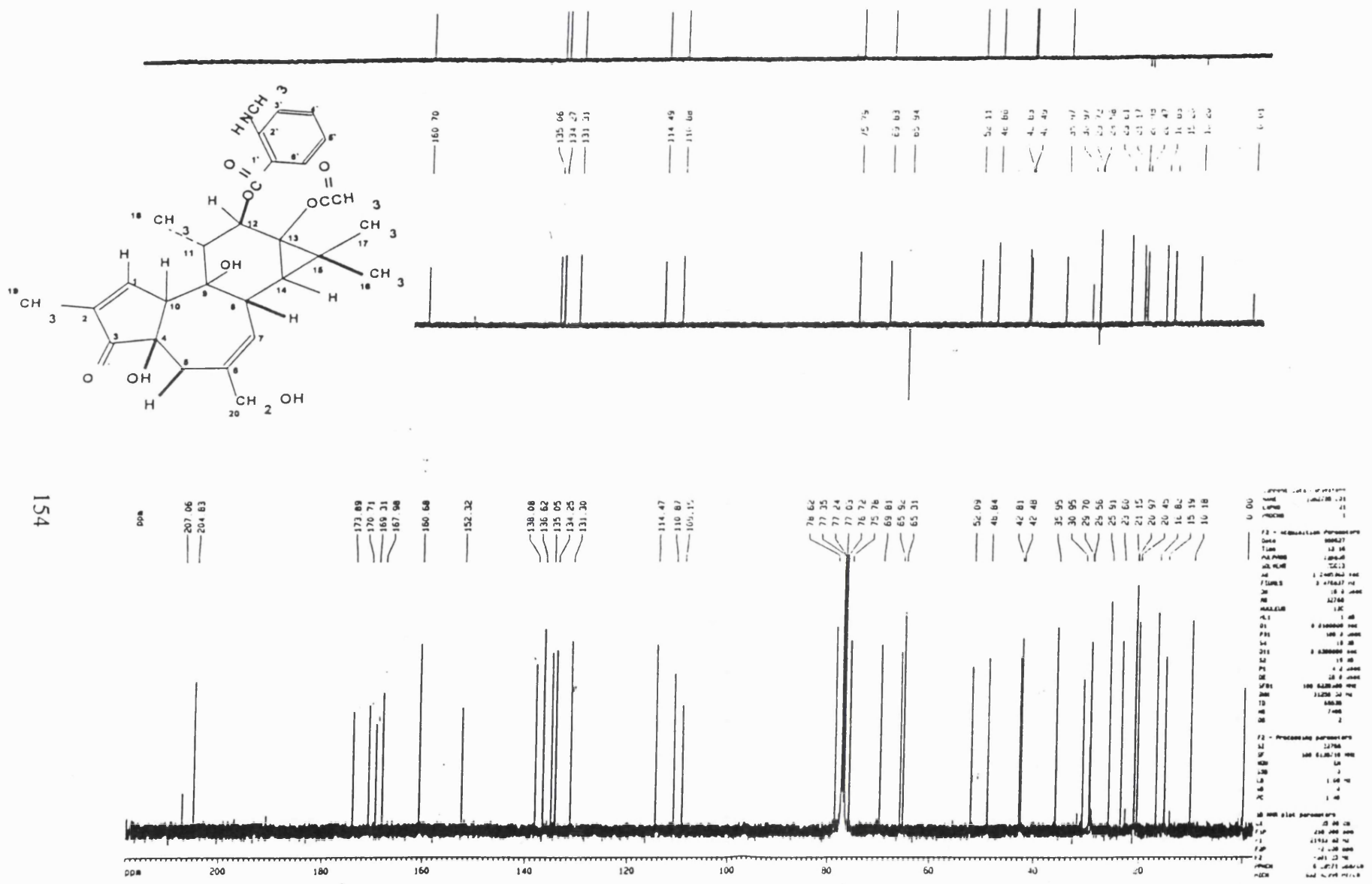


Figure 3.32 ^{13}C NMR Spectrum (100MHz, $CDCl_3$) of Sapintoxin-D, 12-O-(2-Methylaminobenzoyl)-Phorbol-13-Acetate

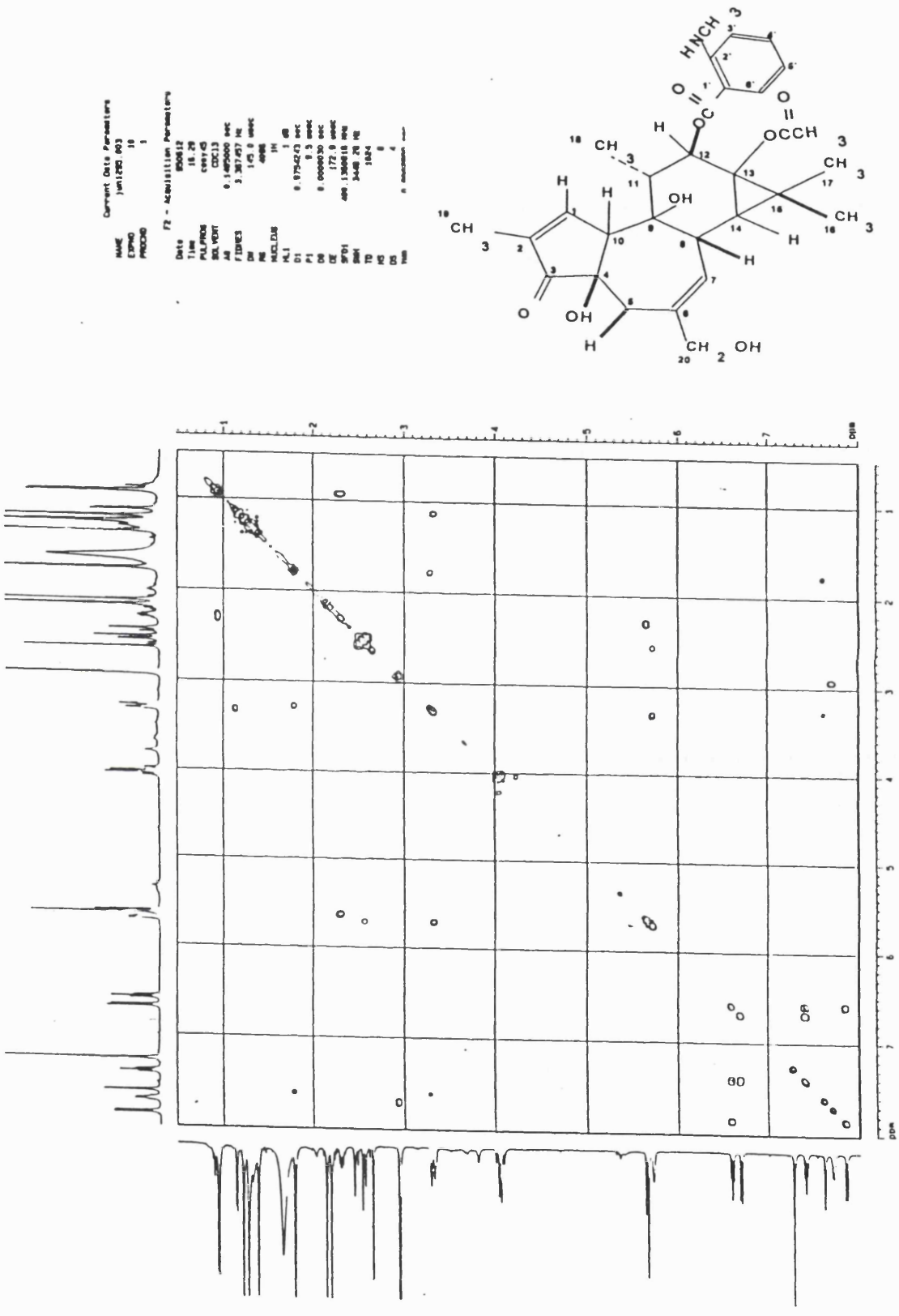
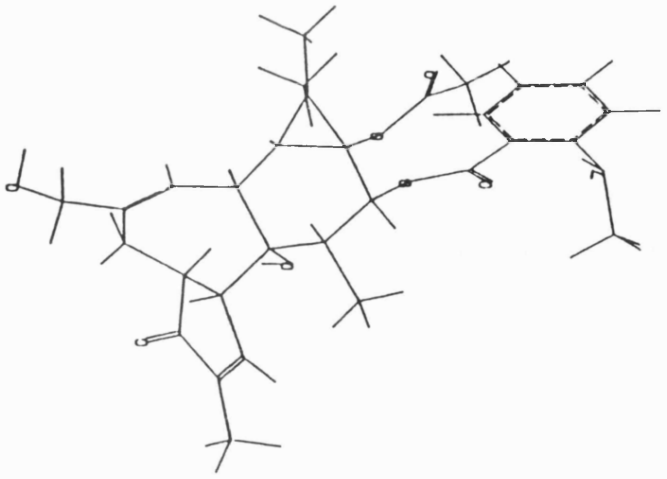
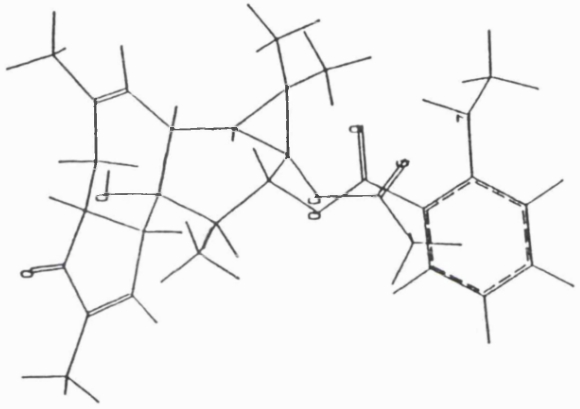


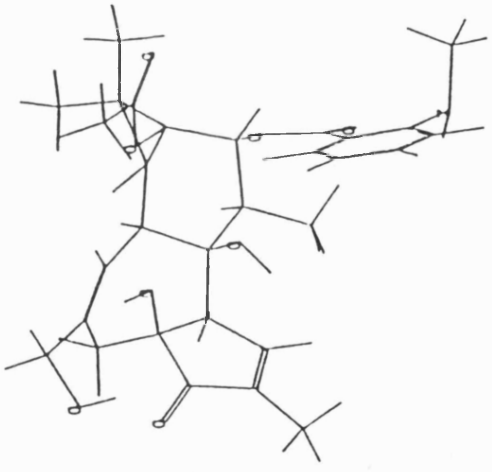
Figure 3.33 COSY H-H NMR Spectrum of Sapintoxin-D, 12-O-(2-Methylaminobenzoyl)-Phorbol-13-Acetate



Sapintoxin-A



Sapintoxin-E



Sapintoxin-D

Figure 3.34 A Computer-generated drawing (Sybyl) of the molecular structure of Sspintoxin-A, sapintoxin-E and sapintoxin-D based on NMR spectral data

CHAPTER FOUR

Biological and Structural Activity Relationship of The Isolated Diterpene Esters

4.1 Introduction

Various *in vivo* and *in vitro* test systems have been employed to evaluate the biological activities of the polyfunctional diterpene esters isolated from the families Euphorbiaceae and Thymelaeaceae. Such tests include tumour induction in mouse skin by a classical protocol (Berenblum 1941), inflammation (Schmidt and Evans 1980b), induction of ornithine decarboxylase in murine epithelial tissue (O'Brien *et al* 1975), activation of protein kinase C (Aitken *et al* 1987) and inhibitory effect on terminal cell differentiation (Yamasaki *et al* 1977 and Diamond *et al* 1978).

The high potency and selective activities of these compounds in a number of test systems have led to their widespread use as pharmacological probes to study the mechanisms of certain diseases including tumour promotion, cell regulation and the immune response.

The naturally occurring and the semisynthetic diterpene esters isolated and prepared during this study were evaluated for biological activities in three *in vivo* and *in vitro* systems. The *in vivo* tests include inflammation and epidermal hyperplasia in mice. *In vitro*, the compounds were tested for their ability to induce aggregation of human blood platelets. The results obtained from these test systems were then correlated on a structural basis. The protocols used for each test system are described in the following section.

4.2 Assay Procedures

4.2.1 Determination of The Pro-inflammatory Activity

Irritancy to mammalian skin is a property common to most esters of the tigliane, daphnane and ingenane hydrocarbons or plants containing these compounds. During this study, the assay procedure for comparative irritancy testing of a series of tigliane and

daphnane esters (Evans and Schmidt 1979a) was adopted to test the diterpenes isolated or synthesized.

Group of six female CD-1 mice each approximately twenty grams were housed in a cage on wood shavings. Standard pelleted food and water was provided. Approximately 1mg of the compound under test was accurately weighed and made up to a 1mg/1ml solution with redistilled acetone. The dilution series was prepared according to the equation :

$$C_m=C_0A^{-m}$$

Where C_0 is the initial concentration, C_m is the concentration after m-dilutions and A is the dilution factor. The dilution factor in all cases was 10 for the pilot assay and 2 for the main assay.

A pilot assay was initially carried out using one cage of six mice. Using a Gilson pipette, a 5µl aliquot from the most dilute concentration was applied to the inner surface of the right ear of two mice. This procedure was repeated for five further dilutions of increasing concentration. The untreated left ear was used as negative control. Two mice received 5µl of acetone as a negative control during the whole procedure. As a positive standard the right ears of six mice in one cage were treated with 0.01nM TPA, 12-O-tetradecanoyl phorbol-13-acetate.

The ears were examined for redness after 15 minutes and then at 15 minutes' intervals until two consecutive examinations indicated that further reddening did not occur. The time taken to achieve maximum erythema was noted. Mice were also examined after 24, 48 and 72 hours to ascertain the chronic inflammatory dose.

Redness of ears was assessed as a quantitative response. The minimum response corresponding to (+) was recorded for increased blood supply to the ear with visible dilation of the major blood vessels. (++) response was recorded for vasodilatation of the major blood vessels with marked redness of the ears in between. If the later response was accompanied by oedema and increased thickness of the ear with severe redness, the response was recorded as +++.

The main pro-inflammatory assay was carried out as follows. Five consecutive dilutions were chosen for the main assay to include one dilution that would be expected to give 100% positive response. A 5µl aliquot of the most dilute solution in the chosen series was applied to one ear of each mouse in a cage. Further four cages of mice were treated similarly with increasing concentrations of the irritant. Mice were examined 15 minutes prior to the expected maximum response then at 15 minute intervals to confirm the time for maximum irritancy. The assay was repeated with a further six cages each containing 6 mice. Redness of the ears was assessed as indicated in the pilot assay.

The total numbers of red ears per dilution were tabulated. The ID_{50}, the irritant dose which induces a response in 50% of the test animals, and the standard deviation (SD) were calculated using the software programme PROBIT (IBM scientific subroutines PROBIT). Table 4.1 lists the results of the irritancy assays carried out on the compounds identified during the course of this investigation.

4.2.2 Induction of Epidermal Hyperplasia

Tumour promoting compounds are capable of inducing an epidermal hyperplasia of mouse skin following a single topical application of the substance (section 1.4.2). Although epidermal hyperplasia is necessary, it is however an insufficient condition for tumour promotion (Mark and Furtenberger 1984). Therefore, a rapid 48 hour hyperplasiogenic assay was set up for the purpose of detecting possible tumour promoting phorbol esters. The protocol used for this short term assay was described in 2.3.6. Refer to Figure 4.1 for test results.

4.2.3 Platelet Aggregation Assay

The assay procedure carried out to evaluate the aggregating activity of the isolated diterpenes was the same procedure used for plant extracts (section 2.3.4). Compounds under investigation were accurately weighed and made up to 5µM/5µl solution using acetone as vehicle. Serial dilutions of 10 times the original concentration were prepared for the main test. The esters were tested in a concentration range of 0.003-73µM.

Acetone was used as the vehicle at a maximum concentration of 0.5% v/v in PRP (platelet rich plasma). At this concentration acetone did not induce aggregation or modify the TPA induced aggregation according to the tests carried out before the experiment. TPA was used as the positive aggregating standard at a concentration of 0.8μM.

4.3 Results and Discussion

In the present study, the naturally occurring and the semi-synthetic diterpene esters were tested using three biological assays. The *in vivo* tests were carried out on CD-1 female mice. Unlike other strains of rodents the CD-1 are considered to be suitable for both irritancy and epidermal hyperplasia. The mouse has been the most susceptible laboratory animal to skin tumorigenesis (Robinson 1986). Other rodents, such as rabbit, rat and guinea-pig do not respond to TPA-induced tumour promotion of initiated skin (Berenblum 1949, Homberger *et al* 1972). The lack of promotion was thought to be due to the presence of the enzyme phorbol-12,13-diester-12-esterhydrase (PDEH) in the epidermis of certain mammalian species (Shoyab *et al* 1982) which converts the phorbol esters to their non-promoting de-esterified analogues. Conversely, Ashman and his team suggested that variations in susceptibility to carcinogenesis may be due to a difference in the ability to repair damaged DNA (Ashman *et al* 1982).

Different stocks and strains of mice have different susceptibilities to skin carcinogenesis (Slaga *et al* 1982). SENCAR however is the most sensitive to skin carcinogenesis and is used by the USA government to test commercial products (Robinson 1986). The relative sensitivity of the mice stocks and strains to complete carcinogenesis is: SENCAR>CD-1>C57BL/6>LACA>ICR/Ha SWISS C3H BALB/c (Slaga *et al* 1982, Ashman *et al* 1982, Slaga 1983, 1984).

The ID_{50} values, obtained from PROBIT analysis of the irritancy test, are summarized in table 4.1. The positive control TPA was the most potent irritant tested in this study having an ID_{50} of $7x10^{-4}$ nM. When the ester substituent at C12 in the daphnane ester mezerein (ID_{50} of $4.5x10^{-3}$ nM) was reduced by one methylene group, the irritant activity was reduced three

fold as observed from the ID_{50} of $16.6x10^{-3}$ in the case of thymelaeatoxin-A. However, if the ester moiety at C12 was replaced with hydrogen as in the case of resiniferonol-9,13,14-orthophenylacetate (ROPA) (ID_{50} of $178x10^{-3}$ nM/ear) the irritant activity decreased ten fold compared to thymelaeatoxin-A and decreased thirty nine fold compared to mezerein. The structurally related fluorescent sapintoxin-A was found to be 1.4 times more active than sapintoxin-D. The ID_{50} values were $42.8x10^{-3}$ nM/ear and $31.3x10^{-3}$nM/ear respectively. The presence of a hydroxyl group on C-4 in sapintoxin-D did not increase the activity as pro-inflammatory agent (Figure 4.2 shows the structures of compounds investigated for irritant activity).

Replacing the primary hydroxyl group at C-20 with an acetate group in phorbol-12,13,20-triacetate and phorbol-13,20-diacetate, or a hydrogen in sapintoxin-E resulted in complete loss of irritant activity at the tested dose levels. These results illustrate the importance of retaining a free primary hydroxyl group at C20 for pro-inflammatory effect. The parent phorbol was an inactive pro-inflammatory agent. The conversion of the A/B-trans linked phorbols into A/B-cis linked types resulted in complete loss of irritant effect as demonstrated by 4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate. This result is consistent with the pro-inflammatory requirements published by Evans and Edwards in 1987.

This investigation indicated that increasing unsaturation and the presence of an aromatic ring as part of the ester moiety at C12 increases the pro-inflammatory activity of the diterpene esters. This activity was dramatically decreased when replaced with hydrogen in the daphnanes as in the case of resiniferonol-9,13,14-phenylacetate orthoester. However, the inflammatory activity was completely abolished when the C13 ester function was replaced with a secondary hydroxyl group in tiglianes as shown by phorbol and its derivatives. Acylation of the hydroxyl group at C20 in phorbol did not induce inflammation at the tested doses of 20μg/ear.

The time to the onset of inflammation (which is the time between the application and the first signs of inflammation) also known as the latency was found to be dose-dependent. The same observation was recorded for the time to peak irritancy. For example, the time to the

onset of inflammation induced by the ID_{50} of thymelaeatoxin-A was one hour reaching its maximum effect four hours from the time of application. Both the times for acute irritancy and peak irritancy decreased dramatically following the application of dose levels 10^3 times greater than the ID_{50} . The variation in the time to peak irritancy of the tested diterpenes could be explained by the proposal that different structures have different diffusion rates to the receptor site (Schmidt and Evans 1980b).

Persistence of inflammation appeared to be related to the nucleus of the irritant. Inflammation induced by the ID_{50} dose level of tigliane derivatives was evident twenty four hours after application, while that induced by daphnanes was not. Increasing the dose levels 10^3 times greater than the ID_{50} resulted in a decrease of both the time for acute and peak irritancy but increased the duration of inflammation. However, increasing the dose level did not induce any instant inflammation. Sapintoxin-D had the shortest time to acute irritancy observed twenty five minutes after application of 5nM to the ear.

Mezerein and thymelaeatoxin-A, both daphnane esters, exhibited persistent inflammatory effect at 5nM dose level lasting for more than seventy hours. In contrast, at an equivalent dose level resiniferonol-9,12,13,14-orthophenylacetate failed to demonstrate a persistent effect for more than twenty two hours. Previous investigations into the skin irritant properties of resiniferonol orthoesters concluded that this type of compound induce a rapid but non-persistent inflammation compared to tiglianes (Schmidt and Evans 1979). The short term inflammation is thought to be a result of a specific receptor site interaction whereas the persistent inflammation is thought to be a result of tissue damage involving the release of inflammatory mediators (Schmidt and Evans 1980, 1978, Williamson *et al* 1981). Observations of the irritant action of the isolated daphnanes support the previous suggestion that irritancy of daphnane orthoesters is mixed aetiology (Gordge *et al* 1992).

Thymelaeatoxin-A and mezerein proved to be potent platelet aggregatory agents. The most potent aggregating agent was found to be TPA having an AD_{50} (aggregating dose inducing 50% platelets aggregation) of 0.207μM after four minutes and 0.053μM after eight

minutes. Sapintoxin-A was 4.6 times less potent than TPA with AD_{50} of 0.25μM. Thymelaeatoxin-A was eight times while mezerein was thirty times less potent than TPA as platelets aggregating agents(at four minutes). Although sapintoxin-D was a more potent pro-inflammatory agent than sapintoxin-A, it was 12 times less potent as an aggregating agent. This observation highlights the fact that C4 tertiary hydroxyl group is not essential for both activities.

Two of the daphnane orthoesters, mezerein and thymelaeatoxin-A, induced a dose dependent aggregation of human blood platelets at the nanomolar level. The third daphnane, resiniferonol-9,13,14-orthophenylacetate was able to induce platelets' aggregation only at a micro molar level despite the fact that all three compounds are resiniferonol derivatives. This distinct response could be explained on a structural basis. In the case of the former two compounds, the resiniferonol nucleus exhibited a very similar unsaturated ester moiety at C12 and an epoxy group between C6 and C7. In addition, the benzylacetate orthoester in these two diterpene esters replaced the phenylacetate orthoester in resiniferonol-9,13,14-phenylacetate. Although previous investigations of the 12-deoxyphorbol esters concluded that an ester function at C12 is not essential for aggregating activity (Williamson *et al* 1981), an ester function at C13 and a free primary hydroxyl group at C20 are essential (Edwards *et al* 1983b, Evans and Edwards 1987). The present study indicates that an ester moiety at C12 dramatically increases the aggregating effect of daphnanes. All three compounds have a free C20 hydroxyl group. The epoxy group linking C6 and C7 appears to play a role in platelet aggregation induced by daphnanes.

