This is to certify that the

thesis entitled

BIOACTIVE CONSTITUENTS OF CURCUMA LONGA, L.

presented by

Geoffrey Nicholas Roth

has been accepted towards fulfillment
of the requirements for

M.S. degree in **Horticulture**

Major professor

Date 8/22/96

BIOACTIVE CONSTITUENTS OF *CURCUMA LONGA*, L.

By

Geoffrey Nicholas Roth

A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Horticulture

1996

ABSTRACT

BIOACTIVE CONSTITUENTS OF *CURCUMA LONGA*, L.

By

Geoffrey Nicholas Roth

Turmeric, the ground rhizome of the tropical monocot *Curcuma longa* Linn., has long been noted for its therapeutic potential, especially in traditional Indian ayurverdic medicine. For example, it possesses significant antioxidant and anti-inflammatory activities and is reported to produce symptom relief in patients with external cancer lesions.

Bioassay-directed fractionation of ethyl acetate extract from turmeric rhizomes yielded three main curcuminoid compounds, which displayed anticancer activity when tested for inhibition of topoisomerase I and II. Curcumin III (3) was the most active curcuminoid, inhibiting the topoisomerases at 25 ppm. Curcumin I (1) and curcumin II (2) inhibited the topoisomerases at 50 ppm. Fractionation of the volatile oil from the rhizomes afforded ar-turmerone (4) which displayed mosquitocidal activity with an LD_{100} of 50 ppm on fourth instar *Aedes aegyptii* larvae.

Bioassay-directed fractionation of hexane extract from the turmeric leaves yielded a diterpene aldehyde, labda 8(17) 12-diene-15,16 dial (5) with antifungal activity against

Candida albicans, at 1 ppm. Similarly, *Candida kruseii* and *Candida parapsilosis* showed activity at 25 ppm and 25 ppm, respectively. In addition, **5** displayed mosquitocidal activity on *Aedes aegypti* larvae with an LD_{100} of 10 ppm.

To my family

ACKNOWLEDGEMENTS

Thanks to my major advisor, Dr. Muraleedharan G. Nair, for his help and encouragement during this project and my guidance committee members, Dr. John Kelly, and Dr. James Miller for their advice and assistance.

A special thanks to Dr. Amitabh Chandra for his overall assistance in the Bioactive Natural Products Laboratory and his assistance in NMR spectral interpretations. Dr. James Nitao deserves thanks for his help with the insecticidal assays. I would also like to express my appreciation to Dr. Long Lee and Kermit Johnson in the Max T. Rogers NMR facility at Michigan State University for their help in obtaining NMR spectra on a very tight schedule.

I have been fortunate to work with many good people in this department and in the BNPL, especially Mark Kelm, Jennifer Miles, Dr. Yu-Chen Chang, Di Zhang, Andy Erickson and Haibo Wang.

A very special thanks to Abigail Holbrook, whose encouragement throughout this project has been invaluable.

Finally, thanks to my parents for always being there for me, no matter what.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

BNPL	Bioactive Natural Products Laboratory
$CHCl_3$	Chloroform
CD	Circular Dichroism
$CDCl_3$	Deuterated Chloroform
$(CD_3)_2CO$	Deuterated Acetone
COSY	Correlated Spectroscopy
DEPT	Distortionless Enhancement of Polarization Transfer
DMSO	Dimethyl Sulfoxide
EIMS	Electron Impact Mass Spectrometry
EtOAc	Ethyl Acetate
FABMS	Fast Atom Bombardment Mass Spectrometry
NMR	Nuclear Magnetic Resonance
PDA	Potato Dextrose Agar
TLC	Thin Layer Chromatography
VLC	Vacuum Liquid Chromatography
δ	Chemical Shift
d	Doublet

dd ..... Doublet of a Doublet

J ..... Coupling Constant

MeOH ..... Methanol

m/z ..... Mass-to-Charge ratio

MIC ..... Minimum Inhibitory Concentration

Rel. Int ..... Relative Intensity

Spp ..... Species

YPDA ..... Yeast Potato Dextrose Agar

LIST OF APPENDICES

CHAPTER I

Introduction

The Plant, *Curcuma longa,* Linn.

The tropical plant, *Curcuma longa,* L., with a long and distinguished human use in Eastern civilization, is native to south and southeast tropical Asia and probably originated in the slopes of hills in the tropical forests that grace the west coast of south India. It can be grown in many tropical conditions, e.g. from sea level to 1500m elevation in the temperature range of 20-30°C, in rainfall, or under irrigated conditions. It thrives in loose, friable loamy or alluvial soils suitable for irrigation and good drainage (Govindarajan, 1980; Verrill, 1940). A member of the Zingiberacae family, akin to ginger, this attractive tropical monocot grows to a height of 1 m with long green stems of parabolic cross section exiting a common rhizome node, and ending in large, ornately ovoid green leaves. The narrow, yellowish-white flowers are present on cylindrical spikes bearing green-white bracts. Since most cultivated varieties are sterile triploids, flowers and fruits are known but rare (Govindarajan, 1980), and the seeds have very low germination. However, this is not a problem since *C. longa* has been propagated vegetatively by the rhizome for thousands of years.

The plant often is referred to by the name of its bright orange rhizome, "turmeric". The sanctity of turmeric color has traced back to the ancient sun-worshiping culture, which began ceremonially using the cheap and easily grown tumeric as an alternative to saffron. The direct English translation of turmeric is "ginger yellow". Its name in the Malay Peninsula was "kunyit", the word for yellow. The genus "*Curcuma*" probably arises from the Arabic name

for turmeric, "kurkum" (Burkill, 1966; Brierley, 1994; Govindarajan, 1980). Of the Curcuma genus, a few species produce the turmeric of commerce; mainly *Curcuma longa* L. (syn. *Curcuma domestica* Valet), and to a smaller extent, *Curcuma aromatica* Salisb, *Curcuma amada* Roxb, *Curcuma zeodaria* Rosb (In India and China) and *Curcuma xanthorrhiza* Rosb (in Indonesia). The subterranial rhizome of *C. longa* is processed into spice. The *C. longa* rhizome consists of a mother rhizome, the stem extension, and many longer secondary rhizomes growing from the mother rhizome. The mother rhizome is known as *C. rotunda* and the secondary rhizomes are known as *C. longa.* This often creates confusion in the classification where the two forms are differentiated for trade (Govindarajan, 1980).

Over thirty varieties of turmeric (*C. longa*) are recognized now and are often associated with a specific state in India. The state of Andhra Pradesh is known for 'Amruthapani', 'Nizamabad', 'Cuddappa', 'Kasturi', 'Chaya', 'Kodur', 'Armoor', 'Duggirala', and 'Tekkurpeta' varieties; Bengal for 'Pattini' and 'Deshi'; Kerala for 'Moovattupuzha','Alleppey', and 'Wynad'; Maharashtra for 'Rajpuri', 'Karhadi' and 'Waigan'; Tamil Nadu for 'Chinnanadan', 'Salem', 'Karur', 'Erode' and 'Perianadan'; and Orissa for 'Behrampuri', 'Koraput', and 'Saveera'. Other varieties include 'Lokhandi halad' (used for dyeing), 'Kuchupudi', 'Sugandham', 'Erode', 'Gadhvi', 'Amoor (CLL 324), 'Duggirala (B,9)', 'Duggirala (CLL 325-B,55)', 'Gorakpur (CLL 316)', 'Karhadi', 'Mydkur (CLL 326-B,52)', 'Rajpore', 'Rajpore (CLL 327)', 'Tekkurpetta (CLL 327)', 'Vontimetta', 'Vontimetta (CLL 322)', and 'Miraj 26' (Shivashankar, 1976; Gopalan, 1976; Govindarajan, 1980). The world's largest turmeric production is the state of Andhra Pradesh, India. In

1973, the 13,600 hectares yield was about 38,000 tonnes of dry turmeric at 2.79 tonnes hectare^{-1}. The state of Madras in India is the second largest producer, followed by the states of Maharashtra, Orissa, Tamil Nadu, Bihar, Kerala, Assam, Mysore, West Bengal, and Tripura (Vervai, 1971).

History of turmeric

In contrast to turmeric's limited use in Western countries as coloring agent for mustard, pickles, and textiles (Survey, 1968), most turmeric is consumed in the countries of origin, where its uses are unlimited. Almost every religious Hindu ceremony makes use of turmeric in one form or another (Kurup, 1979). Addition of lime to turmeric produces a deep brownish-red material known as "kum-kum", which is the material used by Indian women to make the auspicious mark on their foreheads (Govindarajan, 1980). Turmeric's use extends back to the time of the Pharaohs, when it was an ingredient in balm of Gilead, and an ingredient in preparation of Egyptian mummies (Verrill, 1940). It was mentioned in ancient Sumerian inscriptions, dating back further than 2000 B.C. Turmeric was one of the principal spices (the scriptural "calamus") besides myrrh and sweet cinnamon which were considered to compose the perfume compounded by Moses at Jehovah's command in the Old Testament Christian Bible (Verrill, 1940). It is mentioned in the Assyrian Herbal (551-479 B. C.), as a medicine for external and internal use, and as a fumigant and beer condiment. Dioscorides wrote about turmeric and called the spice "cyperis". Theophrastus referred to it as "cypeiros", and noted its similarities to saffron (Miller, 1969). The plant also has a long history of folk medicinal use in India and the Orient.

The plant has been used as a stimulant, tonic, stomach soother, fever alleviator, dropsy cure, and for cleaning foul ulcers (Lindley, 1838). Turmeric paste is applied to facilitate scabbing in chicken pox and small pox (Kuttan, 1985). The rhizome has been used as a blood purifier, liver stimulant, and to remove catarrhal formations leading to jaundice of the liver and gallbladder, which is possible since curcumin dissolves cholestrin, a component of bile and gallstones (Harris, 1972; Vevai, 1971). The rhizome has been used for hazy vision, eye inflammation, night blindness, subnormal temperature, body pain, rheumatism, to regain consciousness, scabies, sores, infantile fistula ani, cough and to promote lactation. The flower is used for sores in the throat, syphilis, dyspepsia, and cholera (Jain et al., 1991). Other reported rhizome uses were as alterative, antiperiodic, antiseptic, carminative, tonic, vermifuge, for anorexia, biliary disorders, coryza, diabetic wounds, hepatic disorders, sinusitis, eosinophilia, and antifertility. Also it is suggested that burning turmeric cures head colds (Kurup, 1979; Waring, 1982). Interestingly, it has been observed that turmeric given to animals causes them to produce male offspring predominantly. In Africa, turmeric is used to prevent pregnancy, and its use to control population may be feasible (Kuttan, 1994).

Turmeric in commerce

India is the largest producer and exporter of turmeric, providing 90% of the world production, which is sold in powdered forms or as whole rhizomes (Shivashankar, 1976). The mother rhizomes are known as "bulbs", and the primary and secondary branches are known as "fingers". In terms of export, west Asian countries prefer 'bulbs', while American and European markets take 'fingers'. In particular, the United States prefers Alleppey

turmeric from the state of Kerala, which is also one of the more expensive varieties of *C. longa* because it has brighter color and better "non-fade" characteristics than other varieties (Survey,1969 ;Govindarajan, 1980). The quality of color desired by the United States market is understandable considering its applications in food coloring.

The United States is the largest user of spice oleoresins; approximately 29 different oleoresins are demanded in the United States annually. Of these oleoresins, eight represent 90% of the volume of oleoresins used in the United States. Among these, turmeric oleoresin is sixth in importance. Its main uses are in mustard paste, pickles and relishes (Govindarajan, 1980; Unterhalt, 1980). It also is used in the manufacture of mixes, soups, canned products, and wrapped confectionary (Wilfred, 1980). The principal yellow pigment in turmeric is curcumin; several national and international patents on the use of curcumin as a food coloring are held by companies such as General Foods Corp. and McCormick & Co., Ltd. (Francis, 1986). The turmeric extract is a suitable replacement for FD&C Yellow #5. As such, it is stable at low pH, and can be used to color dry beverage mixes, pudding mixes, confectionary products, and foods where good clarity is needed (Andres, 1982). Its stability at low pH has, however, caused some concern in quality control of the citrus industry, where it was used to adulterate orange juice (Petrus, 1984). Also, turmeric can be used in combination with annatto extracts to impart yellow hues to cereal products. Food coloring powder (turmeric extract, silica gel, and propylene glycol) and an oil-soluble liquid from turmeric for food coloring (turmeric extract and vegetable oil) are produced for this purpose (Freund, 1985). Under the FDA regulation, certain color additives are certified or exempt from certification, and turmeric and turmeric oleoresin are two additives that fall in this category (Hallagan,

1991). Many additives that are exempt from certification fall under the category of natural colors, as in the case of turmeric. Turmeric's close match to FD&C yellow No. 5 and FDA exemption has allowed it to be used in even more products such as canned beverages, dairy products, sauces, cheeses, salad dressings, and margarine. In some color blends, it is purposely utilized for its antioxidant properties (Lauro, 1991).

Microbial diseases and pests of turmeric

Interestingly, for all of its antimicrobial and insecticidal/insect repellant activities, turmeric has plenty of pests and needs constant protection. (Anjaneyulu, 1968; Govindarajan, 1980; Rao, 1977; Vevai, 1971). The main insect pest of turmeric is the cigarette (or "tobacco") beetle, *Lasioderma serricorne. Lasioderma* caused damage in the form of weight loss in the turmeric rhizome. The infestation was identified by characteristic emergence of holes visible on infested rhizomes. Dissection of these rhizomes revealed as few as three and as many as thirty beetle larvae, each one in its own "cell". Other pests include the common shoot borer *Dichocrocis punctiforalis,* that causes damage to pseudostems and rhizomes, and the leaf roller caterpillar (or "skipper"), *Udaspes folus.* These two pests are controlled by spraying 0.05% dimethoate or phosphamidon. Another pest, the scale insect (or "stores") *Aspidiotus hartii* causes damage to the turmeric rhizome in the field and storage. It is controlled by dipping rhizomes in 0.05% malation or dimethoate. (Regupathy, 1976; Govindarajan, 1980). Other insect pests include the rhizome flies and fly maggots (*Calobata Spp., Formosina flavipes),* hairy caterpillars (*Diacrisia obliqua),* thrips (*Panchaetothrips indica),* lacewing bugs (*Stephanitis typicus),* scale insects (*Aspidiotus curcumae),* leaf beetles

(*Lema praeusta and Lema signatipennis and Lema semi regularis),* red pumpkin beetles (*Aulacophora intermedia),* mealy bugs, and jassids (*Tettigoniella ferruginea)* (Vevai, 1971). In addition, a dry rot of the rhizome was reported due to the association of *Fusarium* and an unidentified nematode (Sarma, 1974).

The most serious microbial disease is the leaf blotch *Taphirna maculans,* which dries up the leaves and affects the rhizome yield significantly. It has been controlled by application of chemical pesticide. A leaf spot caused by *Collectotricum capsici* is a fungal disease that also effects rhizome yield by drying up the affected leaves. It has been controlled by spraying pesticide preventively or at onset of infection. Another disease (leaf soft rot) is caused by *Pythium graminicolum,* which passes from turmeric to its seed, and affects turmeric by rapid and total loss of the crop, starting with leaf drying, softening of pseudostems, and rhizome decay. It has been controlled by treating seed with pesticide prior to planting, or in the field (Govindarajan, 1980). Other diseases include athracnose leaf spot (*Colletotrichum zingiberis),* collar rot (*Corticium rolfsii),* leaf blotch (*Taphrina deformans),* rhizome rot, and leaf rust (*Puccinia curcumae)* (Vevai, 1971).

To some degree, incidence of various pests and diseases of turmeric in India has been contingent on the area in India where the turmeric is being produced. For instance, Andhra Pradesh was plagued by shootborers, lacewing bugs, thrips, skippers, leaf spot, leaf blotch, leaf soft rot and leaf rust; Assam by leaf blotch; and Bihar by the hairy caterpillar, leaf spot and leaf blotch. Other states including Delhi, Gujara, Haryana, Malabar, Tamil Nadu, Kerala, Maharashtra, Salem, Sangli, Mysore, Orissa, Punjab, Uttar Pradesh and West Bengal have also been plagued by a variety of pests and microbial diseases. These included leaf spot, leaf

blotch, leaf soft rot, leaf rust, skippers, shootborers, lacewing bugs, scales, thrips, rhizome flies, leafbeetles, red pumpkin beetles, and hairy caterpillars.

Although they were not strictly considered pests, but rather are a nuisance to processors and present potential health risks, certain microbes have contributed to a large contamination in harvested turmeric rhizomes, and are worth mentioning. Turmeric has had a large incidence of aerobic thermophiles and mesophilic spores, in addition to a small population of non-coagulase type *Staphylococci* and *Clostridium perfringens,* although *Salmonella* were absent (Krishnamurthy, 1971). Trade samples of turmeric bulbs contained high amounts of coliforms, but the low incidence of coliforms in general was not considered a health risk (Krishnamurthy, 1973). Also, large amounts of yeast and mold were found in turmeric powder (Mattada, 1974).

Bioactive Compounds of Turmeric

For hundreds of years, spices (especially turmeric) were used in Asia and around the world to protect food from spoilage and mask the rancid taste of spoiled food. Antioxidant and antimicrobial qualities of turmeric have been found responsible for this activity. About 0.5% of turmeric considerably reduced formation of peroxides in groundnut oil in accelerated stability tests (Rimpler, 1970). Turmeric showed significant antioxidant activity when added to olive, soybean, sesame and linseed oils (Rimpler, 1970) . Turmeric is used commonly with many fish preparations. A dip treatment of turmeric on headless white shrimp did not develop black melanoses and odor as observed in the control. Shrimps treated with the turmeric dip were void of melanoses and gave an appealing and spicy aroma (Govindarajan, 1980;

blotch, leaf soft rot, leaf rust, skippers, shootborers, lacewing bugs, scales, thrips, rhizome flies, leafbeetles, red pumpkin beetles, and hairy caterpillars.

Although they were not strictly considered pests, but rather are a nuisance to processors and present potential health risks, certain microbes have contributed to a large contamination in harvested turmeric rhizomes, and are worth mentioning. Turmeric has had a large incidence of aerobic thermophiles and mesophilic spores, in addition to a small population of non-coagulase type *Staphylococci* and *Clostridium perfringens*, although *Salmonella* were absent (Krishnamurthy, 1971). Trade samples of turmeric bulbs contained high amounts of coliforms, but the low incidence of coliforms in general was not considered a health risk (Krishnamurthy, 1973). Also, large amounts of yeast and mold were found in turmeric powder (Mattada, 1974).

Bioactive Compounds of Turmeric

For hundreds of years, spices (especially turmeric) were used in Asia and around the world to protect food from spoilage and mask the rancid taste of spoiled food. Antioxidant and antimicrobial qualities of turmeric have been found responsible for this activity. About 0.5% of turmeric considerably reduced formation of peroxides in groundnut oil in accelerated stability tests (Rimpler, 1970). Turmeric showed significant antioxidant activity when added to olive, soybean, sesame and linseed oils (Rimpler, 1970) . Turmeric is used commonly with many fish preparations. A dip treatment of turmeric on headless white shrimp did not develop black melanoses and odor as observed in the control. Shrimps treated with the turmeric dip were void of melanoses and gave an appealing and spicy aroma (Govindarajan, 1980;

Hirahara, 1974).

Turmeric was a better antioxidant than BHA (butylated hydroxyanisole) (Kuttan, 1994). At least one component of turmeric that contributed to the antioxidant action was the turmeric anti-oxidant protein (TAP), isolated from the aqueous extract of turmeric (Selvam, 1995). The TAP was heat stable. It showed a concentration-dependent inhibitory effect on the promoter-induced lipid peroxidation. Inhibition at 50% was observed at a TAP concentration of 50 μg/ml. Up to 50% of the initial activity of Ca^{2+} -ATPase from rat brain was protected by the lipid-peroxidant-induced inactivation by the TAP. This protective effect was shown to be associated with binding of turmeric and -SH moieties (Selvam, 1995). In addition, it was demonstrated that turmeric possibly lowered lipid peroxidation by promoting high levels of activity of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in male rats (Reddy, 1994). Curcumin is an effective scavenger of reactive oxygen species, and decreased formation of inflammatory agents such as prostaglandins and leukotrienes (Huang, 1991; Reddy, 1994). The presence of curcumin in turmeric makes turmeric an ideal dietary antioxidant (Reddy, 1994).

Turmeric has potential in cancer prevention. Feeding turmeric to mice prevented tumor formation normally caused by benzopyrene, 3 - methyl cholanthrene, and 3'-methyl-4-dimethylaminobenzene (Polasa, 1991; Reddy, 1994). Turmeric inhibited mutagenicity of cigarette smoke condensates and tobacco extracts (Nagabhushan, 1987). Curcumins, the yellow components of turmeric, inhibited cancer produced by a number of chemicals on skin, stomach, and guinea pig pouch (Kuttan, 1994). Turmeric showed anticancer activity in vitro using Dalton's lymphoma cells, and against lymphocytes at a concentration of 4 $\mu g \cdot ml^{-1}$.

Hirahara, 1974).

Turmeric was a better antioxidant than BHA (butylated hydroxyanisole) (Kuttan, 1994). At least one component of turmeric that contributed to the antioxidant action was the turmeric anti-oxidant protein (TAP), isolated from the aqueous extract of turmeric (Selvam, 1995). The TAP was heat stable. It showed a concentration-dependent inhibitory effect on the promoter-induced lipid peroxidation. Inhibition at 50% was observed at a TAP concentration of 50 μg/ml. Up to 50% of the initial activity of Ca^{2+} -ATPase from rat brain was protected by the lipid-peroxidant-induced inactivation by the TAP. This protective effect was shown to be associated with binding of turmeric and -SH moieties (Selvam, 1995). In addition, it was demonstrated that turmeric possibly lowered lipid peroxidation by promoting high levels of activity of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in male rats (Reddy, 1994). Curcumin is an effective scavenger of reactive oxygen species, and decreased formation of inflammatory agents such as prostaglandins and leukotrienes (Huang, 1991; Reddy, 1994). The presence of curcumin in turmeric makes turmeric an ideal dietary antioxidant (Reddy, 1994).

Turmeric has potential in cancer prevention. Feeding turmeric to mice prevented tumor formation normally caused by benzopyrene, 3 - methyl cholanthrene, and 3'-methyl-4-dimethylaminobenzene (Polasa, 1991; Reddy, 1994). Turmeric inhibited mutagenicity of cigarette smoke condensates and tobacco extracts (Nagabhushan, 1987). Curcumins, the yellow components of turmeric, inhibited cancer produced by a number of chemicals on skin, stomach, and guinea pig pouch (Kuttan, 1994). Turmeric showed anticancer activity in vitro using Dalton's lymphoma cells, and against lymphocytes at a concentration of 4 $\mu g \cdot ml^{-1}$.

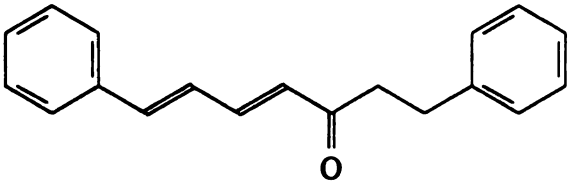
Also, turmeric extract inhibited cell growth in Chinese hamster ovary cells at 4 ppm. A cytotoxic effect of turmeric was found within 30 minutes at 30° C (Kuttan, 1985). Ethanolic extracts of turmeric relieved symptoms in patients with external cancerous lesions (Kuttan, 1987). The yellow coloring material of turmeric, the curcumin, was implicated for the therapeutic potentials of turmeric (Kuttan, 1994). Curcumin had radical-scavenging antioxidant activity against lipid peroxidation in various media, suppressed free-radical-induced oxidation of methyl linoleate in solutions and aqueous emulsions, and was a comparable as an antioxidant to isoeugenol (Noguchi, 1994). Also, turmeric was reported to have higher antioxidative properties than BHT (butylated hydroxytoluene) as indicated by the antioxidant index (AI) (Lee, 1982; Noguchi, 1994). Turmeric's components also inhibited lipid peroxidation induced by ascorbic acid and ferrous sulphate in erythrocyte membrane (Salimanth, 1986).

The anti-inflammatory activity of turmeric and curcumin was as good as aspirin or ibuprofen, and was successfully used to treat arthritic patients (Kuttan, 1994). Two major principles of anti-inflammatory drug activity are available; those inhibiting synthesis of prostaglandins by interfering with the cyclooxygenase system (such as salicylic acid), and glucocorticosteroids which inhibit the cyclooxygenase pathway and the lipoxygenase pathways. Curcumin inhibited 5-lipoxygenase activity in rat peritoneal neutrophils and 12-lipoxygenase and cyclooxygenase activities in human platelets (Ammon, 1993).

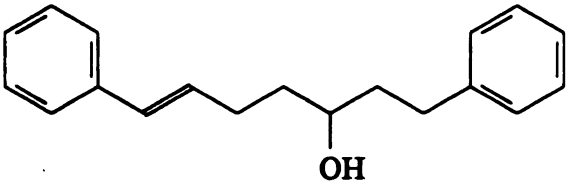
Curcumin, the anti-inflammatory agent in turmeric, has the caffeic acid moiety and is largely responsible for the inhibition of lipid peroxidation. Two caffeic acid molecules joined through a methylene bridge results in a bis-desmethoxy-curcumin. It has more anti-oxidant

activity than curcumin, ferulic acid, caffeic acid, p-hydroxy cinnamic acid, 0-hydroxy cinnamic acid, cinnamic acid, or 3,4,5-trimethoxy cinnamic acid. Methylation of hydroxy groups reduced its antioxidant character (Sharma, 1976). Sodium curcuminate, tetrahydro curcumin, curcumin, phenyl butazone, and triethyl curcumin in decreasing order were found effective in carrageenin-induced rat paw edema and cotton pellet granuloma tests. Ferulic acid and diacetyl curcumin also were tested, but found devoid of anti-inflammatory activity. Interestingly, another plant from the Zingiberaceae family, *Curcuma xanthorrhiza*, yielded three non-phenolic diarylheptanoids, alnustone, *trans* -1,7-diphenyl-1-hepten-5-ol, and *trans, trans*-1,7-diphenyl-1,3-heptadien-5-ol, with anti-inflammatory activity (**Figure I**). These compounds were related to the curcuminoids, but only contained one carbonyl group, and displayed significant anti-inflammatory effects in the assay of carrageenin-induced hind paw edema in rats (Claeson, 1993). The activity of these compounds indicated that phenol groups are not necessary for the anti-inflammatory activity. Curcumin inhibited TPA and arachidonic acid induced epidermal inflammation in mice more than chlorogenic, caffeic, and ferulic acids. Specifically, 3, 10, 30 or 100 μM of curcumin, in vitro, inhibited metabolism of arachidonic acid to 5-hydroxyeicosaterabaenoic acid (5-HETE) by 40, 60, 66, or 83%, respectively, and to 2-HETE by 40, 51, 77 or 85% (IC_{50} = 5-10 μM) (Huang, 1991). However, sodium curcuminate was not anti-pyretic or analgesic, and did not inhibit the arachidonic-acid-dependent pathway of platelet aggregation. Therefore, it is not likely that the anti-inflammatory activity of curcumin derivatives is mediated by inhibition of prostaglandin synthetase enzyme. In any case, the anti-inflammatory activity of turmeric

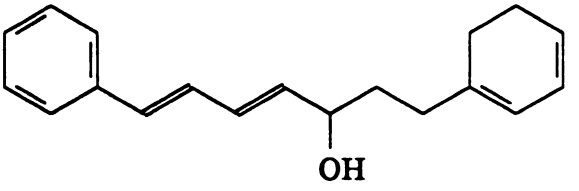
Figure 1



alnustone



trans-1,7-diphenyl-1-hepten-5-ol



trans,trans-1,7-diphenyl-1,3-heptadien-5-ol

explains its effective use against pain and inflammation in Indian herbal medicine (Mukhopadhyay, 1982).

Curcumin inhibited the response of blood neutrophils to superoxide anion (Srivastava, 1989; Satoskar, 1986). Its scavenging effects on active oxygen radicals was reported to be stronger than vitamin E, (Zhao, 1989) and it protected DNA from peroxidative injury (Shalini, 1987). Turmeric has become increasingly important in diet for preventing cancer and genotoxicity through its action as a potent antioxidant. Antimodulatory effects of turmeric and curcumin on different levels of benzopyrene-induced DNA (BP-DNA) adducts in rat liver have been studied by ^{32}P-postlabelling experiments. Turmeric at 0.1, 0.3 and 3% and curcumin at 0.03% levels in the diet significantly reduced the levels of BP-DNA adducts (Mukundan, 1993). Turmeric may prove effective in treating cancer, since it is very non-toxic to humans. About 50 g of turmeric per day was not toxic to humans; it is nonmutagenic, non-carcinogenic and non-teratogenic. It was effective in reducing animal tumors, and both turmeric and curcumin inhibited the fibrosis induced by ethanol and carbon tetrachloride in animals. Turmeric showed excellent preventative activity against carbon tetrachloride-induced liver injury *in vivo* and *in vitro*. It has been proposed that turmeric may be useful for humans by preventing liver disease, including cancer, in people who drink large amounts of alcohol (Kuttan, 1994; Kuttan, 1985; Kiso, 1983). Turmeric showed no signs of toxicity (or spermatotoxic effects) in mice at doses of $3g \cdot kg^{-1}$, although CNS stimulation was observed (Qureshi, 1992).

The properties of turmeric go beyond antioxidant and anti-inflammatory activities. Curcumin inhibited platelet aggregation induced by arachidonate, adrenaline and collagen

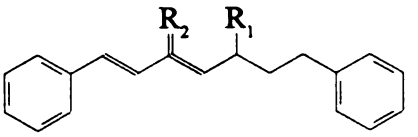
(Srivastava, 1994). Also, topical applications of curcumin inhibited TPA-induced tumors on mouse skin better than chlorogenic acid, caffeic acid or ferulic acid, at 10 μmol concentrations (Huang, 1988). Curcumin inhibited chemical carcinogenesis, (Kuttan, 1989) and its components were noted for antimicrobial activities. An alcohol extract of turmeric inhibited the growth of *Sarcina, Gaffkya, Corynebacterium, Clostridium* strains at 0.5-5 $mg \cdot ml^{-1}$ concentration. Curcumin and essential oil of the rhizome had activities at 5-100 ppm on these strains (Lutomski, 1974). In vitro study of antibacterial activity of turmeric indicated that the sodium salt of curcumin was "antimicrobial at 1 ppm." The essential oil fraction exhibited similar types of activity at high concentrations, with the whole oil being more active than the purified compounds (Ramprasad, 1956; Chopra, 1961; Munasiri, 1987).

Curcumin, when illuminated (with unspecified wavelength light), exerts potent phototoxic effects in micromolar amounts against gram-positive bacteria (Dahl, 1989). Specifically, curcumin was phototoxic to *Salmonella typhimurium* and *Escherichia coli* (Tonnesen, 1987). Although turmeric showed inhibitory effects on the growth of intestinal and pathogenic bacteria in vitro (Chopra 1956; Shankar, 1979), turmeric extracts were less active against gram-positive and gram-negative bacteria than penicillin and streptomycin (Basu, 1971). The antibacterial quality has been attributed to curcumin (Chopra, 1956; Shankar, 1979). Turmeric showed antifungal activity against *Aspergillus parasiticus*, which produces a very potent mycotoxin, aflatoxin. This toxin is a major contaminant of food, and produces severe liver diseases including cancer, and eventually may result in death. Poultry are very susceptible to this toxin; an intake of 100 mg turmeric/day/bird is recommended as a preventative measure against the fungus (Kuttan, 1994).

Turmeric possesses insecticidal activity. It was active as a repellant against the grain borer (Jilani, 1990), the red flour beetle (Jilani, 1988), the housefly *Musca domestica* (Singh 1991), ants (Viswanath, 1981), and *Tripilium castaneum* (Su, 1982). Also, growing turmeric under coconut trees reportedly protected them from white ants (Kuttan, 1994). Alcoholic extracts of turmeric showed anti-protozoal activity against *Entamoeba histolytica* (Dhar, 1968; Gopalan, 1976). Turmeric showed activity against roundworms, threadworms, and other intestinal parasites (Abbiw, 1990). It is antihelmenthic not only in the body, but also when applied topically (Kuttan, 1994). Turmeric proved an effective cure for scabies, and when used as a topical paste treatment on 814 people, cured the patients in 97% of the cases within 3 to 15 days of treatment (Charles, 1992). Combinations of demethoxycurcumin (curcumin II) and bisdemethoxycurcumin (curcumin III) were nematicidal against *Toxocara canis,* but the curcuminoids were ineffective when applied independently (Kiuchi, 1993). Interestingly, five di-phenylheptanoids (**1-5, Figure 2**) isolated from a related plant, *Curcuma comosa,* demonstrated nematicidal activity against *Caenorhabditis elegans.* These compounds are similar to curcumins, except for absence of methoxy and hydroxy substituents in their aromatic rings (Jurgens, 1994).

The volatile oil from turmeric rhizomes possesses other bioactivities as well. The main component, ar-turmerone, exhibited antivenom effects. Brazilian people used cut rhizome slices against insect bite and for allergic reactions from contact with caterpillars. Aqueous extract of the rhizomes was considered to eliminate neuromuscular inhibition from the neurotoxin of *Naja naja siamensis* (cobra) bite (Cherdchu, 1978). Fractions consisting of ar-turmerone removed hemorrhagic activity from *Bothrops jararaca* venom. This was

Figure 2



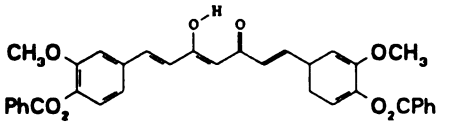
1. R_1 = H, $OCOCH_3$; R_2 = H
2. R_1 = O; R_2 = H
3. R_1 = H, OH; R_2 = H
4. R_1 = H, OH; R_2 = H
5. R_1 = O; R_2 = OH

shown by mixing 10 μg venom (equivalent to 10X the minimum hemorrhagic dose) with 100 μl of saline and ar-turmerone at pH=7, and injecting the solution intradermally into mice. Also, in vitro studies indicated that hexane extracts from turmeric inhibited proliferation of natural killer activity (NK) of human lymphocytes in a dose-dependent manner. An inflammatory reaction is the result of cell population interactions, including lymphocytes (Ferreira, 1992), so this indicated that anti-inflammatory properties of turmeric may have to do with inhibition of lymphocyte activation. Ar-turmerone was as potent as the original crude extract.

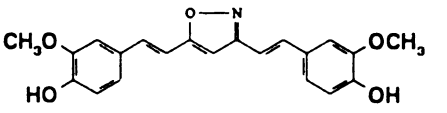
Turmeric reduced cholesterol in blood by reducing cholesterol uptake from the gut. It inhibited serum LDL peroxidation, which can lead to atherosclerotic lesions, and hence turmeric may prevent coronary and heart problems (Kuttan, 1994). Specifically, curcumin increased HDL and decreased LDL. Turmeric is also an antidiabetic; the action may be due to stimulation of pancreas cells, and increased insulin production (Kuttan, 1994). In addition, Curcumin was found to contribute to lowering of blood sugar in diabetics (Ramprasad, 1956; Nagabhushan, 1987).

Curcumin was a modest inhibitor of HIV-1 and HIV-2 proteases (Sui, 1993). These proteases are encoded by HIV viral genomes and are responsible for processing the precursors produced from *gag* and *pol* genes into proteins needed for replication and production of mature viruses (Farmerie, 1987). Inactivation of the HIV-1 protease yields non-infectious virions (Kohl, 1988). The curcuminoids were assayed for anti-HIV protease activities (Sui, 1993). The curcuminoids **1-4** and the boron-curcuminoid complexes **5-9** (**Figure 3**) exhibited activity against the HIV proteases (Sui, 1993). Curcumin-boron

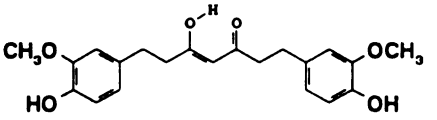
Figure 3



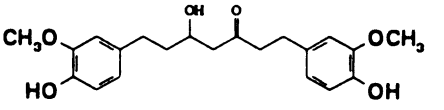
1



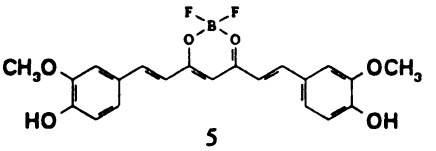
2



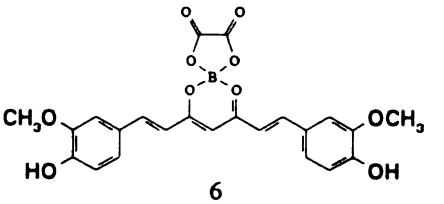
3



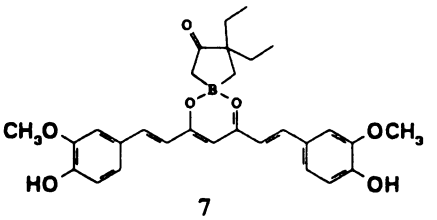
4



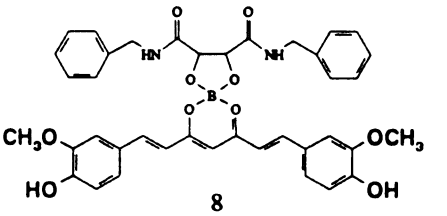
5



6



7



8

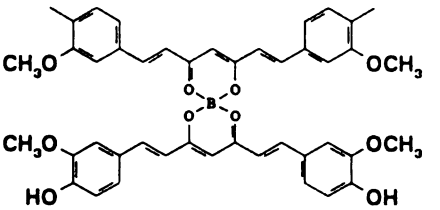
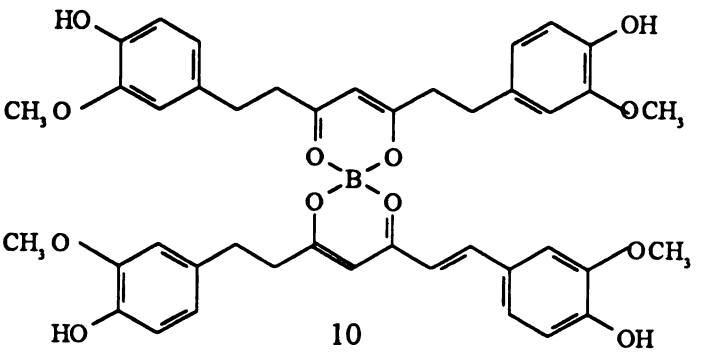
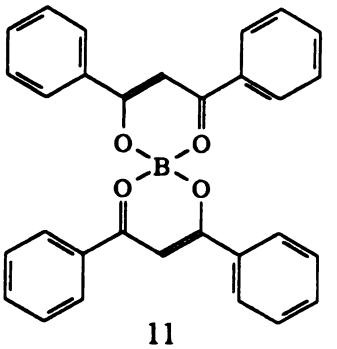


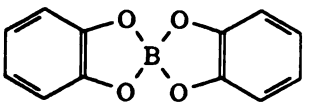
Figure 4



10

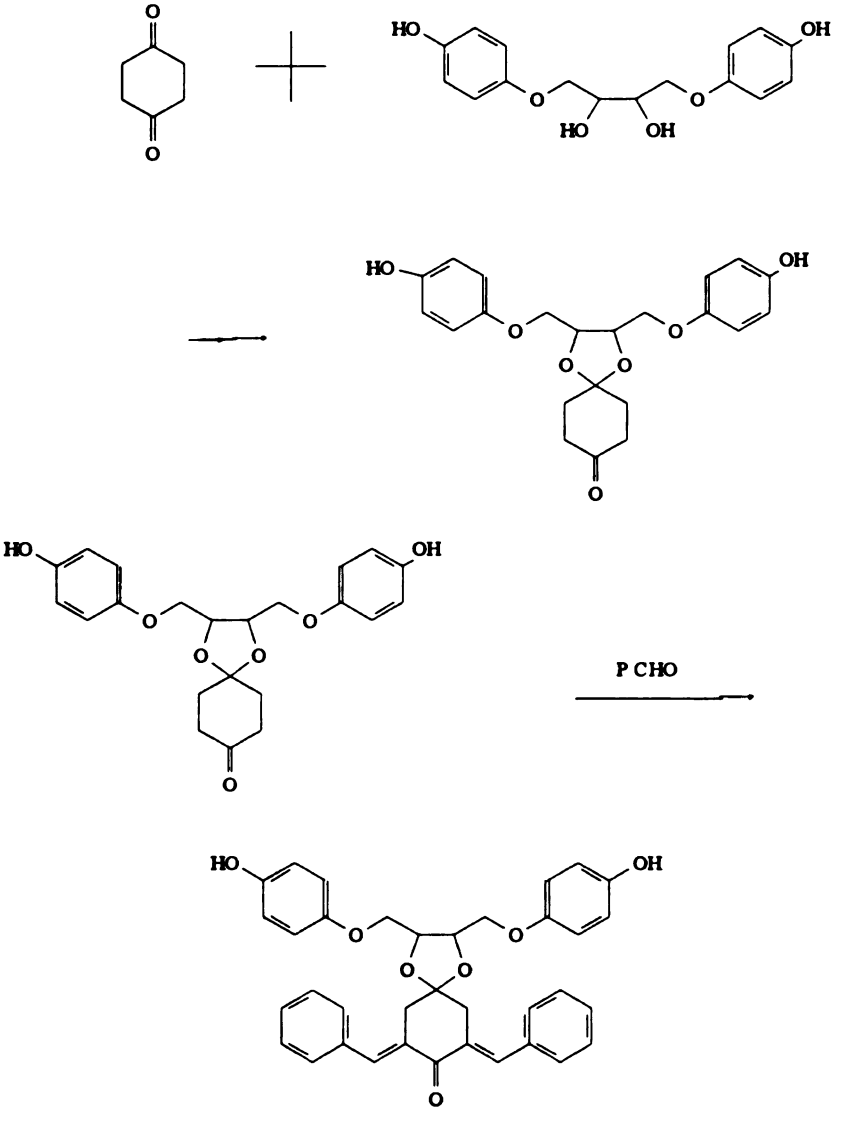


11



12

Figure 5



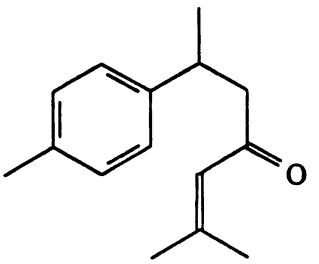
13

complexes exhibited higher activity than curcuminoids against the HIV proteases (Sui, 1993). To insure that activity was not due to boron alone, and to investigate the importance of the boron-curcuminoid complex in the inhibitory activity, compounds **11-12 (Figure 4)** were assayed against the HIV-1 and HIV-2 proteases (Sui, 1993). No activity for these compounds was observed, indicating that the boron entity itself was not responsible for the activity (Sui, 1993). However, the boron complex of curcuminoid (**10**) did not show activity against HIV proteases (Sui, 1993). Compound **13 (Figure 5)** was synthesized and assayed against the HIV proteases. It displayed no activity against the proteases. Therefore, it indicated the importance of the boron-curcuminoid complex in the inhibition of the proteases (Sui, 1993).

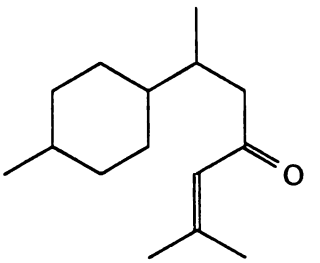
Chemical composition of turmeric

Govindarajan (1980) reported that the composition of the turmeric rhizome varies with variety, growth conditions, maturity of rhizome and time of harvest, although there are at least a few components that were consistently present. Turmeric contained 2.5-6% curcuminoids and 3-5% essential oil, which is composed of 58% turmerones (Alexander, 1973) , 25% zingiberene, 1% phellandrene, 1% cineole, 0.6% sabinene, and 0.5% borneol (Shivashankar, 1976) **(Figure 6)**. Different sources gave conflicting reports on these ingredients, but a few of them are well documented. It is reported that next to ginger oil, turmeric oil was the best source of zingiberene (Survey, 1969). It was proposed that ar-turmerone is an artifact formed during the steam distillation process of the volatile oil, but work by Govindarajan showed this to be false; he reported that ar-turmerone and turmerone

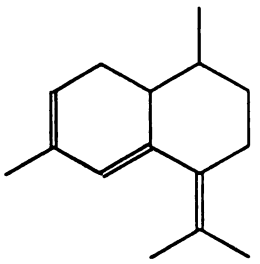
Figure 4



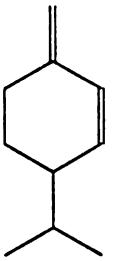
ar-turmerone



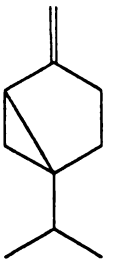
turmerone



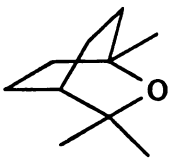
zingiberene



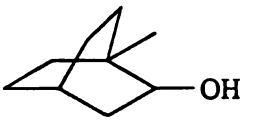
phellandrene



sabinene



cineole

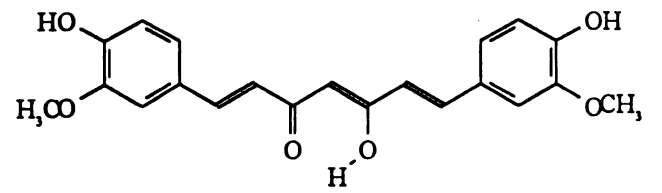


borneol

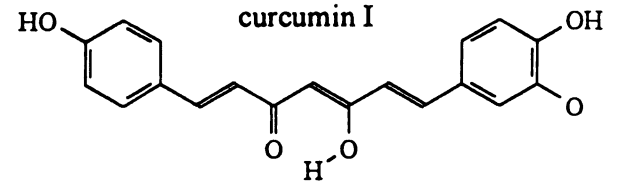
existed in the rhizome in the proportion of 2.5:1. Ar-turmerone was reported to have antivenom activity (Ferreira,1992). However, the majority of bioactivity claims of turmeric's use in commerce are due to the presence of curcuminoids. The rhizomes of turmeric yield three major phenolic pigments, curcumin I, curcumin II, and curcumin III (**Figure 7**). Comparative studies reported that all three curcuminoids inhibited superoxide production and tumor growth; curcumin III was the most active. Also it exhibited higher activity in cytotoxicity assays (Anto, 1994). At least two other curcuminoids were identified from turmeric, (Nakayama, 1992) curcumin IV and curcumin V (**Figure 7**). Another related compound, dihydrocurcumin, an unsymmetrical diaryl heptanoid, was isolated from *C. longa* (Ravindranath, 1980) (**Figure 7**). Only curcumin I, II and III have reported activities.

Mayer reports that the structure of curcumin was elucidated in the early 1900s; in 1870 Daube isolated crystalline curcumin, and Perkin and Philps obtained curcumin by precipitating lead curcuminate from the alcohol extract of turmeric (Mayer, 1943). In 1897, Ciamician and Silber proposed that curcumin was a diferuloyl methane, and Mayer (1943) vouches that this idea was confirmed by von Kostanecki and Lampe, who identified the structure by studying degradative products of curcumin. Boiling curcumin with alkali caused it to degrade to vanillic and ferulic acids. Curcumin reacted with alkali to give protocatechuic acid, with permanganate to give vanillin, and with acetic anhydride to give acetyl-curcumin. The structure of curcumin was elucidated to be diferuloyl methane in the enolic form (Mayer, 1943). Lampe (1919) synthesized curcumin by condensing carbomethoxy feruloyl chloride with ethyl acetoacetate to give an ester. This ester was hydrolyzed to the diketone followed by the condensation with another carbomethoxy feruloyl chloride to give the diferuloyl

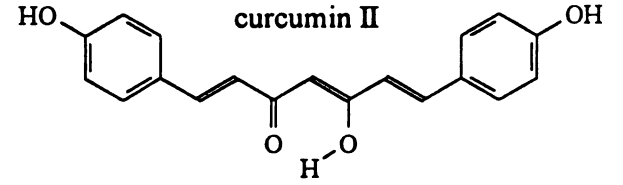
Figure 7



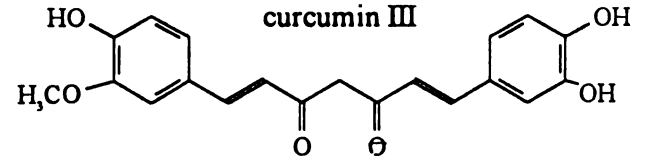
curcumin I



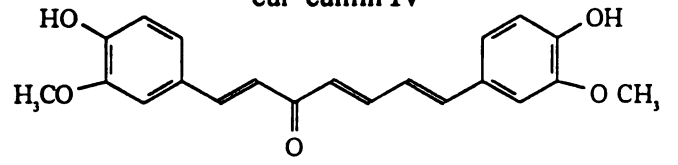
curcumin II



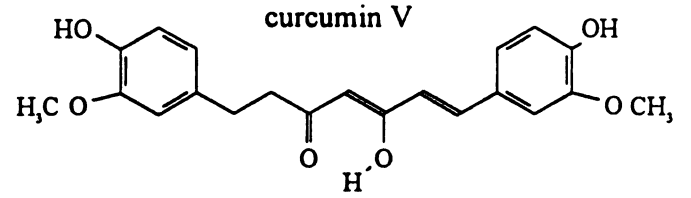
curcumin III



cur cumin IV



curcumin V



dehydrocurcumin

compound (Mayer, 1943). This product was then hydrolyzed to curcumin. Pabon (1964)improved a synthesis of curcumin from acetyl acetone and vanillin reported by Pavolini, and obtained an 80% yield of curcumin.

Srinivasan (1952,1953) found that a characteristic reaction of curcumin with boric acid resulted in a red color. Fluorescent orange color occurred with impure curcumin. These impurities were later investigated. Using column chromatography with silica gel and benzene mobile phase, he separated curcuminoids into three components. The first component eluted was curcumin I (reddish-orange prisms), followed by amorphous orange material (curcumin II) and a yellow plate-like compound (curcumin III). Characteristic reactions of the latter two compounds with acid and alkali showed that all three compounds were related. The first compound, curcumin I, had two methoxy groups. The second compound (curcumin II) had one methoxy group. The third compound (curcumin III) had no methoxy groups. These three curcumins were obtained from natural sources, the rhizome extracts, in good yield by preparative thin layer chromatography (Roughly, 1973). NMR spectra of the curcuminoids at low temperature indicated that in chloroform they existed in the enolic form (Roughly, 1973). The relative amounts of the three curcuminoids from turmeric taken from various harvests were determined by thin-layer chromatography and found in the following ratios; 60:30:10, 47:24:29, 49:29:22, and 42:24:34 for curcumin I, curcumin II and curcumin III, respectively (Perotti, 1975; Jentzsch, 1959; Krishnamurthy, 1976). It is not yet known if these differences are due to cultivars.