These findings provide further explanation to the lack of aggregating effect of the previously investigated daphnane esters. Resiniferatoxin and tinyatoxin did not aggregate platelets at concentrations as high as 70μM (Williamson *et al* 1980). In addition to the aromatic ester group at C20 both compounds exhibit a double bond between C6 and C7. It could be concluded that the ability to induce platelet aggregation is affected by the degree of oxygenation of the daphnane nucleus.

In agreement with the essential structural requirement for platelet aggregation, the novel

compound isolated during this study sapintoxin-E was unable to induce platelet aggregation at 50μM concentration due to the absence of a primary hydroxyl group at C-20. Furthermore, the semi-synthetic 4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate was inactive as a platelet aggregating agent due to the cis-configuration of the link between the A/B rings in the tigliane nucleus.

Treatment of the mouse skin with TPA induced dramatic morphological changes in the epidermis, when compared with the normal untreated skin, (Figure 4.1.b, pp. 169). Within forty eight hours, the stimulation of epidermal proliferation induced by TPA and mezerein was evident from the increase in the number of cell layers from one in the untreated skin (Figure 4.1.a, pp. 169) to three or more (Figure 4.1 b, pp. 169, Figure c & d, pp. 170). Increased epidermal keratinization associated with the increase in the epidermal thickness was seen to be dose dependent.

Phorbol and phorbol-12,13,20-triacetate failed to induce significant hyperplasia of CD-1 mouse epidermis at doses of 100nM however extensive keratinization was detected (Figure 4.1 i & j, pp. 173). In addition. 4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate and the novel compound sapintoxin-E were unable to induce epidermal hyperplasia of the mouse epidermis (Figure 4.1g & h, pp. 172). Microscopical examination of the epidermal layer of the mouse skin treated with sapintoxin-E demonstrated changes in the morphology of the cell layer adjacent to the dermis (Figure 4.1h, pp. 172) suggesting that C20 substituted tiglianes might be biologically active at the cellular level at micromolar concentrations. This proposal is supported by the report of the marginal promoting effect of phorbol in STS mouse skin and the confirmed leukaemogenic effect in SWR mice (Armuth *et al* 1979). Further evidence was obtained from investigation by Doege and Hecker which concluded that stimulation of biochemical effects by cryptic tumour promoters of the phorbol ester type may take place indirectly by generating low dose levels TPA via metabolic activation and directly in relatively high doses (Doege and Hecker 1987). These findings indicate that whilst non irritant phorbol esters are not as potent as TPA, they may exert weak biological effects which at physiological dose levels are not detected. When used as negative controls in any

system their effect, if any, must be determined within the dose-range of the experiment.

In the present study, biological activities of a range of daphnanes and tiglianes were compared in three biological systems. Comparing the results obtained from the biological tests reveal that only pro-inflammatory diterpenes exhibit a full biological spectrum at the tested dose levels. Reports on skin tumours are always associated with inflammatory reactions and epidermal hyperplasia not only in the second but also in the first stage of promotion (Marks and Furstenberger 1984). However, irritant and hyperplastic agents are not necessary tumour promoters (Brooks *et al* 1989). Considering the distinct structural differences between tigliane and daphnane nuclei, significant structural requirements for different biological activities can be summarised. The cyclopropane ring-D in tiglianes is not essential for pro-inflammatory action, platelets aggregation or epidermal hyperplasia. Increasing unsaturation of the ester function at C12 in both tiglianes and daphnanes increase both inflammatory and aggregating effects (Table 4.1, pp. 167 and Figure 4.2, pp. 168). Replacement of the tetradecanoate in TPA with acetate in phorbol-12,13-diacetate resulted in complete loss of biological activities at the tested dose levels.

12-deoxyphorbols were reported to be potent platelet-aggregating agents as well as inducers of long lasting erythema on the mouse ear (Schmidt and Evans 1980b, Williamson *et al* 1981). In contrast resiniferonol-9,13,14-orthophenylacetate, a 12-deoxydaphnane derivative, exhibited weak irritant and aggregatory effects. Resiniferatoxin and tinyatoxin, the closely related C20 substituted daphnane esters, were reported to induce rapid inflammation lasting for less than twenty four hours. These two daphnane esters do not induce aggregation of human blood platelets (Williamson *et al* 1980).

Comparable biological activates induced by the same dose levels of mezerein and thymelaeatoxin-A reflect their close structural relationship. On the other hand, structurally related sapintoxin-A and sapintoxin-D exhibited similar irritant potencies to the mouse skin but different platelet aggregating effects (Table 4.1, pp. 167 & Figure 4.2, pp. 168). Both sapintoxins induced epidermal hyperplasia of the mouse skin but only sapintoxin-D was found to be a second stage promoter (Brooks *et al* 1989). Therefore it could be concluded

that a 4β-hydroxyl group is not essential for irritant and platelet aggregation but it could play a role in tumour-promotion.

In summary, TPA exhibited a wide range of biological activities *in vivo* and *in vitro* systems. The use of a range of structurally related compounds allows a more selective approach to biological activities induced by different derivatives. This approach allows more specific understanding of the environment associated with each biological effect.

Compound	ID_{50}		Peak irritancy		SD	X^2 (Degree of freedom)	Duration of irritancy (Hr)
	nM/ear	ng/ear	Time (Hr)	Degree			
Mezerein	0.0045	3	8.5	+	2.15	0.1799 (4)	15
Thymeleaetoxin -A	0.0166	10.42	4	+	0.98	1.847 (4)	13
Resiniferonol-9,13,14-orthophenylacetate	0.1780	78.8	5	+	0.2146	0.4710	2
TPA	0.0007	0.43	6.5	+	0.2006	5.343 (4)	22
Sapintoxin-A	0.0428	22.38	5	+	0.160	1.15 (4)	27
Sapintoxin-D	0.0313	16.87	4.8	+	2.800	1.864 (4)	27
Sapintoxin-E	39.500	>20	>72	_	_	_	_
Phorbol	57	>20	>72	_	_	_	_
Phorbol-12,13-diacetate	44	>20	>72	_	_	_	_
Phorbol-12,13,20-triacetate	40	>20	>72	_	_	_	_
12-O-(Methylaminobenzoyl)-4α-Deoxy-5-hydroxy phorbol-5,13,20-triacetate	32	>20	>72	_	_	_	_

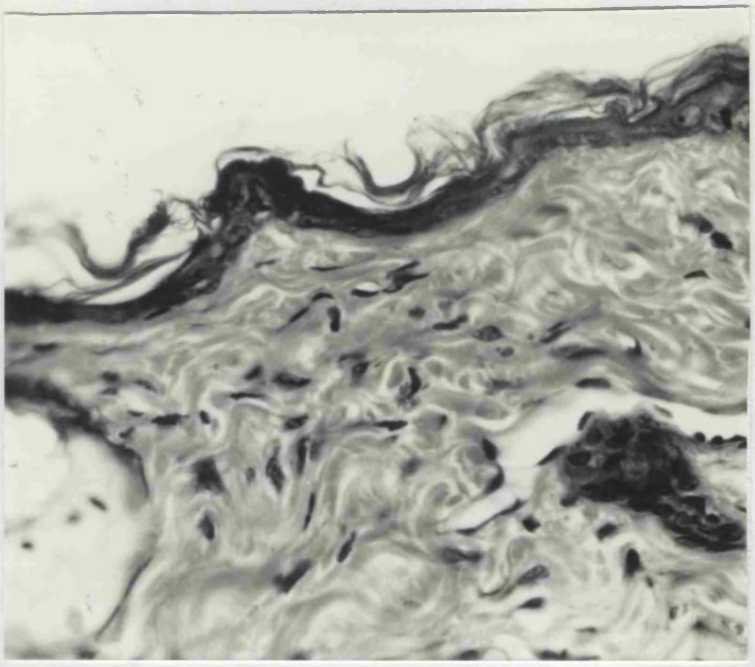
Data represent the mean of four experiments
SD=Standard deviation, X^2 and degrees of freedom were calculated using PROBIT Analysis
ID_{50}= Irritant dose which induces a response in 50% of the test animals

Table 4.1 Results of the Irritancy Assays of the Isolated Diterpene Esters

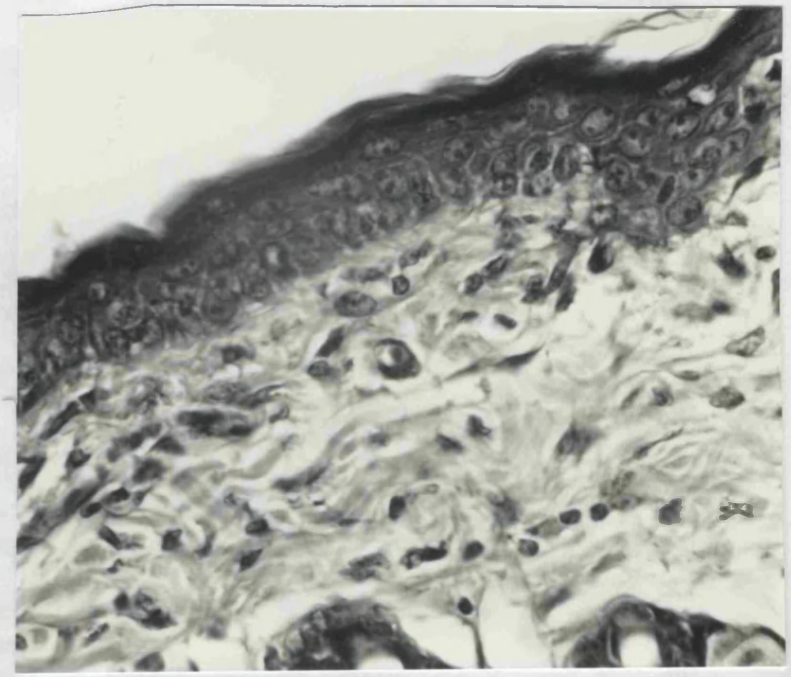
Table 4.2 Results of Platelet Aggregating Assays of the Isolated Diterpene Esters

Compound	% Aggregation		AD_{50} µM		SD	X^2 (Degrees of Freedom)
	4 Minute	8 Minute	4 Minute	8 Minute		
Mezerein	40	60	6.27	0.618	10.86	11.8 (3)
Thymeleaetoxin -A	15	35	1.80	0.480	3.52	8.2 (2)
Resiniferonol-9, 13,14-orthophenyl acetate	15	20	>7	>7	_	_
TPA	20	60	0.207	0.053	7.80	9.9 (5)
Sapintoxin-A	40	70	5.390	0.240	2.48	19 .16 (4)
Sapintoxin-D	25	55	48.800	3.010	1.74	3.7 (2)
Sapintoxin-E	0	0	>1	>1	_	_
Phorbol	0	0	>1	>1	_	_
Phorbol-12,13-diacetate	15	45	73.06	8.100	16.9	3.10 (2)
Phorbol-12,13,20-triacetate	0	0	>73	>73	_	_
12-O-(methylamino benzoyl)-4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate	0	0	>70	>70	_	_

Data represent the mean of four experiments
SD = Standard deviation, X^2 and degrees of freedom were calculated using PROBIT Analysis
AD_{50} = Dose inducing 50% platelet aggregation



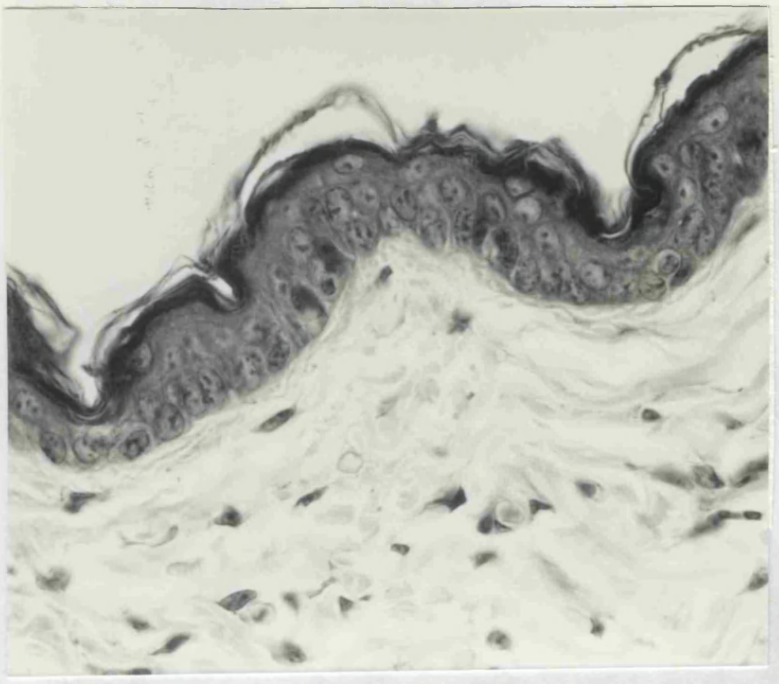
A: Normal Mouse Skin



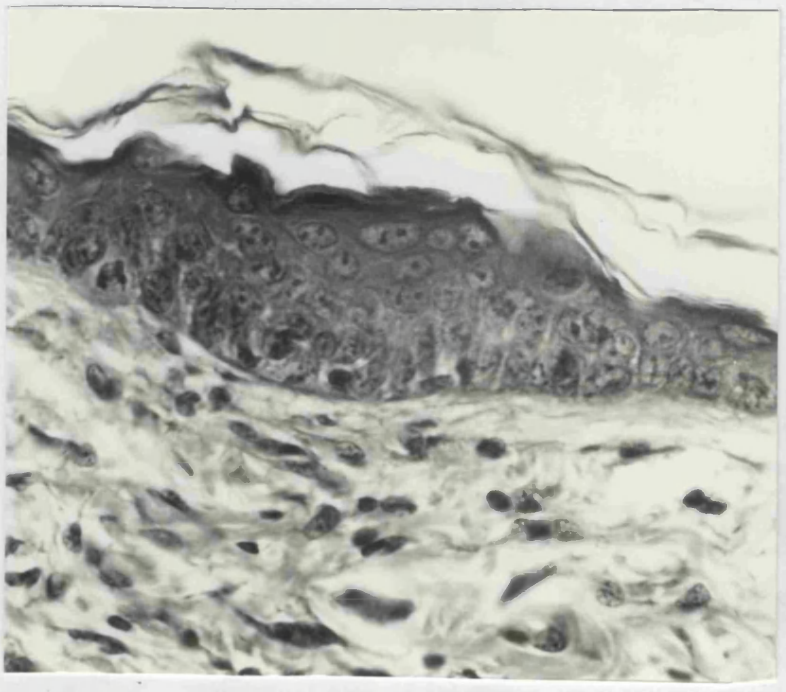
B: Hyperplasia induced by 8pM of TPA

Figure 4.1 Histological Appearance of Female CD-1 Mice Skin After 48 Hours of a Single Treatment with Tigliane and Daphnane Derivatives

All compounds were applied topically dissolved in 200µl acetone
Stain: Haemotoxylin and Eosin Magnification x400

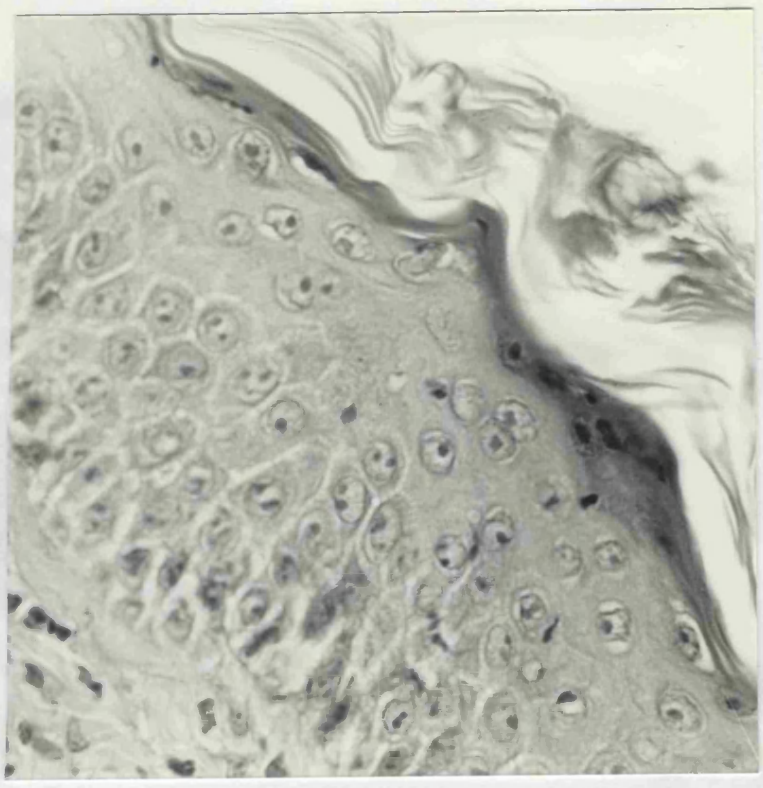


C: TPA 0.2nM

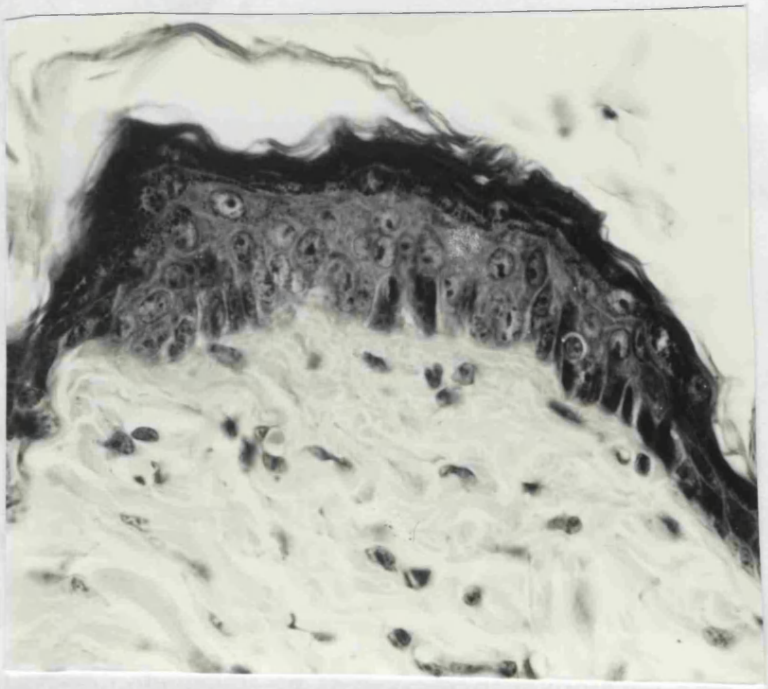


D: TPA 20nM

Figure 4.1 Continued
Hyperplasia and increased keratinization induced by different doses of TPA

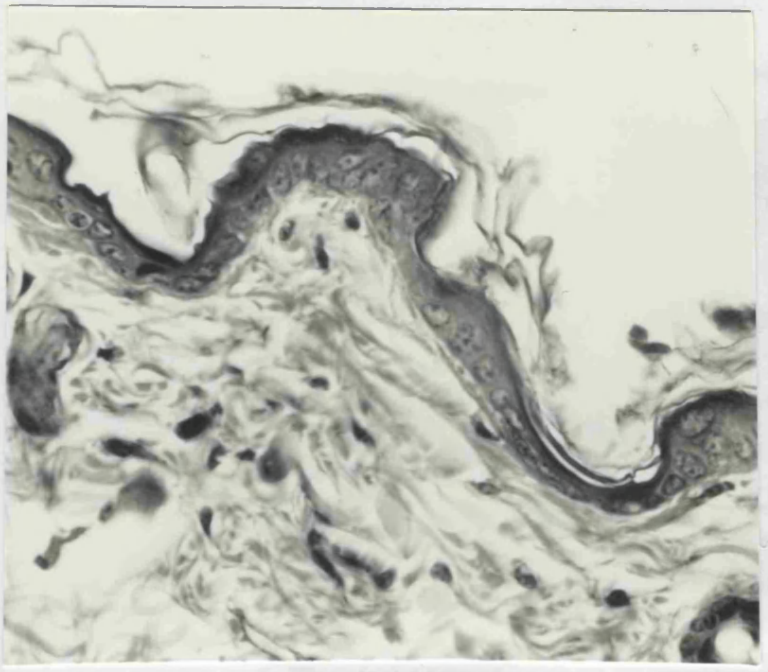


E: TPA 100 nM

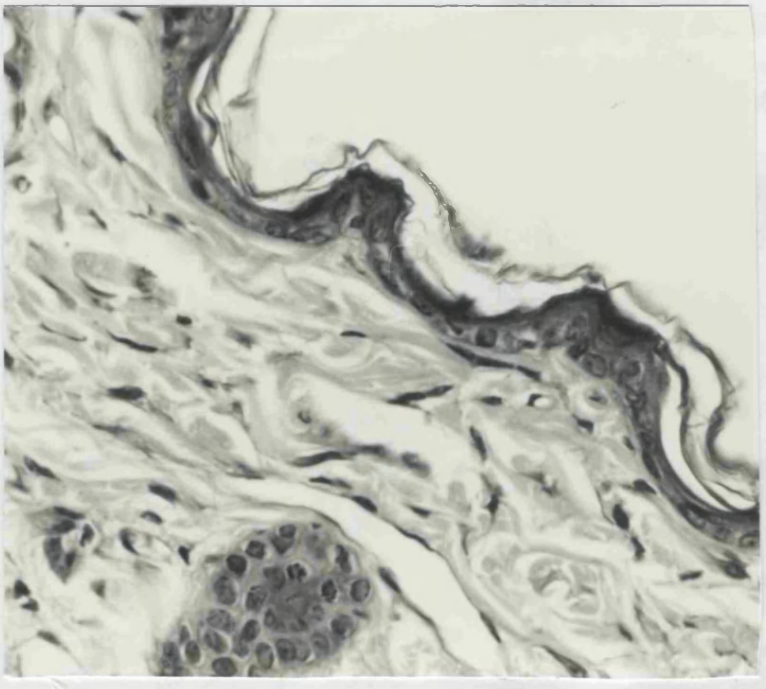


F: Mezerein 2 nM

Figure 4.1 Continued
Hyperplasia and increased keratinization induced by TPA and mezerein

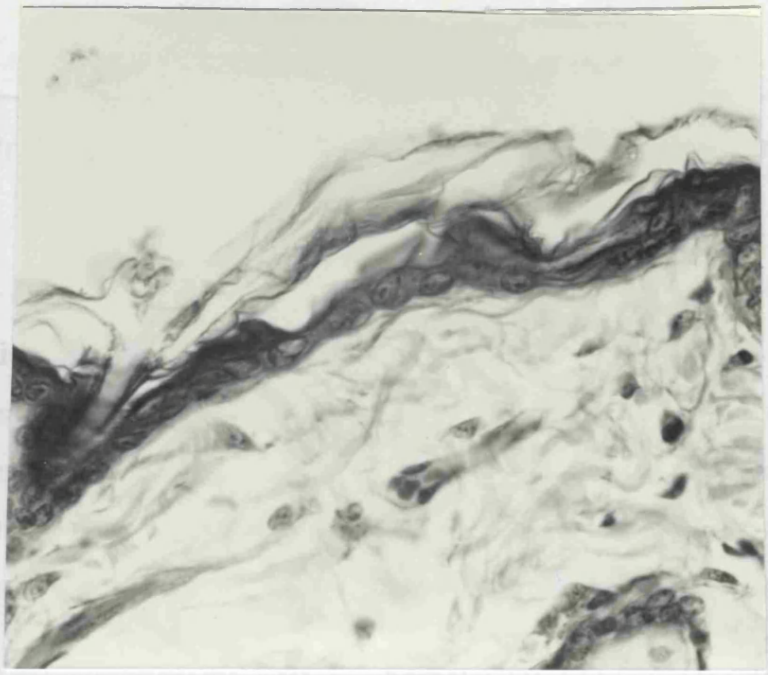


G: Sapintoxin-E 100nM

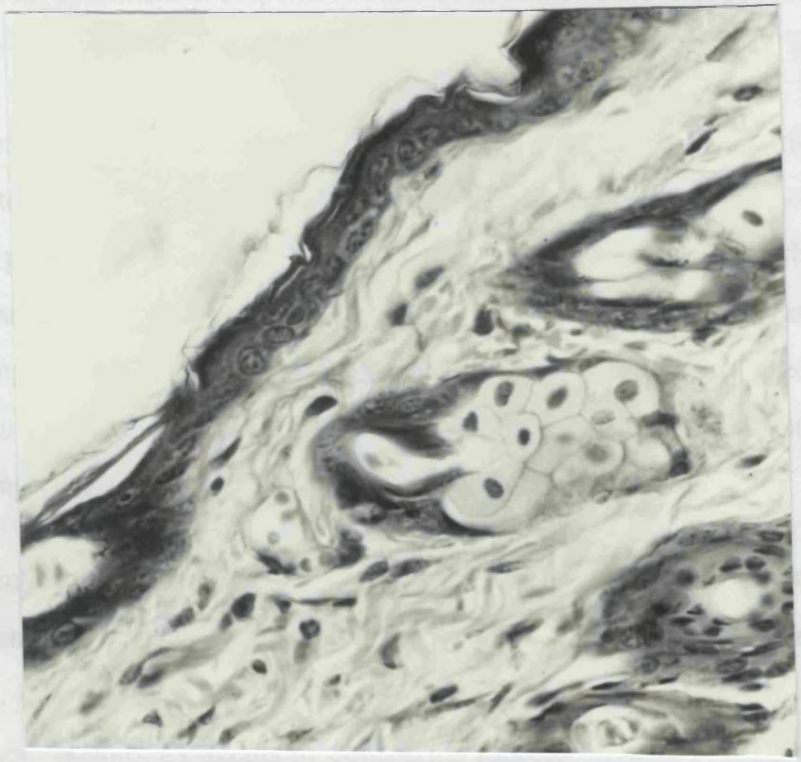


H: 12-O-(2-Methylaminobenzoyl)-4α-Deoxy-5-hydroxyphorbol-5,13,20-triacetate 100nM, SAP-AC

Figure 4.1 Continued
Increased keratinization induced by Sapintoxin-E and 4α-sapintoxin-B-diacetate



I: Phorbol 100nM



J: Phorbol-12,13-20-triacetate

Figure 4.1 continued
Slight increase in kratininzation induced by phorbol and phorbol-12,13,20-triacetate

CHAPTER FIVE

A New Procedure to Detect Potential HL-60 Cell Differentiators

5.1 Introduction

The human promyelocytic leukemia cell line HL-60 can be induced to differentiate *in vitro* into a number of different cell types by a variety of agents. Studies on such cell line has proved invaluable in different research areas. These include new therapeutic approaches for leukemia patients serving as a model for studying specific cellular oncogen expression.