Kelkar and Rao (1934) published analysis on steam-distilled turmeric oil which showed that the oil was a mixture of sesquiterpene ketones and alcohols (Rao, 1934). The

major fraction of the oil, distilled at 158 to 165° C at 11 mm of Hg, was shown to be a mixture of ketones. Rupe (1936) showed that turmeric aroma is largely due to the sesquiterpene ketone ar-turmerone, which made up 40% of the volatile oil of turmeric. Boiling point, optical rotation, derivative analysis and studies involving degradation to known compounds facilitated the characterization of ar-turmerone. Ar-turmerone was purified early by Rupe and Gassman (Rupe, 1936), and more recently by Alexander and Rao (Anjaneyulu, 1968). A method for purifying ar-turmerone from turmeric oil obtained from turmeric rhizome by hexane extraction (Khalique, 1968) involved treating the sesquiterpene ketone portion of the oil (from fractional distillation) with chromium (IV) oxide in acetic acid at low temperature and conversion to crystalline 2,4-dinitrophenyl hydrozone. Isolation of the crystals, followed by exchange with m-nitrobenzaldehyde gave ar-turmerone. Ar-turmerone was one of the first natural sesquiterpene ketones to be characterized and assigned a structure (Rupe, 1936; Govindarajan, 1980). Further purification by thin-layer chromatography gave pure ar-turmerone with an optical rotation of +84 ° (Alexander, 1973). Ar-turmerone also was synthesized by condensation of its degradation products, acetone and curcumone (Rupe, 1936). As mentioned, other sesquiterpene ketones and alcohols and low-boiling terpenes have been identified in the volatile oil of turmeric. With the exception of turmerone, however, it was not possible to purify these components (Rupe, 1934; Rupe, 1936; Govindarajan, 1980). The structure of tumerone has not been identified conclusively, since the position of the double bonds in the ring still are not known (Govindarajan, 1980). A synthesis has not been reported for turmerone.

A significant amount of commercially important compounds have been realized from turmeric rhizomes, some with excellent bioactivities. However, the studies have been concentrated on antioxidative and anti-inflammatory activities. It is obvious that not all the medicinal claims attributed to turmeric have been investigated, especially considering its use in Indian folk medicine. New compounds continue to be discovered in turmeric, but few have been studied for bioactivity. The Bioactive Natural Products Laboratory in the Department of Horticulture at Michigan State University is involved actively in research on plants with folklore medicinal value, by assaying plant natural products against some important human and agricultural pests. Based on the research hypothesis that the tropical plant *Curcuma longa* L. produces some bioactive secondary metabolites which are either novel or possess novel bioactivities, we have studied turmeric to identify additional compounds with as yet undescribed biological activities.

CHAPTER II

Bioactive Compounds From Turmeric Rhizomes

Abstract

Curcumin I (1), curcumin II (2) (demethoxy curcumin) and curcumin III (3) (bisdemethoxy curcumin), the three main diaryl heptanoids from *Curcuma longa,* have demonstrated anti-inflammatory and antioxidant activities. Curcumin III was the most active, followed by curcumin II, and curcumin I. We have isolated these curcuminoids from turmeric rhizomes, and evaluated their biological activities. Curcumin I, curcumin II and curcumin III showed topoisomerase I and II inhibition at 50, 50 and 25 ppm concentrations, respectively.

The volatile oil from turmeric rhizomes yielded a sesquiterpene ketone, ar-turmerone. We have isolated ar-turmerone from the volatile oil, and evaluated it for novel bioactivity. It displayed mosquitocidal activity on *Aedes aegyptii* with LD_{100} at 50 ppm concentration.

Introduction

In Indian folk medicine, turmeric has been used to combat a variety of ailments, such as small pox (Kuttan, 1985), rheumatism, scabies, eye inflammation, body pain, sores, and cough (Jain et al., 1991), to name just a few. Many of these reputed claims have been substantiated. Turmeric has been used as a fever alleviator (Lindley, 1838), and it was verified that it has anti-inflammatory activity similar to aspirin or ibuprofen (Kuttan, 1994). Turmeric also has been used for centuries , especially in Asia, to prevent food spoilage, and studies on the antioxidant and antimicrobial properties of turmeric verified this activity. Curcumin, a main coloring agent present in turmeric, proved to be a better antioxidant than BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene) as rated on the antioxidant index (Lee, 1982). Also, curcumin scavenges active oxygen radicals better than vitamin E (Zhao, 1989).

In Thai folk medicine, *Curcuma* spp. rhizomes were used as a cobra poison antidote (Cherdchu, 1983). The local people claimed the plant to be effective orally for poisonous snake bite. This activity was substantiated in rats, mice and dogs. (Tejasen, et al., 1969 a, b; Tejasen, et al., 1970; Tejasen et al., 1978; Cherdchu, 1978). Ar-turmerone from turmeric abolished both the hemorrhagic activity of *Bothrops jararaca* venom and the lethal effect of *Crotalus durissus terrificus* venom in mice (Ferreira, 1992).

Although the anti-inflammatory, antioxidative, and antivenom properties of turmeric

components have been substantiated, many medicinal and therapeutic claims attributed to turmeric remain noninvestigated. In this chapter we report novel activity for some of the turmeric metabolites isolated and characterized from the rhizomes. Three main curcuminoids from turmeric, curcumin I, curcumin II and curcumin III, exhibited topoisomerase I and II inhibition, curcumin III exhibiting the best activity. In addition, the sesquiterpene ketone ar-turmerone was found to display mosquitocidal activity.

Experimental

Plant Materials - Turmeric plants were grown in large plastic pots, using a mixture of 50% loamy field and 50% bacto soils. Rhizomes were obtained from a grocer in Detroit, Michigan. Several rhizomes were planted 3 inches below the surface of the soil in each pot. The pots were kept in the BNPL greenhouse at Michigan State University. The plants were watered every two to three days, and fertilized with 20-20-20 Peters brand fertilizer. The turmeric plants were harvested for the rhizomes every nine months. Rhizomes were collected, washed with water, lyophilized, milled and kept in the -20 ° C freezer in ziploc bags. Some of the fresh rhizomes were replanted for additional production.

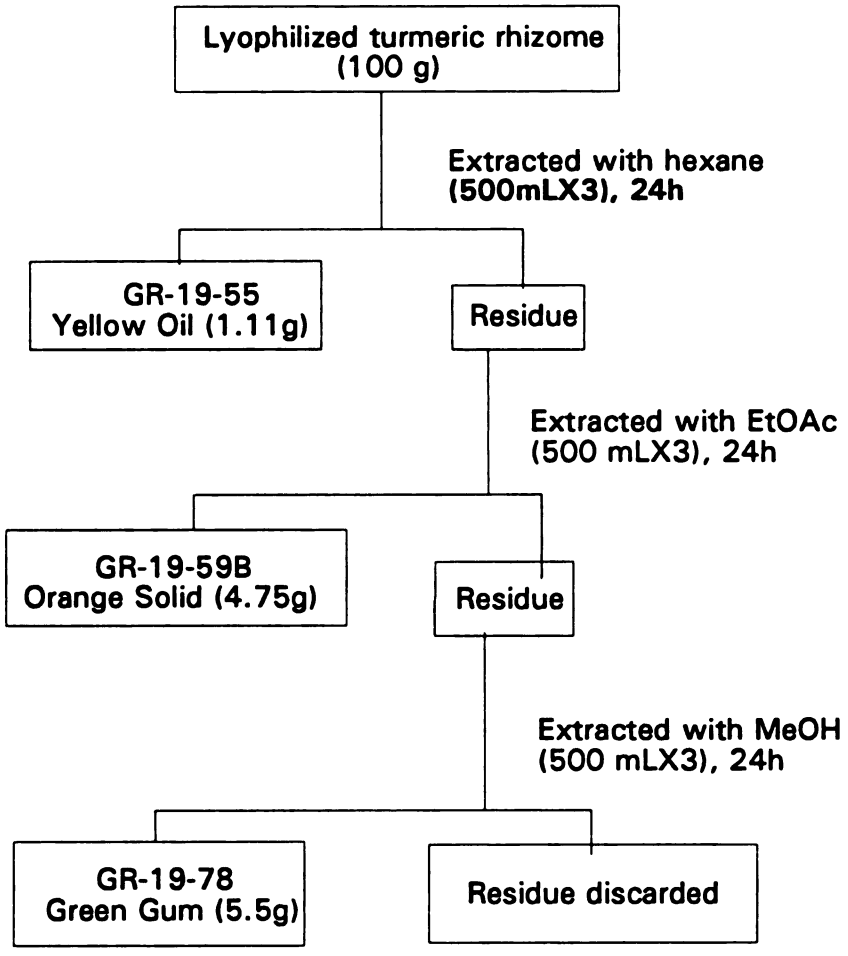
Chemicals and cell culture media.- YMG medium (yeast extract 4 $g \cdot L^{-1}$, maltose 10 $g \cdot L^{-1}$, glucose 4 $g \cdot L^{-1}$ and agar 12 $g \cdot L^{-1}$), PDA (potato dextrose agar; potato infusion 200 $g \cdot L^{-1}$, dextrose 20 $g \cdot L^{-1}$, agar 15 $g \cdot L^{-1}$), Emmons medium (neopeptone 10 $g \cdot L^{-1}$, glucose 20 $g \cdot L^{-1}$ and agar 15 $g \cdot L^{-1}$), NG medium (NaCl 3.0 $g \cdot L^{-1}$, bacto peptone 2.5 $g \cdot L^{-1}$, cholesterol 1 $ml \cdot L^{-1}$ of 5 $mg \cdot ml^{-1}$ stock solution, $CaCl_2$ 1 $ml \cdot L^{-1}$ of 1 M stock solution, $MgSO_4$, 1 $ml \cdot L^{-1}$ of 1 M stock solution and potassium phosphate buffer 25 $ml \cdot L^{-1}$ of stock solution of KH_2PO_4

11.97 g•100ml^{-1} and K_2HPO_4 2.0 g•100ml^{-1}) and YPDA medium (yeast extract 20 g•L , peptone 10 g•L^{-1}, dextrose 20 g•L^{-1} and adenine sulphate 2ml•L^{-1} of 0.5% stock solution) were prepared were prepared as published (Nair et al., 1989) in our lab. The ingredients were purchased from Difco Lab (Detroit, Michigan, USA). The anticancer standards, camptothecin and etoposide were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin, USA). Sterile saline was prepared by dissolving NaCl (8.5 g•L^{-1}) in an appropriate volume of deionized water.

Isolation and identification of Curcumin I (1), *curcumin II* (2), *and curcumin III* (3)- The extraction procedure for *Curcuma longa* rhizomes is summarized in **Figure 2.1**. Lyophilized and pulverized turmeric rhizomes (100g) were extracted successively with hexane, ethyl acetate, and methanol in an extraction column. Each solvent was used in 500 ml aliquots (3) over a 24-h period. Each extract was evaporated to dryness, yielding 1.11 g of hexane extract, 4.75 g of ethyl acetate extract, and 5.50g of methanol extract. The ethyl acetate extract exhibited activity on the topoisomerase inhibition assays. A TLC analysis of

32

Figure 2.1 Procedure for extraction of *C. longa* rhizome



the EtOAc extract showed three yellow and UV flourescing bands as major components in the extract. The EtOAc extract (1.6g), was purified by VLC using 71.6g silica gel. The fractions collected were: hexane (100 ml) [fraction 1] and 4:1 hexane:$CHCl_3$ (30mL) [fraction 2] and 1:1 hexane:$CHCl_3$ (50 mL) [fraction 3]; 1:1 hexane:$CHCl_3$ (50 ml) [fraction 4] and 1:1 hexane:$CHCl_3$ (50 ml) [fraction 5] and $CHCl_3$ (50 mL) [fraction 6]; $CHCl_3$ (50 ml) [fraction 7] and $CHCl_3$ (50 ml) [fraction 8] and $CHCl_3$ (50 ml) [fraction 9]; $CHCl_3$ (50 ml) [fraction 10] and $CHCl_3$ (50 ml) [fraction 11]; $CHCl_3$ (50 ml) [fraction 12] and $CHCl_3$ (50 ml) [fraction 13] and $CHCl_3$ (50 ml) [fraction 14] and $CHCl_3$ (50 ml) [fraction 15] and $CHCl_3$ (35 ml) [fraction 16] and $CHCl_3$ (50 ml) [fraction 17] and $CHCl_3$ (100 mL) [fraction 18]; $CHCl_3$ (100 ml) [fraction 19] and 4:1 $CHCl_3$:MeOH (50 mL) [fraction 20]; 4:1 $CHCl_3$:MeOH (50 ml) [fraction 21] and 1:1 $CHCl_3$:MeOH (50 mL) [fraction 22]; 1:1 $CHCl_3$:MeOH (50 ml) [fraction 23] and 1:1 $CHCl_3$:MeOH (50 ml) [fraction 24] and 1:1 $CHCl_3$:MeOH (50 ml) [fraction 25] and MeOH (50 ml) [fraction 26] and MeOH (50 ml) [fraction 27] and MeOH (50 ml) [fraction 28] and MeOH (150 ml) [fraction 29]. Based on TLC results, fractions 1-3, 4-6, 7-9, 10-11, 12-18, 19-20, 21-22, and 23-29 were combined to form fractions 1-8. Based on TLC, fraction 7 and 8 were combined to form GR-19-126 (.914 g). Purification of GR-19-126 (266.5 mg) by preparative TLC using a 30:1 $CHCl_3$:MeOH mobile phase yielded fractions A-E. Fractions B, C and D, the three curcuminoids, were collected separately. Repeated purification of fractions B, C and D by TLC using a 9:1 $CHCl_3$/Acetic acid mobile phase afforded pure compounds, curcumin I, curcumin II, and curcumin III, with yields of 79.0, 42.2, and 29.0 mg- these yields were used to calculate the % dry weights of these compounds as 0.8, 0.4, and 0.3%, respectively. The presence of curcumin I (**1**), curcumin II

(2), and curcumin III (3) was confirmed by ^{1}HNMR studies (**Appendices I, II, and III** for ^{1}H NMR spectra of the curcuminoids). Preparative silica gel TLC plates were purchased from Analtech Inc. (Newark, Delaware, USA).

Spectra - NMR spectra were recorded at the Max T. Rogers NMR facility at Michigan State University on Varian VXR 300 and 500 MHz spectrometers (Varian, California, USA) at ambient temperature. Mass spectra were acquired at the Michigan State University Mass Spectrometry Facility on a JEOL HX-110 double focusing mass spectrometer (JEOL, Tokyo, Japan).

Compound (1).: $C_{21}H_{20}O_6$; ^{1}HNMR ($CDCl_3$): δ 3.93 (6H, s, 2 OMe), 5.77 (1H, s, 1-H), 6.46 (2H, d, *J*=16 Hz, 3, 3'-H), 6.91 (2H, d, *J*=8.4 Hz, 9, 9'-H), 7.03 (2H, d, *J*= 2Hz, 6-6'-H), 7.10 (2H, dd, *J*=2, 8 Hz, 10, 10' -H), 7.56 (2H, d, *J*=16 Hz, 4, 4'-H). These ^{1}HNMR assignments were identical to the published data for curcumin I, (Roughly, 1973). No additional spectral data on compound 1 were obtained.

Compound (2).: $C_{20}H_{18}O_5$; ^{1}HNMR ($CDCl_3$): δ 3.95 (3H, s, OMe), 5.89 (1H, s, 1-H), 6.48 (2H, d, *J*=16 Hz, 3, 3'-H), 6.86 (2H, d, *J*=8 Hz, 7'-9'-H), 6.93 (1H, d, *J*=8 Hz, 9-H), 7.05 (1H, d, *J*=2, 6-H), 7.12 (1H, dd, *J*=2, 8 Hz, 10-H), 7.47 (2H, d, *J*=8, 6'-10'-H), 7.59 (1H, d, *J*=16Hz, 4-H), 7.61 (1H, d, *J*=16 Hz, 4'-H). These ^{1}HNMR assignments were identical to the published data for curcumin II (demethoxy diferuloyl methane) (Roughly, 1973).

Compound (3).: $C_{19}H_{16}O_2$; ^{1}HNMR ($(CD_3)_2CO$): δ 5.97 (1H, s, 1-H), 6.68 (2H, d, *J*=16 Hz, 3,3'-H), 6.89 (4H, d, *J=8* Hz, 7, 7', 9, 9'-H), 7.56 (4H, d, *J=8* Hz, 6, 6', 10, 10'-H),

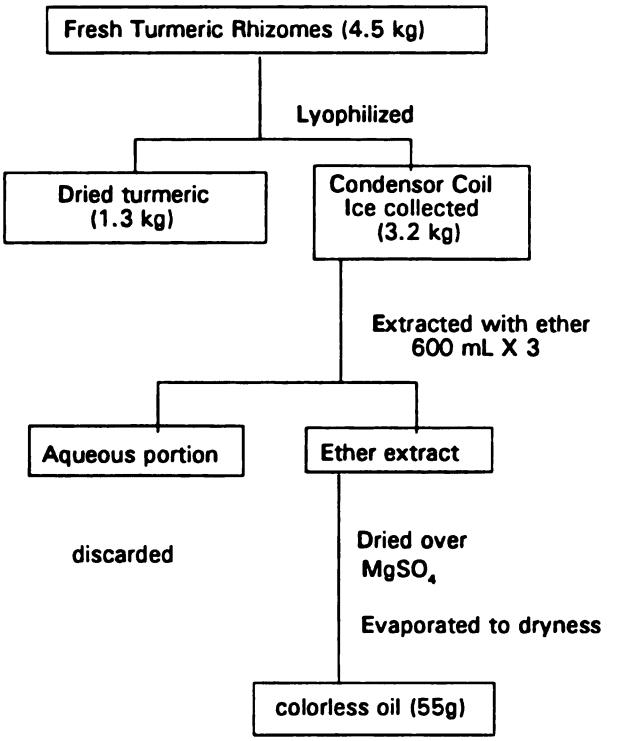
7.62 (2H, d, *J*=16 Hz, 4, 4'-H). The ^{1}HNMR assignments were identical to the published data for curcumin III (bis demethoxy diferuloyl methane) (Roughly, 1973).

Ar-Turmerone **(4):** The isolation procedure for the compound is summarized in **Figure 2.2.** A colorless oil was obtained from the ice accumulated on the condensor coil in the lyophilizer when rhizomes were freeze dried. Purification of the colorless oil from the rhizomes (464.4 mg) by TLC, using a 50:1 hexane: acetone mobile phase yielded fractions A-E. Fraction C (3.8 mg) exhibited mosquitocidal activity and was identified as ar-turmerone from ^{1}H and ^{13}CNMR and EIMS spectral studies (**Appendices IV, V, and VI**).

Compound **4.:** $C_{15}H_{20}O$ ^{1}HNMR ($CDCl_3$): δ 1.22 (3H, d, *J*=6.9 Hz, H-12), 1.84 (3H, s, H-13), 2.09 (3H, s, H-14), 2.29 (3H, s, H-1), 2.59 (1H, dd, *J=8,* 16 Hz, H-8), 2.69 (1H, dd, *J*=6.6 ,16 Hz, H-8), 3.27 (1H, m, H-7), 6.00 (1H, s, H-10), 7.10 (4H, br s, H-2, 3, 4, 5). ^{13}C ($CDCl_3$): δ 20.55 (C-13), 20.83 (C-1), 21.84 (C-12), 27.49 (C-14), 35.13 (C-7), 52.54 (C-8), 123.94 (C-10), 126.51 (C-4,5), 128.94 (C-2,3), 135.39 (C-15), 143.53 (C-11), 154.92 (C-6), 199.71 (C-9). EIMS: *m/z* (rel. Int.); 216 (63), 201 (30), 119 (84), 91 (13), 83 (100).

Antimicrobial assay - Antifungal and antibacterial assays of compounds **1,2,3** and **4** were conducted according to the reported procedure (Nair et al., 1989). Cultures of *Fusarium oxysporum* (MSU-SM-1322), *Fusarium moniliforme* (MSU-SM-1323), *Gleosporum spp.* and *Rhizoctonia spp.* were raised on potato dextrose agar (PDA) medium in petri dishes. Cultures of *Candida albicans* and *Aspergillus flavus* (MSU strains) were cultured on YMG medium in petri dishes. Cultures of *Staphylococcus epidermidis* (ATCC

Figure 2.2 For the Extraction of Volatile Oil



25923), *Streptococcus aureus* (MS strain), and *Escherichia coli* (ATCC 25922) cultures were grown on Emmons medium in petri dishes.

Anticancer (topoisomerase inhibition) assay - Topoisomerase assays of compounds **1,2,3** and **4** were conducted according to the reported procedure of Chang, et al. (1995). Mutant *Saccharomyces cerevisae* cell cultures JN394, JN394 $_{t\text{-}1}$ and JN 394 $_{t2\text{-}5}$ were provided by Dr. John Nitiss at St. Jude Children's Research Hospital (Nitiss et al., 1993). JN394 is hypersensitive to topoisomerase I and II poisons because of deletions that irradicated the *RAD*52 repair pathway. JN394$_{t\text{-}1}$ is isogeneic to JN394 except that the top1 gene is deleted. The deletion of this gene causes a lack of response to topoisomerase I poisons. JN 394$_{t2\text{-}5}$, that contains the top2-5 gene, is resistant to topoisomerase II poisons, but it responds to topoisomerase I poisons. Topoisomerase I and II are enzymes that alter the DNA by catalyzing a three-step process. This involves cleavage of one strand of DNA by topoisomerase I, or two strands of DNA by topoisomerase II, movement of a DNA segment through this break, and then resealing of the DNA strand (s) (Stryer, 1988). The mutant yeasts were raised on YPDA medium (20 ml) in petri dishes.

The cell suspension for bacteria and mutant yeast and spore suspension for fungi were prepared by suspending the bacteria or yeast or spores from a fully grown culture in a petri dish in 10 ml of sterile saline solution, and transferring the suspension to a sterile culture tube. The cell or spore suspension concentration was adjusted to 10^6 colony forming units per milliliter (CFU/ml) to yield a stock solution. Serial dilutions of the stock culture were

made in sterile saline by innoculating the proper media with the approximate dilutions to determine the required CFU/ml. Bioassays were conducted by lawning 100 µl of the desired cell or spore suspension containing 10^6 CFU/ml on petri dishes of the corresponding medium. The test compound was spotted carefully on the bioassay plates at varying concentrations, along with 25µl of DMSO alone as control. The plates were allowed to dry in a laminar flow hood, and then incubated at 27° C for 72 h. The zone of inhibition was measured in mm.

Mosquitocidal and nematicidal assays - Aedes aegyptii mosquito larvae were used to test for mosquitocidal activity of compounds **1,2,3** and **4,** according to an established procedure of Nair et al. (1989). *A. aegyptii* eggs were provided through the courtesy of Dr. Alex Raikhel of the Department of Entomology at Michigan State University. Approximately 200 mosquito eggs were placed in 500 ml of degassed, deionized water prepared by sonication. About 5 mg of bovine liver powder was added to the water as a food source. After four days, 4th instar mosquito larvae were transferred to 980 µl of the water in a test tube. 20 µl of the test material at the desired concentration in DMSO was added to each tube, which was shaken lightly to insure a homogeneous test solution. Each tube was covered and left at room temperature. 20 µl of DMSO was used as the control. The larval mortality was recorded at 2-, 4-, 6-, 24-, and 48-h intervals. The assay was conducted in triplicate.

Panagrellus redivivus and *Caenorhabditis elegans* were used to test for nematicidal activity of compounds **1,2,3** and **4** according to the published procedure of Nair, et al. (1989). A stock culture of nematodes in NG media in a scintillation vial was maintained at 14 day integrals designed to provide uniform young nematodes. 10-20 nematodes in 48 µl of the

growth media were transferred to a 96-well tissue culture plate. 2 µl of 50% aqueous DMSO solution of test material was added to this. 2 µl of 50% aqueous DMSO was used as control. The experiment was completed in triplicate.

Gypsy moth, forest tent caterpillar, corn earworm and tobacco hornworm assays- Dry diet for each larval type was weighed out in a scintillation vial, one for each test compound. Dry diet ingredients for the gypsy moth consisted of wheat germ (36g), casein (7.5g), Wesson's salt mix (2.4g), sorbic acid (0.6 g), methylparaben (0.3 g), and vitamin mix Hoffman-LaRoche #26862 (3g). The dry diet mix used for each treatment weighed 0.845g. The corn earworm diet was obtained from North Carolina State, and the amount used for each treatment was 0.94g. The tobacco hornworm diet also was obtained from North Carolina State, and 0.95 g was used for each treatment. For each treatment, the test compound was dissolved in DMSO, so that 20 µl of the resulting solution containing the test compound added to the dry diet gave the desired concentration of the test compound. 20 µl of DMSO served as the control. To this, agar solution was added to bring the total wet weight to 5.0 g, and the mix was homogenized with a spatula. The mixture was poured immediately (before jelling), equally into 15 micro beakers. The beakers were randomized and allowed to cool down for 3 h. One insect larvae of the type being tested was transferred to each of the 15 micro beakers with a small paint brush. The beakers were capped and stored in a tray in an incubation chamber at the desired temperature for six days. At the end of this period, each larvae was weighed. The average weight of each sample was compared to the average weight of the control to determine bioactivity of the test compound. Data were analyzed using Dunnet's test; means of the treatments were compared with the control.

Results and Discussion

Rhizomes of *C. longa* were extracted sequentially with hexane, ethyl acetate and methanol. Volatile oil was extracted from ice accumulated on condenser coils on the freeze dryer in which the rhizomes were lyophilized. Preliminary bioassays were performed on these extracts to test for antimicrobial, anticancer, mosquitocidal, nematicidal, and gypsy moth, forest tent caterpillar, tobacco hornworm and corn earworm growth inhibition.

The ethyl acetate extract inhibited the growth of *Saccharomyces cerevisae* mutants JN394, JN 394 $_{t-1}$ and JN394$_{t2-5}$ at 250 ppm concentration (**Table 2.1**) The hexane extract, an oily residue, demonstrated anticancer activity at 250 ppm. The volatile oil and hexane extract demonstrated mosquitocidal activity at 250 ppm. Since a literature search showed that the curcuminoids inhibited certain types of cancer, anticancer assays were directed on the fractions obtained from the EtOAc extract. Purification of the EtOAc fraction afforded compounds **1, 2** and **3,** the curcuminoids. Purification of the volatile oil yielded compound **4**. The curcuminoids **1, 2** and **3** were evaluated for anticancer activity, and gave zones of inhibition at 50, 50, and 25 ppm, respectively (**Table 2.1**). Mosquitocidal activity was then evaluated for compounds **1-4**. Compound **4** exhibited LD_{100} at 50 ppm on *Aedes aegypti* in 48 h. Compounds **1** and **2** exhibited minor activity, each with LD_{100} at 250 ppm in 48 h. Compound **3** showed no mosquitocidal activity. The MeOH extract exhibited no anticancer activity or mosquitocidal activity. In addition, none of the extracts or the volatile oil exhibited activity against nematodes, or caused growth inhibition in the gypsy moth, forest tent caterpillar, tobacco hornworm or corn earworms.

Table 2.1 Preliminary anticancer bioassay results for EtOAc and Hexane extracts and compounds recorded as zone of inhibition in mm.

Test material	JN 394	JN 394_{t-1}	JN 394_{t2-5}
Crude EtOAc extract	16.7	19.0	14.3
Crude Hexane extract	12.7	20.0	23.7
Curcumin I	13	16	15
curcumin II	14	12	15
curcumin III	15	18	13

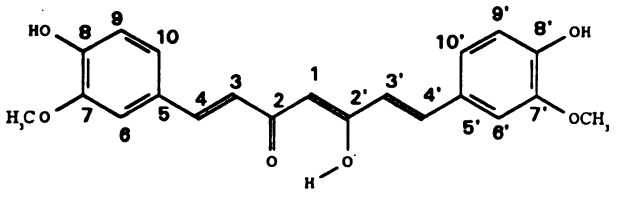
The preliminary identity of the three curcuminoids was obtained by comparison with the chromatographic data published earlier (Govindarajan, 1980). The structures were confirmed further by detailed spectral studies. The structures of **1-3** are shown as **Figure 2.1**. Since curcuminoids **1** and **3** are symmetrical, only half of the signals were evident in their ^{1}HNMR spectra.

In compound **1**, the assignment of H-3 and H-4, as well as the H-3' and H-4' protons as *trans* to each other, was based on the large coupling constant between them. The signals for H-4 and H-4' appeared further downfield than H-3 and H-3', because these protons were deshielded by the aromatic rings in the curcuminoids. The singlet at 5.77 ppm was assigned to the H-1, because the methylene protons at C-1 in the curcuminoids are enolyzed to the α-β-unsaturated carbonyl and appear as the singlet. The singlet at 3.93 ppm, integrated for six protons, was assigned to the methoxy protons at the C-7 and C-7' positions.

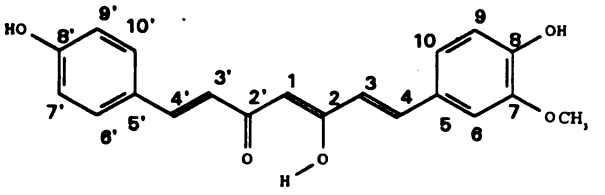
Compound **2** lacked the symmetry evident in compounds **1** and **3**. However, the assignments of H-3, H-3', H-4, H-4' and H-1 were made by the same rationale as for compound **1**. Compound **2** showed two doublets at 6.86 and 7.47 ppm, respectively, with a coupling constant of 8 Hz to each other. These doublets integrated for two protons each and were assigned to H-6' and H-10', and H-7' and H-9', respectively. The singlet at 3.95 ppm, integrated for three protons, was characteristic of a methoxy group at C-7.

For compound **3**, the assignments of H-3, H-3', H-4, H-4' and H-1' were made by the same reasoning as for compound **1**. The ^{1}HNMR spectrum of **3** gave two aromatic protons at 6.89 and 7.56 ppm, respectively, These protons were ortho to each other as evident from ther 8Hz coupling constant. In Pople notation, this is characeristic of a **AA'XX'** or **AB**

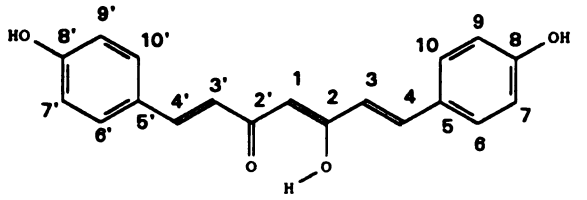
Figure 2.1 Curcuminoids



Curcumin I



Curcumin II



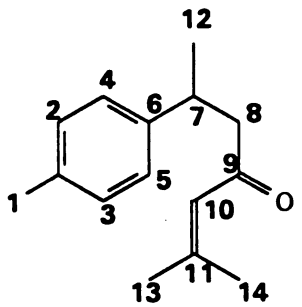
Curcumin III

system, and is indicative of a para-disubstituted aromatic ring (Silverstein, 1991). Also, these doublets integrated for four protons each. The protons at H-6, 6', 10, and 10' corresponded to the doublet at 7.47 ppm, indicating they were deshielded further than the remaining aromatic protons.

The three main curcuminoids from turmeric, curcumin I (**1**), curcumin II (**2**), and curcumin III (**3**), all were isolated previously rhizomes of *Curcuma longa* (Roughley, 1973). The spectra of all three curcuminoids were confirmed by ^{1}HNMR studies.

The structure of compound **4** is **Figure 2.4.** ^{1}HNMR and ^{13} CNMR spectra of compound **4** revealed that it contained 20 protons and 15 carbons. The three singlet integrated for three protons each at 1.84, 2.09, and 2.29 ppm, respectively, indicated that it had three quaternary methyl groups. Also, the singlet at 2.29 ppm for 3 protons suggested an aromatic methyl.group. The aromatic ring deshielded the methyl protons, hence, the chemical shifts for these methyl protons were found further in the downfield region than a regular aliphatic methyl group.. The singlets at 2.09 and 1.84 ppm were typical of allylic methylene protons and were assigned to C-14 and C-13, respectively. The protons at C-14 were in close proximity to a carbonyl group and appeared further downfield. A doublet at 1.22 ppm integrated for three protons, indicative of a methyl group at C-12. A one-proton singlet in the region at 6.00 ppm was assigned to C-10. Also, a broad singlet at 7.10 ppm indicative of an **AB** system was due to H-2, H-3, H-4, and H-5 protons. The ^{13}CNMR spectrum of compound **4** supported the ^{1}HNMR assignments. The proton and carbon NMR data and the proposed structure for compound **4** was confirmed further by the EIMS spectrum. The molecular ion was evident with 63% intensity at m/z 216.

Figure 2.4 Ar-Turmerone Structure

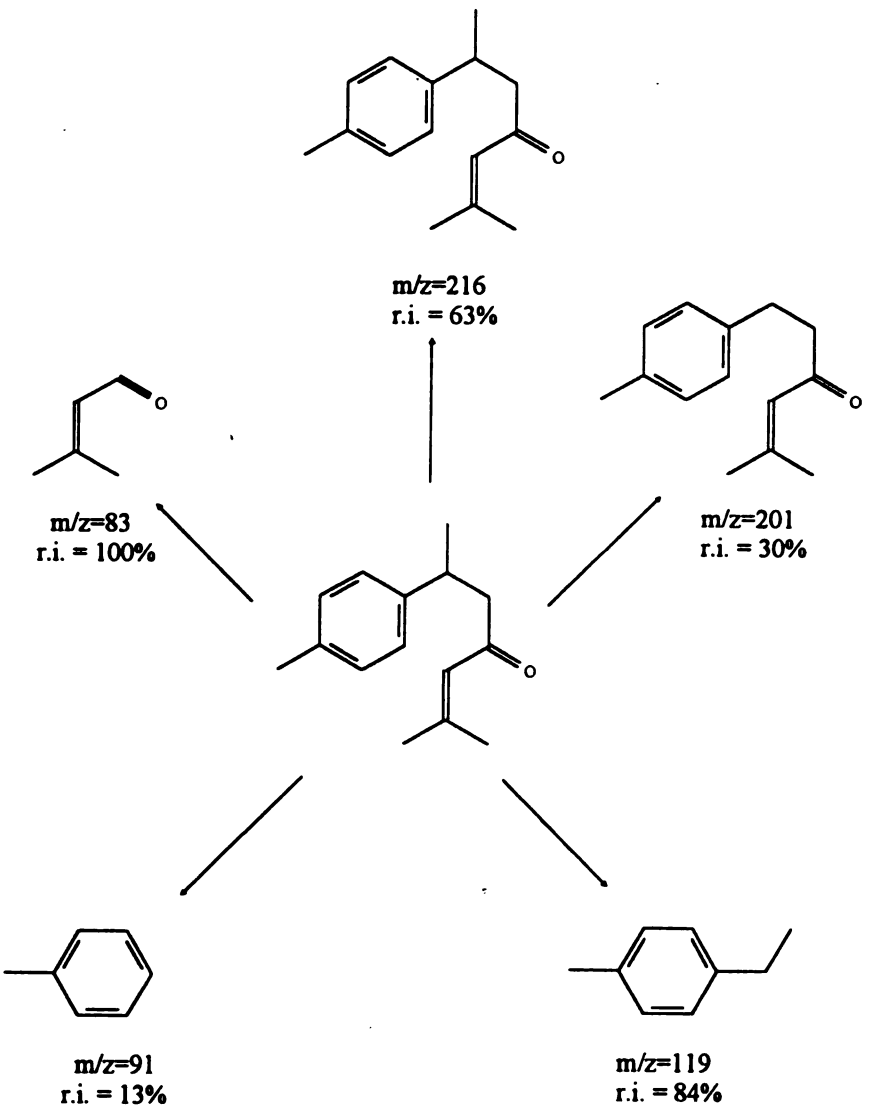


The EIMS spectrum had a base peak at m/z 201, representing the loss of a methyl group (M^+-15) by sigma-bond dissociation (α-cleavage). A peak at m/z 119 represented another sigma-bond dissociation which commonly occurs at tertiary carbons. This peak was also particularly stable due to the aromatic ring and the resulting resonance. Additional sigma-bond dissociation and the potential fragments generated from compound **4** are shown in **Figure 2.5**. The ^{1}HNMR, ^{13}CNMR, and EIMS spectral data of compound **4** were identical to the published spectral data of compound **4**, a sesquiterpene ketone from the rhizome of *C . longa* (Rao, 1934), and identified compound **4** as ar-turmerone. Spectral data of this compound were published recently (Ferreira, 1992). The yield of pure ar-turmerone from the rhizome in our research was much lower than previously reported value. Ar-turmerone is very volatile and hence it is possible that the rotatory evaporation and desiccation steps evaporated most of the compound along with the solvent, resulting in an extremely low yield of the compound.

Curcumin I (**1**), curcumin II (**2**) and curcumin III (**3**) all showed anticancer activity, with curcumin III being most active at 25 ppm concentration. Curcumin I (**1**) and curcumin II (**2**) exhibited mosquitocidal activity on *Aedes aegyptii* at 250 ppm. Ar-turmerone (**4**) displayed LD_{100} at 50 ppm against *Aedes aegyptii*.

Curcumin already is in use by the food industry as a safe coloring agent. This may facilitate the acceptance of curcumin III as an effective chemotherapeutic cancer preventative. In any case, curcumin is known to be a safe and nontoxic natural product, and may have potential for use in chemotherapy. Also, ar-turmerone has proved to be an effective

Figure 2.5 EIMS Fragmentation of Ar-Turmerone



mosquitocidal agent, and may be useful to control populations of *A. aegyptii* and manage it as a disease vector. All of the activities reported in this chapter are novel. Further studies need to be carried out to elucidate the mechanism of the activities associated with these three curcuminoids and ar-turmerone.

CHAPTER III

An Antifungal Terpenoid from the Leaves of *Curcuma longa*

Abstract

Curcuma spp. plants are an integral part of traditional and modern Indian Ayuverdic medicine, and have provided some important antifungal agents. Surprisingly, because of its commercial importance in food industry, turmeric has not been investigated thoroughly for medicinal or agricultural use. Therefore, our laboratory assayed turmeric extracts against some important agricultural and human pathogens. Lyophilyzed *Curcuma longa* leaves were extracted sequentially with hexane, ethyl acetate and methanol. Bioassay-directed fractionation and purification by solvent partitioning and reverse phase preparative chromatography of the hexane extract afforded an antifungal compound, **5**. Detailed NMR studies revealed that this compound is a diterpene dial. The structure was confirmed to be labda-8(17),12-diene-15,16-dial. It showed antifungal activities on several yeasts including *Candida albicans, Candida kruseii, and Candida parapsilosis,* at 25, 25 and 1 ppm concentrations, respectively. Also, it displayed mosquitocidal activity against *Aedes aegyptii* with an LD_{100} at 10 ppm concentration. This is the first report of the isolation and characterization of a diterpene from *C. longa,* and the biological activity for labda-8(17),12-diene-15,16-dial.

Introduction

Plants from the Zingiberaceae family play an important part in historical and contemporary Ayurverdic Indian medicine (Jain, et al., 1991). Various members of this family yield several antimicrobial agents (Bauer, 1988; Ghosh, 1980; Banerjee, 1976). Recently, several fungi have become resistant to existing fungicides, and some of the antimicrobial agents from plants in the Zingiberaceae family may be useful in managing these fungal pathogens. An effective antifungal agent, the methyl ester of para-coumaric acid, was isolated from rhizomes of the herbaceous Indian plant *Costus speciosus* Sm (Bauer, 1988). This compound inhibited the growth of *Aspergillus niger, Cladosporium cladosporioides, Colletotrichum gloeosporioides, Curvularia spp* ,and *Penicillium spp* (Bauer, 1988). Plants of the *Curcuma* genus also yielded antifungal agents. The ethyl acetate extract of the rhizomes of *Curcuma amada* showed antifungal activity against *Candida albicans, Epidermophyton floccosum, Trichophyton rubrum, Aspergillus niger, Aspergillus flavus, Penicillium notatum, Helminthosporium oryzae, Botrytis alli, Paecilomyces spp., Fusarium spp., Saccharomyces cerevisae, Pythium spp., Phytophthora parasitica, Rhizopus spp., Mucor spp., Curvularia spp.*, and *Sclerotium spp* (Ghosh, 1980). The essential oil of rhizomes of *Curcuma caesia* displayed activity against *Aspergillus niger, Aspergillus flavus, Penicillium lilacinum, Pencillium javanicum, Trichoderma viride, Curvularia oryzae, Helminthosporium oryzae, Pestalotia lapagericola, Microsporum gypseum*, and *Trichophyton mentagrophytes* (Banerjee, 1976).

Among pathogenic yeast types, *Candida* species are the most common and stubborn

of infectious agents in humans (Montes, 1985). Although a number of treatments existed for yeast infections, many of them involved oral administration, and their bad taste made them unattractive to patients. There is always a need for new treatments to combat yeast infection since diagnostic problems can lead to systematic infection and death. Considering the discovery of effective antifungal agents from Zingiberaceae family, particularly the *Curcuma* genus, our laboratory assayed extracts from *C. longa* L. against some important agricultural and human pathogens. In this chapter, we report the isolation of an antibiotic diterpene, labda-8(17),12-diene-15,16-dial, from the leaves of *C. longa.* This is the first report of the isolation and characterization of labda-8(17),12-diene-15,16-dial from *C. longa* and its antibiotic and mosquitocidal activities.

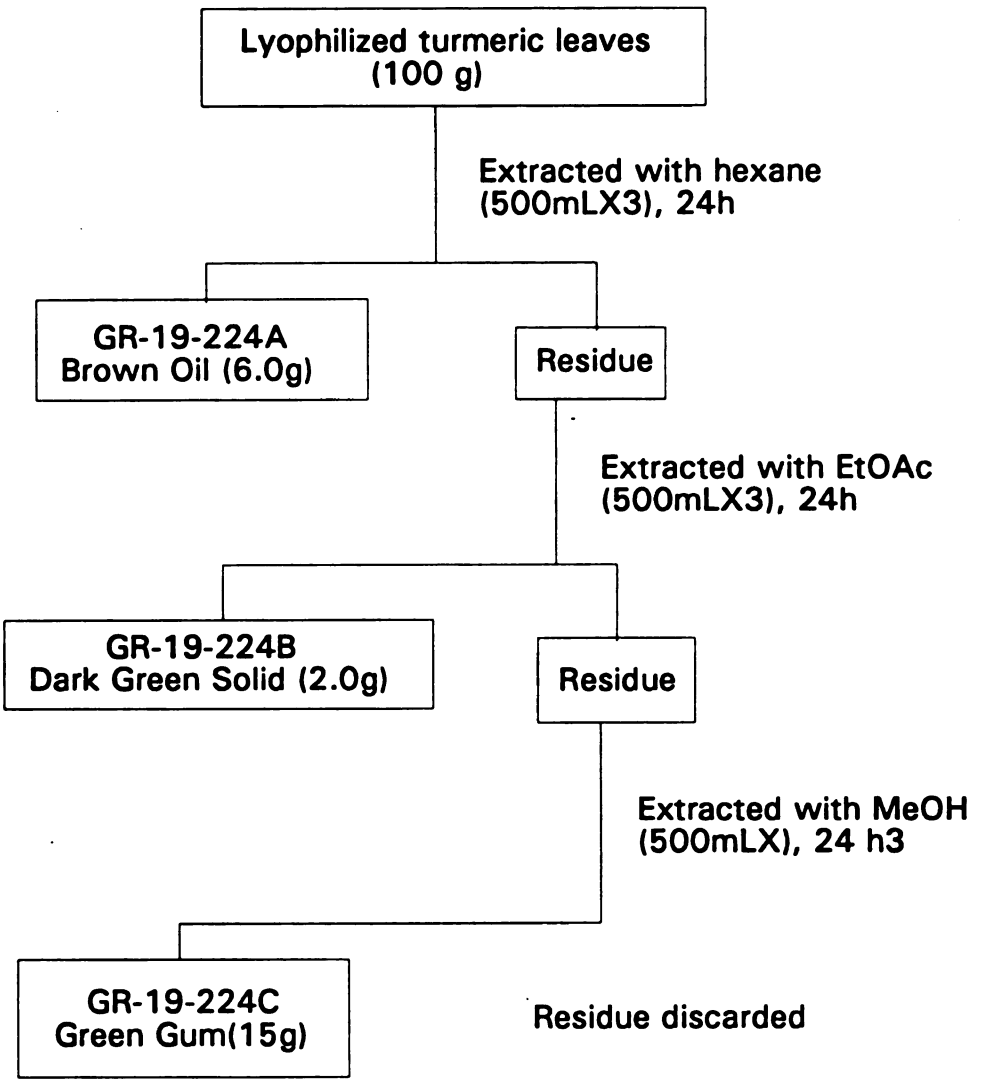
Experimental

Plant Materials - Turmeric plants were grown in large plastic pots, using a mixture of 50 % loamy field soil and 50% bacto soil. The *C. longa* rhizomes were obtained from a grocer in Detroit, Michigan. Several rhizomes were planted just below the surface of the soil in each pot. The plants were watered every two to three days, and fertilized with 20-20-20 Peters brand fertilizer. The turmeric plants were harvested for rhizomes in nine-month cycles. Leaves were collected every two weeks for a three-month period, lyophilized and milled, and kept in a -20 °C freezer until extraction..

Chemicals and cell culture media- YMG medium (yeast extract 4 $g \cdot L^{-1}$, maltose 10 $g \cdot L^{-1}$, glucose 4 $g \cdot L^{-1}$ and agar 12 $g \cdot L^{-1}$), PDA (potato dextrose agar; potato infusion 200 $g \cdot L^{-1}$, dextrose 20 $g \cdot L^{-1}$, agar 15 $g \cdot L^{-1}$), Emmons medium (neopeptone 10 $g \cdot L^{-1}$, glucose 20 $g \cdot L^{-1}$ and agar 15 $g \cdot L^{-1}$), NG medium (NaCl 3.0 $g \cdot L^{-1}$, bacto peptone 2.5 $g \cdot L^{-1}$, cholesterol 1 $ml \cdot L^{-1}$ of 5 $mg \cdot ml^{-1}$ stock solution, $CaCl_2$ 1 $ml \cdot L^{-1}$ of 1 M stock solution, $MgSO_4$, 1 $ml \cdot L^{-1}$ of 1 M stock solution and potassium phosphate buffer 25 $ml \cdot L^{-1}$ of stock solution of KH_2PO_4 11.97 $g \cdot 100ml^{-1}$ and K_2HPO_4 2.0 $g \cdot 100ml^{-1}$) and YPDA medium (yeast extract 20 $g \cdot L^{-1}$, peptone 10 $g \cdot L^{-1}$, dextrose 20 $g \cdot L^{-1}$ and adenine sulphate 2$ml \cdot L^{-1}$ of 0.5% stock solution) were prepared were prepared as published (Nair et al., 1989) in our lab. The ingredients were purchased from Difco Lab (Detroit, Michigan, USA). Sterile saline was prepared by dissolving NaCl (8.5 $g \cdot L^{-1}$) in an appropriate volume of deionized water.

Extraction and isolation of the antifungal compound **5** - The initial extraction method for compound **5** is summarized in **Scheme 3.1.** Lyophilized and milled turmeric leaves (100 g) were extracted successively with hexane, ethyl acetate and methanol in an extraction column. Each solvent was used in 3X500 ml aliquots over a 24 h period. Each extract was evaporated to dryness, yielding 6.0 g of hexane extract (GR-19-224A), 2.0 g of ethyl acetate extract (GR-19-224B), and 15.0 g of methanol extract (GR-19-224C). The hexane extract exhibited activity against *C. albicans.* The TLC of the hexane extract exhibited a UV-fluorescing major component in the extract. The hexane extract GR-19-224A (2.5 g) was dissolved in hexane (300 ml) and partitioned with methanol (3 X 100 ml) in a separatory

Scheme 1 Extraction of Leaf



funnel. The methanol extracts then were combined and extracted further with hexane (3 X 100 ml). The hexane and methanol fractions were evaporated to dryness separately, yielding a light yellow oil and a dark green solid, respectively. TLC analysis showed that most of the fluorescing compound was in the methanol-partitioned fraction (1.8 g). A small amount of this compound was still present in the hexane-partitioned fraction and then was extracted further with MeOH (10X5 ml). The MeOH fraction was evaporated to dryness, and combined with the original methanol fraction, yielding a total of 2.1 g of GR-19-273B. The yield of the hexane fraction, GR-19-273A, was 440 mg. Methanol:water (7:3) was used to dissolve GR-19-273B (1.8 g) (14 ml X 3), in order to precipitate the chlorophyll. The suspension was centrifuged, and supernatant was collected, and removal of solvent *in vacuo* yielded 1.38 g of a brown oil (GR-19-274A). A portion of this oily residue (560 mg) was purified by VLC using 85:15 methanol:water as the mobile phase, and fractions were collected in aliquots of 50[I], 50[II], 25[III], 30[IV], 35[V], 40[VI], and 40[VII] ml, respectively. The final elution was with chloroform in 60ml[VIII] and 200 ml[IX] aliquots, altogether to yield fractions I-IX. Bioassays revealed that fractions III-VII were antifungal, and were combined to yield an oily residue (128 mg).. Repeated purification of this oily residue by reverse phase preparatory TLC, using a 7:1 methanol:water mobile phase, gave a UV-flourescing band, GR/19/277A . Elution of this band afforded an oily compound **5** (21.1 mg). **(Appendices VII, VIII, IX, X, XI, XII, XIII** for spectra of compound **5)**.

Spectral - NMR spectra were recorded at the Max T. Rogers NMR facility at

Michigan State University on a Varian VXR 500 MHZ spectrometer (Varian, California, USA) at ambient temperature. Mass spectra were acquired at the Michigan State University Mass Spectrometry Facility on a JEOL HX-110 double focusing mass spectrometer (JEOL, Tokyo, Japan). Reverse phase preparative TLC plates were purchased from Whatman Inc. (Clifton, New Jersey).