Tumour promoting agents, such as phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), induce the HL-60 cells to differentiate into mature cells (Huberman and Callaham 1979). Treatment of suspension cultures of HL-60 cells with TPA induced terminal differentiation into macrophage like cells (Rovera *et al* 1979a).

The existing techniques used for quantitative evaluation of differentiation activity of diterpene esters and other compounds are generally based on the use of radio labeled compounds particularly (6-^{3}H) thymidine. This method necessitates the use of well established but expensive facilities. In a small laboratory, where resources are limited, large scale screening procedures would be neither practical nor affordable. An effective simple and rapid procedure was therefore required to assess biological activities of both plant extracts and pure compounds.

An aim of the present study was to develop such an assay and use it to determine the differentiation abilities of a range of plant extracts and diterpenes of the daphnane and tigliane type. A further objective was to establish the structure-activity relationship of the possible tumour promoting agents by comparing this test system with other *in vivo* and *in vitro* assays.

5.2 Materials and Methods

5.2.1 Cell Culture and Routine Maintenance

The cell line HL-60 was donated by professor P. Parker (ICRF) and was grown in RPMI-1640 medium (supplied by Gibco, UK). The medium was supplemented with 10% heat -inactivated foetal calf serum (HI-FCS), 2mM glutamine, penicillin 50U/ml and gentamycin 50 µg/ml (all supplied by Gibco, UK). The medium was stored at 4°C and fresh 5% HI-FCS was added on monthly basis to maintain healthy cells. Cells were grown in plastic tissue culture flasks (supplied by Flow laboratories Ltd) at 37°C in a humidified atmosphere of 5% CO_2.

Twice weekly passage of the cell culture maintained the concentration of the cells number between 0.5-8 $x10^5$ cells/ml. Under these conditions, viability of the cells was more than 95%, as assessed by trypan blue exclusion test previously described in 2.4, and doubling time of 40 hours. Spontaneous differentiation determined by adherence, morphology and Gimsa stain was never more than 1%. All procedures were carried out under aseptic conditions in a laminar air flow cabinet (supplied by Flow Labs Ltd).

After thirteen passages the cell culture was replaced by a new batch to eliminate any increase in spontaneous differentiation. Waste culture and contaminated plastic equipment were disposed off by decontamination with hypochloric solution and autoclaving.

5.2.2 Preliminary Tests for Cell Differentiation

For preliminary cell differentiation studies 100µl of TPA (supplied by Sigma, UK), phorbol and resiniferonol-9,12,13-orthophenylacetate in supplemented medium were added to 100µl of HL-60 cell culture to give final cell density of $2x10^5$ cells/ml equivalent to $4x10^4$ cells/200µl per well. Two fold serial dilutions were carried out along the breadth of two adjacent columns of the micro titer-plate to obtain fourteen concentrations in the range of 10µM to $3x10^{-4}$ µM. The twelfth column was left as a drug-free control. Gentle pipetting of the cell culture in the micro titer plate was carried out to ensure good mixing and uniform distribution of cell numbers in each well. TPA plates were covered with lids and incubated

for twenty four, forty eight, seventy and ninety six hours in an incubating chamber. Plates containing phorbol and resiniferonol-9,13,14-orthophenylacetate were incubated for forty eight hours. After each incubation period cell cultures were examined under the microscope for macrophage-like differentiation.

Cell suspension was counted in a haemocytometer, subsequently decanted off and wells were gently and carefully washed twice with an already prepared one strength of phosphate buffer saline (1xPBS), (supplied by Gibco, UK). 1xPBS was prepared from 10xPBS (supplied by Gibco, UK) by diluting with deionized water then warmed at 37°C in a water bath prior to use. Warming the buffer prevents detaching the adherent cells from the bottom of the wells.

Adherent cells were trypsinized with 50µl EDTA/Trypsin (supplied by Gibco, UK). The plate was shaken for five minutes then examined under the microscope for resuspension. It was also incubated for ten minutes after which cells were completely detached from the well's bottom. Cells were resuspended in 150µl RPMI-1640 medium supplemented with 20% FCS to stop the enzymatic activity of trypsin, final volume 200µl/well. The cells were left for five minutes at room temperature before counting in a haemocytometer. Gimsa stain (supplied by Sigma, UK) was used to confirm macrophage pattern in trypsinized cells. Refer to results in Table 5.1, pp. 183 and Table 5.2, pp. 184.

5.2.3 Quantitation of Dye Binding and Development of the Staining Procedure

The relationship between dye binding by cell monolayers in micro-titer system and the number of cells was established by comparing the optical density of the released dye with the number of cells. This was achieved by setting up two identical micro-titer plates containing serial dilutions of cell suspension treated with 20nM TPA. The first row-A of each plate was used as negative control. The first half of row-A, which consisted of six wells, was seeded with TPA-free cell suspension mixed with 5µl of acetone. The second half contained 200µl of supplemented RPMI-1640 medium. Suspension of the HL-60 cell line was counted in a haemocytometer to give $6x10^5$ cells/ ml of culture medium. 200µl aliquots

were seeded in well B-1. Subsequent wells, except 2H, were filled with 100µl supplemented RPMI-1640 medium. Two fold serial dilutions were performed along the breadth of the micro-titer plate to obtain seven cell concentrations. 100µl of supplemented RPMI-1640 medium containing 20 nM TPA was added to each well to give final cell concentrations of $3x10^5$ cells/ml in 1B and 0.00036 $x10^4$ cells/ml in 2H, which are equivalent to $6x10^4$ and $0.000732x10^4$ cells/200µl well

respectively. Considerable care was exercised in maintaining good mixing in order to ensure uniform cell numbers in each well. The procedure was carried out in triplicate . Column 12 was left as a blank control. The two plates were covered with lids and incubated in 5% CO_2-air at 37°C chamber for twenty four hours. After the incubation period, macrophage like differentiation of the cell culture was assessed by microscopic examination to identify adherence, appearance of pseudopodia of the nucleus, cell clumping and adherence to the base of the wells of the micro titer plates.

Both cell suspension and resuspended adherent cells from plate one were counted in a haemocytometer. Cell suspension in plate two was decanted off, wells were gently washed twice with 300µl 1xPBS then left to dry. Adherent cells were fixed with 100µl methanol and the plate was again left to dry. Stock solution of eosin B di-sodium salt (supplied by Sigma) was prepared in distilled water. Final concentrations of the staining solutions were 1% and 0.5%. Fixed cells were stained with 100µl of 0.5% aqueous eosin solution for fifteen minutes. The stain was decanted off and the excess was washed off four times in 1% acetic acid bath. After washing, the stained plate was inverted and left to dry overnight at room temperature. The dye was released from the stained cells by adding 200µl of 5mM sodium hydroxide solution.

After thirty minutes absorbencies (optical density OD) of the dye solutions in each well were read at 490 nm by an MR700 micro plate reader (supplied by Dynatech Labs. Inc.) against air as a blank. The same procedure was repeated using 1% eosin solution. Staining procedure was carried out using 0.5% and 1% eosin solution. In all procedures, 100µl of the

staining solutions were added to the fixed cells in each well of the 96 micro titer plate then left to develop for 30 or 60 minutes (refer to Table 5.3, pp. 185).

To optimize the procedure, plates incubated for forty eight, seventy two and ninety six hours were counted, stained and their optical density was determined at 450, 490 and 550 nm. The number of adherent cells counted were plotted against optical density (Table 5.4, pp. 186)

5.2.4 Assay Procedure for Potential HL-60 cell Differentiation

The phorbol esters sapintoxin-A, sapintoxin-D, sapintoxin-E, 12-O-(2-methylamino-benzoyl)-4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate, Thymelaeatoxin-A, mezerein and resiniferonol-9,13,14-orthophenylacetate were isolated as described in Chapter 3. Phorbol, phorbol-12,13-diacetate, phorbol-12,13,20-triacetate were purified as described in Chapter 6. Test samples were dissolved in acetone to give final concentration of 10μM and kept at -20°C. Working solutions were freshly prepared by diluting the stock solutions (10μM) with a supplemented RPMI-1640 medium.

Cell suspension was disposed of then wells were washed twice with 300μl 1xPBS. Plates were then left to dry at room temperature. Adherent cells were fixed with 200μl methanol. After leaving at room temperature for two hours, or until methanol has evaporated, plates were stained with 0.5% aqueous eosin solution and left for one hour. Stain was discarded of by inverting the plates. Excess eosin was washed off four times in 1% acetic acid bath. After washing, stained plates were inverted and left to dry at room temperature.

Dye was extracted from the stained adherent cells by addition of 200μl of 5mM sodium hydroxide to each well and was allowed to digest the stain for 30 minutes. The optical density of the released dye solution in each well was measured at 490 nm by MR7009 micro- titer plate reader after blanking against cell free wells.

For inhibition of TPA-induced differentiation test samples were diluted in RPMI-1640 medium containing 20nM TPA. Serial dilutions and staining procedure was carried out

according to the method described above. All procedures were repeated three times and conducted in triplicate wells.

5.2.5 Calculation of Percentage Cell Differentiation

For counted plates, percentage of adherent HL-60 cells was calculated according to the following equation:

$$\% \text{ Differentiated cells} = \frac{\text{Adherent cell numbers}}{\text{Total cell density}} \times 100 \quad \text{...Equation 5.1}$$

For stained plates optical density readings were converted into percentage of differentiated cells using equation 5.2.

$$\% \text{ Differentiated cells} = 100 \times \log \frac{\text{OD of wells containing drug treated HL-60 cell}}{\text{OD of drug free cells + OD of cell free wells}} \quad \text{... Equation 5.2}$$

5.3 Results and Discussion

The objective was to establish a method to evaluate the potential of selected tigliane and daphnane derivatives to induce differentiation of HL-60 cells in a micro titer system. This investigation was based on the quantitation of released dye associated with stained differentiated cells and measuring the absorbance at an appropriate wavelength.

Over the initial four day study, control cells treated with acetone as a vehicle exhibited a steady growth. No change in the adherent cell population or morphology were observed (Table 5.1, pp. 183). The cell density increased five fold by day four. In contrast, cultures treated with daphnane or tigliane derivatives showed no increase in cell density over the four day study (Table 5.1, pp. 183). In addition, cells became increasingly adherent to the well bottoms of the micro- titer plates. Adherent cells were clumped in groups, increased in size when compared to the control cells and exhibited macrophage-like morphology. These changes were used as indicators of differentiation. Previous research indicated that HL-60 cell line was unable to progress beyond the G1 phase of the cell cycle in response to TPA treatment (Rovera *et al* 1980).

Counting cell cultures incubated over the range of four days revealed that the

doubling time was approximately forty hours. During the forty eight hour incubation period the diterpene-treated cultures were exposed to one doubling cycle. The effect of incubation period on cell differentiation induced by TPA is summarized in Table 5.1, pp. 183. After seventy two hours incubation period cells adhered quite strongly to the well bottoms. It was not possible to detach them by vigorous pipetting of the cell suspension. This effect was even stronger when cells were incubated for ninety six hours.

Eosin is a weak acidic protein dye (James and Tas 1984) producing pink-red colour. The micro-titer plate mini reader is essentially a colorimeter through which a single beam passes through a rapidly rotating filter wheel. Figure 5.1, pp. 187 shows that the correlation between the number of adherent cells per well and the optical density of the stained cells measured at 490 nm is a linear relationship. The correlation coefficient was found to be 0.9 indicating good correlation. However, at cell numbers above $2.5x10^5$ cells/ml the correlation began to drift slightly. The size of the test culture, $2x10^5$ cell/ml correlated well with the linear relationship. Preliminary experiments indicated that the relationship between cell numbers and the optical density of eosin stained cells correlated more closely when measured at 490 nm. Optical density (OD) measurements at 450 nm were higher then those carried out at 490 nm. However, OD values measured at 550 nm were lower than those measured at 450 and 490 nm, (Table 5.4, pp. 186).

For cell free wells, staining with 1% eosin solution produced high optical density readings which was significantly different from unstained blank wells resulting in high OD values. Repeated washing with 1% acetic acid failed to remove the excess stain. In contrast, excess 0.5% eosin solution was easily washed off with four washes. Experiments carried out, to determine the concentration of eosin solution and the development time of staining procedure, indicated that the relationship between the concentration of the dye and absorbance, recorded as optical density, was found to be directly proportional (Table 5.3, pp. 185). However, plates stained with 1% eosin solution produced high OD values compared to those stained with 0.5%. Results obtained from the latter concentration correlated more closely with the data obtained from cell counting. Absorbencies of cell free wells stained

with 1% eosin solution were comparable to those containing diterpene-free HL-60 cells. The results of different staining times for 0.5% and 1% eosin solution are listed in Table 5.3, pp. 185. The dye-cell binding was insignificant after fifteen minutes over the concentration range examined. Staining the micro-titer plate for fifteen minutes was insufficient for binding with cells' protein and was easily washed off with 1% acetic acid solution.

The 200µl aliquots of 5mM sodium hydroxide solution was able to extract the protein-bound dye over thirty minutes after which time the absorbance readings of the wells became stable. Absorbance readings recorded at one, two and twenty four hours later failed to show any increase in the amount of dye released. Furthermore, the dye appeared to be completely extracted with the first aliquot of sodium hydroxide solution. Replacement of the first dye extraction with a new aliquot of the eluting solvent indicated that no measurable dye remained bound to the fixed cells.

TPA was the most potent diterpene ester tested. In forty eight hours, at a concentration of 2.4 nM this compound induced 37% of the cell population to differentiate which increased to 67% in day four. Maximum differentiation of 98-90% was observed at concentrations of 10-1 µM at forty eight and ninety six hours. At 0.3nM TPA induced slow differentiation detected only in day three and reaching 55% in day four (Table 5.1, pp. 183).

Mezerein and thymelaeatoxin-A, 12-hydroxydaphentoxin derivatives, were as potent as TPA in inducing differentiation of HL-60 cells (Table 5.5, pp. 188 & Figure 5.2, pp. 189). This indicated that the cyclopropane ring-D in tiglianes is not necessary for differentiating HL-60 cells. However, resiniferonol-9,13,14-orthophenylacetate, induced a maximum differentiation of 40% at 10µM (Table 5.5, pp. 188, Figure 5.2, pp. 189). The difference in the relative potencies of the tested daphnane derivatives suggested that the presence of an ester moiety at C12 is necessary for full efficiency. At concentrations of 20 nM and greater both sapintoxin-A and sapintoxin-D induced 30% of the HL-60 cells to differentiate with a maximum effect achieved at 10µM. In contrast, previous studies of the differentiating effect of 12-deoxyphorbol-13-phenylacetate, a 12-deoxy tigliane derivative, reported maximum effect at doses of 100nM and greater (Ryves *et al* 1994). These observations indicate that

although the presence of an ester moitey at C-12 increases the differentiation potency, it is not an absolute requirement for this activity.

The phorbol, in addition to its diacetate and triacetate derivatives, 4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate and sapintoxin-E were inactive in this biological system at all tested concentrations up to 10μM. The phorbol did not exhibit inhibitory effect on cell proliferation. With the exception of phorbol, the lack of activity of these compounds could be attributed to the presence of an ester or methyl group at C-20 suggesting that the primary hydroxy at C-20 is essential for differentiating activity.

Microscopic examination indicated that the tigliane and daphnane diterpene esters, under investigation, did not inhibit TPA-induced differentiation. At 10,5 and 2.5 μM/ml daphnane diterpenes exhibited cytotoxic effects confirmed by the trypan blue exclusion test.

The correlation between cell counts and their optical densities indicated the validity of the assay as a screen for potential differentiating agents. Although the percentage of adherence obtained from counting the cells and measuring absorbencies are reproducible, the effect of tested compounds on total cell density could not be predicted.

The microdilution assay described above provided a rapid and economic method to test a range of diterpenes for their ability to induce HL-60 cell differentiation. This *in vitro* test is very practical for screening a large number of compounds at micro molar concentrations.

Table 5.1 Time Course of HL-60 Cell Differentiation Induced by TPA

Conc. uM	0 hours	24 hours			48 hours			72 hours			96 hours		
	Total	Adherent	Total	% Differentiated	Adherent	Total	% Differentiated	Adherent	Total	% Differentiated	Adherent	Total	% Differentiated
0	27	2	49	4	4	76	5	4	107	4	10	140	7
10	25	9	23	40	28	29	98	53	63	83	76	78	98
5	26	7	26	28	26	27	98	50	57	86	79	81	98
2.5	27	6	23	25	28	29	97	39	53	73	82	85	97
1.25	25	5	25	23	28	31	90	40	57	69	69	75	92
0.63	26	3	24	14	28	32	86	37	54	68	71	79	91
0.32	27	3	24	13	31	39	79	40	62	64	73	82	89
0.16	23	3	25	14	24	38	63	39	56	69	63	74	86
0.079	25	9	48	18	21	37	57	29	54	54	55	65	84
0.039	28	8	43	20	29	59	50	24	51	46	61	75	81
0.0197	26	6	38	17	32	66	49	20	50	46	57	73	79
0.0098	23	4	36	11	28	61	45	21	54	40	54	68	79
0.0049	24	4	40	10	24	61	40	23	56	41	64	88	73
0.0025	25	2	33	7	23	62	37	21	53	39	63	95	67
0.0012	27	3	53	7	8	76	11	21	66	35	45	81	56
0.0006	28	2	43	5	3	66	5	19	71	27	53	92	58
0.0003	29	2	61	4	3	65	5	16	75	22	48	89	55

Data represent the mean of three experiments carried out in triplicates
Cells were counted in a haemocytometer (10000 cells/ml)
Total cell density calculated by adding the adherent and suspension cell population
% Differentiated cells calculated by dividing adherent cell population by total cell density

Table 5.2 The Effect of Diterpene Esters on HL-60 Cells

Controlled Cells			Diterpene Esters					
		Conc. uM	Phorbol		TPA		ROPA	
Total	% Differentiated		Total*	% differentiated	Total*	% Differentiated	Total*	% Differentiated
126	4.76	10.0000	124	5	29	98	54	40
132	4.5	5.0000	0	8	27	98	55	28
		2.5000	109	8	29	97	53	26
		1.2500	0	4	31	90	58	23
		0.6300	0	5	32	86	53	19
		0.3100	0	5	39	79	63	16
		0.1600	0	5	38	63	55	10
		0.0780	0	3	37	57	97	7
		0.0390	0	4	59	50	89	6
		0.0197	0	8	66	49	87	6
		0.0099	0	8	61	45	90	7
		0.0048	0	4	61	40	100	5
		0.0024	0	6	62	37	98	4
		0.0012	0	5	76	11	92	5
		0.0006	0	4	66	5	97	6
		0.0003	0	8	65	5	95	5

* Total cell density following 48 hours incubation period calculated by adding suspended and adherent populations quoted (x 10000 cells/ml)
% Differentiated cells were calculated by dividing the adherent population by the total cell density.
Data represent the mean of three experiments carried out in triplicate
TAP = 12-O-tetradecanoyl phorbol-13 acetate
ROPA = resiniferonol-9,13,14-orthophenyl acetate

Table 5.3 The Effect of Dye Concentration on the Optical Density of Stained HL-60 Cells

	1% Eosin solution				0.5% Eosin solution		
No of cells **	OD* of cells stained for			No of cells **	OD* of cells stained for		
	1 Hour	0.5 Hour	0.25 Hour		1 Hour	0.5 Hour	0.25 Hour
60	1.9	0.95	0.52	65	1.5	0.62	0.35
52	0.98	0.89	0.32	59	1	0.55	0.3
38	0.98	0.82	0.3	42	0.91	0.54	0.32
20	0.89	0.75	0.28	26	0.62	0.4	0.29
15	0.79	0.62	0.25	13	0.51	0.32	0.26
10	0.75	0.65	0.2	10	0.5	0.26	0.1
5	0.6	0.12	0.18	4	0.15	0.13	0.12
3	0.5	0.11	0.1	4	0.12	0.08	0.11
0	0.3	0.05	0.01	0	0	0	0