Compound **5**. ^{1}HNMR ($CDCl_3$):δ 0.71, 0.80, and 0.87 (each 3H, s, 18, 19, 20 CH_3), 1.04 (1H, m, *J*= 4, 13 Hz, 1-H), 1.11 (1H, dd, *J*= 3, 13 Hz, H-5), 1.17 (1H, m, H-3), 1.33 (1H, m, *J*= 4, 13 Hz, H-6), 1.40 (1H, m, H-3), 1.49 (1H, m, *J*=3.3, 14 Hz, H-2), 1.57 (1H, m, H-2), 1.67 (1H, m, H-1), 1.73 (1H, m, H-6), 1.88 (1H, dd, *J*= 1.5, 11 Hz, H-9), 2.00 (1H, m, H-14), 2.31 (1H, m, H-11), 2.40 (1H, m, H-14), 2.48 (1H, m, H-11), 3.35 (1H, d, *J*= 17 Hz, H-14), 3.42, (1H, d, *J*= 17 Hz, H-14), 4.34 (1H, d, *J*= 1 Hz, H-17), 4.84 (1H, d, *J*= 1 Hz, H-17), 6.74 (1H, t, *J*=6.5 Hz, H-12), 9.38 (1H, s, H-16), and 9.61 (1H, t, *J*=1.5, H-15). ^{13}CNMR ($CDCl_3$): δ 14.4 (C-20), 19.3 (C-2), 21.7 (C-19), 24.1 (C-6), 24.7 (C-11), 33.6 (C-18 , C-4), 37.8 (C-14), 39.2 (C-1), 39.3 (C-7), 39.6 (C-10), 42.0 (C-3), 55.4 (C-5), 56.5 (C-9), 107.8 (C-17), 134.8 (C-13), 148.0 (C-8), 159.9 (C-12), 193.5 (C-16), and 197.3 (C-15). EIMS: *m/z* (rel. Int.); 302 (M^+ 39) 287 (7), 284 (5), 273 (9), 137 (100), 69 (56), 55 (36).

Circular Dichroism (CD) of compound **5**

Circular Dichroism Analyses. - The CD analysis of compound **5** was carried out using a JASCO J-710 71CD-ORD spectropolarimeter (Jasco, Incorporated, Japan). The spectra

were plotted on a DeskJet 855C Hewlett Packard plotter (Hewlett Packard Corporation, Palo, Alto, California). Nitrogen (99.99%) was generated by a nitrogen generator model NG-150 (Peak Scientific, Chicago, Illinois) at a rate of 15 $L \cdot m^{-1}$. Pure labda 8(17), 12- diene-15,16-dial was dissolved in EtOH (1.4 mg/1 ml), and the CD was determined under the following conditions: scan mode (wavelength), bandwidth (0.5 nm), sensitivity (50 mdeg), response (1 s), wavelength range (200-400 nm), step resolution (1 nm), scan speed (200 nm/min), and accumulation (1) (**Appendix XIII**).

Antimicrobial assay - Antifungal and antibacterial assays of compound **5** were conducted according to the reported procedure (Nair et al., 1989). Cultures of *Fusarium oxysporum* (MS-SM-1322), *Fusarium moniliforme* (MS-SM-1323), *Gleosporum spp.* and *Rhizoctonia spp.* were raised on potato dextrose agar (PDA) medium in petri dishes. Cultures of *Candida albicans, Candida kruseii, Candida parapsilosis* and *Aspergillus flavus* (MS strains) were raised on YMG medium in petri dishes. Cultures of *Staphylococcus epidermidis* (ATCC 25923), *Streptococcus aureus* (MS strain), and *Escherichia coli* (ATCC 25922) cultures were grown on Emmons medium in petri dishes.

Anticancer (topoisomerase inhibition) assay - Topoisomerase assay of compound **5** was conducted according to the reported procedure (Chang et al., 1995). Mutant *Saccharomyces cerevisae* cell cultures JN394, JN394 $t\text{-}_1$ and JN 394 $t_{2\text{-}5}$ were provided by Dr. John Nitiss at St. Jude Children's Research Hospital (Nitiss et al., 1993). JN394 is hypersensitive to topoisomerase I and II poisons because of deletions that irradicated the RAD52 repair pathway. JN394 $t\text{-}_1$ is isogeneic to JN394, except that the top1 gene is

deleted. The deletion of this gene causes a lack of response to topoisomerase I poisons. JN 394 $t_{2\text{-}5}$, that contains the top2-5 gene, is resistant to topoisomerase II poisons, but it responds to topoisomerase I poisons. The mutant yeasts were cultured on YPDA medium in petri dishes containing 20 ml of media.

The cell suspension for bacteria and mutant yeast and spore suspension for fungi were prepared by suspending the bacteria or yeast or spores from a fully grown petri-dish culture in 10 ml of sterile saline solution, and transferring the suspension to a sterile culture test tube. The cell or spore suspension concentration was adjusted to 10^6 colony forming units per milliliter (CFU/ml) by conducting a serial dilution of the stock culture and by plate count, to determine the CFU/ml of the test organism. Bioassays were conducted by lawning the desired cell or spore suspension (100 µl) on petri dishes of the corresponding medium. The test compound in DMSO then was spotted carefully on the bioassay plates at desired concentrations along with 25 µl of DMSO which served as a control. The plates were allowed to dry in a laminar flow hood, and then incubated at 27° C for 72 h. The zone of inhibition was measured in mm.

Mosquitocidal assay - *Aedes aegyptii* mosquito larvae were used to test for mosquitocidal activity of crude leaf extracts and pure compounds according to the established procedure of Nair et al. (1989). *A. aegyptii* eggs were provided by Dr. Raikhel at Michigan State University, Dept. of Entomology. Approximately 200 mosquito eggs were placed in 500 ml of degassed, deionized water prepared by sonication. About 5 mg of bovine liver

powder was added as a food source for the larvae. After four days, 4th-instar mosquito larvae were transferred in 980 µl of deionized water in a test tube. 20µl of compound **5** at the desired concentrations in DMSO was added to each tube and left at room temperature. 20µl of DMSO alone served as control. The mortality of the larvae was recorded at 2-, 4-, 6-, 24-, and 48-h intervals. The assay was conducted in triplicate.

Nematicidal assays - Panagrellus redivivus and *Caenorhabditis elegans* were used to test for nematicidal activity of crude leaf extracts and compound **5** according to the established procedure of Nair, et al. (1993). A stock culture of nematodes in media was maintained at 14 day intervals to provide uniform young nematodes. 10-20 nematodes in 48µl growth media were transferred to wells in a 96-well tissue-culture plate. 2µl of 50% aqueous DMSO solution of test material was added to this. 2 µl of 50% aqueous DMSO alone was used as control. The experiment was conducted in triplicate.

Gypsy moth, forest tent caterpillar, corn earworm and tobacco hornworm assays - These assays were conducted using artificial diets. Dry diet ingredients for the gypsy moth consisted of wheat germ (36g), casein (7.5g), Wesson's salt mix (2.4g), sorbic acid (0.6 g), methylparaben (0.3 g), and Hoffman-LaRoche #26862 vitamin mix (3g). 0.845g of dry diet mix was used for each treatment. The corn earworm diet was obtained from North Carolina State, and 0.94 g was used for each treatment. The tobacco hornworm diet also was obtained from North Carolina State, and 0.95 g was used for each treatment. Dry diet was weighed out in a scintillation vial, one for each test compound. The test compound was dissolved to a desired concentration in DMSO, so that 20 µl of the solution was added to the diet. 20µl

of DMSO alone served as the control. Agar solution was added to bring the total weight to 5.0 g, and the mix was homogenized with a spatula. The mixture was poured immediately (before jelling) and equally into 15 micro beakers. The beakers were randomized and allowed to cool for 3 h. One insect larvae of the type being tested was transferred to each of the 15 micro beakers with a small paint brush. The beakers were capped, and transferred to a tray in an incubation chamber at 28 °C for six days. At the end of this period, each larvae was weighed, and the average (in mg) for each sample was compared against weight of the control to determine bioactivity. Data were analyzed using Dunnet's test, where means of the treatments are compared with the control.

Results and Discussion

Leaves of *C. longa* were extracted sequentially with hexane, ethyl acetate and methanol. Preliminary bioassays were performed on these extracts to test for antimicrobial, anticancer, mosquitocidal, nematicidal, and Gypsy moth, forest tent caterpillar, tobacco hornworm and corn earworm growth inhibition.

Hexane extract was found to inhibit *C. albicans* at 250 ppm concentration. In addition, it exhibited activity against *A aegyptii* with LD_{100} at 250 ppm in 48 h. The hexane extract showed only minor activity against *Rhizoctonia spp.* Preliminary anticancer assays of the hexane extract exhibited marginal activity on *all three* mutant yeast strains, rendering it impossible to distinguish if the extract was inhibiting topoisomerase, or exhibiting antifungal activity, or both. Hence the topoisomerase inhibition assay was not studied further using this extract. The hexane extract did not exhibit activity against any of the other microbial

pathogens tested, including *Fusarium oxysporum* (MS-SM-1322), *Fusarium moniliforme* (MS-SM-1323), *Gleosporum spp,. Rhizoctonia spp, Aspergillus flavus* (MS strains) *Staphylococcus epidermidis* (ATCC 25923), *Streptococcus aureus* (MS strain), and *Escherichia coli* (ATCC 25922). The methanol and ethyl acetate extracts did not show any antimicrobial, anticancer or mosquitocidal activities. In addition, none of the three solvent extracts exhibited activity against nematodes or caused growth inhibition in the gypsy moth, forest tent caterpillar, tobacco hornworm or corn earworm assays.

Purification of the hexane extract afforded compound **5** as the active compound. It was evaluated for antifungal activity on *C. albicans, C. kruseii, and C. parapsilosis* at 100, 50, 25, 10, and 1 ppm concentrations. Activity was observed at 1 ppm for *C. albicans*, and at 25 ppm for *C. kruseii*, and *C. parapsilosis*. Mosquitocidal activity was evaluated for compound **5** at 100, 50, 25, 12.5, 10, 6.25, and 1 ppm concentrations. Compound **5** had LD_{100} at 100 ppm on *A. aegyptii* in 12 h. In 48 h, compound **5** exhibited LD_{100} on *A. aegyptii* at 10 ppm. The mechanism of this activity has not been determined. However, upon application of the test material to the mosquitoes, they began to behave in a frenzied manner. Within 20 min, the mosquitoes gathered at the water- air interface, until death occurred, and sank to the bottom of the assay tube.

The ^{1}HNMR, ^{13}CNMR, and DEPT spectra of compound **5** showed that there were 30 protons and 20 carbons, respectively. In the ^{1}HNMR spectrum, a singlet appeared at 9.38 ppm, integrated for one proton, was characteristic of an aldehydic group adjacent to an olefinic moiety. Also, a proton triplet at 9.61 ppm, with a coupling constant of 1.5 Hz was

typical of an aldehydic group adjacent to a methylene group. It was assigned to C-15 in compound **5**. The C-14 methylene protons appeared as two separate doublets, at 3.37 and 3.43 ppm, respectively. These protons were deshielded by the adjacent aldehyde group. Two doublets in the olefinic region at 4.35 and 4.84 ppm integrated for one proton each and were assigned to C-17. A single proton triplet in the olefinic region at 6.74 ppm was assigned to C-12. Also, the ^{1}HNMR spectrum showed three singlets at 0.71, 0.80, and 0.87 ppm, respectively, for C-18,19 and 20 methyl groups.

^{13}C NMR supported the presence of two aldehydic carbons at C-15 and 16, two aliphatic methylene carbons at C-11 and 14, one aliphatic methine carbon at C-12, one exocyclic methylene carbon at C-17, and three aliphatic methyl carbons at C-18,19 and 20, respectively, in compound **5**. The DEPT spectrum of **5** showed 4 quaternary carbon signals at 134.8, 148.0, 39.6 and 33.6 ppm, respectively. The signal at 134.8 ppm was in agreement with the reported value for olefinic carbons vicinal to an aldehydic substituent (Silverstein, 1991), and was assigned to C-13. The signal at 148 ppm was assigned to C-8. The two remaining quaternary carbons at 33.6 and 39.6 ppm, were assigned to C-4, and C-10, respectively.

The HMQC spectra proved very helpful in elucidating the structure of compound **5.** It confirmed the assignments of protons and carbons at residence at C-1, C-2, C-3, C-6 and C-7. The COSY spectrum further proved these assignments and showed the proton couplings between C-1 and C-2, and C-2 and C-3. Similar HMQC analysis provided assignments of C-6 and C-7 at 24.1 and 39.3 ppm, respectively. The COSY data showed the connectivities of C-5 to C-6, and C-9 to C-11 (**Figure 3.2**). From the NMR data, the molecular formula

Figure 3.1 Structure of
Labda 8(17)-12-diene, 15,16 dial

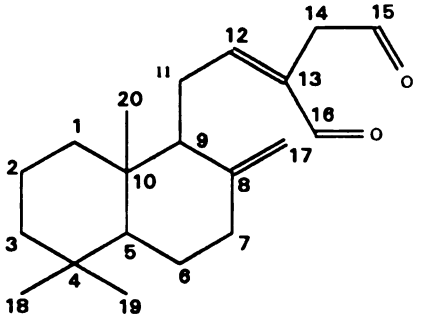


Figure 3.2 COSY Correlations for Labda 8(17)-12-diene, 15,16 dial

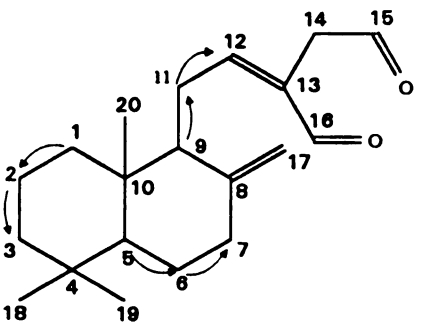
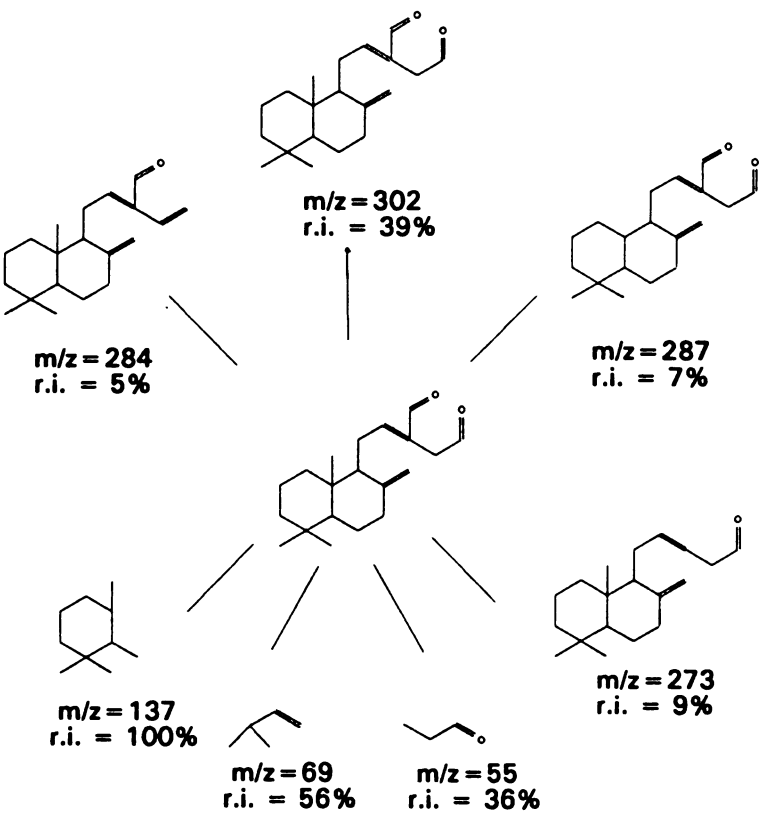


Figure 3.3-Possible EIMS Fragmentation Patterns For Labda 8917)-diene - 15,16-dial

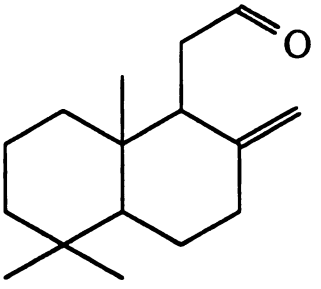


of the compound was calculated as $C_{20}H_{30}O_2$. The EIMS of compound **5** gave the molecular ion at m/z 302. Also, the FABMS spectra confirmed the molecular weight as 302, as indicated by the MH^+ peak at m/z 303.

The EIMS gave a peak at m/z 287, indicative of a sigma bond dissociation (α-cleavage) of a methyl group, (M^+-15). The peaks at m/z 284 and 273 were resulting from the loss of water (M^+-18), and an aldehydic group, respectively. The peak at m/z 137 was 100% intense. The peak at m/z 69 resulted from an α-cleavage at the C-14 carbonyl to give a stable acylium ion, and the peak at m/z 55 signified two sigma ring bond dissociations with concomitant double bond formation (**Figure 3.3**). Based on the spectral data, compound **5** was confirmed to be the diterpene labda 8(17),12-diene-15,16- dial (**Figure 3.1**).

The molar elipticity of compound **5** was at the extrenum of -17.23 mdeg at 320 nm (**Appendix XIII**). Compound **5** with a negative optical rotation was isolated previously from the seeds of *Alpinia galanga* (Morita, 1987). The positive form of 8 (17), 12 -labd-diene-15,16-dial was isolated previously from rhizomes of *Alpinia speciosa* (Itokawa, 1980). Also, Morita (1987) determined the absolute configuration of 8 (17), 12 -labd-diene-15,16-dial from *A. galanga* by the ozonolysis of the diterpene 8 (17), 12-labd-diene-15,16- dial, yielding compound **6** (**Figure 3.4**). The CD spectrum of **6** gave a molar ellipticity of -2.79 at 289 nm (Morita, 1987). The sign value of the ellipticity would be the same for compound **6** as in the case of the natural product from *A. galanga*, since no chiral centers were lost or introduced during the ozonolysis. The negative value of the ellipticity of compound **5** indicated that both compound **5** and 8 (17), 12 -labd-diene-15,16-dial from *A. galanga* are identical in their configuration. Also, due to the double bond and aldehydic group in

Figure 3.4



6

conjugation, a higher ellipticity value was obtained for **5** than for **6**. This is the first report of the CD for labda 8(17) 12-diene-15, 16-dial.

. Compound **5** exhibited activity against *C. albicans, C. kruseii* and *C. parapsilosis* at 1, 25, and 25 ppm concentrations, respectively. *Candida* is the causative agent in vaginal yeast infections. As such, there are relatively few antibiotics available to combat this pathogen. It is possible that this natural product or its analogues may be useful in the future as a topical treatment to combat yeast infections. Compound **5** exhibited an LD_{100} at 10 ppm on *A. aegyptii* larvae The mosquitocidal activity may be exploited in the future to combat growing populations of *A. aegyptii* and manage them as vectors of human disease. These activities are novel. The mechanisms of these bioactivities are unknown, and warrant further research.

CHAPTER IV

Summary and Conclusions

Fresh rhizomes collected from the greenhouse-grown *Curcuma longa* Linn plants were lyophilized. The volatile oil was extracted from the ice accumulated on the condenser coil of the lyophilizer. Lyophilized rhizomes of *C. longa* L. were extracted sequentially with hexane, ethyl acetate, and methanol, separately. A similar process was conducted for the lyophilized leaves. Preliminary bioassays were carried out on all these extracts at 250 ppm concentrations to determine the presence of antibacterial, antifungal, anticancer, mosquitocidal, and nematicidal activities. All crude extracts were tested for anticancer activity in the form of topoisomerase inhibition, using mutant *Saccharomyces cerevisae* cultures. These crude extracts also were evaluated for growth inhibition of gypsy moth larvae, *(Lymantria dispar)*, forest tent caterpillar larvae (*Malacosoma dystria)*, tobacco hornworm larvae *(Manduca sexta)*, and corn earworm larvae *(Helicoverpa zea)*.

The ethyl acetate extract from the rhizome displayed good anticancer activity. The essential oil from the rhizome and the ethyl acetate extract exhibited activity on anticancer and

mosquitocidal assays, respectively. The crude methanol extract from the rhizome displayed no antimicrobial, insecticidal, anticancer, or growth inhibition activity when tested on *Aspergillus spp., Botrytis spp., Fusarium moniliforme, Fusarium oxysporum, Gloesporum spp., Rhizoctonia spp., Candida albicans, Streptococcus spp., Staphylococcus spp., Escherichia coli, Aedes aegyptii* mosquitoes, *Panagrellus redivivus* Goody nematodes, *Caenorhabditis elegans* nematodes, *Saccharomyces cerevisiae* mutant cultures JN394, JN 394t-1, JN394t2-5, *Lymantria dispar, Malacosoma dystria, Manduca sexta,* or *Helicoverpa zea.*

Bioassay-directed fractionation was carried out to isolate the active compounds from rhizomes of *C. longa*. Rhizomes yielded three anticancer compounds, curcumin I (**1**), curcumin II (**2**), curcumin III (**3**). Structures of compounds **1-3** were confirmed using ^{1}HNMR experiments, and these compounds were evaluated for anticancer activity (topoisomerase inhibition) (**Chapter II**). Curcumin III gave the most potent anticancer activity, represented by the inhibition of topoisomerase I and II enzymes at 25 ppm concentration. However, curcumin I and curcumin II inhibited topoisomerase I and II at 50 ppm concentration.

The volatile oil of the rhizome yielded a mosquitocidal sesquiterpene ketone, ar - turmerone (**4**). The structure of compound **4** was confirmed using ^{1}HNMR, ^{13}CNMR, and EIMS experiments (**Chapter II**). Compound **4** was evaluated for mosquitocidal activity (**Chapter II**). It displayed an LD_{100} at 50 ppm on *A. aegyptii*.

The hexane extract from the leaves exhibited good antifungal and mosquitocidal activities. The crude leaf methanol and ethyl acetate extracts displayed no antimicrobial,

insecticidal, anticancer, or growth inhibition activity when tested on *Aspergillus spp.*, *Botrytis spp.*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Gloesporum spp.*, *Rhizoctonia spp.*, *Candida albicans*, *Streptococcus spp.*, *Staphylococcus spp.*, *Escherichia coli*, *A. aegyptii* mosquitoes, *Panagrellus redivivus* Goody nematodes, *Caenorhabditis elegans* nematodes, *Saccharomyces cerevisiae* mutant cultures JN394, JN 394t-1, JN394t2-5, *Lymantria dispar*, *Malacosoma dystria*, *Manduca sexta*, or *Helicoverpa zea* (**Chapter III**).

Bioassay-directed fractionation was carried out to isolate the active compound from the leaf extracts of *C. longa*. The hexane extract yielded an antifungal diterpene labda 8(17) diene-15, 16,-dial, compound **5**. The structure of compound **5** was confirmed using ^{1}HNMR, ^{13}CNMR, DEPT, HQMC, COSY, EIMS and FABMS experiments (**Chapter III**). Compound **5** was evaluated for antifungal activity on *C. albicans*, *C. kruseii*, and *C. parapsilosis*. Compound **5**, labda 8(17) 12-diene-15,16- dial, was antifungal against *C. albicans* at 1 ppm. Also, this diterpene inhibited the growth of *C. kruseii* and *C. parapsilosis* at 25 ppm. It also displayed an LD_{100} at 10 ppm in mosquitocidal assays against *A. aegyptii*.

The most effective mosquitocidal component was the labdane type diterpene, labda 8(17) 12-diene -15,16-dial, compound **5**, which displayed an LD_{100} at 10 ppm in mosquitocidal assays, compared to the activity of ar-turmerone, compound **4**, which displayed an LD_{100} at 50 ppm on *A. aegyptii*. Interestingly, ar-turmerone caused mortality in 18 h at 50 ppm, while labda 8(17) 12-diene -15,16-dial took 48 h at 10 ppm concentration to cause mortality.

The mechanism for the inhibition of these compounds has not been determined. However, some observations regarding inhibition displayed by the curcuminoids can be made

based on their structural differences. Curcumin III displayed greater anticancer activity than either curcumin II, or curcumin I, and curcumin II displayed more activity than Curcumin I. A literature search revealed that this trend is not unusual for most of the activities reported for curcuminoids. The lack of a methoxy substituent in a curcuminoid increased its activity. Also, removal of both methoxy substituents further enhanced activity as displayed by curcumin III. It is possible that the elimination of the methoxy group(s) increased the phenolic nature of the curcuminoid, and hence it can act as an effective antioxidant or a free radical scavenger. Additional research is required to confirm this hypothesis.

The work reported in this thesis has provided additional insight into known compounds with novel activities. Also, this is the first report of the isolation of compound 5, (-) labda 8(17)-12- diene - 15,16-dial from *C. longa.*

Literature Cited

Abbiw, D.K. (1990) Useful Plants of Ghana: West African uses of wild and cultivated plants. Intermediate Technology Publications. Royal Botanic Gardens. Kew, England. 129.

Alexander, J. (1973) The Chemistry of ar-turmerone, atlantone and related monocyclic sequiterpenoid ketones. *Flavour Industry*. Sep. 390-3.

Ammon, H., Safayhi, H., Mack, T., and Sabieraj, J. (1993) Mechanism of antiinflammatory actions of curcumine and boswellic acids. *Journal of Ethnopharmacology*. **38.** 113-119.

Amphawan, A., Pongsawad, N., (1986) Antimicrobial activity of Thai natural products-Proceeding of the 4th Conference on Health Science. Chiang Mai Univ., Chiang Mai, Thailand. 25-6.

Andres, C. (1982) Yellow and reddish-orange colors have good stability and solubility. *Food Processing*. **43**. no.1-6. 44-5.

Anjaneyulu, V., and Krishnamurthy, D. (1986) Turmeric in Andhra Pradesh. *Indian Spices*. **5**(1). 8.

Anto, R.J. (1994) A comparative study on the pharmacological properties of natural curcuminoids. *Amala Research Bulletin*. **14.** 60-65.

Basu, A. P.(1971) *Indian Journal of Pharmacology*. **33**. 131.

Bandara, B. M., Hewage, C. M., Karunaratne, V., and Adikaram, N. (1988) Methyl ester of para-coumaric acid: antifungal principle of the rhizome of *Costus speciosus*.

Planta Medica. 1988. 477-478.

Bielig, H.H., Dreyer, H., and Askar, A. (1977) Blei-und Cadmiumgehalt von Gewurzen". Dtsch. *Lebensm. Rundsch.* **73**(2). 43.

Brierly, J. (1994) Spices - The Story of Indonesia's Spice Trade. Oxford University Press. Oxford.

Burkill, I. (1966) A Dictionary of the Economic Products of the Malay Peninsula, Singapore. Governments of Malaya and Singapore.

Chang, Yu-Chen, and Nair, Muraleedharan, G. (1995). Metabolites of Daidzein and Genistein and their Biological Activities. *J.of Nat. Prod.* **58**. 1901- 5.

Charles, V., and Charles, S. (1992) The use and efficacy of *Azadirachta Indica* ADR ('Neem') and *Curcuma longa* ('Turmeric') in scabies. *Trop. Geogr. Med.* **44**. 178-181.

Cherdchu, C., Srisukawat, K., Ratanbanangkoon, K. (1978) Cobra neurtotoxin inhibiting activity found in the extract of *Curcuma sp.* (Zingiberaceae). *Journal of the Medical Association of Thailand.* **61**. 544.

Cherdchu, C., Srisukavat, K., Ratanabanangkoon, K. (1983) Cobra neurotoxin inhibition activity found in the extract of *Curcuma* spp. (Zingeberacaeae). *South East Asian Journal of Tropical Medicine and Public Health.* **14**. 176-180.

Chopra, I.C. (1961) Review of Research on Indian Medicinal and Allied Plants. *Indian Council of Agricultural Research*. 32.

Chopra, R.N. (1969) Supplement to Glossary of Indian Medicinal Plants. Publications and Information Directorate, New Delhi. 21.

Claeson, P., Panthong, A., Tuchinda, P., Reutrakul, V., Kanjanapothi, D., Taylor, W. and Santisuk, T. (1993) Three non-phenolic diarylheptanoids with anti-inflammatory activity from Curcuma xanthorrhiza. *Planta Medica*. **59**. 451-4.

Dahl, T., McGowan, W., Shand, M., Srinivasan, V., (1989) Photokilling of bacteria by the natural dye curcumin. *Archives of Microbiology*. **151**(2). 183-5.

Dhar, M.L. (1968) *Indian Journal of Experimental Biology*. **6**. 232.

Farmerie, W.G., Loeb, A.D., Lasavant, N.C., Hutchinson, C.A., Edgell, M.H., and Swanstrom, R. (1987) Expression and processing of the AIDS virus reverse transcriptase in *Escherichia coli*. *Science*. **236**. 305.

Ferreira, L. (1992) Antivenom and biological effects of ar-turmerone isolated from Curcuma longa. *Toxicon*. **30**. Iss 10. 1211-8.

Francis, F.(1986) Handbook of Colorant Patents. University of Massachusetts, Dept. of Food Science and Nutrition. Food and Nutrition Press, Inc. Westport, CT. 80-83.

Freund, P. (1985) Natural Colors in Cereal-Based Products. *Cereal Foods World.* **30** no. 1-6. 271-3

Ghandi, R.P., vig, O.P, and Mukerji, S. (1958) A new synthesis of ar-turmerone. *Sci. Cul*. **23**. 483.

Gopalan, C. (1976) Medicinal Plants of India, Vol 1. Indian Council of Medicinal Research 316.

Govindarajan, V. (1980) Turmeric-Chemistry, Technology, And Quality. *CRC Critical Reviews in Food Science and Nutrition*. CRC Press, Inc. Boca Raton, FL. **12**. Issue3.

Hallagan, J. (1991) The Use of Certified Food Color Additives in the United States. *Cereal Foods World.* **36**. no. 7-12. 945-948.

Harris, B.C. (1972) The Compleat Herbal; being a description of the origin, the lore, the characteristics, the types and prescribed uses of medicinal herbs, including an alphabetical guide to all common medicinal plants. Barre Publishers. Barre, MA.184.

Hirahara, F., Takai, Y., Iwao, H. (1974) Antioxidative activity of various spices on oils and fats. I. Antioxidative activity towards oxidation on storage and *heating. Japanese Journal of Nutrition.* **32**. (1) 1-8, 23.

Huan, M., Smart, R., Wong, C., Conney, A. (1988) Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-)-tetradecanoylphorbol-13-acetate. *Cancer Research.* **48**. 5941-5946.

Huang, M., Lysz, T., Ferraro, T., Abidi, T., Laskin, J., and Conney, A. (1991) Inhibitory Effects of Curcumin on in Vitro Lipoxygenase and Cycloxygenase Activities in Mouse Epidermis. *Cancer Research.* **51**. 813-819.

Itokawa, H., Morita, M., Mihashi, S. (1980) Labdane and bisnorlabdane type diterpenes from *Alpinia speciosa* K. Schum. *Chemical and Pharmaceutical Bulletin.* 3452.

Jain, S. K. (1991) Medicinal Plants of India: vol 2. Reference Publications, Inc.615.

Jentzch, K., Spiegl, P., and Kamitz, R. (1970) Qualitative und quantitative Unterschungen uber Curcuma farbstoffe in Verschiedenen Zingiberaceedrogen, 2 Teil. Quantitative Unterschungen. *Sci. Pharm.Vienna.* **38.** 50. *Chem. Abst.* **73** 28961.

Jilani, G., Saxena, R. (1990) Repellent and feeding deterrent effects of turmeric oil, sweetflag oil, neem oil, and a neem-based insecticide against lesser grain borer (Coleoptera: Bostrychidae). *Journal of economic entomology*. **83**(2). 629-634.

Jurgens, T., Frazier, E., Schaeffer, J., Jones, T., Zink, D., Borris, R. (1994) Novel Nematocidal agents from *Curcuma comosa*. *J. of Nat. Prod.* **57**. 2. 230-5.

Khalique, A., and Das, N.R. (1968) Examination of *Curcuma longa* L., Part II-Constituents of the essential oil. *Sci. Res.* East Regional Lab(Pakistan). **5**(1). 44.

Kiso, Y., Suzuki, Y., Watanabe, N., Oshima, Y. and Hikino H. (1983) Antihepatotoxic principles of Curcuma longa rhizomes. *Journal of Medicinal Plant Research*. **49**. 185-7.

Kiuchi, F.,Goto, Y., Sugimoto, N., Akao, N., Kondo, K., and Tsuda, Y. (1993) Nematocidal activity of turmeric: Synergistic action of curcuminoids. *Chem. Pharm. Bull.* **41**(9). 1640.

Krishnamurthy, M. A., Patel, J.D., and Parthasarathy, N. (1971) Enumeration of micro-organisms in spices and spice mixtures. J. *Food Sci. Technol.* **8**(4). 191.

Krishnamurthy, N., Mathew, A.G., Nambudiri, E.S., Shivasankar, S., Lewis, Y.S., and Natarajan, C.P. (1976) Oil and oleoresin of turmeric. *Trop. Sci.* **18**(1). 37.

Krishnamurthy, M. A., Patel, J. D., Parthasarathy, N., and Nair, K.K. S. (1973) Some types of coliforms, aerobic mesophilic spore formers, yeasts, and molds present In spices. *Journal of Plantation Crops*. (India). **1** (supplement), 200.1973.

Kurup, P.N. V. (1979) Hand Book of Medicinal Plants. Central Council for Research

in Ayurveda and Siddha. 78. Kuttan, R. (1994) Turmeric - A goldmine for biological research. *Amala Research Bulletin.* **14**(20). 80-81.

Kuttan, R., Bhanumathy, P., Nirmala, K., and George, M. (1985) Potential Anticancer activity of turmeric (*Curcuma longa). Cancer Letters.* **29**. 197-202.

Kuttan, R., Soudamini, K. (1989) Inhibition of chemical carcinogenesis by curcumin. *Journal of Ethnopharmacology.* **1-2**. 227-33

Kuttan, R., Sudheeran, P., Josph, C. (1987) Turmeric and curcumin as topical agents in cancer therapy. *Tumori.* **73**(1). 29-31.

Lauro, G. (1991) A Primer on Natural Colors. *Cereal Foods World.* **36.** no. 7-12. 949-953.

Lee, C. (1982) Studies on the antioxidative activities of spices grown in Taiwan. II. *Journal of the Chinese Agricultural Chemical Society.* **20** (½) 61-66, 15.

Lindley, J. (1838) Flora Medica: A botanical Account of all the More Important Plants Used in Medicine. Longman, Orme, Brown, Green and Longmans. 562.

Lutomski, V., Kedzia, B., Debska, W. (1974) Effect of an alcohol extract and active ingredients from curcuma longa on bacteria and fungi. *Planta Medica.* **26**. 9-19.

Mattada, R.R., Sankaran, R., Thangamani, and Ramanathan, L.A. (1974) Control of microbial contamination in species. *Indian J. Microbiology.* **14**(3). 139.

Mayer, F. (1943) The Chemistry Natural Coloring Matters, Transl. And revised by Cook, A.H., Reinhold, New York. 93.

Miller, J. (1969) The Spice Trade of the Roman Empire. Clarendon Press, Oxford.

Morita, Hiroshi, and Itokawa, Hideji. (1988) Cytotoxic and Antifungal Diterpenes from the Seeds of *Alpinia galanga*. *Planta Medica*. 117-120.

Mory, 1989. ANQ, vol 2. 126.

Mukhopadhyay, A., Basu, N., Ghatak, N., and Gujral, P. (1982) Anti-inflammatory and irritant activities of curcumin analogues in rats. *Agents and Actions*. **12**. 4.

Mukundan, M., Chacko, M., Annapurna, V., and Krishnaswamy, K. (1993) Effect of turmeric and curcumin on BP-DNA adducts. *Carcinogenesis*. **14**. 493-6.

Munasiri. (1987) *Journal of Food Science*. **52.**

Nair, M.G., Putnam, A.R., Mishra, S.K., Mulks, M.H., Taft, W.H., Keller, J.E., and Miller, J.R. (1989) Faeriefungin: a new broad-spectrum antibiotic from *Streptomyces Griseus* var. *Autotrophicus*. *Journal of Natural Products*. **52**. No. 4. 797-809.

Nagabhushan, M., Amonkar, A., and Bhide, S. (1987) *In vitro* antimutagenicity of curcumin against environmental mutagens. *Fd Chem. Toxic*. **25** (7). 545-7.

Nakayama, R. (1993) Two curcuminoid pigments from Curcuma domestica. *Short Reports. Phytochemistry*. **33**. No. 2. 501-2.

Noguchi, N., Komuro, E., Niki, E., Willson, R. (1994) Action of curcumin as an antioxidant against lipid peroxidation. *Journal of the Japan Oil Chemists' Society*. 1994. **43** (12) 1045-1051.

Pabon, H.J.J. (1964) Synthesis of curcumin and related compounds. *Rec. Trav. Chim. Pays-Bas*. **83**. 379. 1964.

Petrus, D. (1984) Orange juice adulteration: detection and quality effects of dilution,

added orange pulpwash, turmeric and sorbate. *Journal of Food Science*. Institute of Food Technologists. **49** (6). 1438-1443.

Polasa, K., Sesikaran, B., Krishna, T., and Krishnaswamy, K. (1991)Turmeric (*Curcuma longa*)-induced reduction in urinary mutagens. *Fd Chem. Toxic*. **29**. 699-706.

Qureshi, S., Shah, A., and Ageel, A. (1992) Toxicity studies on *Alpinia galanga* and *Curcuma longa*. *Planta Medica*. **58**. 124-7.

Ramprasad, C. (1956) *Journal of Science and Industry Research*. Col 15c. 239.

Ravindranath, V., and Satyanarayana, M. (1980) *Phytochemistry*. An unsymmetrical diarylheptanoid from Curcuma longa. **19**. 2031-2.

Rao, M. And Reddy, V. (1977) Effect of different levels of nitrogen, phosphorous, and potassium on the yield of turmeric (Curcuma longa L.). *J. Plantation Crops*. **5**(1). 60.

Reddy, A., and Lokesh, B. (1994) Effect of dietary turmeric (*Curcuma longa*) on iron-induced lipid peroxidation in the rat liver. *Fd. Chem. Toxic*. **32** (3). 279-283.

Regupathy, A., Santharam, G., Balasubramaniam, M., and Arumugam, R. (1976) Occurrence of the scale Aspidiotus hartii C. (Diaspididae: Homoptra) on different types of turmeric (Curcuma longa, Linn). *J. Plantation Crops*. **4**. 80.

Rimpler, H., Hansel, R., and Kochendoerfer, L. (1970) Xanthorrhizol, ein neues Sesquiterpen aus *Curcuma xanthorrhiza, Z. Naturforsch.* **25b**. 995.

Roughly, P.J. and Whiting, D. A. (1973) Experiments in the biosynthesis of curcumin. *J.Chem Soc. Perkin Trans.* I, 2379.

Rupe, H., Clar, G., Pfau, A. St., and Plattner, PL. (1934) Volatile plant constituents.

II. Turmerone, the aromatic principle of turmeric oil (in German). *Helv. Chim. Acta.* **17**.372. ; *Chem Abst.* **28**. 4708.

Rupe, H. And Gassmann, A. (1936) Ar-Turmerone from *Curcuma* oil. *Helv. Chim Acta.* **19**. 569. ; *Chem Abstr.* **30**, 6381.

Salimanth, B., Sundaresh, C., Srinivas, L. (1986) Dietary components inhibit lipid peroxidation in erythrocyte membrane. *Nutr Res.* Pergamon Press **6** (10) 1171-1178.

Sarma, Y. R., Nambiar, K., Nair, C. (1974) Brown rot of turmeric. *Journal of Plantation Crops.* **2**. 33.

Satoskar, R., Shah, S., Shenoy., S. (1986) Evaluation of anti-inflammatory property of curcumin (diferuloyl methane) in patients with postoperative inflammation. *International Journal of Pharmacol Ther Toxicol.* **24**(12). 651-4.

Selvam, R., Subramanian, L., Gayathri, R., and Angayarkanni, N. 1995. The anti-oxidant activity of turmeric (*Curcuma longa). Journal of Ethnopharmacology.* **47** 59-67.

Shalini, V., Srinivas, L. (1987) Lipid peroxide induced DNA damage: protection by turmeric (Curcuma longa). *Mol Cell Biochem.* **77**(1). 3-10.

Shankar, R.N.B. Effect of turmeric fractions on the growth of some intestinal and pathogenic bacteria in vitro. *Indian Journal of Experimental Biology.* New Delhi, Council of Scientific and Industrial Research.

Sharma, O. (1976) Antioxidant activity of curcumin and related compounds. *Biochemical Pharmacology.* **25**. 1811-1812.

Shivashankar, S.; Lewis, Y.; Natarajan, C.; Krishnamurthy, N.; Mathew, G.;

Nambudiri, E. (1976) Oil and oleoresin of turmeric. *Tropical Science*. Central Food Technological Research Institute. Mysore-570013, India.**18**. 37-45.

Silverstein, R. M., Bassler, G. C., Morrill, T. C. (1991) Spectrometric Identification of Organic Compounds. 5th edition. John Wiley and Sons, Inc. New York, New York.

Singh, D. (1991) Repellant and insecticidal properties of essential oils against the housefly, *Musca domestica*. *Insect Science and its Application*. Nairobi, Kenya. Science Press.**12**(4).

Stryer, L. (1988) Biochemistry, 3rd ed. 977-978. W.H. Freemand and Company, New York.

Srinivasan, K. R. (1952) The colouring matter in turmeric. *Curr. Sci*. **21**. 911.

Srinivasan, K. R. (1953) Chromatographic study of the curcuminoids in *Curcuma longa*. *J.Pharm. Pharmacol*. **5**. 448.

Srivastava, K. (1995) Curcumin, a major component of food spice turmeric (*Curcuma longa*) inhibits aggregation and alters eicosanoid metabolism in human blood platelets. *Prostaglandins Leukotrienes And Essential Fatty Acids*. **52**. 223-7.

Srivastava, R. (1989) Inhibition of neutrophil response by curcumin. *Agents and Actions*. **3-4**. 298-303.

Su, H.C.F.(1982) Isolation, purification and characterization of insect repellants from *Curcuma longa* L. Effects on *Tribolium castaneum*. *Journal of Agr. and Food Chem.* Washington, D.C. American Chem. Soc. **30**. 290.

Sui, Z., Salto, R., Li, J., Craik, C. and de Montellano, P. Inhibition of the HIV-1 and

HIV-2 proteases by curcumin and curcumin boron complexes. *Bioorganic & Medicinal Chemistry.* **1** (6). 415-422.

Tejasen, P., Chantaratham, A., and Kanjanapothi, D. (1969) The antagonistic effect of a medicinal plant (*Curcuma sp., Zingiberaceae)* to Thailand cobra venom (*Naja naja siamensis). Part* I. *Chiang Mai Medical Bulletin.* **8a**. 165.

Tejasen, P., Chantaratham, A., and Kanjanapothi, D. (1969) The antagonistic effect of a medicinal plant (*Curcuma sp., Zingiberaceae)* to Thailand cobra venom (*Naja naja siamensis).* Part II. *Chiang Mai Medical Bulletin.* **8b.** 229.

Tejasen, P., Chantaratham, A., and Kanjanapothi, D. (1969) The antagonistic effect of a medicinal plant (*Curcuma sp., Zingiberaceae)* to Thailand cobra venom (*Naja naja siamensis). Chiang Mai Medical Bulletin.* **9a**. 56.

Tejasen, P. And Thongtharb, C. (1978) The antagonistic effect against cobra envenomization: comparison of medicinal plants, corticosteroid and antivenine. *Chiang Mai Medical Bulletin.* **17**. 159.

Tonnesen, H., deVries, H., Karlsen, J., Beijersbergen van Henegouwen, G. (1987) Studies on curcumin and curcuminoids. IX: Investigation of the photobiological activity of curcumin using bacterial indicator systems. *Journal of Pharm Science.* **76**(5). 371-3.

Unterhalt, B. (1980) Turmeric and its use in mustard. *Zeitschrift fuer Lebensmittel Untersuchung und Forschung.* **170** (6) 425-428.

Vevai, E. (1971) Know your crop, its pest problems and control -32; Ginger and turmeric. *Pesticides.* **5**(5), 32,

Verrill, A. (1940) Perfumes and Spices. L. C. Page & Company. Boston, MA. 48-51, 287

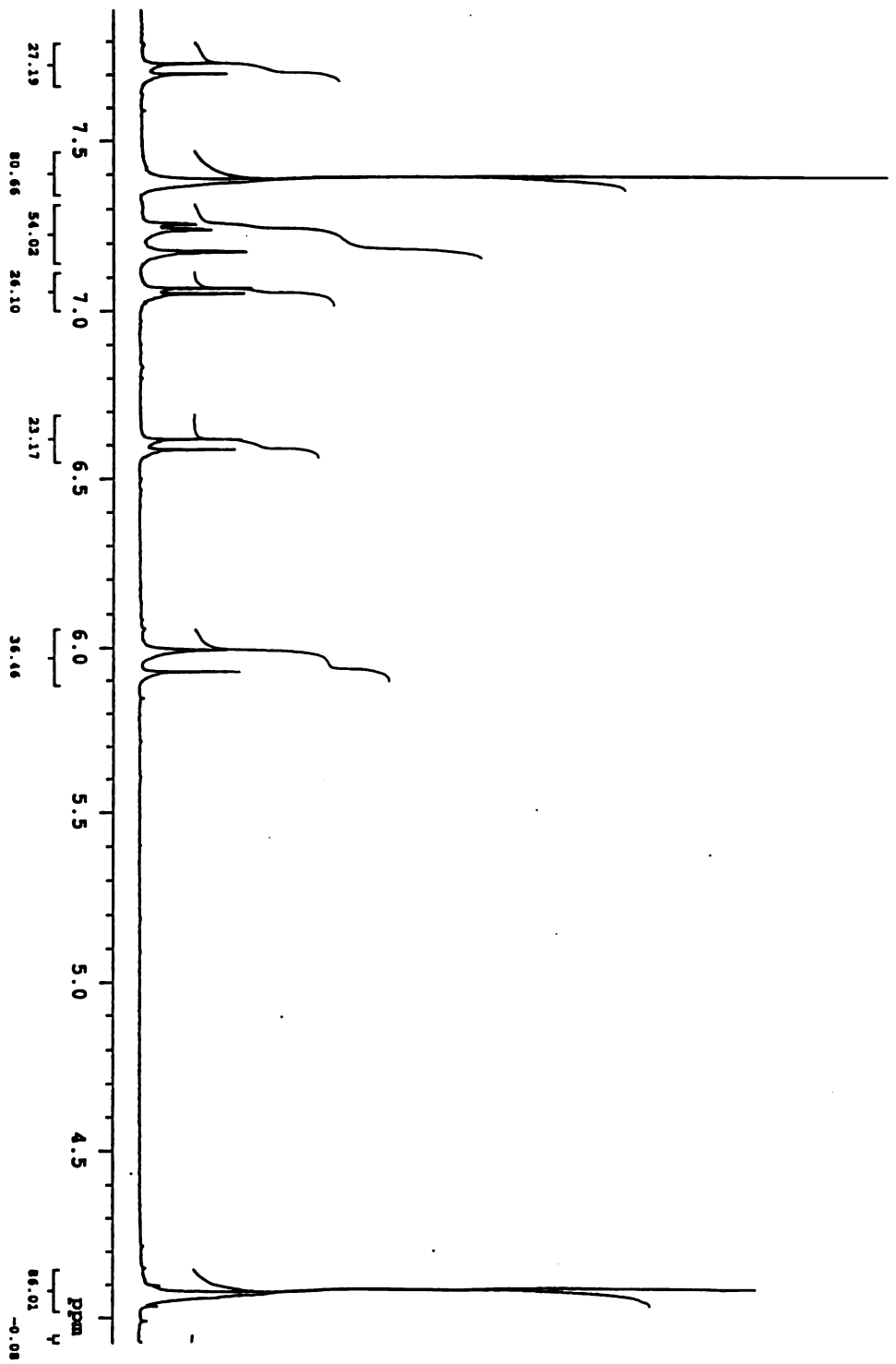
Viswanath, B.N.(1981) Turmeric powder as an ant repellant feeding on foods. *V.A.S. Technical Series -India*, The University. **37**.313.

Waring, E.J. (1982) Bazaar Medicines and Common Medical Plants of India. Bishen Singh Mahendra Pal Singh. 150.

Wilfred, J. (1980) Turmeric- Developments in Food Colors 1. Organics Division, Imperial Chemical Industries, Ltd. Elsevier Applied Science Publishers. London.215.

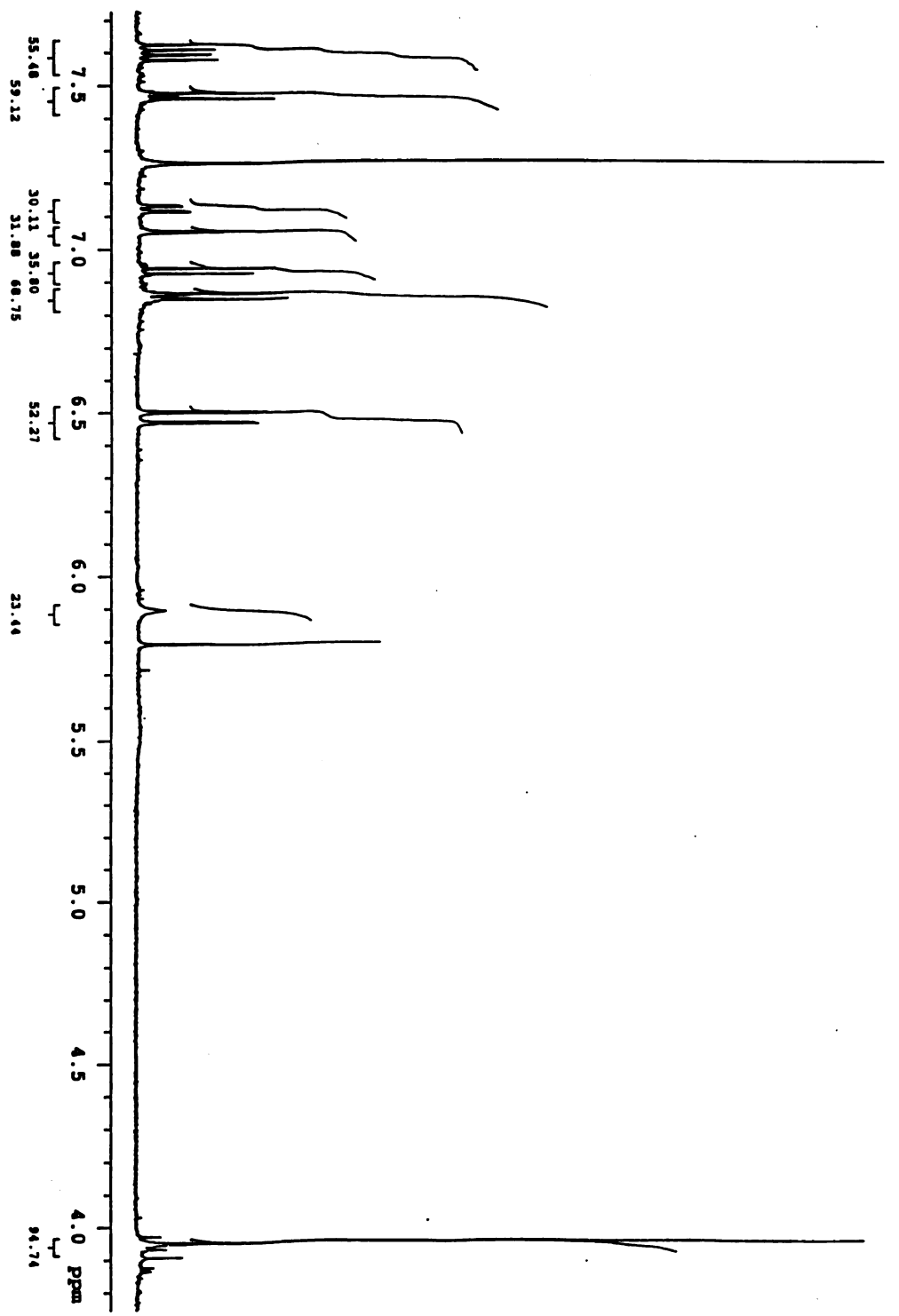
Zhao, B., Li, X., He, R., Cheng, S., Xin, W. (1989) Scavenging effect of extracts of green tea and natural antioxidants on active oxygen radicals. *Cell Biophysics.***14**(2) 175-85.