Data represent the mean of three experiments carried out in triplicate

Microtiter plates were incubated for 48 hours

** Cells were counted in a haemocytometer, cell population quoted (X10000cells/ml)

* Optical density was measured at 490 nm

Table 5.4 Correlation of Optical Density and Number of Stained HL-60 Cells

No of stained cells*	OD at			% Differentiation		
	450nm	490nm	550nm	450 nm	490nm	550nm
35	1.92	1.62	0.89	67	97	83
20	1.50	1.30	0.70	56	88	73
16	1.02	0.87	0.6	39	70	66
12	0.78	0.64	0.35	27	57	43
8	0.63	0.54	0.30	18	50	36
4	0.41	0.17	0.13	0	0	0
3	0.30	0.12	0.09	0	0	0
0	0.01	0.00	0.00	0	0	0

* Adherent cell population (X10000cell/ml)
Data represent the mean of 4 experiments carried out in triplicates
Adherent cells were stained with 0.5% eosin solution for 1 hour
% Differentiated cells = $100 \times \log \frac{\text{OD of diterpene treated cells}}{\text{OD of diterpene free cells}}$

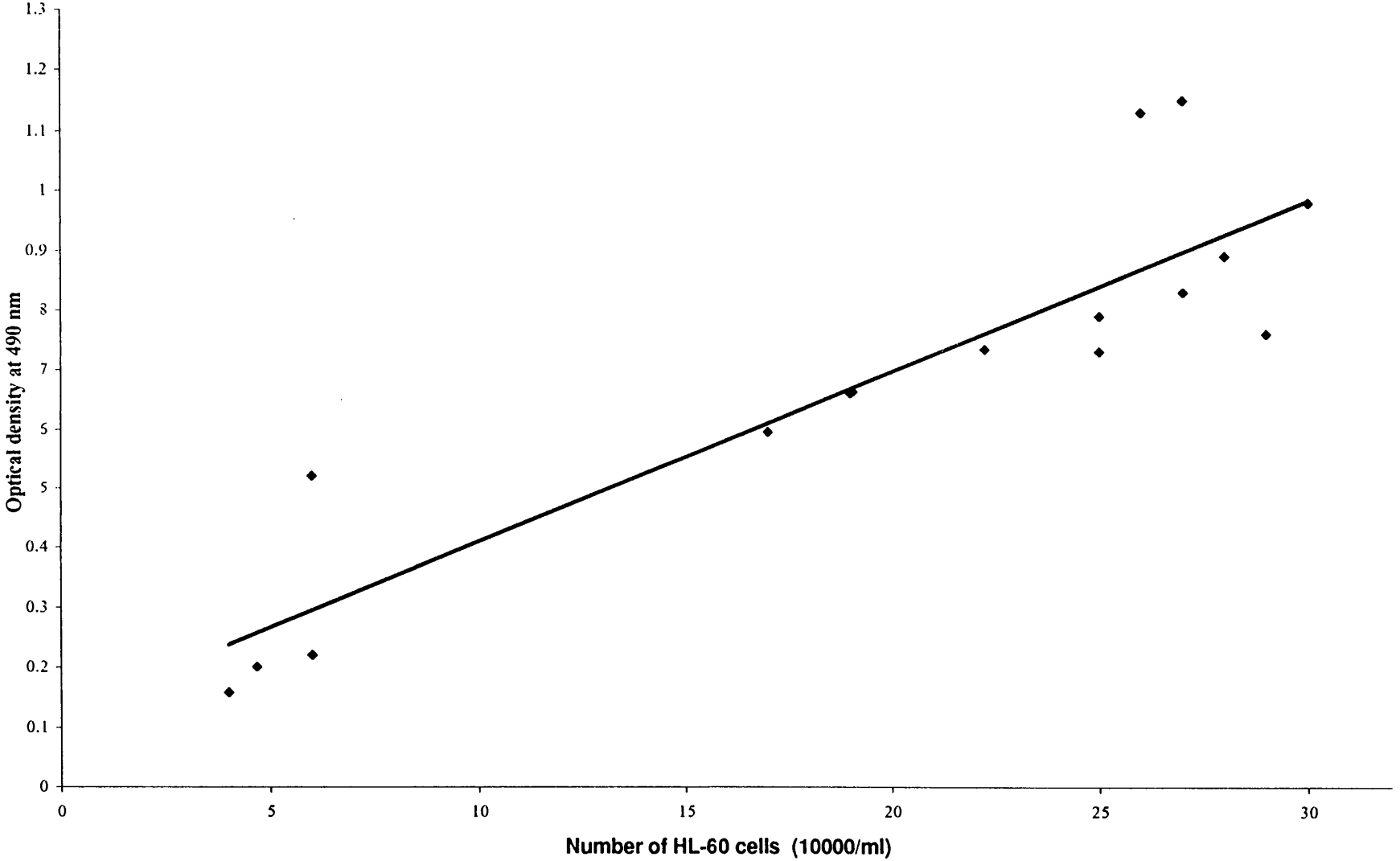


Figure 5.1 Correlation of Optical Density and Number of HL-60 Cells

Line best fit determined by linear regression
Standard deviations were in the range of 0.4-0.9
Correlation coefficient was 0.9

Conc. uM	% Differentiated cells										
	TPA*	SAP-A*	SAP-D*	SAP-E*	SAP-AC*	TH-A*	MEZ*	*ROPA	PH*	PHDA*	PHTA*
10	93	97	85	10	6	98	95	42	11	10	11
5	93	93	84	9	6	95	92	35	11	9	8
2.5	90	89	73	6	4	89	87	30	9	6	7
1.25	83	80	70	5	1	80	85	28	6	6	9
0.63	78	73	65	1	3	76	79	15	5	4	5
0.31	72	66	59	0.08	0.06	68	73	9	4	3	5
0.15	70	62	57	0.08	0.08	55	67	5	4	1	4
0.078	66	54	50	0.09	0.02	52	60	4	4	0	2
0.04	64	48	42	0.05	0	49	56	4	2	0	0
0.02	60	32	31	0.01	0	45	51	0	1	0	0
0.0098	49	20	12	0	0.01	32	46	0	1	0	0
0.0049	36	12	5	0	0	18	30	0.02	0	0	0
0.0024	22	5	5	0	0	12	22	0.03	0	0	0
0.0012	16	0	0	0.01	0	10	18	0	1	0	0
0.0006	7	0	0.01	0	0.01	8	10	0	0	0	0
0.0003	5	0	0	0.02	0	4	7	0	0.04	0	0

The data represent the mean of three experiments carried out in triplicates
Micro-titer plates were incubated for 48 hours, adherent cells were stained with 0.5% eosin for 1 hour
Optical density of the released dye was measured at 490 nm
*TPA= 12-O-tetradecanoylphorbol-13-acetate PH=Phorbol PHDA = Phorbol-12,20-diacetate PTA = Phorbol-12,13,20-triacetate
SAP-A = Sapintoxin-A, SAP-D = Sapintoxin-D, SAP-E = Sapintoxin-E, SAP-AC = 4-Alpha-sapintoxin-B-5,20-diacetate
THY-A = Thymelaeatoxin-A, ROPA = Resiniferonol-9,13,14-orthophenyl acetate, MEZ = Mezerein
% Differentiation = 100 X log $\frac{\text{OD of diterpene treated cells}}{\text{OD of diterpene free cells}}$

Table 5.5 Induction of HL-60 Cell Differentiation by Diterpene Esters

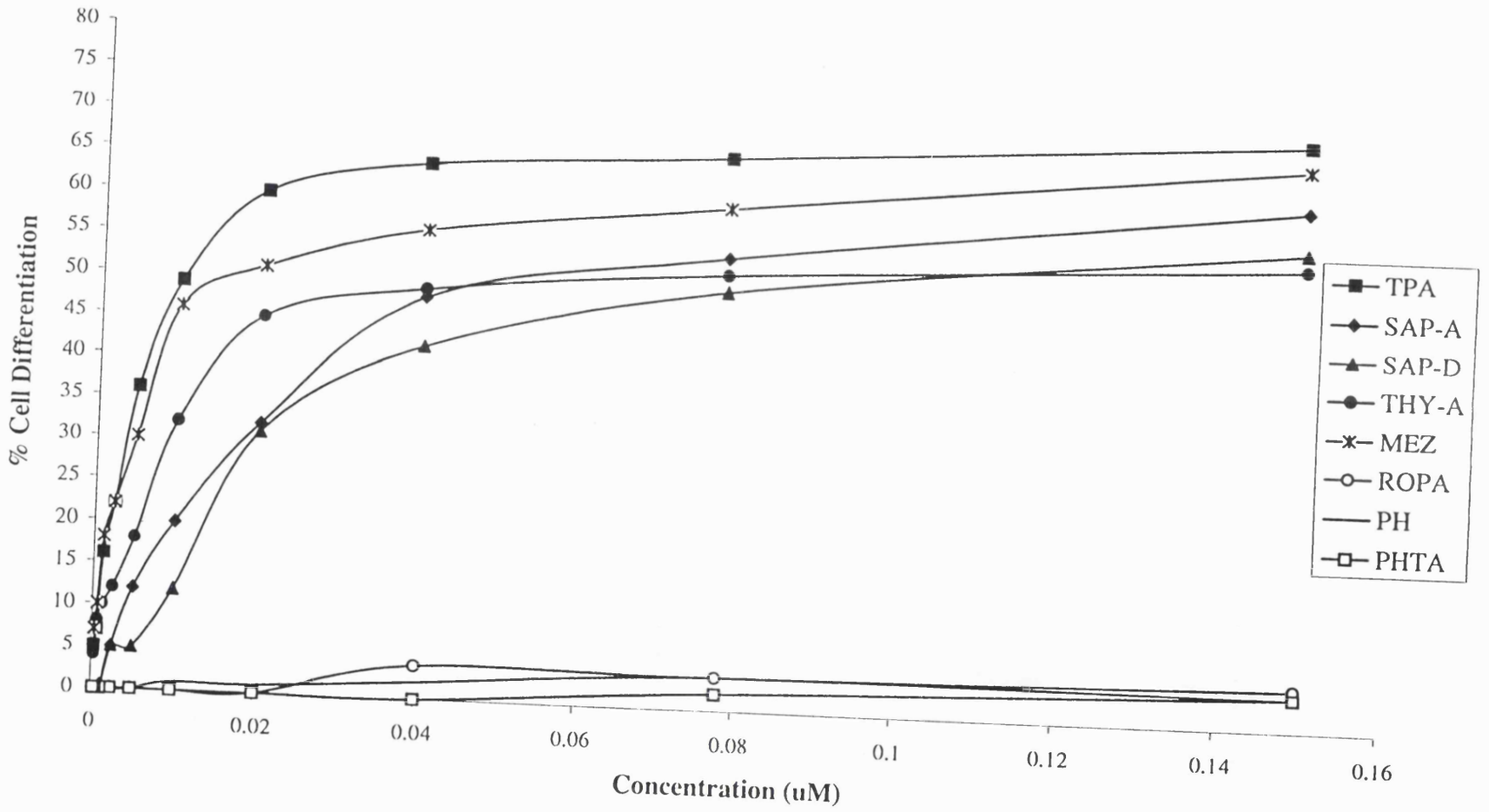


Figure 5.2 Induction of HL-60 Cell Differentiation by Diterpene Esters

For chemical names refer to Table 5.5, page 188

CHAPTER SIX

Analysis and Purification of Tigliane and Daphnane Derivatives Using Normal Phase HPLC and Adsorption Thin-Layer Chromatography

6.1 Introduction

The irritant and tumour-promoting constituents of the families Euphorbiaceae and Thymelaeaceae, phorbol esters, have been widely used as probes in biochemical studies. Although these compounds are active at micro molar concentrations they tend to exist in a very complex mixtures.

Biologically guided fractionation accompanied by thin-layer (TLC) and open column chromatography are widely used to detect and purify phorbol esters. Both TLC and open column chromatography are labourious, inadequate for complete separation and require multiple solvent systems to resolve many potential phorbol esters. In 1973, Evans and Kinghorn described a micro-method for identification of ingenol and phorbol acetates based on colour reaction on TLC chromatograms. Other methods used to analyse and detect the polyfunctional diterpene esters include gas-liquid chromatography (Kinghorn and Evans 1974) and Craig liquid-liquid distribution (Hecker and Schmidt 1974).

Chromatographic methods used to purify phorbol esters include dry column chromatography (Ocken 1969), rotational counter current chromatography (RLCC) (Pieters and Vlietinick 1986), droplet counter current (DCC) (Marshal and Kinghorn 1981), reversed phase high pressure liquid chromatography (RP-HPLC) (Rao *et al* 1974, Bauer *et al* 1983) and O'Keffe liquid-liquid distribution (Hecker and Schmidt 1974).

The techniques are either time consuming or involve the use of aqueous solvent systems which affect the stability of the sensitive diterpene esters. In addition, toxic effects of these compounds impose health hazards to scientists working with them. Therefore it was necessary to develop a rapid, sensitive, reproducible, highly resolved and safe method that could be used to analyse and purify phorbol esters. In order to meet these requirements HPLC and thin-layer chromatography were used to analyse seventeen

daphnane and tigliane derivatives. The analytical data was used to purify microgrammes quantities of diterpene esters. Their identities were revealed by comparing their R_f and R_t values to the standards and were later confirmed by data from NMR and mass spectra.

6.2 Materials and Methods

6.2.1 Preparation of the Test Samples

Samples under investigation were either purified from plant extracts, purchased or semi-synthesized. TPA, phorbol-12,13,20-tributyrate, phorbol-12,20-dibenzyl-13-acetate and phorbol-13,20-diacetate were purchased from Sigma, UK. Resiniferatoxin, 12-deoxyphorbol-13-phenylacetate and 12-deoxyphorbol-13-phenylacetate-20-acetate were isolated from *Euphorbia poissonii*. Resiniferonol-9,13,14-orthophenyl acetate and thymelaeatoxin-A were isolated from *Thymelaea hirsuta* (refer to Section 3.1). Sapintoxin-A, sapintoxin-D and sapintoxin-E were isolated from *Sapium indicum* (refer to Section 3.3.1). Sapintoxin-C was isolated from *Sapium indicum* (Brooks 1989) while the semi-synthetic 4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate was isolated from *Sapium insigne* (refer to Section 3.3.2). Phorbol was isolated from Croton oil. Phorbol-12,13,20-triacetate was prepared by incubating 5mg of phorbol with 6ml acetic anhydride:pyridine mixed at a ratio of 1:2 for 12 hours at room temperature. The reaction mixture was purified on the semi-preparative HPLC column.

Samples were dissolved in HPLC-grade chloroform to give final concentration of 100 mg/ml. All samples were filtered through 0.25 µm filter (supplied by Millipore, UK) to remove any silica gel particles.

6.2.2 Thin-layer Chromatography

For analytical TLC, 0.25 mm aluminium-backed silica gel 60 F_{254} plates (Merck, supplied by BDH UK) were developed at temperateure between 20-25°C in a mobile phase consisting of a mixture of chloroform:methanol mixed at a ratio of 99.2:0.8. Developed plates were allowed to dry at room temperature after which spots were visualized under the ultraviolet light. Plates were sprayed with 60% sulphuric acid and heated at 110°C for 15 minutes. The spots were visualized in day light and under both 254 nm and 365 nm ultraviolet lamp.

6.2.3 Analytical and Preparative HPLC

The HPLC system used consisted of two pumps by, Altex model 110A, an Altex controller (supplied by Altex, USA) and Waters 991 photodiode-array detector (supplied by Millipore UK). The analytical HPLC was performed on an 250 mm long Apex Prepsil silica column, 4.6 mm in internal diameter and particle size of 5µm (supplied by Jones, UK). The semi-preparative HPLC column was an 250 mm long Apex Prepsil 10mm in internal diameter and particle size of 8 µm (supplied by Jones, UK). Samples were injected manually using a micro-syringe into a 20µl loop.

Solvents were Super Purity HPLC-grade (supplied by Romil Chemicals Ltd, UK) degassed prior to use by sonicating (sonicator supplied by Dawe) each litre for twenty minutes. Samples were eluted using a mixture of chlorofom:methanol at a ratio of 99.2:0.8 and at a flow rate of 1ml/min. Absorbencies were detected between 220 and 400 nm. HPLC procedures were carried out at temperature between 20-25°C and elutes were collected manually. Data was processed using PDA software version 6.22. Chromatograms were printed using Waters 5200 printer plotter (supplied by Waters Association).

6.3 Results and Discussion

Analysis of pure daphnane and tigliane derivatives on analytical TLC silica gel plates revealed that each group has a characteristic colour reaction when the plate was sprayed with 60% sulphuric acid and heated for 15 minutes, for R_f values and visualization refer to Table 6.1, pp 196. The analysed tigliane derivatives fall into three groups: the phorbol, the 12-deoxy phorbol and the 4-deoxy phorbol derivatives (refer to Figure 6.1.a., pp. 205 for chemical structures).

Examining the TLC chromatograms in day light revealed that the analysed tigliane derivatives produced orange-brown colour however, each group had characteristic fluorescence under the 356nm ultraviolet lamp. The most noticeable was the blue ultraviolet fluorescence produced by the nitrogen-containing phorbols known as sapintoxins. This fluorescence was clearly visible under the ultraviolet light even before spraying with the reagent. Daphnanes produced black spots on TLC chromatograms when

viewed in day light. Under the 365nm ultraviolet light 12-hydroxy daphentoxin derivatives (mezerein and thymelaeatoxin-A) (Figure 6.1.b, pp. 206) produced bright yellow fluorescence while the resiniferonol derivatives (resiniferatoxin and resiniferonol-9,13,14-orthophenylacetate) produced bright orange fluorescence (Table 6.1, pp. 196).

The migration of the analysed diterpene esters on TLC plates appeared to be a function of the ester and the oxygen moieties at carbons 4, 12, 13 and 20. Compounds bearing an acetyl or a methyl group at C-20 have higher R_f values than those bearing a primary hydroxy group. The presence of a hydroxy group on C-4 (as in the case of sapintoxin-D and phorbol) and the cis-link between ring A and ring B (as in the case of 4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate) decreases the migration distance and hence increases the retention time of the phorbol derivatives. Increasing the length of the unsaturated alkene at C12 decreases the migration distance of both tiglianes and daphnanes, demonstrated by the R_f and R_t values of thymelaeatoxin-A, mezerein and resiniferonol-9,13,14-orthophenylacetate, resiniferatoxin, phorbol tributyrate and phorbol-12,20-dibenzyl-13-acetate. These observations indicated that the tertiary hydroxy group at C-4 has a role in the adsorption of these diterpenes on silica gel. For example the R_f value of the phorbol derivative sapintoxin-D is lower than that of the 4-deoxy phorbol derivative sapintoxin-A. The highly oxygenated phorbol was strongly adsorbed on silica gel and consequently lower R_f and high R_t values were observed (Table 6.1, pp. 196).

Optimum R_f values less than or equal to 0.3 for HPLC analysis (Hostettman, Hostettman and Marston 1986) were achieved with chloroform:methanol as eluents mixed at a ratio of 99.8:0.2. The purity of the compounds was evaluated on analytical HPLC column coupled with photodiode array (PDA) detector. Although this detector shares many elements with a conventional UV/VIS detector, the essential difference is that it can record the entire spectral range (190-800 nm) during analysis, thus monitoring the chromatogram at selected wavelengths whilst recording the spectra of the eluates simultaneously.

The diode-array detectors were coupled to a computer to analyse the large amount

of data generated during the HPLC separation. Post-run analysis of the chromatogram provided extremely sophisticated methods for visualizing the data. The spectrum index option provided information on the composition of the sample under investigation and the spectra of each component (Figure 6.2.A, pp.207, Figure 6.2 E, pp. 211) which enabled the detection of contaminants. The contour plot (refer to Figure 6.2.C, pp. 209) and the three-dimensional topographical plot (Figure 6.2.B, pp. 208) provided information on the purity of each component of the analysed sample. The library feature enabled comparison of experimental spectra with standards. Contaminants were recognised and quantified by comparing peak areas hence quantification of purity. Integration and quantification of elution peaks can then be achieved, once peak area has been calibrated with standard amount of the references. Increasing the sensitivity of the detector to 0.001 AUFS enabled the detection of nanogrammes of the test samples, which was not feasible on TLC, despite the difficulties from the signal to noise ratios. The method was scaled up to a semi-preparative column to analyse and detect irritant diterpene esters (refer to Table 6.1, pp. 196 for analytical data).

In addition to the characteristic UV blue fluorescence produced by nitrogen-containing phorbol derivatives on TLC the HPLC spectrum exhibited a unique UV profile, showing two UV maxima on chromatogram analysis and spectrum index (Figure 6.2 D, pp. 210 & Figure E, pp. 211). Investigation of the purity of 12-deoxy phorbol-13-phenyl acetate (DOPP) and 12-deoxy phorbol -13-phenyl acetate-20-acetate (DOPPA), stored at -20°C for five years, suggested that the latter compound had partially disintegrated into the de-acetylated derivative. Both compounds were purified on the semi-preparative column. Proton-NMR and mass spectra were used to confirm the identity of the isolates (Table 6.2, pp. 197 & Figure 6.3, pp. 198).

Data generated by TLC and HPLC analysis was used to isolate compounds from semi-pure biologically active fractions. Semi-preparative HPLC was used to purify 400µg resiniferatoxin from 800µg impure fraction. The identity of the isolate was confirmed by running FAB and EI mass spectra (Table 6.4, pp. 199).

The semi-preparative HPLC column was used to purify diterpenes from

hydrolysed croton oil. The isolate produced brown-orange colour on TLC, indicating the presence of a phorbol derivative. The NMR and mass spectral data (Table 6.5, pp. 199) identified the isolate to be phorbol. Data from ^{1}H-NMR and mass spectra confirmed that its acetylation reaction product was phorbol-12,13,20-triacetate (Table 6.6, pp. 202). TPA was purified from solutions stored under nitrogen at -20°C for more than 2 years. The identity of the isolate was confirmed from FABMS and ^{1}H-NMR spectral data (Table 6.7, pp. 203).

Optimum separation of the diterpene esters was achieved by injecting 1- 2 mg of the sample into the semi-preparative column. Application of more than 2mg resulted in poor resolution. Analytical R_t values were reproduced on the semi-preparative column using a flow rate of 4ml/min. However, the optimum semi-preparative separation was achieved at a flow rate of 1ml/min; higher rates resulted in tailing of the peaks' down slopes and overlapping of diterpenes with polar contaminants.

The use of HPLC in analysing and purifying the diterpene esters proved to be reproducible, rapid and a highly sensitive chromatographic technique. The high yield and resolution of the analytical and the semi-preparative HPLC compared to other chromatographic methods suggested the use of a similar method in biochemical, pharmacological and toxicological studies (Ryves *et al* 1994). Colour reaction on TLC plates could be used as a guide for classification of the diterpene under investigation. However, the complexity of different systems containing diterpene esters whether plant extracts or biological extracts suggests that the use of a single purification or analytical method is not possible. Additional advantages for using the HPLC include reduction of toxicity, usually associated with the exposure of the chemists to the samples, and minimizing the decomposition of the sensitive compounds during purification procedures.

Although the cost of the high performance liquid chromatography and the diode-array detector could be considered as a disadvantage, their use greatly simplified the analysis and the purification of the tigliane and daphnane derivatives. It is more likely that this method described here would be increasingly used in the future to isolate new members of the irritant diterpene esters.

PE	TLC			HPLC		
	Day Light	365nm UV	R_f	Analytical R_t (min)	Semi-prep R_t (min)	UV Maxima (nm)
TPA	orange-brown	yellow	0.26	8.98	16.18	240
DOPP	orange-brown	orange	0.05	4.18	19.38	242
DOPPA	orange-brown	orange	0.03	3.63	13.47	243
SAP A	orange-brown	blue	0.39	4.17	11.15	255 & 360
SAP C	orange-brown	blue	0.17	4.22	11.39	255 & 355
SAP D	orange-brown	blue	0.11	8.59	14.71	250 &355
SAP E	orange-brown	blue	0.302	3.58	11.39	254 & 360
SAP-AC	orange-brown	blue	0.06	3.49	12.91	247 & 360
PHORBOL	orange-brown	orange	0 (0.92*)	8.19	14.93	242
PTA	orange-brown	orange	0.31	3.64	12.15	243
PDA	orange-brown	orange	0.074	3.95	12.60	245
PTB	orange-brown	orange	0.49	3.46	13.95	248
PDBA	orange-brown	orange	0.41	3.64	13.65	242
MEZ	grey	bright yellow	0.26	4.00	22.62	240 sh &365
THYA	grey	bright yellow	0.43	5.84	13.44	283
ROPA	grey	bright orange	0.16	5.57	14.73	244
RX	grey	bright orange	0.47	9.23	18.63	242 & 280sh

R_f values and TLC colours after spraying with 60% H_2SO_4& heating at 110°C for 15 minutes. Data represent the mean of 4 experiments. Standard deviations range between 0.2-.92.
Normal phase HPLC, flow rate 1ml/min. Mobile phase : $CHCl_3$:CH_3OH (99.8:0.2) (*99:1) .
Tigliane derivatives, Phorbol type (TPA, PH, PDA, PTA, PDBA, PTB & Sap-D), 12-deoxyphorbol type (DOPP, DOPPA), 4-deoxyphorbol type (Sap-A, C, E & SAP-AC).
Daphnane derivatives, 12-hydroxydaphentoxin type (MEZ & Thy-A), resiniferonol type (ROPA & RX).
For chemical names and structures of the analysed compounds please refer to Figure 6.1.a, page205 and figure 6.1.b, page 206.

Table 6.1 Summary of HPLC and TLC profiles of tigliane and daphnane derivatives

Table 6.2 Mass and ^{1}H-NMR spectral data of 12-deoxyphorbol-13-phenyl acetate (DOPP):

EI mass spectrum run at 30eV exhibited a mass ion at 466 mass units corresponding to $C_{23}H_{34}O_6$. The mass was confirmed by running FAB mass spectrum using MNOBA +Na as a matrix.

FABMS exhibited a base peak at 489 mass units corresponding to $C_{23}H_{34}O_6Na$

High resolution FAB spectrometry exhibited:

Measured mass = 466.23 Daltons

Actual mass = 466.58 Daltons

The EI mass spectrum exhibited the following fragment ions:

M/Z	% Abundance	Inference
466	20	$M^{+\cdot}$
413	10	$M^{+\cdot} - (C_4H_5^{+})$
353	20	$M^{+\cdot} - (C_4H_5^{+\cdot} + CH_2COHOH^{+})$
331	10	$M^{+\cdot} - (C_6H_5CH_2OCO^{+})$
313	7	$M^{+\cdot} - (C_6H_5CH_2OCO^{+\cdot} + H_2O)$
242	71	$M^{+\cdot} - (C_6H_5CH_2OCO^{+\cdot} + H_2O + C_5H_{11}^{+\cdot})$
199	35	$M^{+\cdot} - (C_6H_5CH_2OCO^{+\cdot} + H_2O + C_5H_{11}^{+\cdot} + CH_3CO^{+\cdot})$
136	17	$C_6H_5CH_2OCO^{+\cdot}$

^{1}H-NMR spectral data: 400MHz, $CDCl_3$, TMS= 0.0000 ppm

PPM	Multiplicity & Integration	J=Hz	Inference
7.5759	1H bs	-	H-1
7.3252	5H m	-	5H aromatics
5.6366	1H d	1	H-7
5.3634	1H bs (D_2O exchanged)	-	OH
4.0027	2H M	-	2H-20
3.6090	2H q	2.3	2H-1′
3.2516	1H bs	-	H-10
2.9503	1H t	5.4	H-8
2.4567	2H m	-	2H-5
2.1809	2H m	-	2H-12
2.0823	1H bs (D_2O exchanged)	-	OH
1.9635	1H d	4.6	H-11
1.7696	3H m	-	3H-19
1.5714	1H s (D_2O exchanged)	-	OH
1.2571	6H s	-	3H-16 & 3H-17
1.0358	3H s	-	3H-18
0.8643	1H d	6.4	H-14

Table 6.3 Mass and ^{1}H-NMR Spectral Data of 12-Deoxyphorbol-13-Phenyl acetate-20-Acetate (DOPPA):

EIMS run at 30 eV exhibited a molecular ion at 507 mass units.

FABMS using MNOBA+Na as matrix exhibited a mass peak at 531 mass units corresponding to $M^{+\cdot}$-1+23.

Measured mass = 508.43 Daltons

Actual mass = 508.62 Daltons corresponding to $C_{30}H_{36}O_7$

The EI mass spectrum exhibited the following fragment ions

M/Z	% Abundance	Inference
507	12	$M^{+\cdot}$-1
465	6	$M^{+\cdot}$- ($CH_3CO^{+\cdot}$)
446	10	$M^{+\cdot}$- ($CH_3CO^{+\cdot}$ + H_2O)
417	10	$M^{+\cdot}$- ($CH_3CO^{+\cdot}$ + H_2O+ $C_2H_5^+$)
399	20	$M^{+\cdot}$- ($CH_3CO^{+\cdot}$ + H_2O+ $C_2H_5^+$ +H_2O)
381	15	$M^{+\cdot}$- ($CH_3CO^{+\cdot}$ + H_2O+ $C_2H_5^+$ +H_2O +H_2O)
372	15	$M^{+\cdot}$- ($C_6H_5OCO^{+\cdot}$)
354	20	$M^{+\cdot}$- ($C_6H_5OCO^{+\cdot}$ + H_2O)
339	30	$M^{+\cdot}$- ($C_6H_5OCO^{+\cdot}$ + H_2O+$CH_3^{+\cdot}$)
312	90	$M^{+\cdot}$- ($C_6H_5OCO^{+\cdot}$ + CH3OCO$^{+\cdot}$)
294	100	$M^{+\cdot}$- ($C_6H_5OCO^{+\cdot}$ + CH3OCO$^{+\cdot}$ +H_2O)
136	98	$C_6H_5OCO^{+\cdot}$

^{1}H-NMR Spectral Data of DOPPA: 400MHz, $CDCl_3$, TMS= 0.0000 ppm

PPM	Multiplicity & Integration	J=Hz	Inference
7.5890	1H bs	-	H-1
7.3324	5H m	-	5H aromatics
5.6810	1H d	3.9	H-7
5.3592	1H bs (D_2O exchanged)	-	OH
4.4400	2H d	3.6	2H-20
3.6109	2H d	2.2	2H-1′
3.2656	1H m	-	H-10
2.9630	1H t	5.2	H-8
2.3921	2H bs	-	2H-5
2.1809	2H m	-	2H-12
2.0454	3H s	-	3H-acetate
1.9490	1H d	6.5	H-11
1.7807	3H m	-	3H-19
1.5670	1H s (D_2O exchanged)	-	OH
1.2537	6H s	-	3H-16 & 3H-17
1.0476	3H d	4.2	3H-18
0.8645	1H d	6.4	H-14

Table 6.4 Mass Spectral Data of Resiniferatoxin (RX):

FABMS using MNOBA+Na as a matrix exhibited a mass ion at 651 mass units.

Measured mass was 651.340 Daltons corresponding to $C_{37}H_{40}O_9Na$

Actual mass was 628.013 Daltons corresponding to $C_{37}H_{40}O_9$

The FAB mass spectrum of resiniferatoxin exhibited the following fragment ions:

M/Z	% Abundance	Inference
651	100	$M^{+\cdot}$ + Na
629	25	$M^{+\cdot}$ + 1
612	12	$M^{+\cdot}$ + 1 - (OH)
518	20	$M^{+\cdot}$ + 1 - (OH+ $C_6H_6O^{+\cdot}$)
447	12	$M^{+\cdot}$ - (OH+ $C_6H_6O^{+\cdot}$+$OCOCH_2^{\cdot}$)
310	75	$M^{+\cdot}$ - (OH+ $C_6H_6O^{+\cdot}$+$OCOCH^{\cdot}{}_2$+)
182	92	$C_6H_3OCH_3OHCH_2OCO^{\cdot+}$
136	84	$C_6H_6CH_2OCO^{\cdot+}$

Table 6.5 NMR and Mass Spectral Data of Phorbol (PH):

FAB mass spectrum using MNOBA+Na as matrix exhibited a mass ion of 387 mass units.

FAB accurate mass measurement revealed that:

Measured mass was 387.1799 Daltons

Accurate mass was 387.184 Daltons corresponding to $C_{20}H_{28}O_6Na$

The FAB mass spectrum exhibited the following fragment ions:

M/Z	% Abundance	Inference
387	70	$M^{+\cdot}$ +Na
364	15	$M^{+\cdot}$
329	12	$M^{+\cdot}$ - (2 H_2O)
311	5	$M^{+\cdot}$ - (3 H_2O)
280	12	$M^{+\cdot}$ - (3 H_2O + $CH_3O^{\cdot}$)
176	32	
154	100	
136	72	
120	17	
91	50	

^{1}H-NMR Spectral Data of Phorbol:

400MHz, D_2O, TMS = 0.0000 ppm, Figure 6.3, page 212.

PPM	Multiplicity & Integration	J=Hz	Inference
7.6180	1H t	1.7	H-1
5.5970	1H d	4.5	H-7
4.0437	1H d	10.2	H-12
3.9350	2H s	-	2H-20
3.1553	1H t	4.9	H-8
3.0831	1H t	2.5	H-10
2.4700	2H m	-	2H-5
1.9321	1H m	-	H-11
1.7464	3H m	-	3H-19
1.2508	3H s	-	3H-17
1.1757	3H s	-	3H-16
1.0626	3H d	6.4	3H-18
0.7337	1H d	5.3	1H-14

^{13}C-NMR Spectral Data of Phorbol:

100MHz, D_2O, TMS= 0.00 ppm, Figure 6.4, page 213.

PPM	Inference
21.00	C-3
161.33	C-1
141.88	C-2
134.28	C-1
131.12	C-7
82.04	C-12
79.74	C-4
74.83	C-6
68.27	C-20
63.17	C-9
58.75	C-10
46.10	C-11
40.28	C-8
38.55	C-5
37.42	C-14
27.07	C-15
24.10	C-17
17.83	C-16
15.47	C-18
10.28	C-19

COSY H-H NMR Spectral Data of Phorbol:

D_2O, TMS = 0.0000 ppm, Figure 6.5, page 214.

PPM-PPM	Correlation (H-H)
7.6180-1.7464	H-1....H-19
7.6180-3.0831	H-1....H-10
5.5970-3.1553	H-7....H-8
5.5970-3.9350	H-7....2H-20
4.0437-1.9321	H-12....H-11
3.1553-0.7337	H-8....H-14
3.0831-1.7464	H-10....3H-19
2.4700-5.5970	H-5....H-7
1.9321-1.0626	H-11....3H-18

NOESY H-H NMR Spectral Data of Phorbol:

D_2O, TMS= 0.0000 ppm, Figure 6.6, page 215

PPM-PPM	Correlation (H-H)
7.6180-1.0626	H-1....3H-18
7.6180-1.7464	H-1....3H-19
7.6180-3.0831	H-1....H-10
5.5970-0.7337	H-7....H-14
5.5970-3.0831	H-7....H-10
5.5970-3.9350	H-7...2H-20
4.0437-1.0626	H-12....3H-18
4.0437-1.9321	H-12....H-11
3.9350-2.4700	2H-20....2H-5
3.1553-1.1757	H-8....H-16
3.1553-1.9321	H-8....H-11
3.0831-7.6180	H-10....H-1
1.1757-1.9321	H-16....H-11
1.1757-0.7337	H-16....H-14

C-H One Bond Correlation NMR Data of Phorbol:

D_2O, TMS= 0.00 ppm, Figure 6.7, page 216.

PPM-PPM	Correlation (C-H)
161.33-7.6180	C-1....H-1
13112-5.5970	C-7....H-7
82.04-4.0437	C-12....H-12
68.27-3.9350	C-20....2H-20
58.75-3.0831	C-10....H-10
46.10-1.9321	C-11....H-11
40.28-3.1553	C-8....H-8
38.55-2.4700	C-5....H-5
37.42-0.7337	C-14....H-14
24.10-1.2508	C-17....3H-17
17.83-1.1757	C-16....3H-16
15.47-1.0626	C-18....3H-18
10.28-1.7464	C-19....3H-19

C-H Long Range Correlation NMR Data of Phorbol:

D_2O, TMS = 0.00 ppm, Figure 6.8, page 217.

PPM-PPM	Correlation (C-H)
17.83-1.2508	C-16....3H-17
24.10-0.7337	C-17....H-14
38.55-3.9350	C-5....2H-20
46.10-1.0626	C-11....3H-18
46.10-3.0831	C-11....H-10
58.75-2.4700	C-10....H-5
63.17-0.7337	C-9....H-14
63.17-4.0437	C-9....H-12
74.83-2.4700	C-6....H-5
79.74-3.0831	C-4....H-10
82.04-1.0626	C-12....H-18
131.12-0.3377	C-7....H-14
131.12-2.4700	C-7....H-5
131.12-3.1553	C-7....H-8
131.12-3.9350	C-7....2H-20
27.07-1.1757	C-15....3H-16
27.07-1.2508	C-15....3H-17

Table 6.6 Mass and ^{1}H-NMR spectral data of phorbol-12,13,20-triacetate (PTA):

Phorbol-12,13,20-triacetate exhibited the following spectral characteristics:

FABMS using MNOBA +Na as matrix

Measured mass was 513.23 Daltons corresponding to $C_{26}H_{34}O_9Na$

Actual mass was 490.045 Daltons corresponding to $C_{26}H_{34}O_9$

The FAB mass spectrum exhibited the following fragment ions:

M/Z	% Abundance	Inference
513	10	$M^+ + Na$
490	10	$M^+ = C_{26}H_{34}O_9$
430	13	$M^+ - (CH_3COOH)$
405	4	$M^+ - (CH_3COOH+CH_3CO^-)$
388	14	$M^+ - (CH_3COOH+CH_3CO^- +CH_2CO^-)$
352	3	$M^+ - (CH_3COOH+CH_3CO^- +CH_2CO^- + 2X\ H_2O)$
311	60	$M^+ - (CH_3COOH+CH_3CO. +CH_2CO^- + XH_2O+C_3H_5^-)$
293	80	$M^+ -(CH_3COOH+CH_3CO^- +CH_2CO^- + 2xH_2O+C_3H_5^- +H_2O)$

^{1}H-NMR Spectral Data of Phorbol-12,13,20-Triacetate:

$CDCl_3$, 500 MHZ, TMS = 0.0000 ppm, Figure 6.9, page 219.

PPM	Integration	J=Hz	Inference
7.5992	1H s	-	H-1
5.7134	1H d	4.2	H-7
5.5300	1H s (D_2O exchanged)		OH
5.3900	1H d	10	H-12

Table 6.6 continued

PPM	Integration	J=Hz	Inference
4.4576	2H q	12	2H-20
3.2503	2H m	-	H-10 & H-11
2.549	1H d	19	H-8
2.4200	2H d	20	H-5
2.0757	9H t	12	3 x CH_3 of acetate
1.7751	3H m	-	3H-19
1.6720	1H s (D_2O exchanged)	-	OH
1.2276	6H m	-	3H-16 & 3H-17
1.0687	1H d	6	H-14
0.8942	3H d	5	3H-18

Table 6.7 Mass and ^{1}H-NMR Spectral Data of 12-*O*-Tetradecanoyl Phorbol-13-Acetate (TPA):

Accurate CI ammonia mass spectrum of TPA exhibited:

Measured mass of 633.521 Daltons corresponding to $C_{36}H_{56}O_8NH_4$

Actual mass of 616.026 Daltons corresponding to $C_{36}H_{56}O_8$

CIMS ammonia exhibited a mass ion of 633 mass units. The mass spectrum exhibited the following fragment ions:

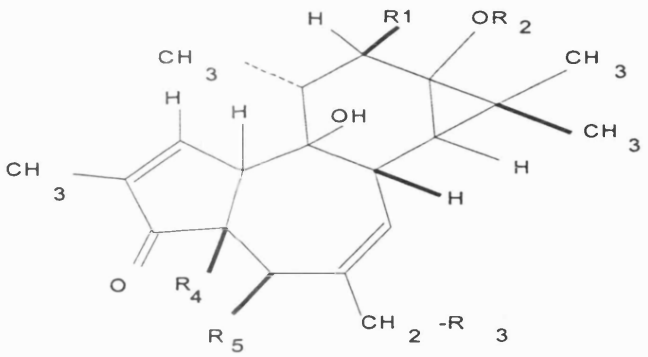
M/Z	% Abundance	Inference
633	5	$M^+ + NH_4$
616	20	M^+
599	50	M^+ -(OH)
567	100	M^+ -(OH+32)
389	45	$M^+ - (C_{14}H_{27}O_2^{\cdot})$
370	15	$M^+ - (C_{14}H_{27}O_2^{\cdot} + H_2O)$
310	92	$M^+ - (C_{14}H_{27}O_2^{\cdot} + H_2O + CH_3COOH)$

^{1}H-NMR Spectral Data of TPA: 400MHz, $CDCl_3$, TMS= 0.0000 ppm, page 219.

PPM	Multiplicity & Integration	J=Hz	Inference
7.5964	1H s	-	H-1
5.6799	1H d	5.4	H-7
5.5491	1H bs	-	OH
5.4102	1H d	-	H-12
4.0075	2H q	7.7	2H-20
3.2359	2H m	-	H-10 & H-11
2.5104	2H m	-	2H-5
2.3273	1H m	-	H-8
2.1790	1H s	-	OH
2.0940	3H s	-	3H acetate
1.7821	3H q	1	3H-19

Table 6.7 continued

PPM	Multiplicity & Integration	J=Hz	Inference
1.5668	1H s	-	OH
1.2520	27H s	-	ester protons at C-12
1.2087	6H s	-	3H-16 & 3H-17
1.0781	1H d	5	H-14
0.8887	3H m	-	3H-18



Tigliane

R_1	R_2	R_3	R_4	R_5	Name of the Compound (Abbreviations)
OH	H	OH	OH	H	Phorbol (PH)
$OCCH_3$	H	$OCCH_3$	OH	H	Phorbol-13,20-diacetate (PDA)
$OCCH_3$	CCH_3	$OCCH_3$	OH	H	Phorbol-12,13,20-triacetate (PTA)
OC	CCH_3	OC	OH	H	Phorbol-12,20-dibenzyl-13-acetate (PDBA)
OCC_3H_7	CC_3H_7	OCC_3H_7	OH	H	Phorbol-12,13,20-tributyrate (PTB)
$OC(CH_2)_{12}CH_3$	CCH_3	OH	OH	H	12-O-Tetradecanoyl phorbol-13-acetate (TPA)
H	CCH_2	OH	OH	H	12-Deoxyphorbol-13-phenyl-acetate (DOPP)
H	CCH_2	$OCCH_3$	OH	H	12-Deoxyphorbol-13-phenyl-acetate-20-acetate (DOPPA)
OC $NHCH_3$	CCH_3	OH	H	H	Sapintoxin-A (SAP-A)
OC $NHCH_3$	CCH_3	OH	OH	H	Sapintoxin-D (SAP-D)
OC $NHCH_3$	CCH_3	H	H	OH	Sapintoxin-C (SAP-C)
OC $NHCH_3$	CCH_3	$OCCH_3$	-H	$OCCH_3$	4 -Sapintoxin-B-5,20-diacetate (SAP-AC)
OC $NHCH_3$	CCH_3	H	H	H	Sapintoxin-E (SAP-E)