APPENDIX I



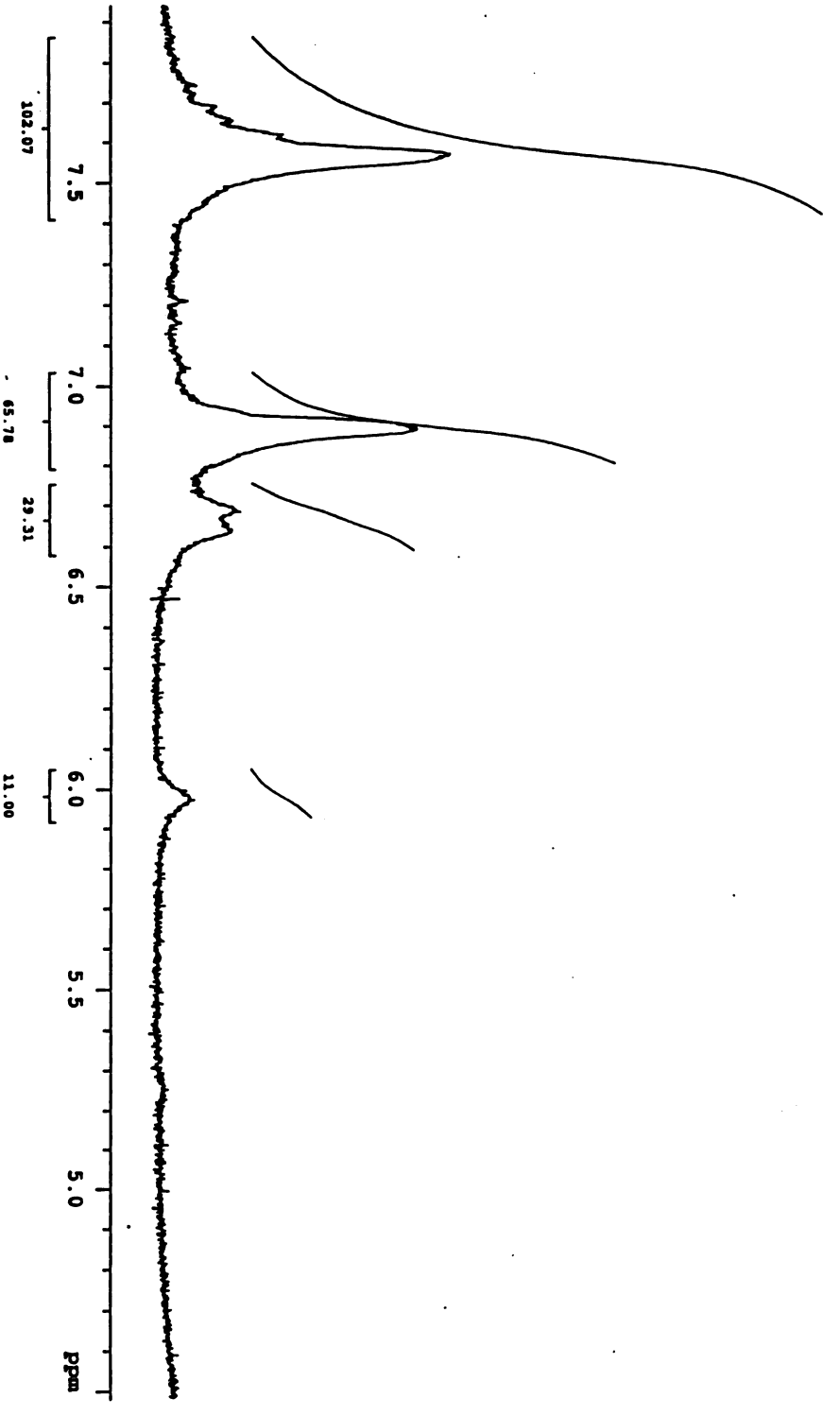
^{1}HNMR Spectrum of Curcumin I

APPENDIX II



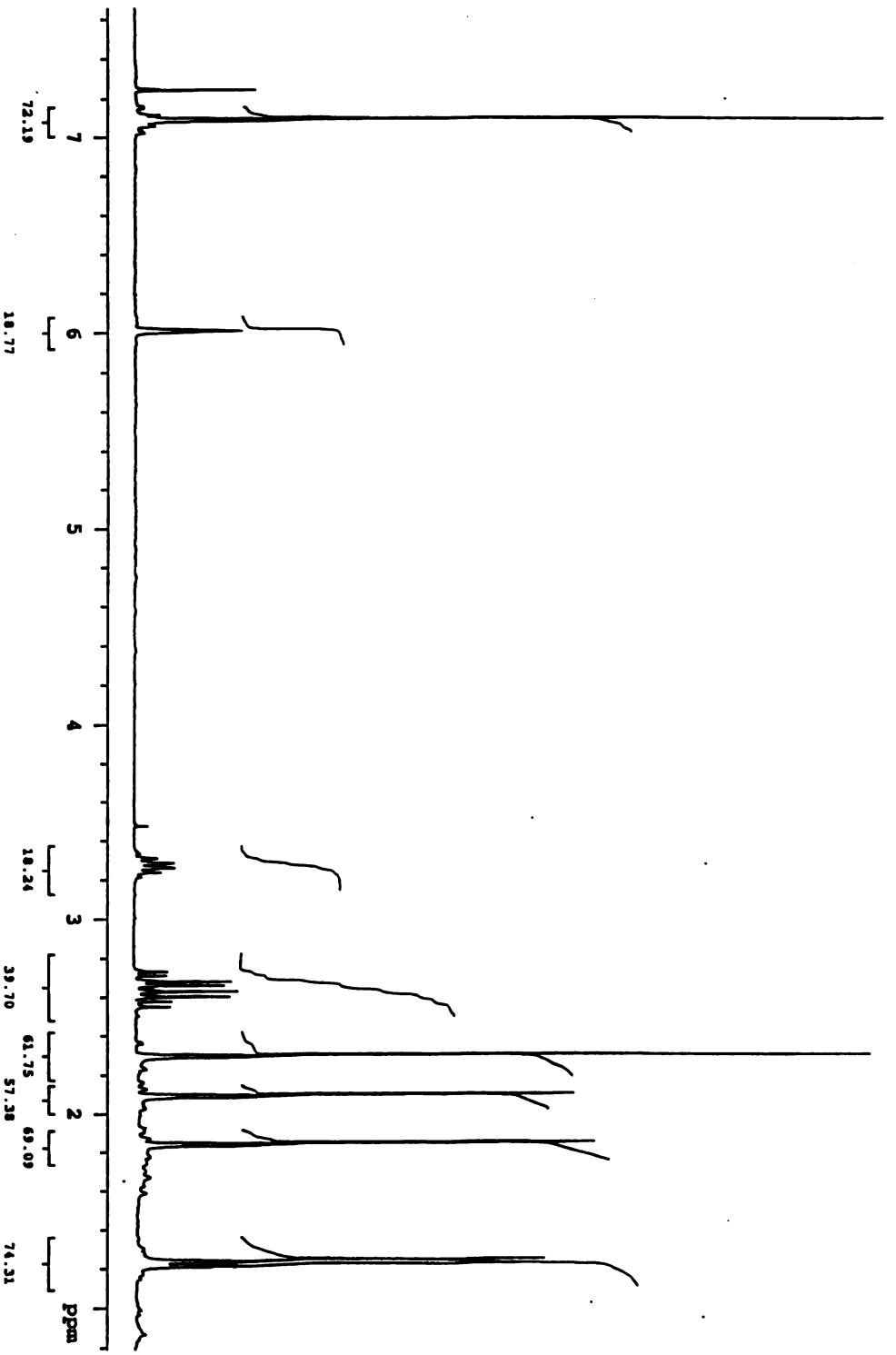
^{1}HNMR Spectrum of Curcumin II

APPENDIX III



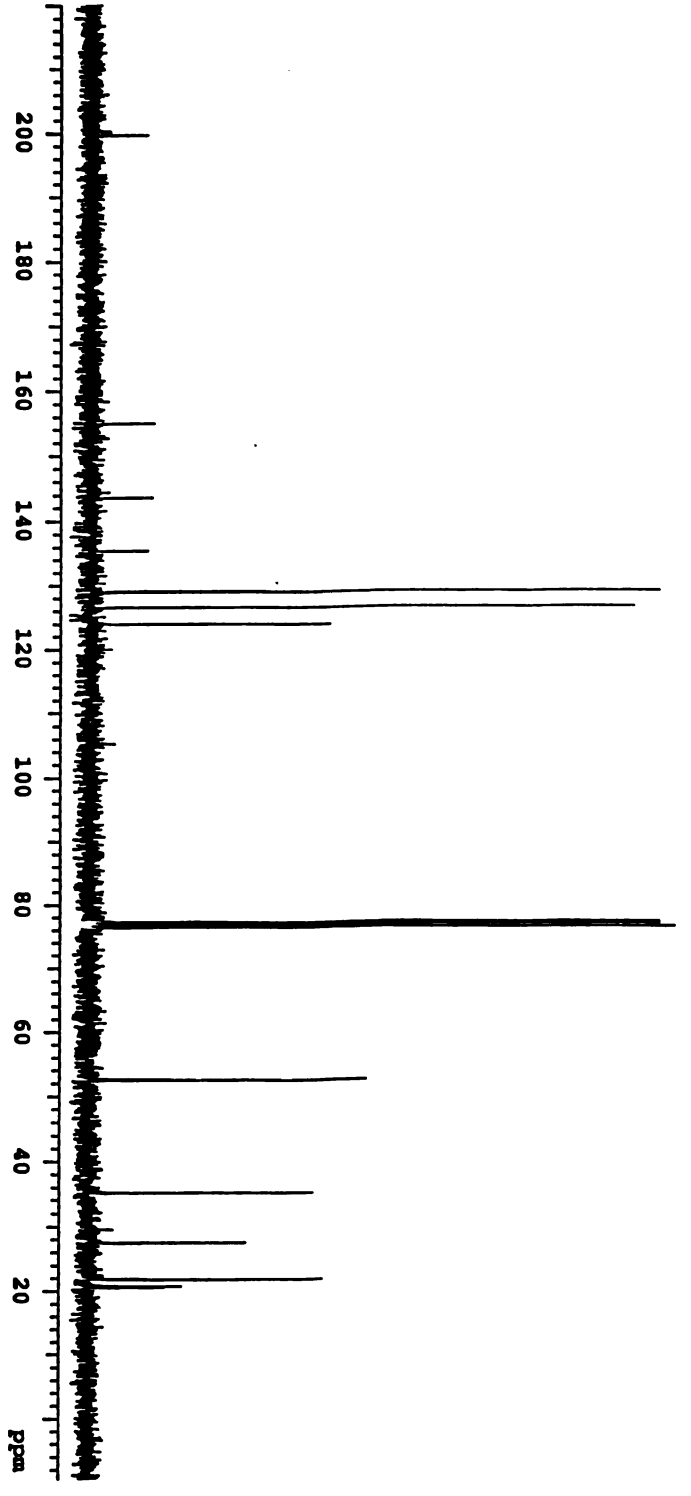
^{1}HNMR Spectrum of Curcumin III

APPENDIX IV



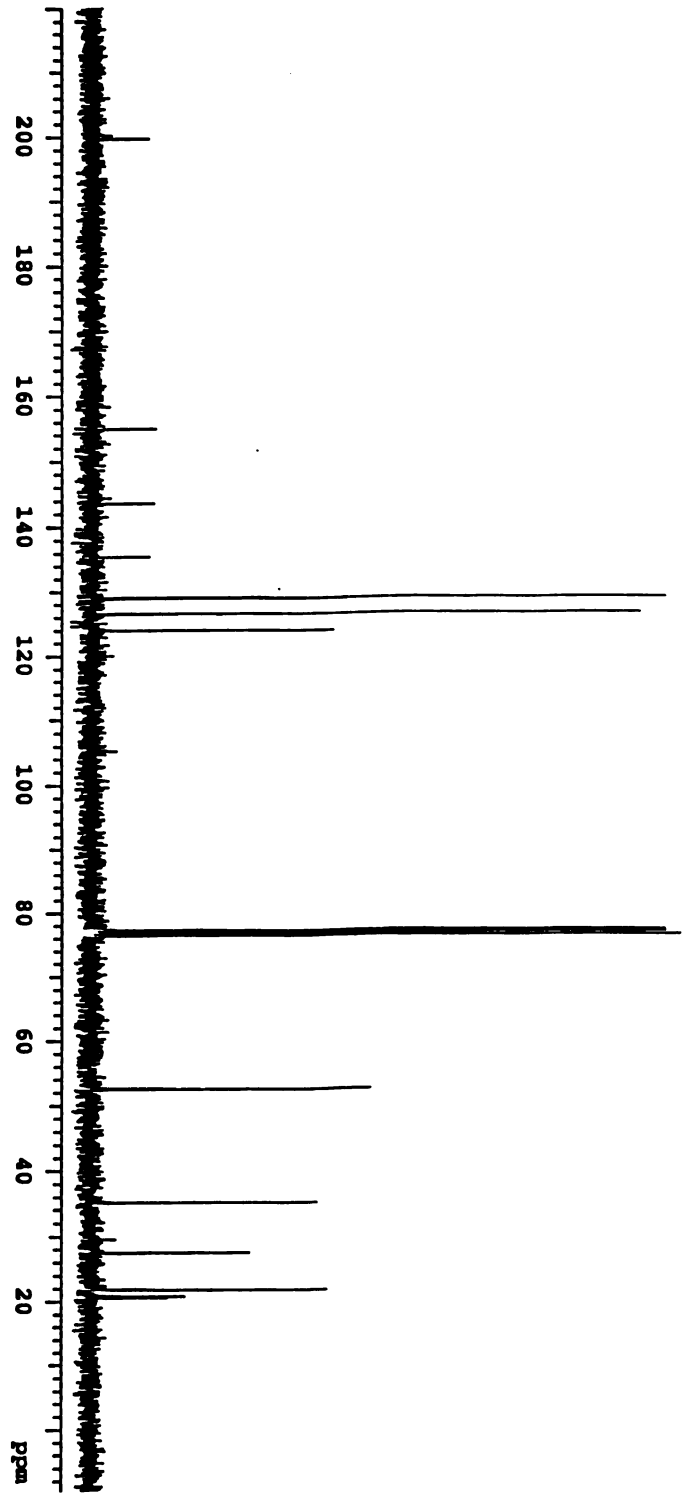
^{1}HNMR Spectrum of Ar-Turmerone

APPENDIX V



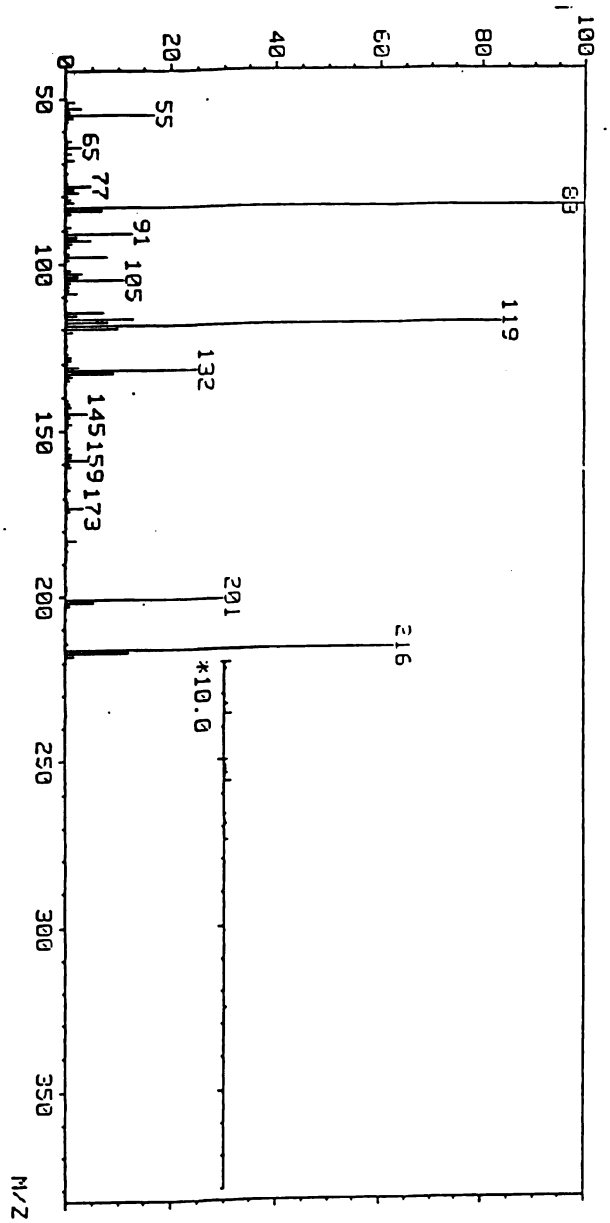
^{13}CNMR Spectrum of Ar-Turmerone

APPENDIX V



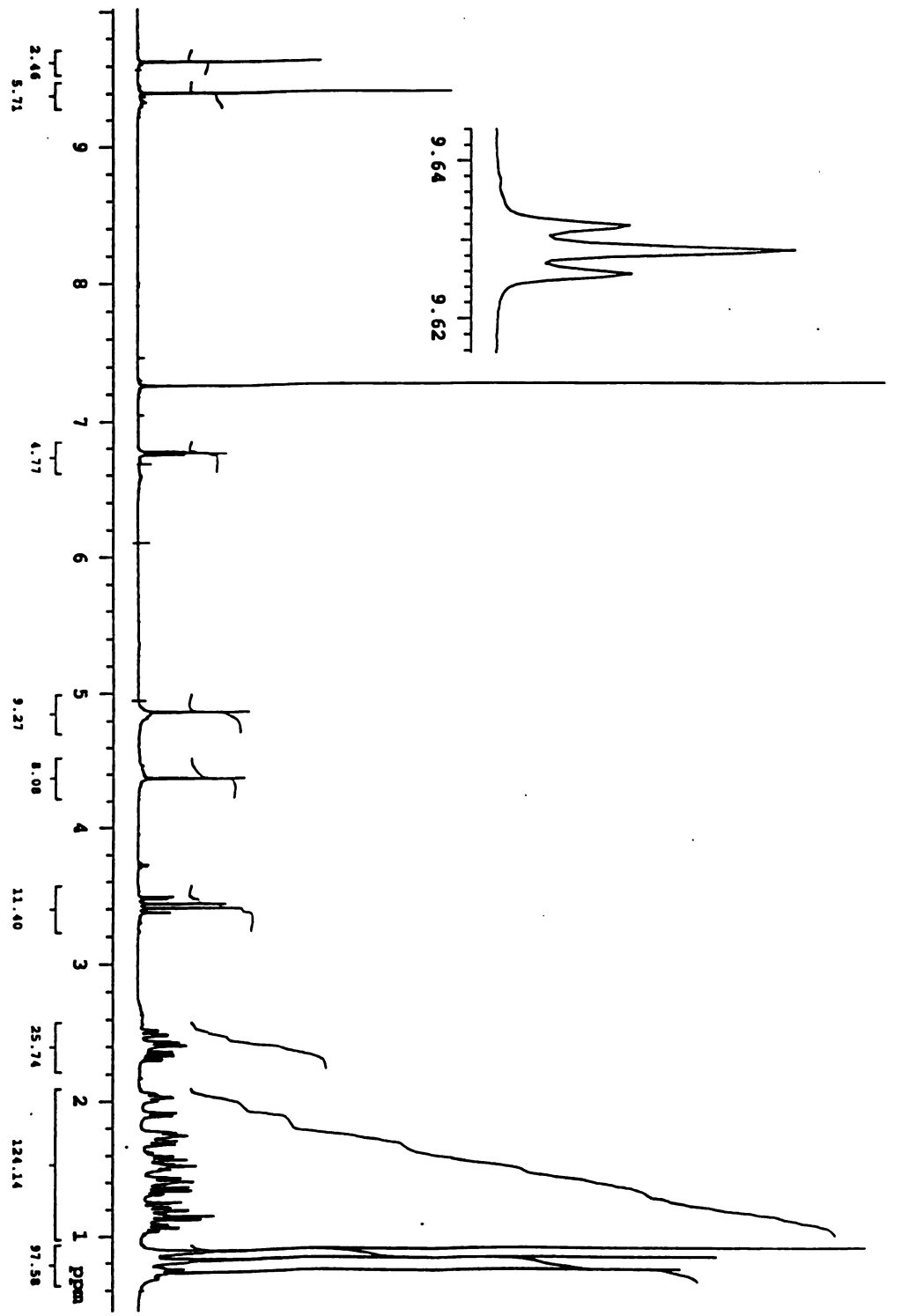
^{13}CNMR Spectrum of Ar-Turmerone

APPENDIX VI



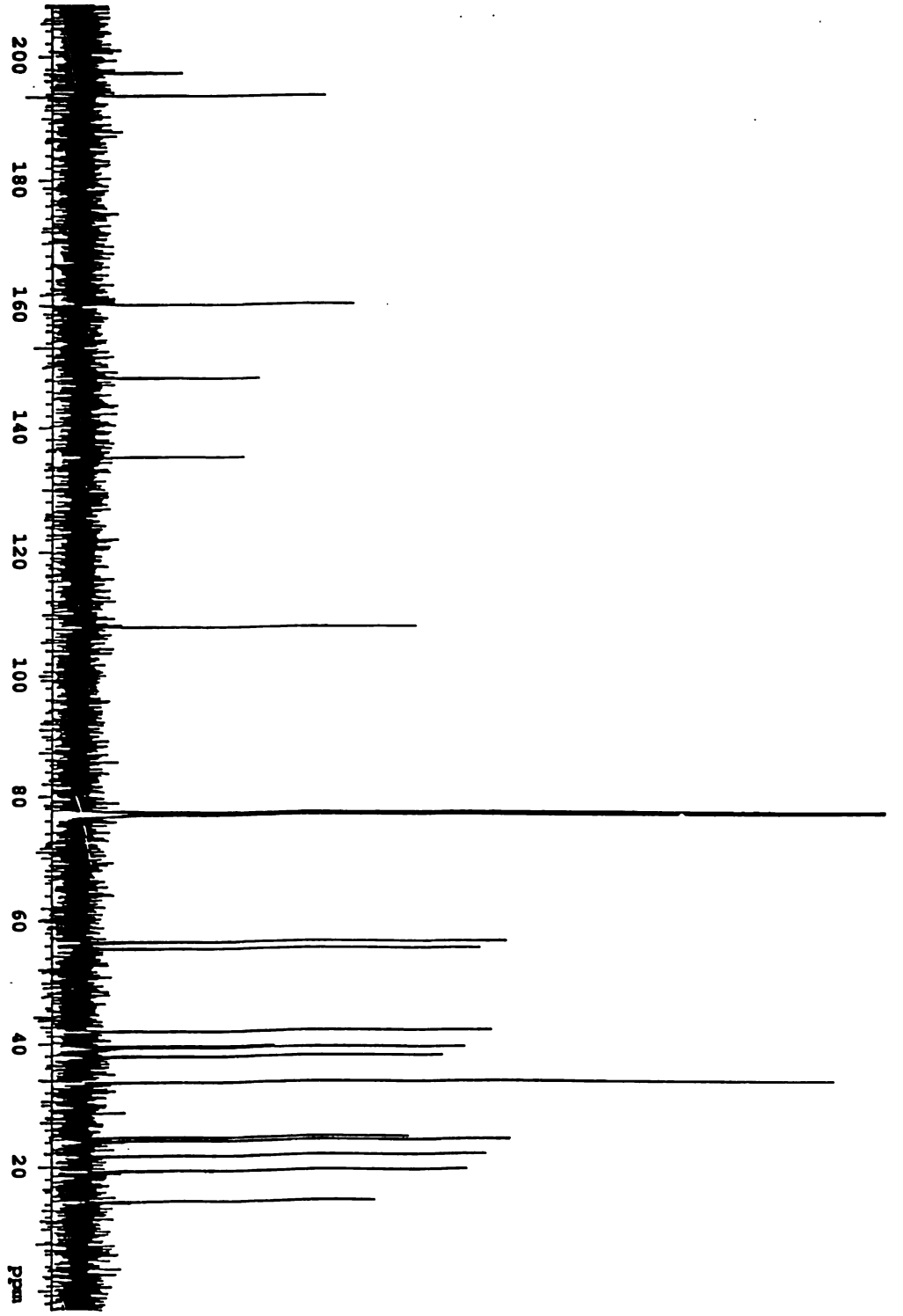
CD Spectrum of Ar-Turmerone

APPENDIX VII



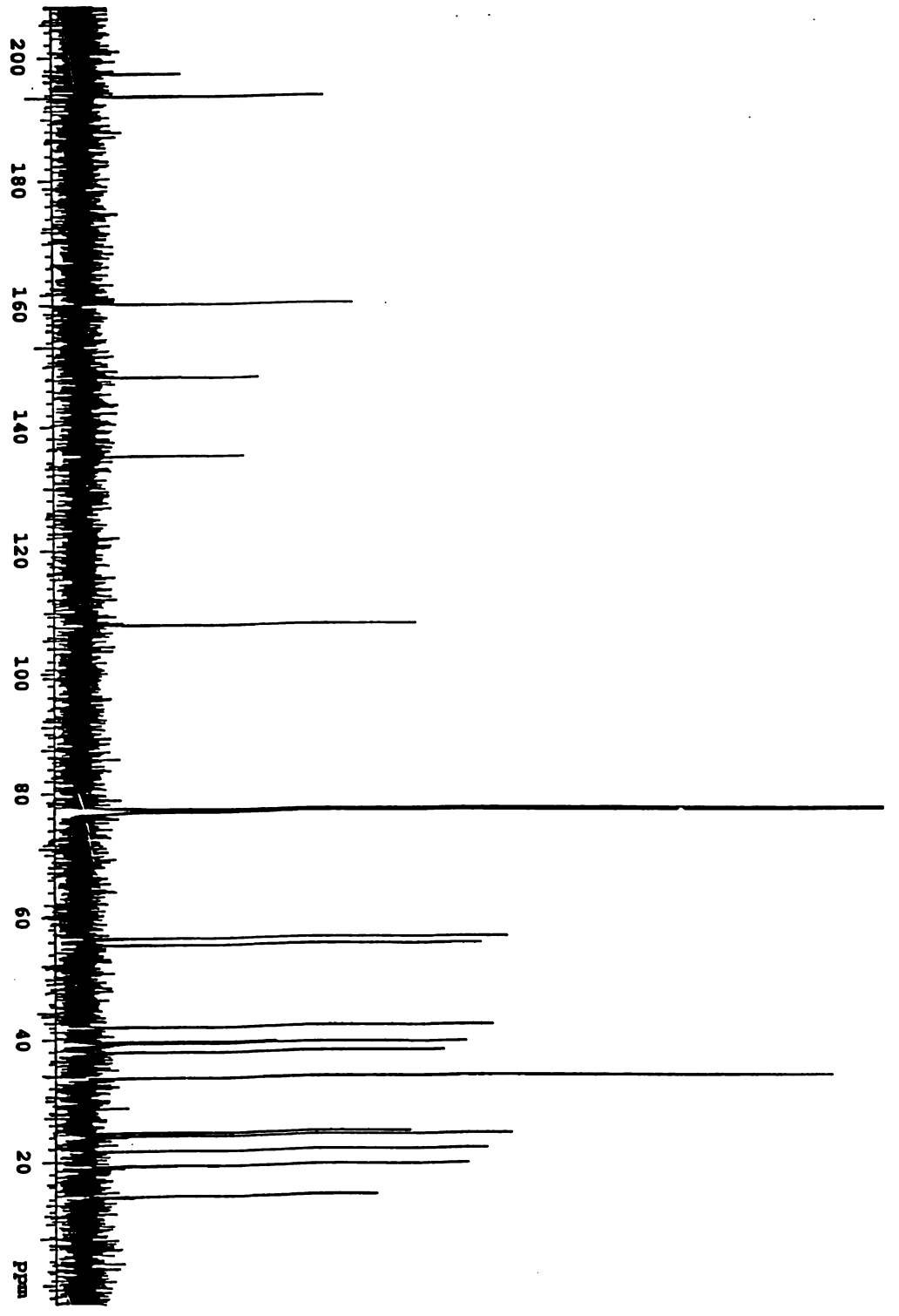
^{1}HNMR Spectrum of Labda 8 (17), 12-diene-15,16 dial

APPENDIX VIII



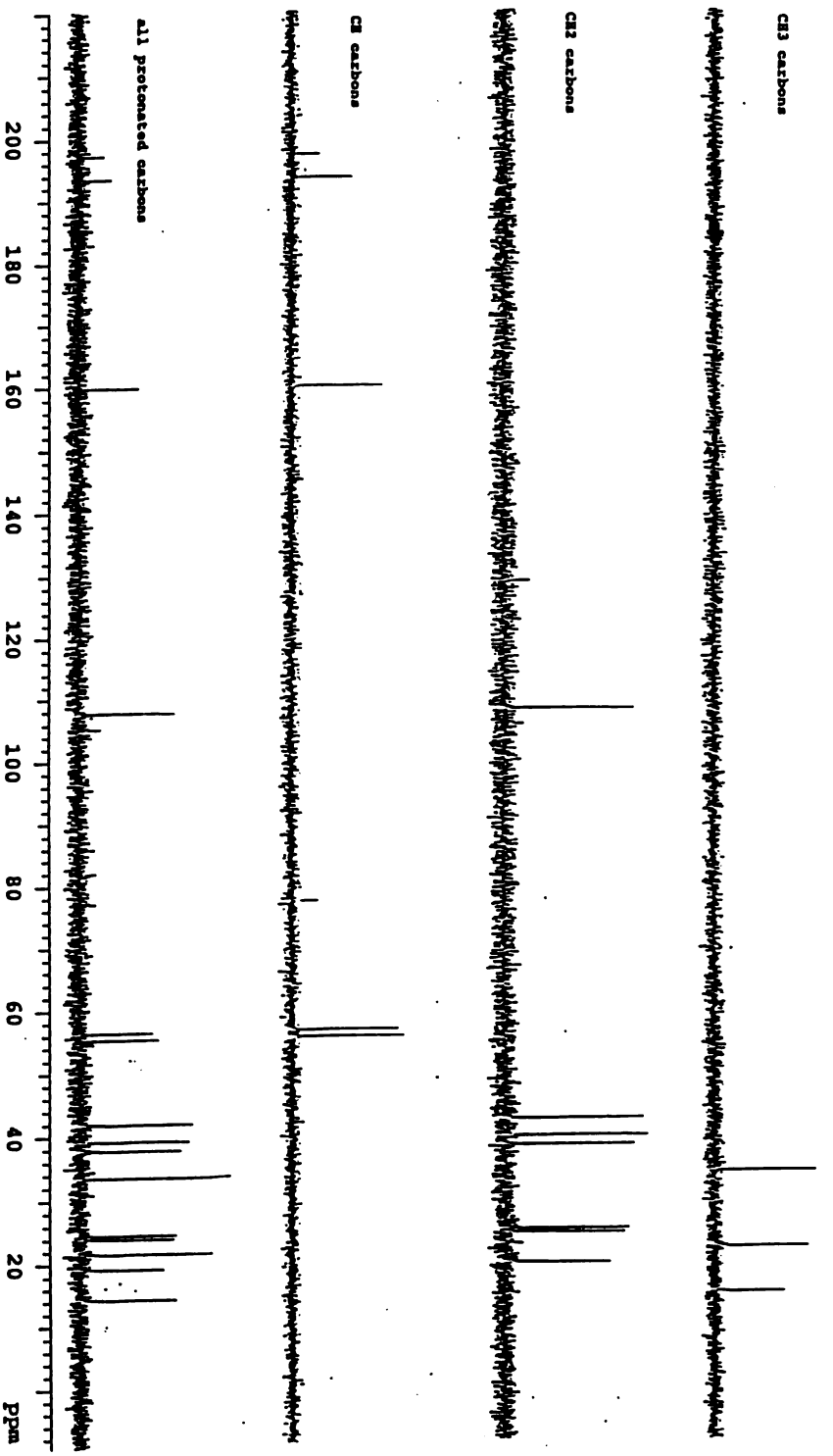