Figure 6.1.A Structures of Tigliane Derivatives Analysed by HPLC and TLC

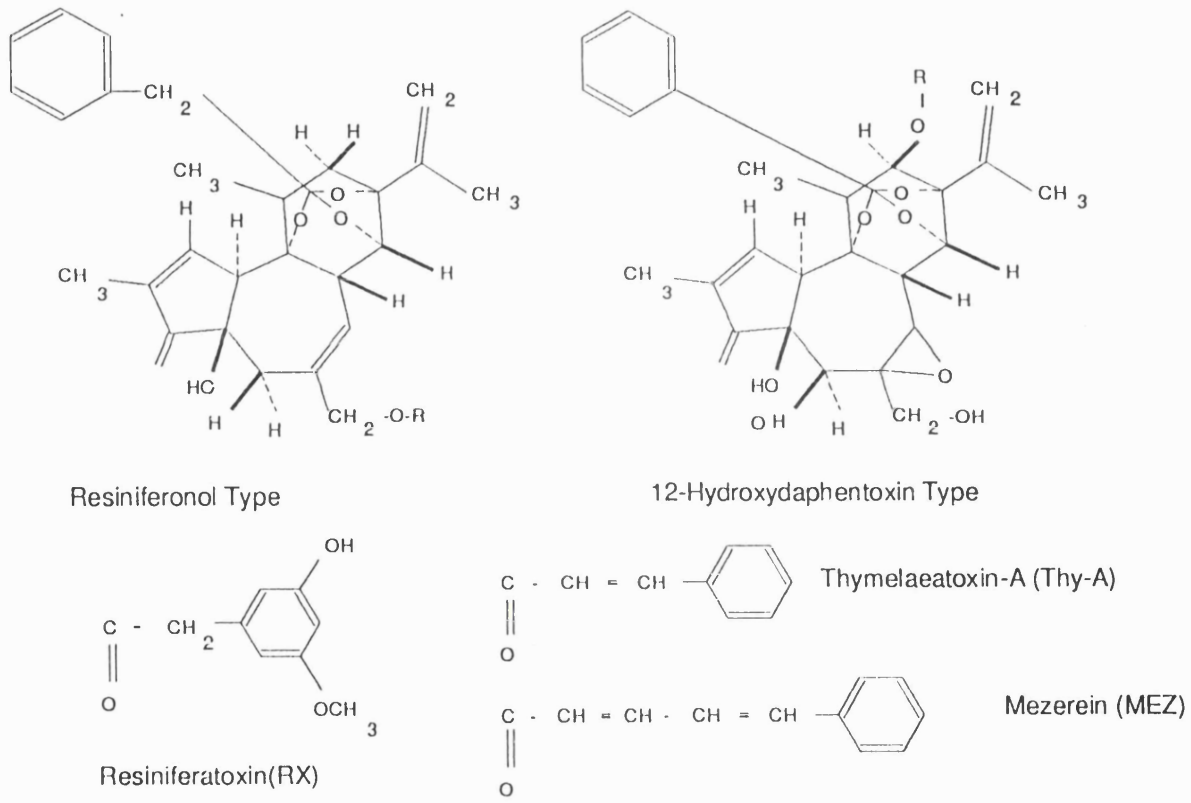


Figure 6.1.B Structures of Daphnane Derivatives Analysed by HPLC and TLC

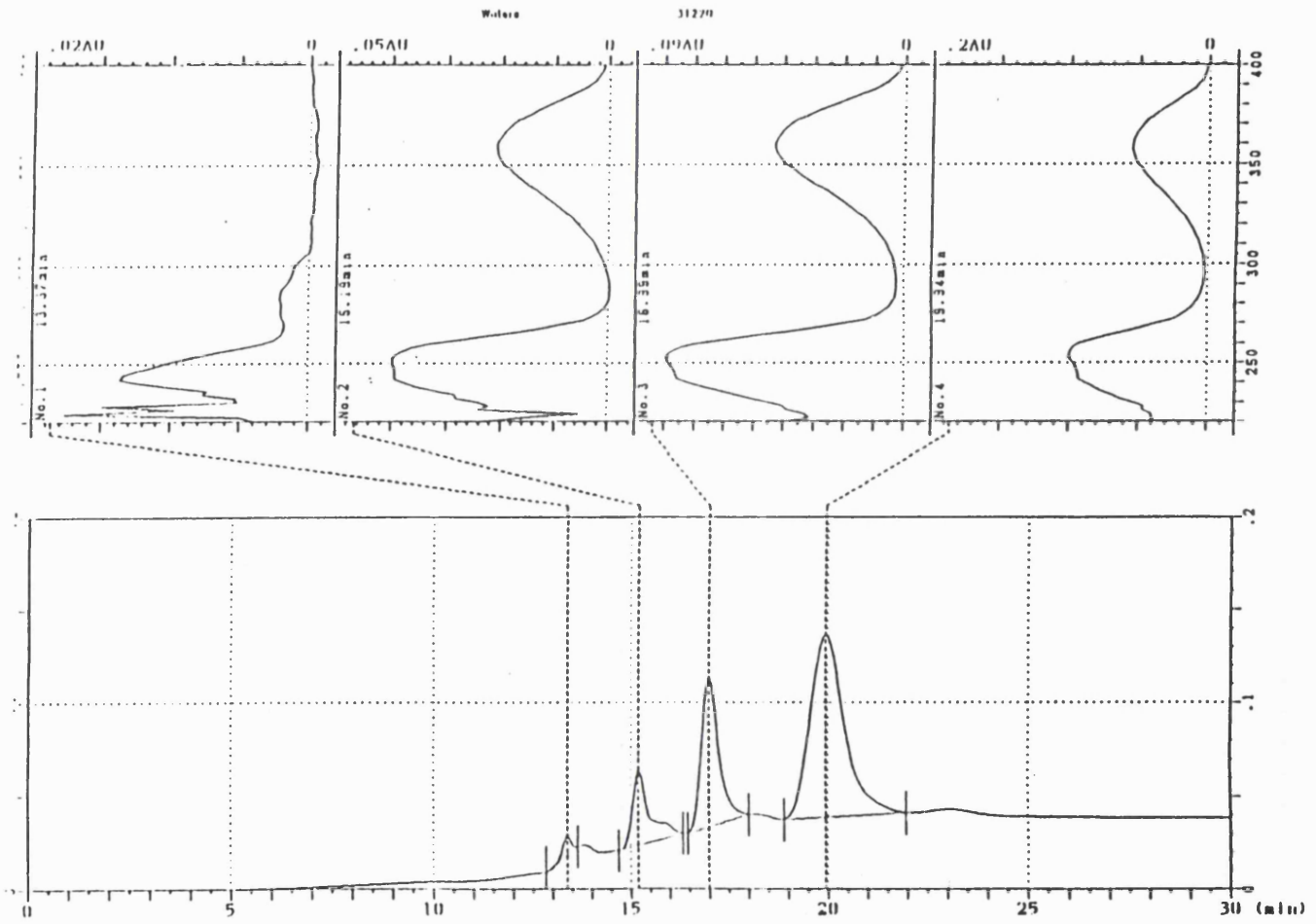


Figure 6.2.A Spectrum Index of a Mixture of Impure Sapintoxins

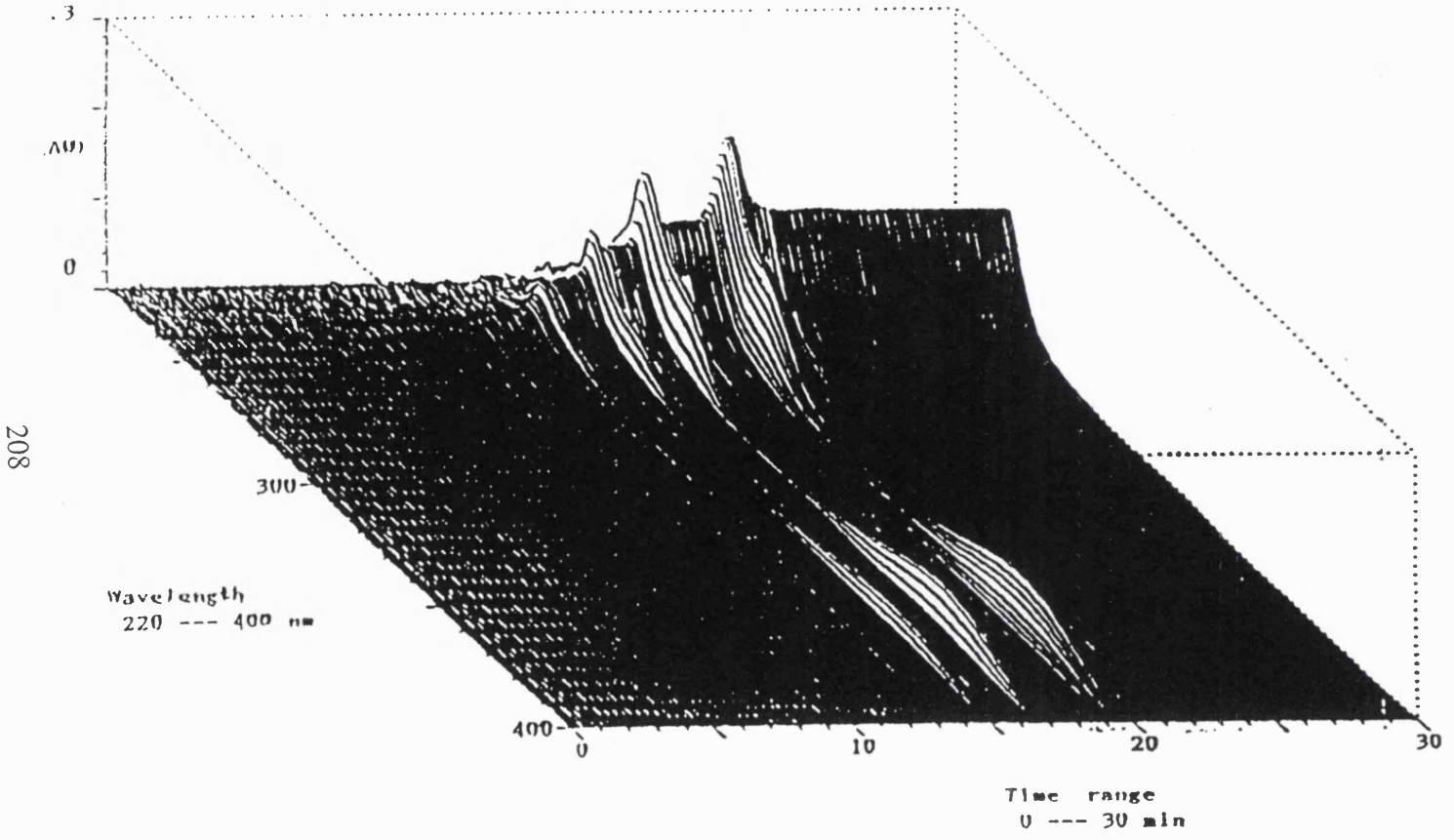


Figure 6.2.B Three Dimensions Plot of Impure Sapintoxins Mixture

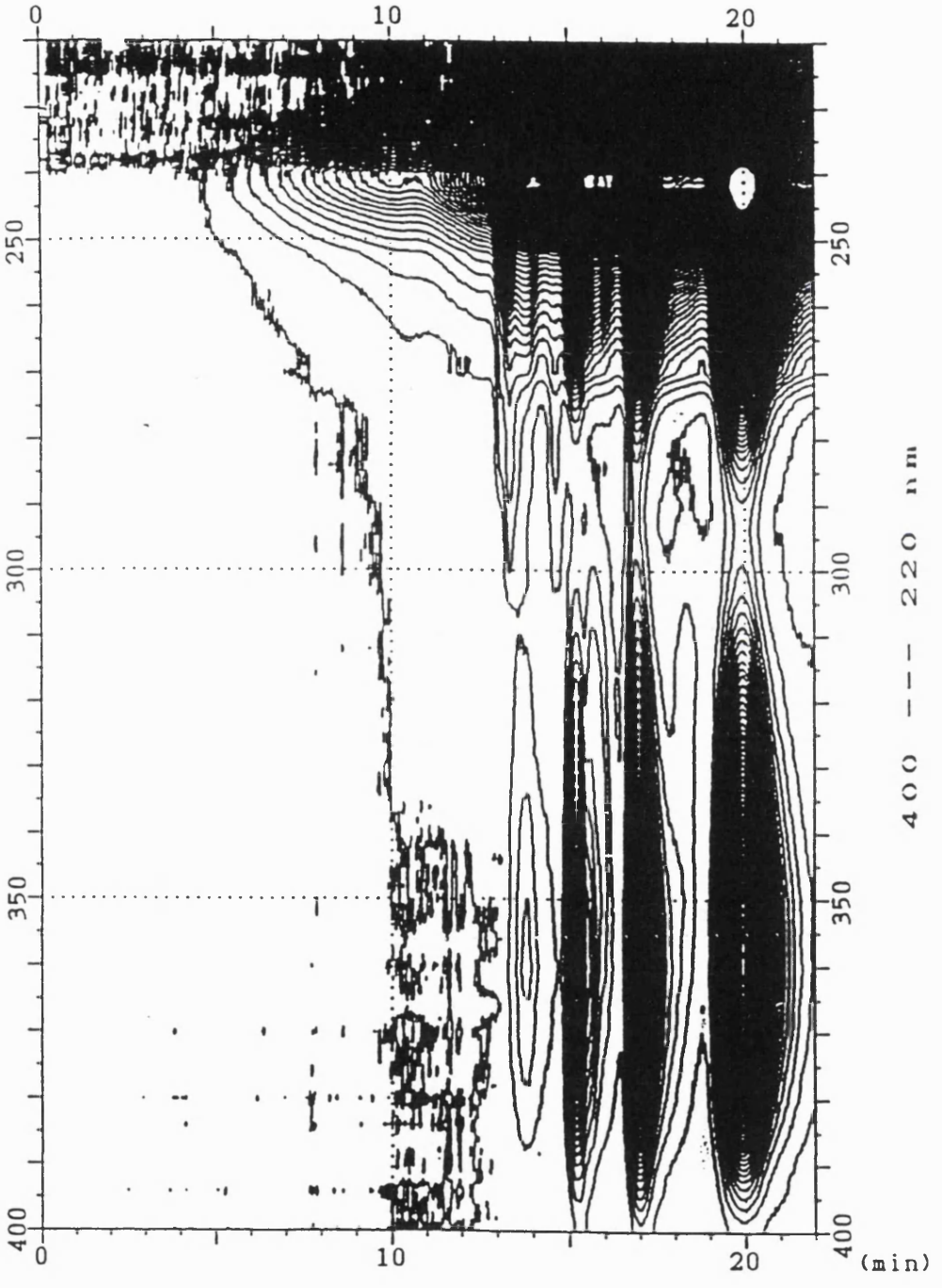


Figure 6.2.C Contour Plot of Impure Sapintoxins Mixture

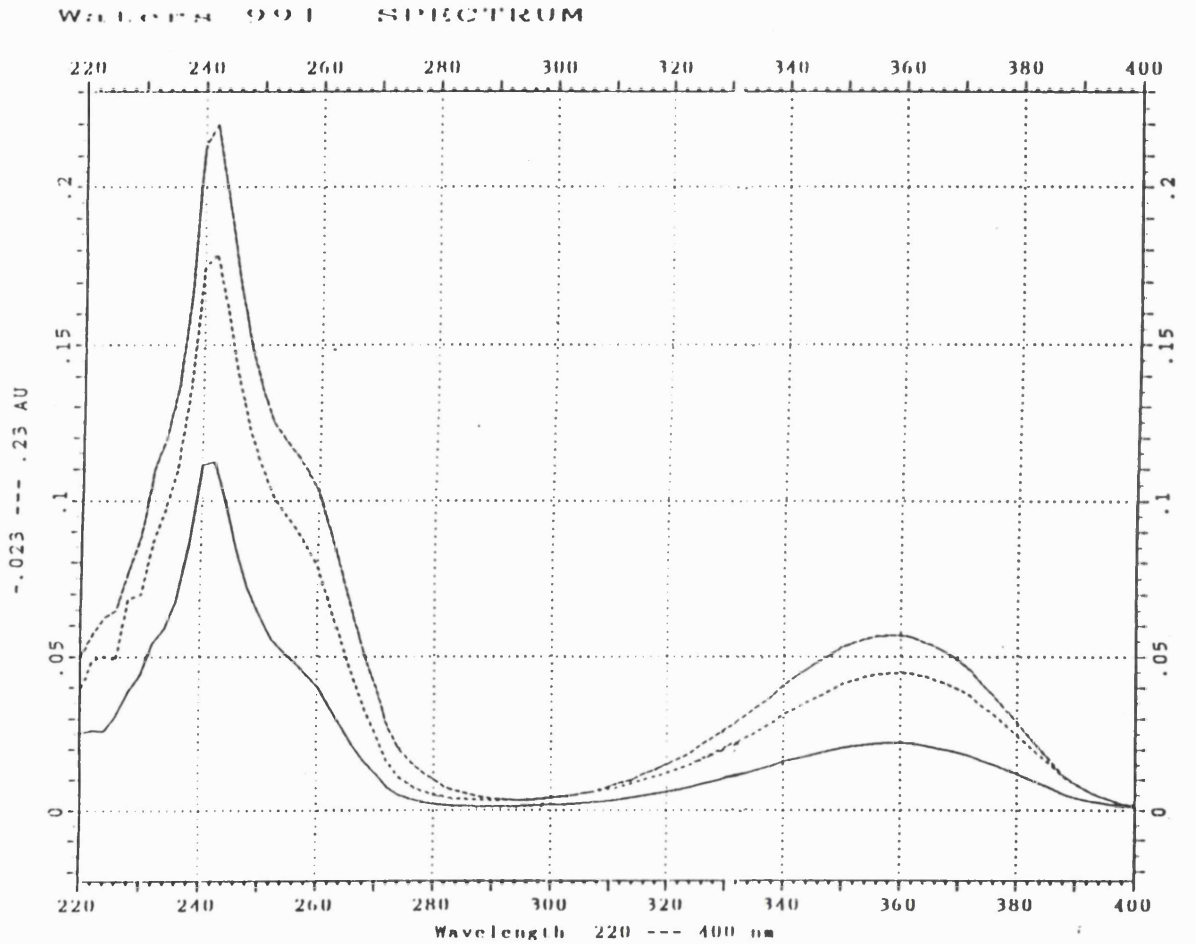


Figure 6.2.D Chromatogram Analysis of Impure Sapintoxins mixture

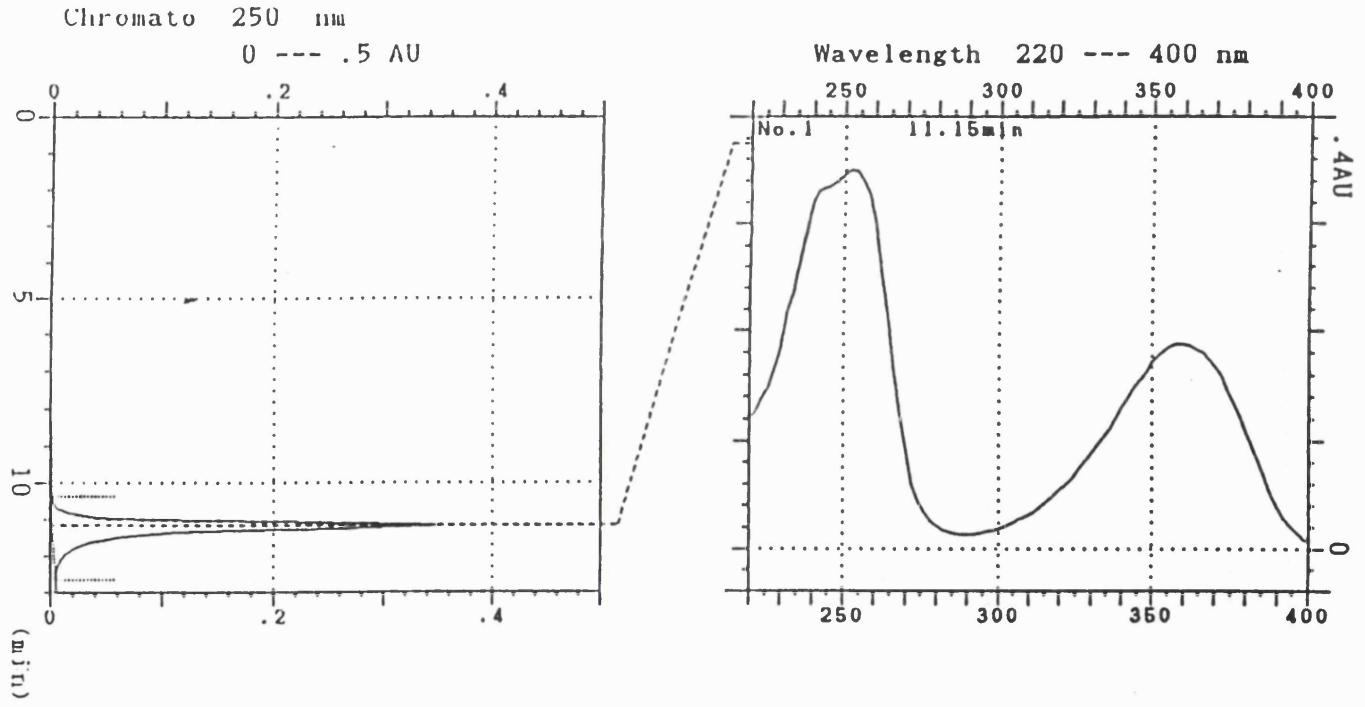


Figure 6.2.E Spectrum Index Plot of Purified Sapintoxin-A Chromatogram

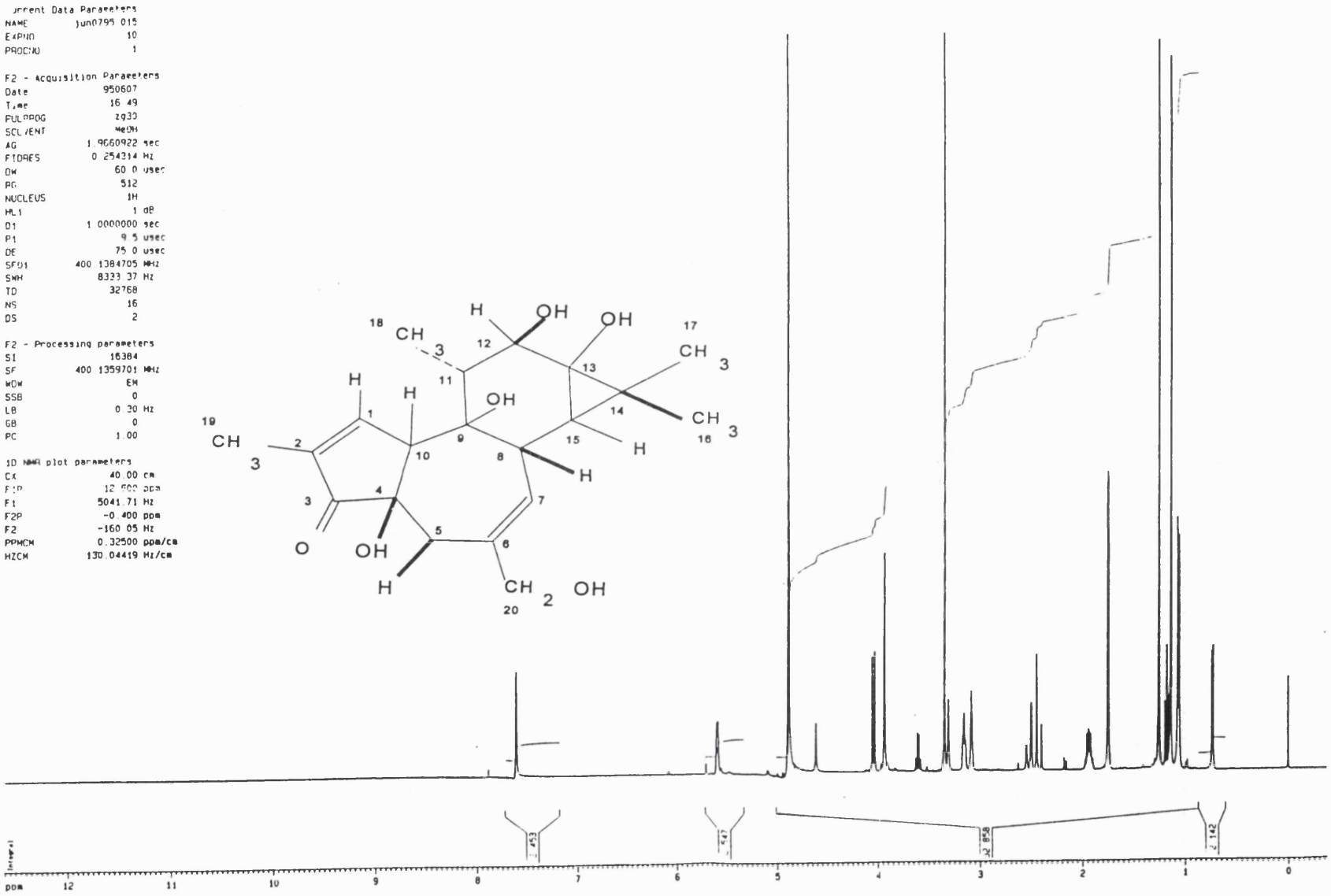


Figure 6.3 ^{1}H NMR (400MHz, D_2O) of Phorbol

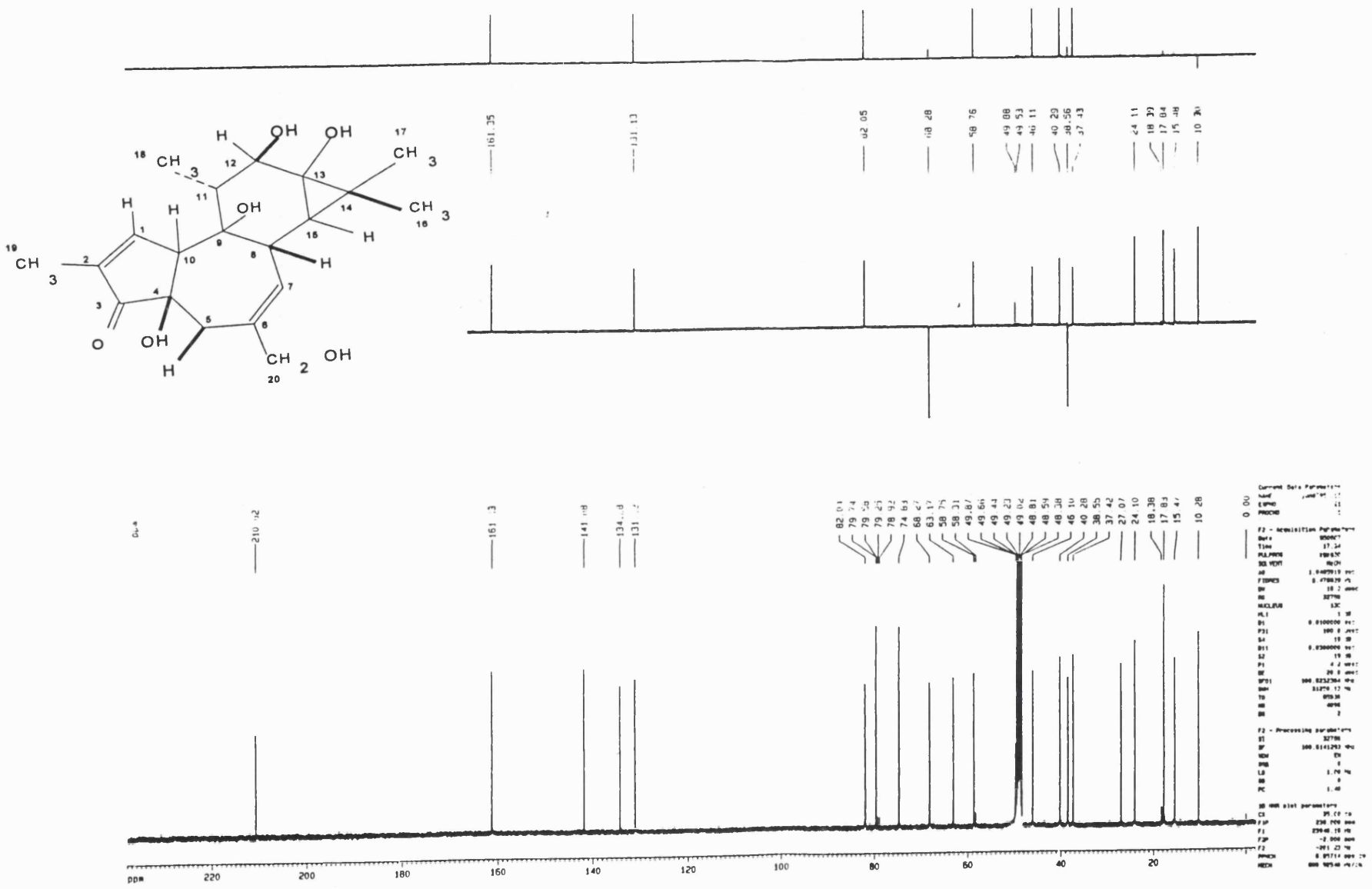


Figure 6.4 ^{13}C NMR (100MHz, D_2O) of Phorbol

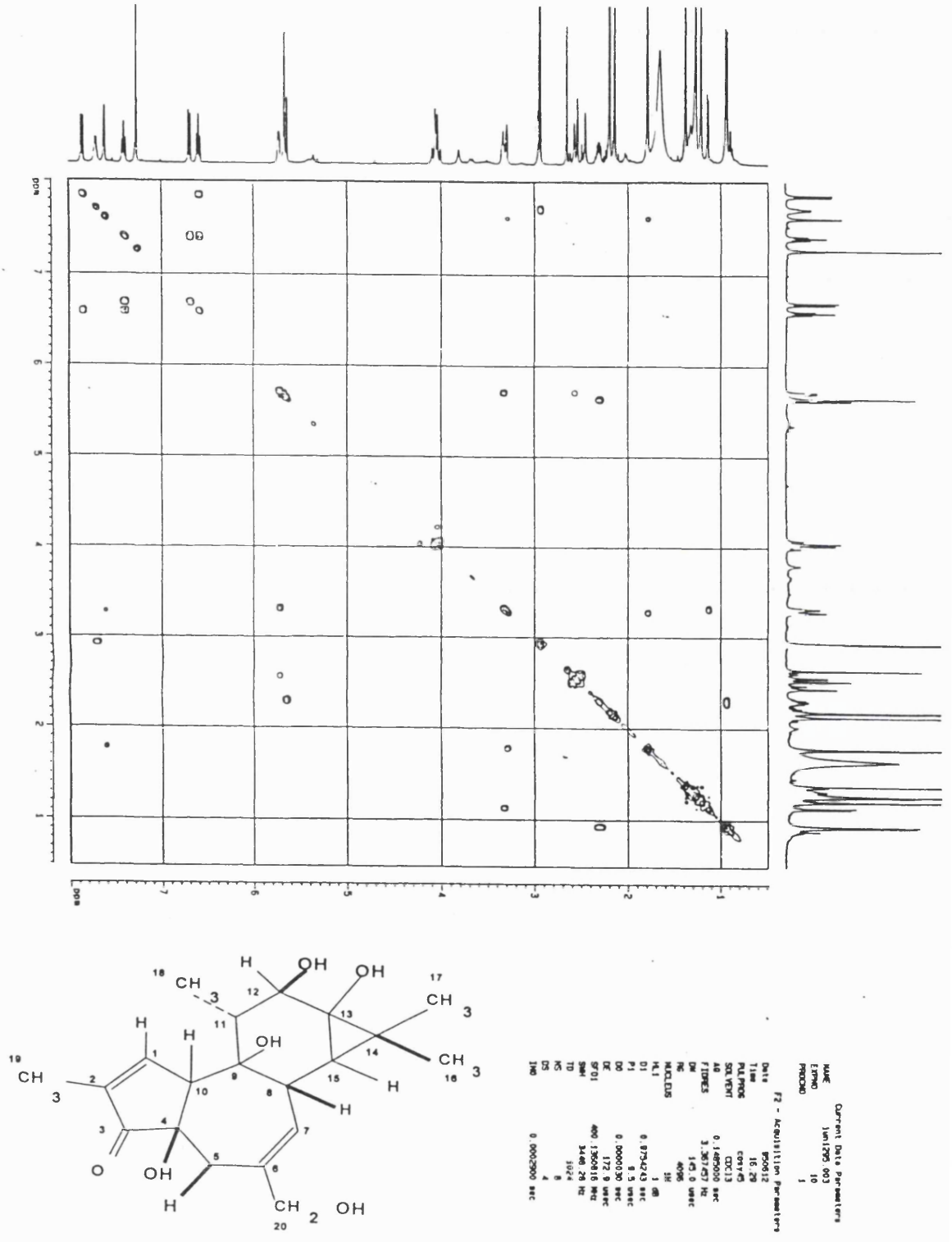


Figure 6.5 COSY H-H NMR Spectrum of Phorbol

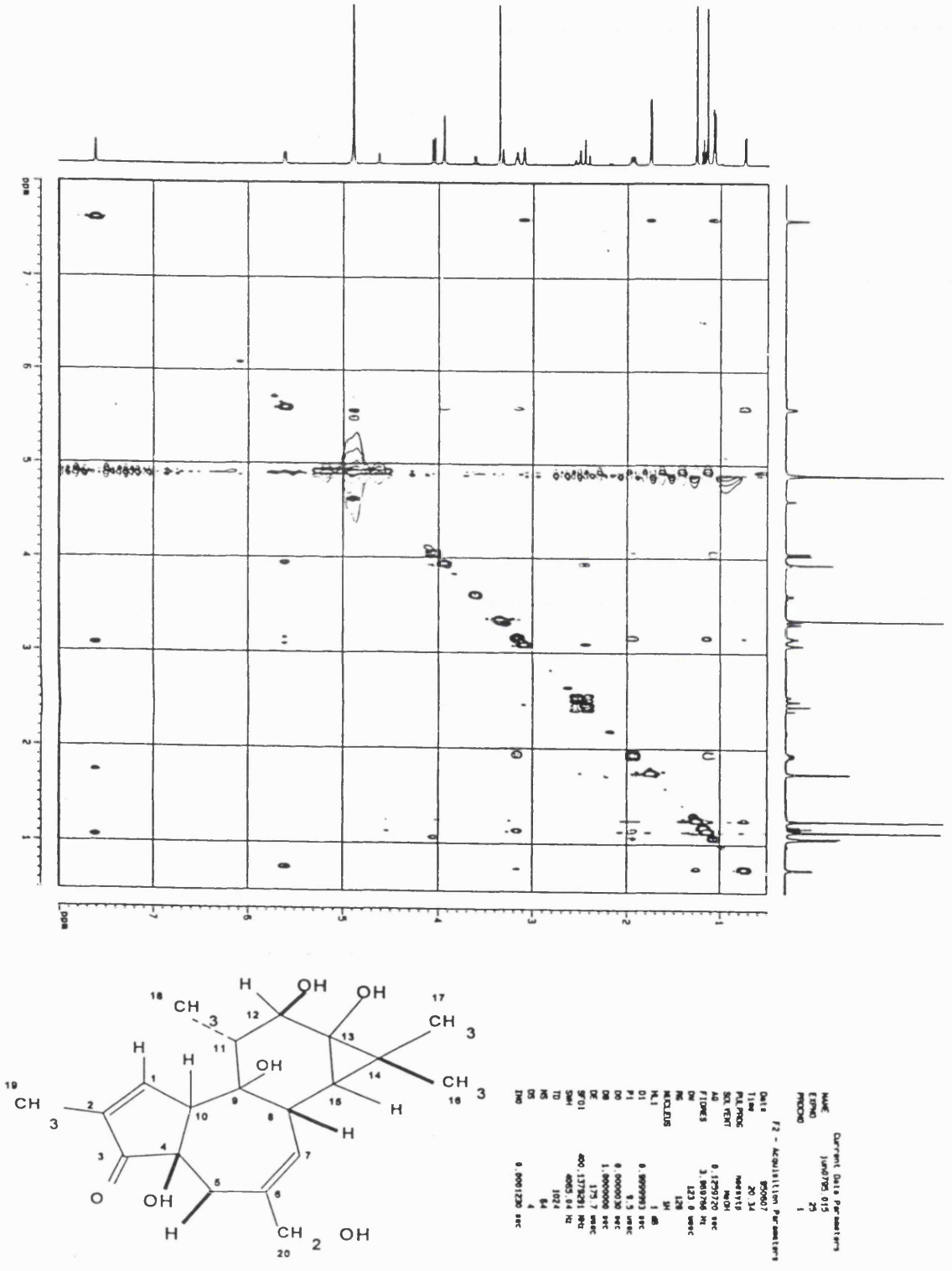


Figure 6.6 NOESY H-H NMR Spectrum of Phorbol

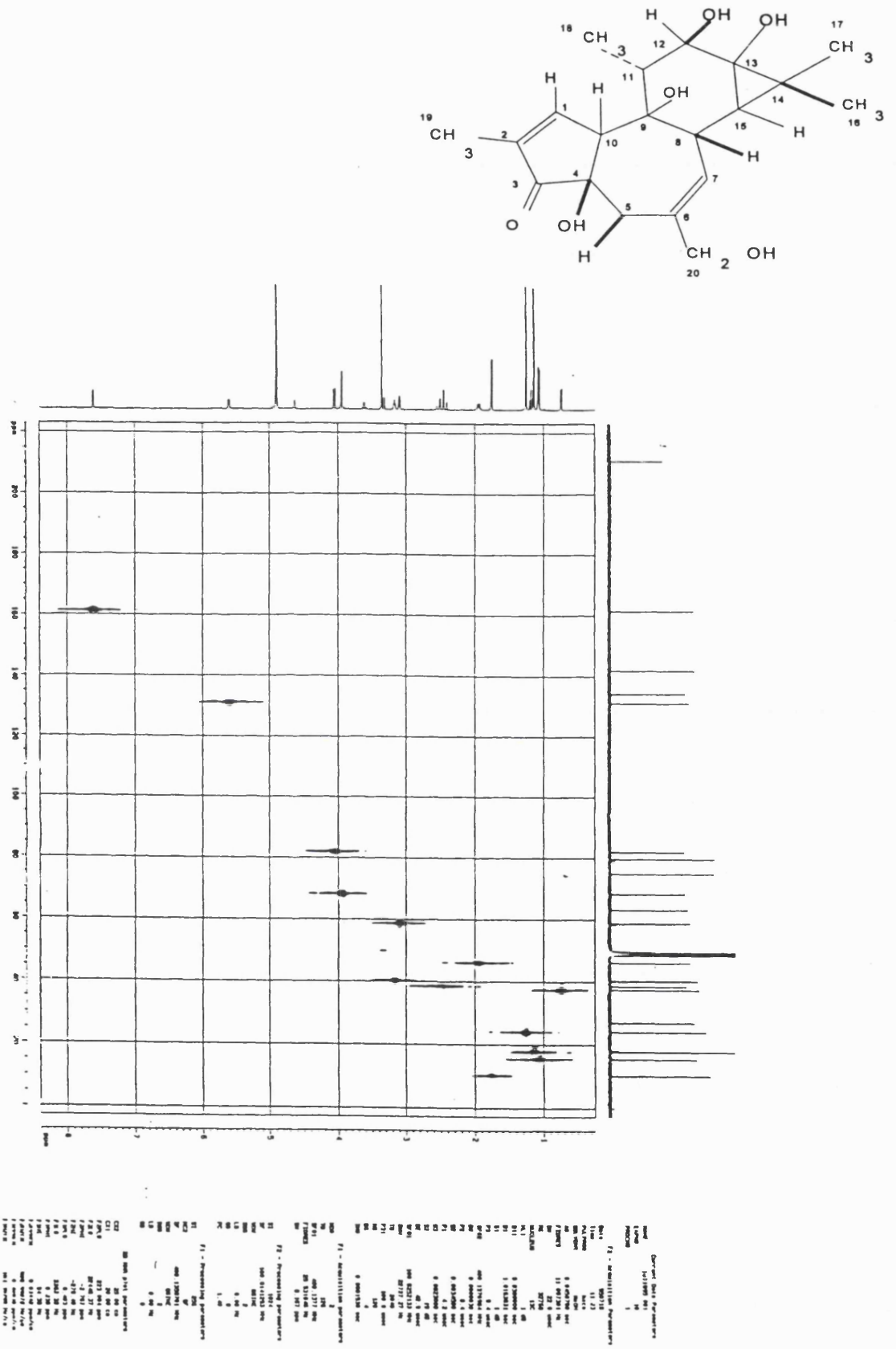


Figure 6.7 C-H One Bond Correlation NMR Spectrum of Phorbol

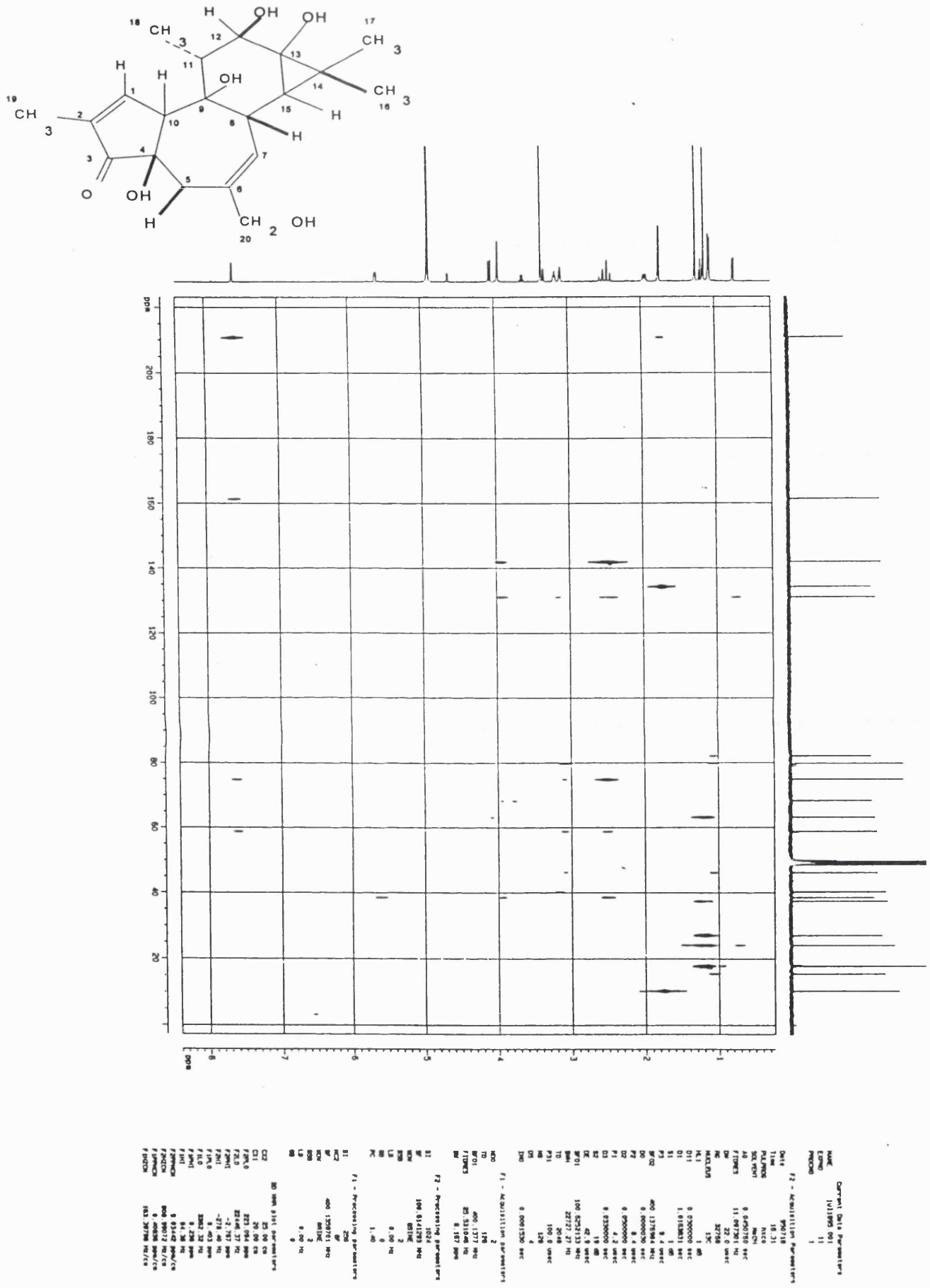


Figure 6.8 C-H Long Range NMR Spectrum of Phorbol

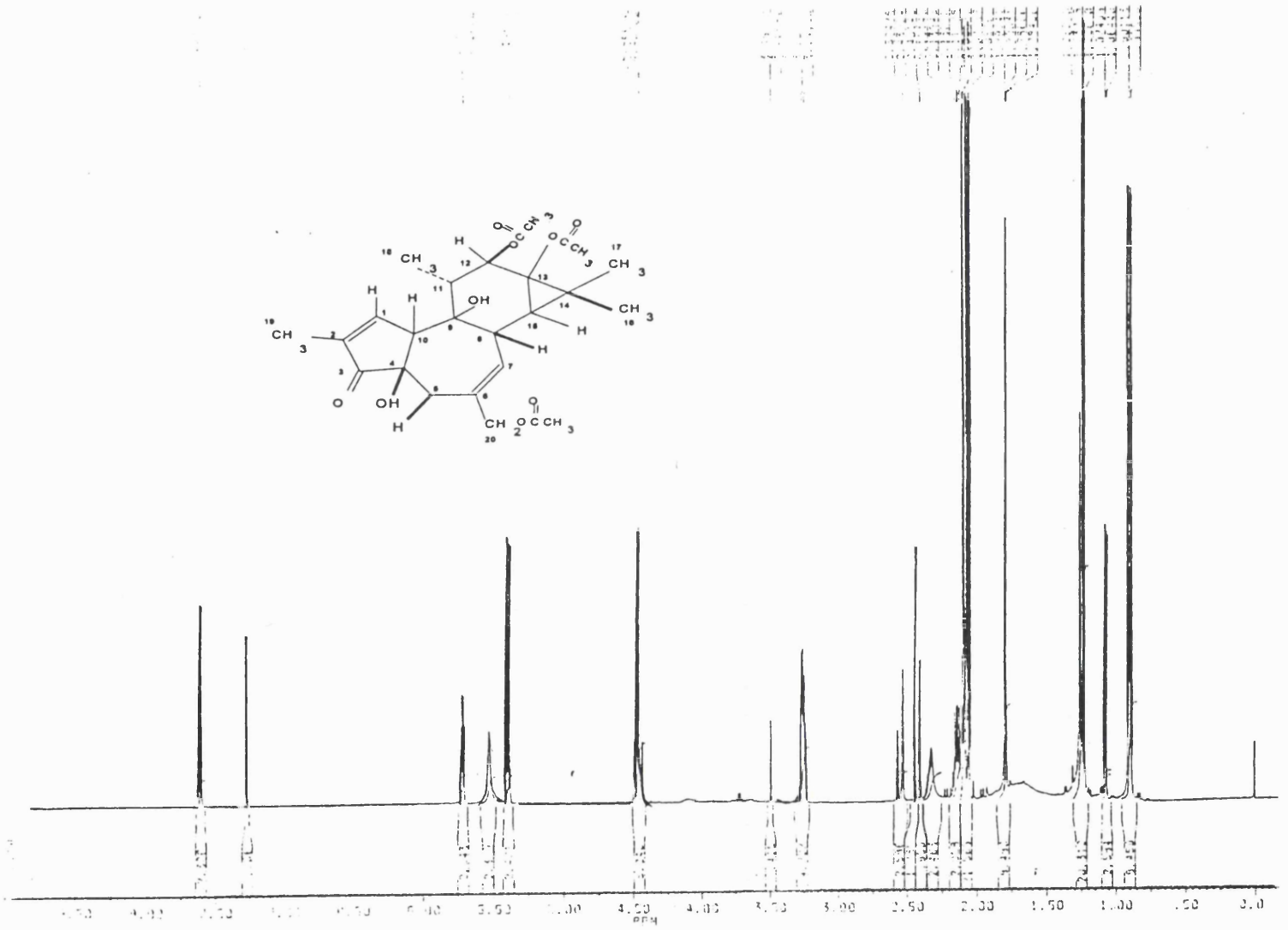


Figure 6.9 ^{1}H NMR Spectrum (500MHz, $CDCl_3$) of Phorbol-12,13,20-Triacetate

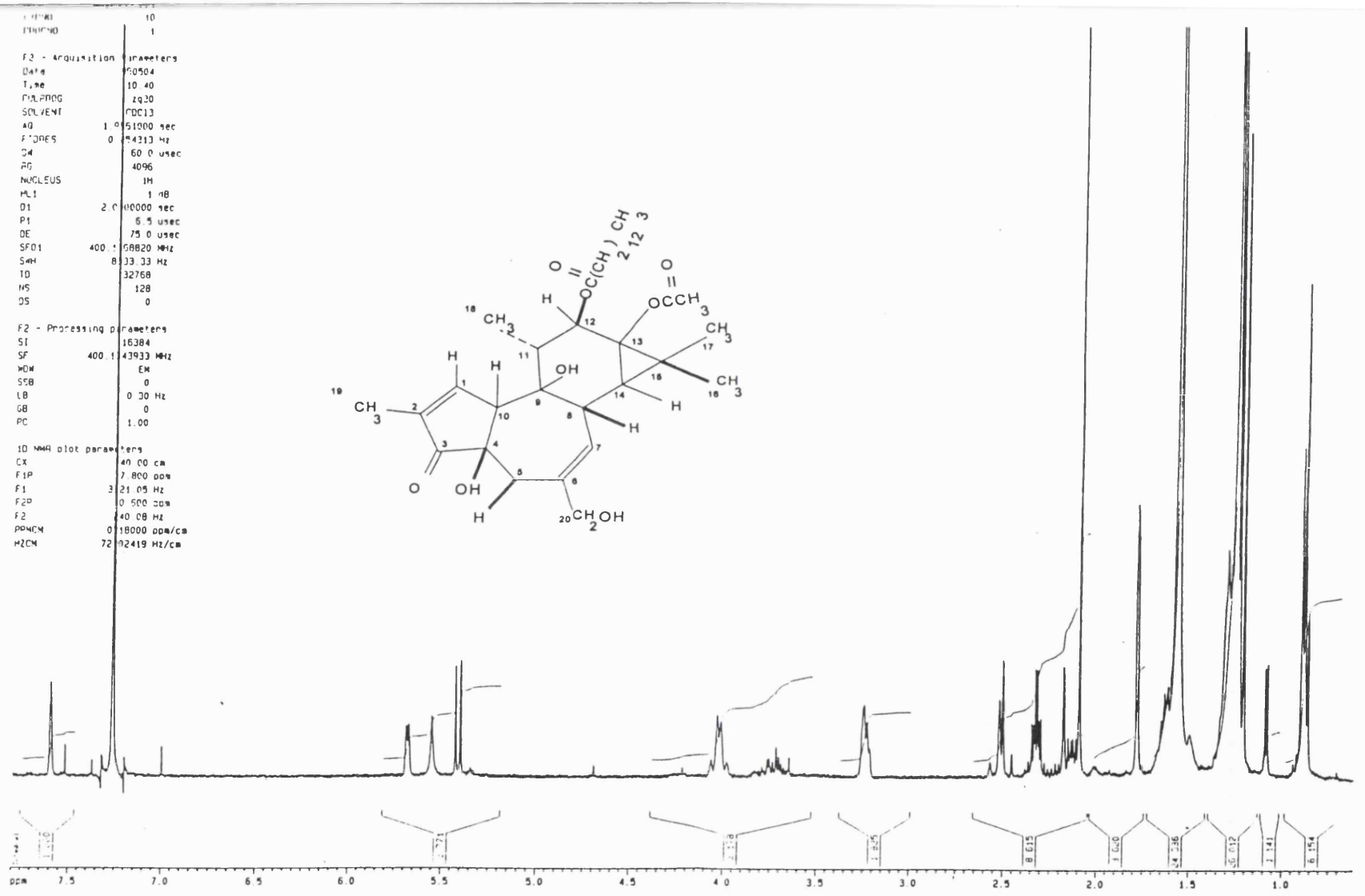
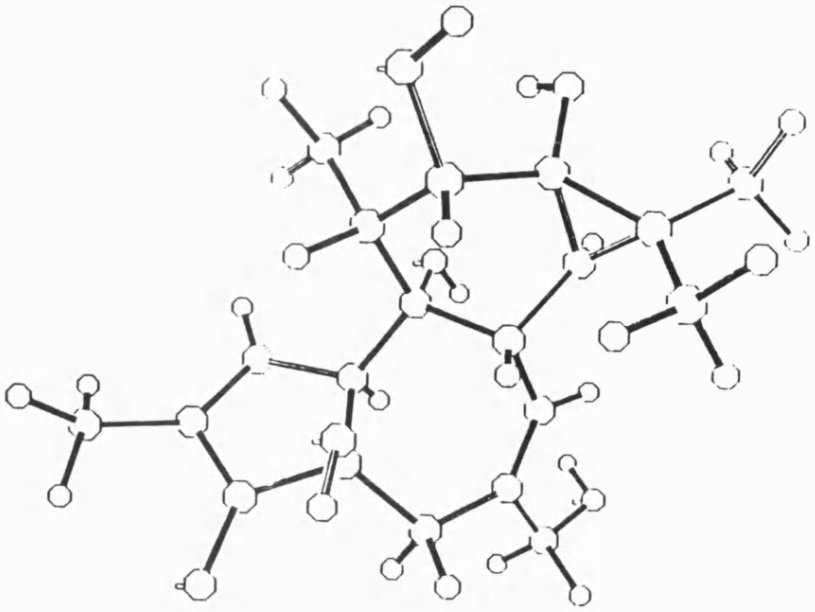
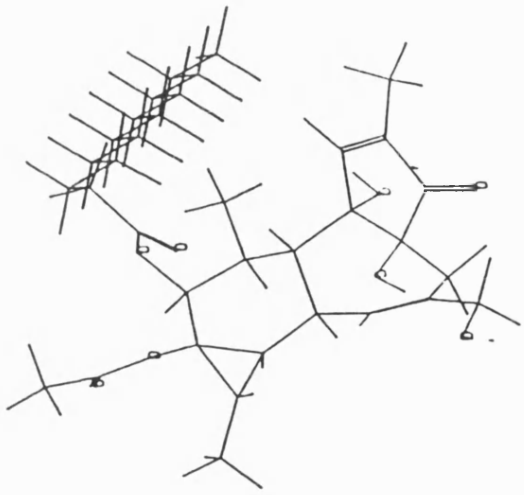


Figure 6.10 ^{1}H NMR Spectrum (500MHz, $CDCl_3$) of TPA, 12-O-Tetradecanoyl phorbol-13-Acetate



Phorbol



12-O-Tetradeconyl phorbol-13-acetate (TPA)

Figure 6.11 A Computer-generated drawing (Sybyl) of the molecular structure of phorbol and TPA based on NMR spectral data

Summary of Conclusions and Recommendations

Cancer is the result of a complex interaction between humans and the surrounding environment. Epidemiological studies concluded that exposure to certain environmental agents is related to specific types of human cancers. Approximately 80% of human cancers are believed to be induced by continuous exposure to exogenous environmental factors.

A group of diterpene esters, commonly reported in the literature as phorbol esters, are skin irritants and mouse skin tumour promoters (Blumberg 1980). The plant families Euphorbiaceae and Thymelaeaceae are the only reported natural sources of these toxic diterpenes.

This study adopted two approaches to assess forty samples from the above families for the presence of pro-inflammatory and tumour-promoting diterpene esters. The first approach involved the use of *in vivo* and *in vitro* tests to screen plant extracts for biological effects. *In vivo*, all tested Thymelaeaceous species were irritant to the mouse skin but not necessarily hyperplasiogenic. Assessments concluded that whilst skin irritancy is a pre-requisite for epidermal hyperplasia skin irritants are not necessarily hyperplasiogenic. *In vitro*, the pro-inflammatory extracts induced differentiation of HL-60 cells and platelet-aggregation of human blood. The second approach employed a number of chromatographic and spectroscopic techniques to isolate, identify and analyze the biologically active constituents.

From the irritant species of the family Thymelaeaceae, three biologically active daphnane derivatives were isolated and had their structures identified. Two irritant diterpene esters, thymelaeatoxin-A and resiniferonol-9,13,14-orthophenyl acetate were isolated from the twigs and leaves of *Thymelaea hirsuta*. Intense irritancy of *Daphne blagayana* was partially due to the presence of the 12-hydroxydaphentoxin derivative, mezerein.

Four nitrogen-containing fluorescent phorbol derivatives, one of which is new, were

isolated from two *Sapium* species of the family Euphorbiaceae. The inflammatory effect, induced by *Sapium indicum*, was attributed to the presence of sapintoxin-A and sapintoxin-D in the oil extract. The novel and the biologically inactive sapintoxin-E was also isolated from *Sapium indicum*. Acetylation of the ultraviolet blue fluorescent compound isolated from *Sapium insigne* yielded the 4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate.

Both flash column and centrifugal liquid chromatography (CLC) reduce repeated preparative TLC which involves long and laborious work. However, flash silica gel column proved to be the best method for fractionating plant extracts. It has many advantages over the CLC. First the flash column is a simple equipment which could be run in any laboratory with a small budget and medium resolution, it separates compounds with an R_f difference of 0.1 - 0.2. It is also more flexible with the range of the eluents resulting in higher resolution. The preparation and separation time are very short, typically 30 minutes. In contrast, preparing the CLC plate required pre-refrigeration of the adsorbent for a minimum of one hour and allowing 24 - 48 hours for the plate to dry. Moreover, both techniques allowed preliminary purification without contamination of expensive HPLC column.

Repeated development of the preparative TLC plates improved the separation and enhanced the resolution. However, this increased the risk of degradation and decreased the yield of the diterpene obtained from plants in small amount. Also both yield and resolution were greatly enhanced by using the semipreparative normal phase HPLC technique. This was particularly useful in purifying microgramme quantities of the diterpenes. High cost could be considered as a disadvantage.

The colour reaction on analytical TLC provided the first indication of the presence of irritant diterpenes. The 60% sulphuric acid is the widely used spraying reagent to detect the toxic diterpenes in plant extracts. However, it is not specific for this group of compounds and others such as cardiac glycosides may interfere. Supporting the TLC, the HPLC profile of tigliane and daphnane derivatives provided a valuable tool for detecting and purifying microgrammes of these esters in plant extracts, chemical reaction mixtures and biological systems.

Electron impact mass spectra run at low voltage of 30 eV at 110°C exhibited mass ions of more than 5% relative abundance. Each group of the diterpenes exhibited characteristic fragmentation pattern which provided information on the acyl substituents. The fast atom bombardment (FAB) with the MNOBA plus sodium as matrix was the best technique to confirm the mass of the isolated tigliane and daphnane derivatives. The laser mass determination technique had no advantage over the FAB.

The ^{13}C-NMR spectra provided information on the chemical groups and the number of carbons and hydrogens in the structure. The substituents were identified from the ^{1}H-NMR spectra. The two dimension-NMR spectra COSY, NOESY, and C-H correlation provided valuable information on the connectivity and the stereochemistry of the substituents. Previously published NMR spectral data were revised and signals were assigned according to the new data from the H-H and C-H connectivity in the two dimension NMR spectra.