^{13}CNMR Spectrum of Labda 8 (17), 12-diene-15,16 dial

APPENDIX VIII



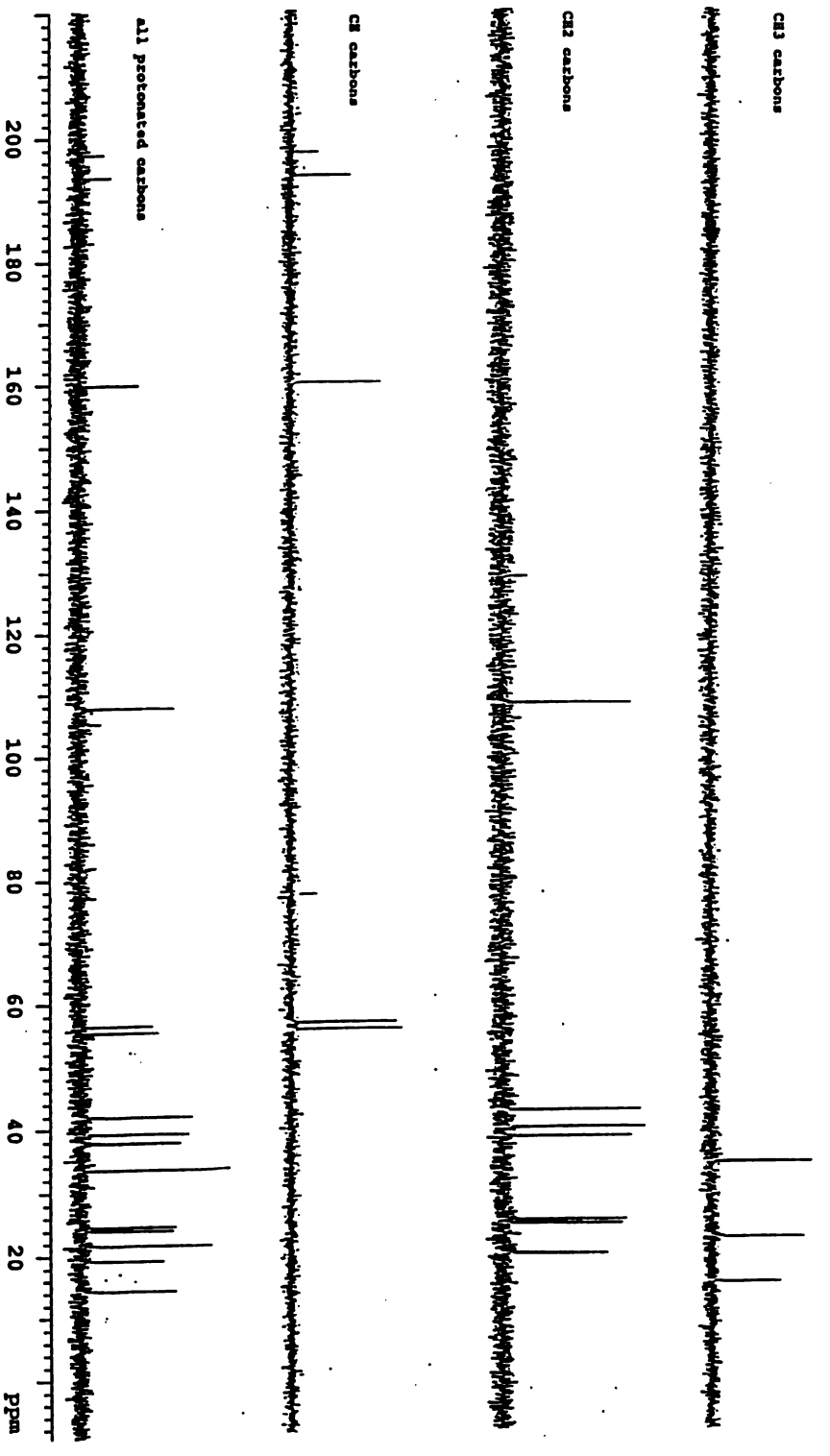
^{13}CNMR Spectrum of Labda 8 (17), 12-diene-15,16 dial

APPENDIX IX



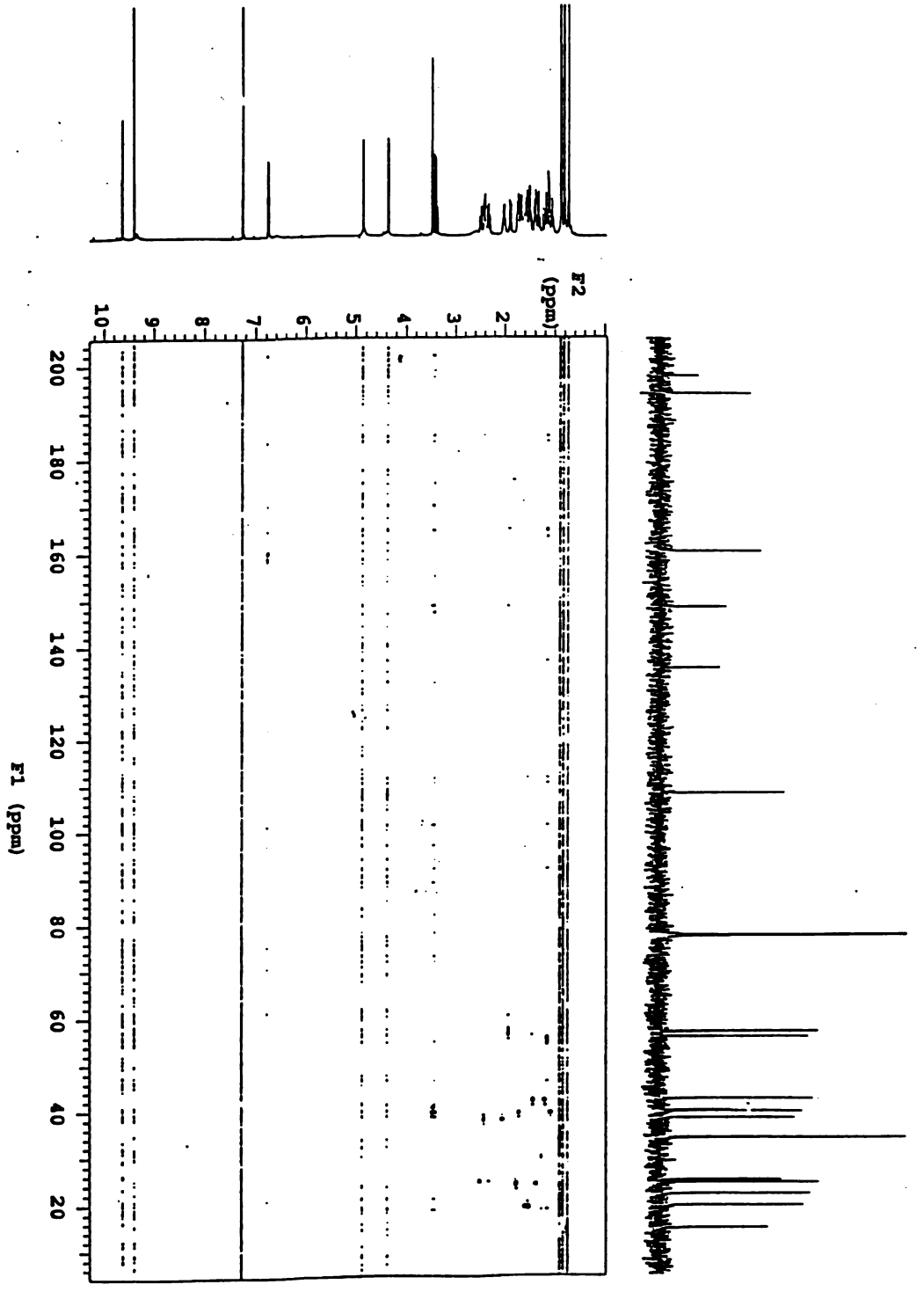
DEPT Specitrum of Labda 8 (17), 12-diene-15,16 dial

APPENDIX IX



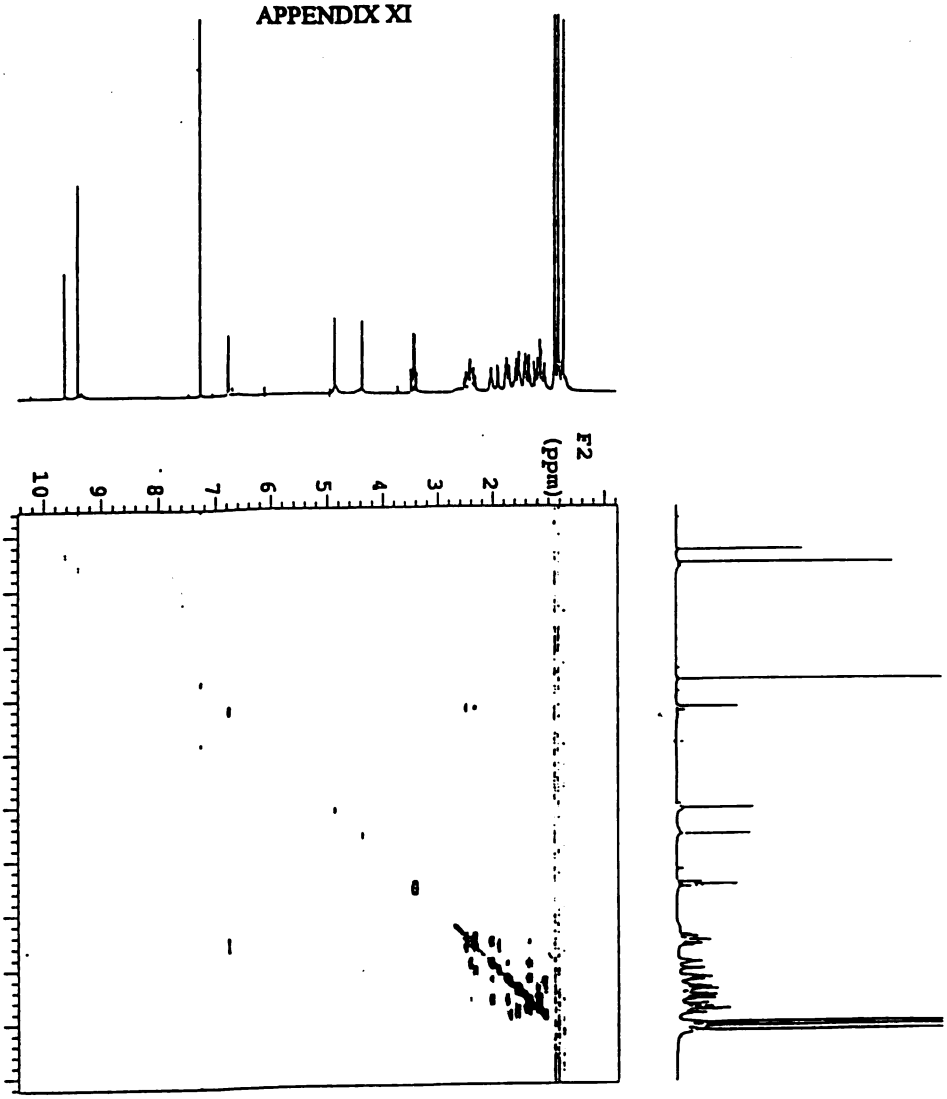
DEPT Spectrum of Labda 8 (17), 12-diene-15,16 dial

APPENDIX X



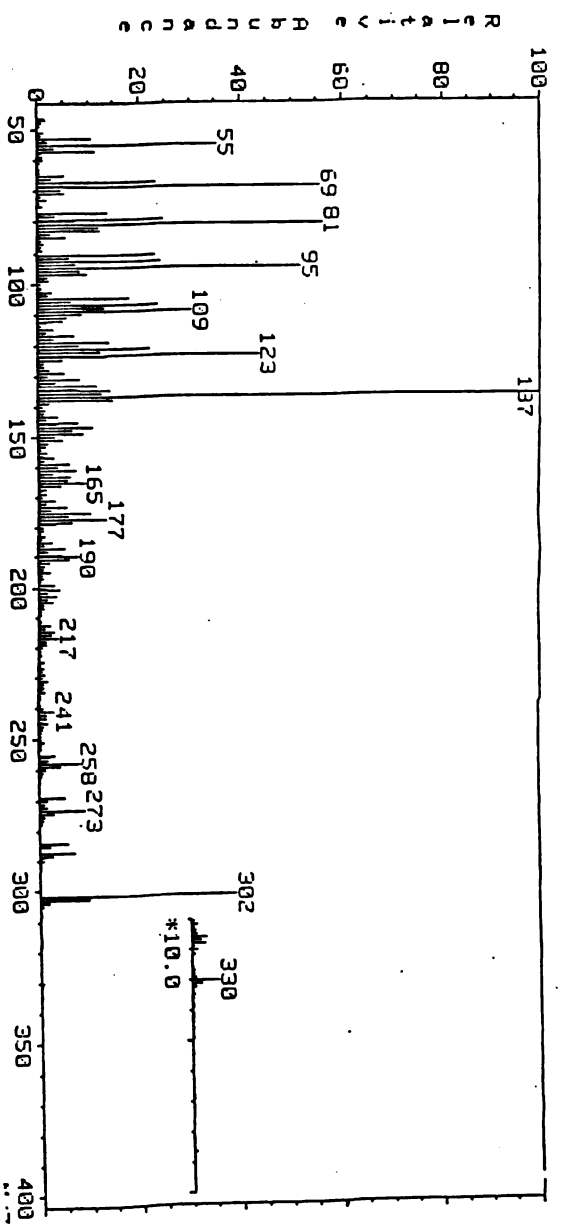
HMQC Spectrum of Labda 8 (17), 12-diene-15,16 dial

APPENDIX XI



COSY Spectrum of Labda 8 (17), 12-diene-15,16 dial

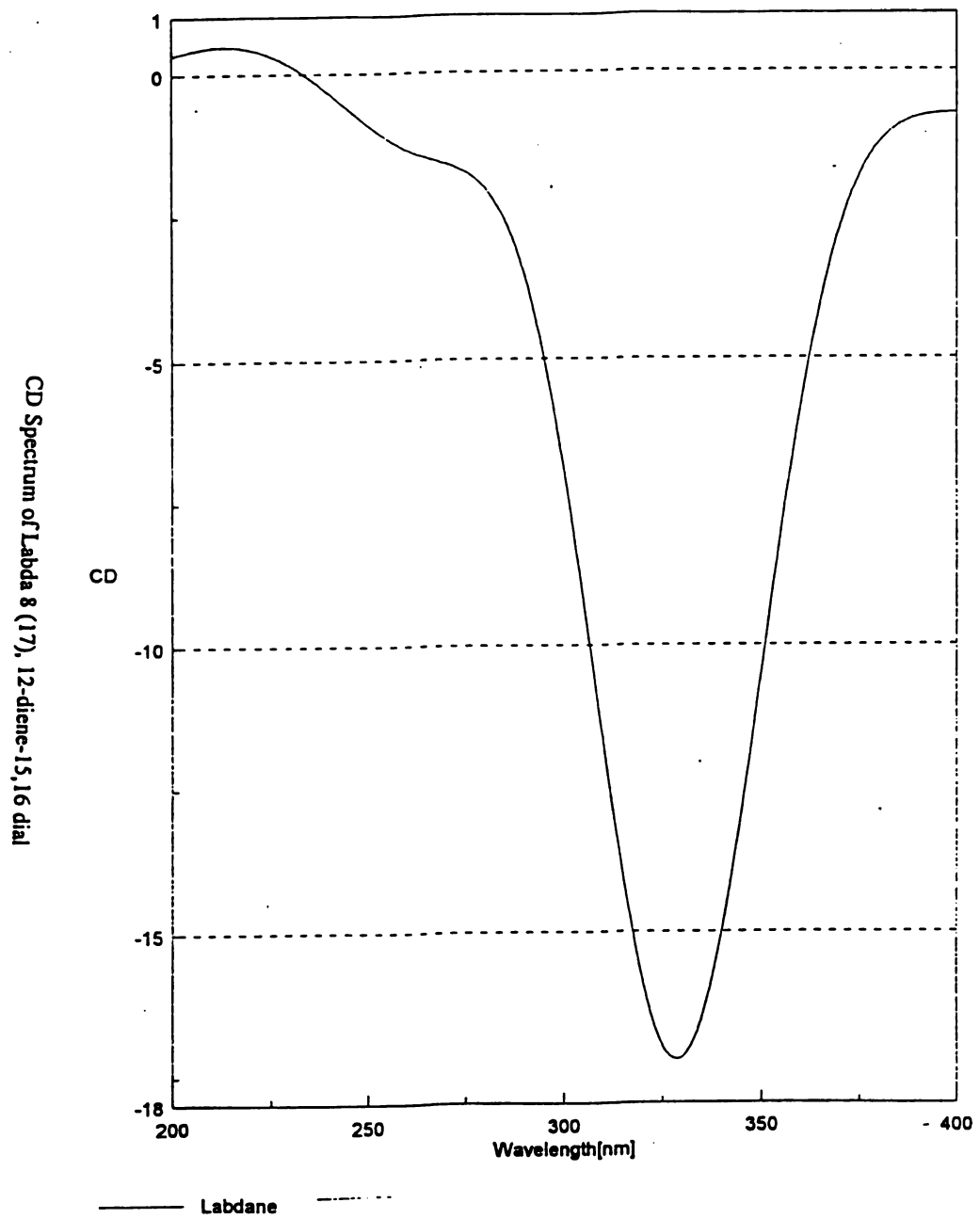
APPENDIX XII



EIMS Spectrum of Labda 8 (17), 12-diene-15,16 dial

APPENDIX XIII

Diterpene from Curcuma longa



CD Spectrum of Labda 8 (17), 12-diene-15,16 dial