Diterpene esters were reported to exert their effects through activation of members of the PKC family. PKC exhibit selective tissue distribution. Therefore, diverse biological responses to different members of phorbol esters could be mediated by specific members of PKC. For example, resiniferonol-9,13,14-orthophenyl acetate did not induce platelet aggregation at 7 μM. However, it was able to induce 24% differentiation of HL-60 cells at concentration of 10μM. Since PKC- α, PKC-β_I and PKC-β_{II} are present in the human platelet and in the HL-60 cells (Rais *et al* 1994, Hashimotok *et al* 1990, Tanaka *et al* 1992), biological effects induced by ROPA could indicate that activation of the above PKC members is not sufficient to induce platelet aggregation and HL-60 cell differentiation at nanomolar concentration and that activation of PKC-δ, PKC-ϵ, PKC-ζ and PKC-η might play an important role in platelet-aggregation.

Although the presence of an ester substituent at C12 in daphnanes increased the potency for induction of cell differentiation and platelet aggregation, it was not an absolute requirement. However resiniferonol-9,13,14-orthophenyl acetate, which lacks the C12 substituent, was a very potent pro-inflammatory agent having an ID_{50} of 0.18 nM/ear. Increasing the length of the unsaturated substituent at C12 increased the pro-

inflammatory activity and the epidermal hyperplasia of daphnanes but decreased the platelet aggregation substantially. The presence of a hydroxy group at C4 in phorbol derivatives substantially increased the irritancy with an opposite effect on platelet aggregation and had no or negligible effect on HL-60 cell differentiation. The primary alcohol at C20 was necessary for full efficacy. Elimination of this chemical group in the phorbol derivative sapintoxin-E abolished biological activities. The same effect was observed when the link between ring-A and ring-B was converted from the trans to the cis conformation in 4α-deoxy-5-hydroxyphorbol-5,13,20-triacetate. The pro-inflammatory activities of the tigliane and daphnane derivatives did not necessarily correlate with their differentiation and aggregation activities. Structural requirements for differentiation of HL-60 cells and aggregation of human blood platelet appeared to be more specific than those required for skin inflammation.

It is possible that the difference in potency, of the tested diterpene esters in different biological systems, is due to either the difference in their availabilities and hence phorbol ester/PKC interaction or due to the difference in the mechanisms of action. Early studies on the irritancy effects of the diterpene esters suggested that tiglianes may act in part by causing general tissue damage whereas the daphnane ortho ester diterpenes may elicit effect by means of a direct action at a receptor site in the skin (Evans and Schmidt 1979a). In their *in vivo* PKC binding studies of the phorbol esters Driedger and Blumberg (1980) have suggested that distinct biological targets may exist for the pro-inflammatory and the tumour promoting effects. In contrast, diterpene ester induced irritancy, with the exception of C20-substituted derivatives, correlated well with the ability to activate PKC *in vitro* (Ellis *et al* 1987). Potency of the HL-60 cell differentiation induction correlated well with the ability to activate PKC from mammalian brain (Aitken *et al* 1989) but not with irritancy or with tumour promotion .

Using the micro dilution assay developed during this study does not only assess the ability to differentiate HL-60 cells, it also provides preliminary information on possible activators or inhibitors of specific PKC isoforms. To establish a complete insight into the differentiation ability of the diterpene esters, ingenol derivatives should be tested for this activity.

The range of structurally related daphnane and tigliane types provided means for studying their behavior in a range of biological and chromatographic systems. Employing the same techniques to study a range of ingenol derivatives will provide the opportunity to establish the aetiology of biological effects of these naturally occurring diterpenes.

The use of HPLC and the advanced NMR techniques provide new opportunities to investigate the chemical and the biological changes in daphnane, tigliane and ingenane derivatives under prolonged storage conditions. The use of the short term *in vitro* assay for detecting HL-60 cell differentiation could apply for mass screening of plant extracts to detect naturally occurring irritant and possibly promoting substances. Correlation of the *in vitro* studies with the epidemiological studies could be applied to assess the risk of exposure to plants and plant products containing these diterpene esters and cancer development. The short term testing for biological activities of diterpene esters could be followed by long term evaluation. In order to evaluate the already known environmental promoters more specific promoting criteria for *in vivo* and *in vitro* tests are urgently needed. Present data from scientific research could be employed to draw up legislation for marketing potentially toxic plant material and potentially toxic plant-based remedies.

PUBLICATIONS

Shehadeh M., Dimetrijivic S., Hynes A., Ryves W.J., Evans F.J. (1993) Phorbol esters as probes of PKC function. ULLA First Summer School, The School of Pharmacy, London, July 1993, Poster 162.

Shehadeh M. and Evans F.J. (1994) Screening of extracts of selected species of the family Thymelaeaceae for skin irritant effects. Journal of Pharmacy and Pharmacology 46S, 1088.

Shehadeh M., Dimetrijivic S., Humer U., Ryves W.J., Evans F.J. (1995) HPLC-PDA, a method for analysing phorbol esters. Proceedings of the 36th annual meeting of the American Society of Pharmacognosy, Natural Products as Prototype Anti-infective: Discovery, Characterization and Development, Poster 138. Mississippi, Oxford Mississippi, USA.

Dimetrijivic S., Shehadeh M., Humer U., Ryves W.J., Evans F.J. (1995) A new method to analyse tiglianes and daphnanes phorbol esters using normal-phase HPLC. Proceedings of the 132nd British Pharmaceutical Conference, oral presentation no. 4, Warwick, UK.

APPENDIX

General Experimental Techniques

3.1 Spectroscopic Methods

3.1.1 Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR)

All spectra were run on a 400 MHz Bruker AMX 400 spectrometer or a 500 MHz Bruker AM 500 spectrometer, unless otherwise specified. Pure compounds were dissolved in deuteriochloroform ($CDCl_3$) (supplied by Sigma). Two drops of the internal standard tetramethylsilane (TMS) were added to the compound solution.

Deuterium exchange experiments were used to identify the OH and NH signals by shaking one drop of deuterium oxide (D_2O) (supplied by Sigma) with the compound solution and then re-running the sample. The OH and NH hydrogen exchanged rapidly with the deuterons, the HDO floated to the surface away from the region examined by the spectrometer and the signals of these groups disappeared from the spectrum.

Chemical shifts are quoted in parts per million and coupling constants (J) in Hertz (Hz). The following symbols are used: (s) represents a singlet, (d) represents a doublet, (dd) represents double doublet, (t) represents a triplet, (q) represents a quartet, (b) represents broad signal and (m) represents a multiplet.

3.1.2 Carbon Nuclear Magnetic Resonance (^{13}C-NMR)

All spectra were recorded on 400 MHz Bruker AMX (D) spectrometer at 100.6228 MHz. Pure compounds were prepared as described in the ^{1}H-NMR using TMS as an internal standard. Chemical shifts are also quoted in parts per million.

Distortionless enhancement polarization transfer technique (DEPT) was used to distinguish between signals for quaternary carbons and all others. Quaternary carbons gave

no signals in the DEPT spectrum and were recognized from the standard ^{13}C-NMR spectrum. The signals for CH and CH_3 carbons appeared up while those for CH_2 carbons appeared down .

3.1.3 Two Dimension NMR Spectroscopy

Strongly coupled protons connectivities were obtained form Correlated Spectroscopy (COSY). Nonbonding proton-proton interactions were obtained form the Nuclear Overhauser and Exchange Spectroscopy (NOESY).

Proton-carbon connectivities were obtained from one bond carbon-hydrogen NMR and long range carbon-hydrogen NMR spectra. All two dimension techniques were run on a 400 MHz Bruker AM spectrometer.

3.2 Mass Spectroscopy (MS)

Electron impact (EI) mass spectra were recorded on a VG Analytical LTD ZAB IF analytical ZAB-SE spectrometer by direct insert at 30-70eV at a maximum of 120°C. Samples were dissolved in a 2-nitrobenzyl alcohol plus sodium matrix (MNOBA+Na).

Chemical ionization (CI) mass spectra were recorded on VG Masslab 12-250 quadrupole instrument using ammonia as ionizing gas at temperatures between 170-220 °C.

Fast-atom bombardment (FAB-MS) spectra were recorded on a VG Analytical Ltd. ZAB IF spectrometer using xenon fast atom source at room temperature.

3.3 Ultraviolet Spectroscopy (UV)

All spectra were obtained from a Perkin-Elmer 402 Ultraviolet-Visible spectrometer using spectroscopic grade methanol (supplied by BDH) and quartz cells.

3.4 Infrared Spectroscopy (IR)

All spectra were obtained from a Perkin-Elmer 298 Infra-red spectrometer. Compounds were dissolved in analar grade chloroform (supplied by BDH). A thin film was

then pressed between two sodium chloride discs.

3.5 Solvents

Unless otherwise specified, general purpose grade solvents (GPR) (mainly supplied by BDH) were used for plant extraction and thin-layer chromatography. Solvents were redistilled and kept in air tight amber glass containers.

HPLC grade solvents were used for HPLC analysis, final purification of compounds and dissolving samples for biological tests.

3.6 Chromatography

3.6.1 Preparative Thin-layer Chromatography (PTLC)

Preparative thin-layer separations were performed on 20 cm by 20 cm glass plates. Plates were spread with 0.5 or 1 mm layer of acetone-washed silica gel GF_{254} with fluorescent indicator (Supplied by Fluka) buffered at PH 7. Plates were activated at 110°C for two hours. Once cool, the plates were washed with acetone and allowed to dry at room temperature. Once completely dry, plates were washed with methanol and allowed to dry before use.

Samples were dissolved in 0.5 ml chloroform or acetone and were applied 2.5 cm from the bottom of each plate, taking care not to apply more than 15mg to the 0.5mm thick plate and not more than 50mg to the 1mm thick plate.

Preparative partition thin-layer plates were prepared with acetone-washed kieselguhr G (Merck) (supplied by BDH) buffered at PH 7. Plates were activated at 110°C for two hours. After cooling, the plates were developed in acetone into their full length in 20% diethylene glycol (digol) in acetone and left to dry at room temperature for 30 minutes before streaking with samples (maximum of 10mg/0.5mm thick plate). After developing, bands were inspected under 254 nm and 365 nm ultraviolet lamps. A narrow strip at one edge was sprayed with 60% sulphuric acid and heated at 110°C for 15 minutes. Bands of interest were then scraped off and extracted with acetone. The eluates were dried under reduced pressure.

The residue was dissolved in 0.5% sodium chloride solution and then partitioned with dichloromethane. The liberated diterpenes in the dichloromethane were washed with water and then dried over anhydrous sodium sulphate. Solvent was evaporated under nitrogen gas and final traces were removed in vacuum desiccator.

3.6.2 High Speed Centrifugal Liquid Chromatography (CLC)

High speed centrifugal liquid chromatography was performed on silica gel discs. The glass discs (supplied by Toshiba) were prepared by slurring the appropriate weight of kieselgel 60 PF_{254} containing gypsum (Merck) (supplied by BDH) in distilled water buffered at PH 7.0. The silica, the distilled water and the buffer were cooled in the refrigerator overnight. The silica slurry was poured onto a rotating glass disc then left to dry for 48 hours at room temperature. The silica was then shaped using the Harrison Research scraping tools. The disc was loaded into the chromatotron with a constant stream of nitrogen and washed with acetone for 20 minutes then allowed to dry. Samples were dissolved in the least polar solvent system and were loaded to the saturated disc in a volume not more than 20ml. Samples were fractionated by gradient elution of increasing polarity and the disc was finally washed with methanol to remove polar impurities. Eluates were collected in 5ml fractions.

3.6.3 Preparation of the Buffer Solution

Kolthoff's borax phosphate buffer was used to buffer chromatography plates at PH 7.0. The buffer solution was prepared by dissolving 14.9gm of borax (supplied by M & B) and 16.6gm of potassium dihydrogen phosphate (KH_2PO_4) (supplied by BDH) in one liter of distilled water. The solution was diluted at a ratio 1:1 with distilled water immediately before to use.

3.6.4 Flash Column Chromatography

Flash column chromatography was carried out according to the technique described by Still *et al* (1978) using a glass flash chromatography columns (May & Baker). The column was dry packed with Sorbsil 60-H silica gel of particle size between 40-60 μm (Rhone-

Poulenc). The silica was saturated with solvents to expel any trapped air forced out by nitrogen gas.

Samples were applied to the top of the silica packed column using the minimum amount of solvent. The nitrogen was turned on to load the sample onto the silica. When the solvent reached the top of the silica, the nitrogen was turned off and the column walls were washed with little solvent to remove any traces of sample. Acid washed sand (supplied by BDH) was poured on top of the silica until a layer of 3cm was formed. The eluting solvent was added to the column and the nitrogen was turned on. Eluates were collected as 5ml fractions (unless otherwise specified).

Where the sample did not have sufficient solubility in the eluting solvent, it was dissolved in suitable solvent and mixed with five times the sample's weight of deactivated silica then evaporated until dry. The mixture is then poured on top of the column. Deactivated silica was prepared by shaking it with 10% of its weight of water until its appearance resembles a dry powder again.

3.6.5 Open Column Chromatography

Column chromatography was carried out in glass columns packed with slurry of silica gel (Merck) (supplied by BDH) having particles size of 0.2-0.5mm for early stages of purification. Biologically active fractions were finally purified on silica gel (Merck) having particles size of 0.06-0.2mm (supplied by BDH).

Samples were applied to the top of the column dissolved in 1-2ml of the starting solvent. If the sample was insoluble in the eluting solvent it was absorbed on deactivated silica (refer to 3.6.4). Samples were eluted with gradient solvent mixtures of increasing polarity. Fractions were collected in glass vials and grouped according to similarities on analytical TLC.

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