BIOASSAY-GUIDED ISOLATION OF POTENTIAL ANTINEOPLASTIC NATURAL PRODUCTS FROM SOUTHWESTERN PLANTS

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Todd Raymond Furbacher

entitled Bioassay-guided Isolation of Potential Antineoplastic Natural Products From Southwestern Plants

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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William A. Remers, Ph.D. — Date

Lynn A. Joens — 12-6-01
Lynn A. Joens, Ph.D. — Date

Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Joseph J. Hoffmann — 12-11-01
Dissertation Director — Date
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STATEMENT BY AUTHOR

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SIGNED: ____________________

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Gratitude must be given to Dr. A. A. Leslie Gunatilaka for his kindness and for his willingness to provide excellent advice and generous support. Being a member of his laboratory proved invaluable.

I also need to express my deepest thanks to the rest of my committee: Dr. William A. Remers, Dr. Lynn A. Joens, and Dr. J. Glenn Songer. Thank you for seeing it through.

Thanks must be extended to Dr. Steven P. McLaughlin for his collection and identification of the many plant species utilized in this work. Mark LeRud must be recognized for his fractionation of *Psorothamnus thompsoniae*. Lyn Rafferty and Arthur Kerschen are to be acknowledged their assistance in conducting the mutant yeast assay and plasmid DNA assay, respectively.

I am indebted to Louis K. Hutter and Meide Pan for their friendship and for their willingness to answer my endless questions with patience and grace..

DEDICATION

To my father, Ray

for being an endless source of inspiration.

To my mother, Marilyn

for your love, support, and patience...especially your patience.

To my brother, Keith

for being everything a big brother should be and more.

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ABSTRACT

This dissertation details the investigation of numerous plants for potential antineoplastic compounds. 144 plants (391 extracts) were prescreened with an assortment of assays. The pre-screens included an *Agrobacterium tumefaciens*/potato disk gall tumor inhibition assay, a *Saccharomyces cerevisiae* mutant topoisomerase assay, and an *Escherichia coli* plasmid scission assay. Bioassay-guided fractionation was conducted on three plants, *Phoradendron juniperinum*, *Psorothamnus thompsoniae*, and *Acourtia thurberi*, using a different assay for each.

Phoradendron juniperinum (Viscaceae) was screened with a plasmid scission assay and the novel compound, 5-caffeoyl-*epi*-quinic acid (**I**) was isolated, the first chlorogenic acid to be reported for the genus. Chemical structure was established using NMR and MS data and published structural information. The EC_{50} for 5-caffeoyl-*epi*-quinic acid-mediated plasmid DNA cleavage was 76 μM.

Fractionation of *Psorothamnus thompsoniae* (Fabaceae) was directed using the potato disk assay. The active component was dalrubone (**II**). *P. emoryi* was fractionated to obtain dalrubone and to search for related compounds. 5-Methoxydalrubone (**III**) was isolated and tested with dalrubone in cell line assays. 5-Methoxydalrubone was active against MCF-7 (IC_{50} = 28.2 μM), while dalrubone (IC_{50} = 1.3 mM) was not. Neither compound significantly inhibited the growth of NCI-H460 or SF-268.

Acourtia thurberi (Asteraceae) was active in the yeast mutant assay. Fractionation yielded the sesquiterpene, 14,15-diacetoxy-,8-hydroxy-,3-(3-methylbutanoyl)-14, 15-epoxy-isocedrene (**IV**). This compound was weakly active against the topoisomerase II sensitive yeast strain, RS321N, with an IC_{12} of 342 μg/mL. The isocedrene was active in the yeast assay but inactive against human topoisomerase IIα.

Ten celastroloids (unsaturated, oxygenated D:A-friedo-nor-oleanane triterpenoids from Sri Lankan Celastraceae) and their derivatives, some of which were also weakly active against RS321N, were tested for activity against human topoisomerase IIα. Demethylzeylasterone (ex. *Kokoona zeylanica*) strongly inhibited topo IIα with an IC_{50} of 17.6 μM. All others, including the structurally similar zeylasterone, possessed no activity at 100 μM. Demethylzeylasterone was determined to be a "catalytic inhibitor," preventing DNA from binding to the enzyme while not interacting with the DNA itself. Demethylzeylasterone selectively inhibits the MCF-7 breast cancer cell line with an IC_{50} of 12.5 μM.

CHAPTER 1

INTRODUCTION

The isolation of biologically active molecules typically involves the selection one of two screening approaches. One approach is a cell-based screen. The biological activity of a compound or mixture is tested against living cells. The cells may be of any type (bacterial or animal) or complexity [free cell culture, tissue or organism (*in vivo*)] as long as they are capable of stable metabolism. The end result of a cell-based screen is a measurement of any change of cell metabolism (growth), appearance/disappearance of secondary messengers, or through the activation of a signal transduction cascade. The other approach is "isolated target" screening. The assays developed with this method of biomolecular screening involve using purified targets (enzymes, organelles, DNA, and membranes). These are non-living assay systems. Each of the approaches has its own merits and problems.

The proliferation of cell culture labs has allowed for widespread use of cell-based screening. The primary advantage of using whole cells (organisms) is that one can determine the likelihood of a compound having an effect on a living system. By using multiple cell lines or strains, one can discover the existence of specificity against one particular type of cell. Antimicrobials are typically discovered through this method of screening. The drawbacks to cell-based screening are numerous. Cells are often affected by the solvents used to introduce the compounds of interest. A molecule might be very active but may be unable to penetrate the cell wall or membrane. A mixture might weakly activate several cellular targets with significant activity disappearing as purification progresses. In a cell-based screen, one must also contend with the metabolism of the test molecule. Cell line screening, historically resulted in numerous false positives. Methodology and equipment have progressed to the point that high throughput assays with cells are now possible. Ideally, a cell line assay should be used as a follow up to an "isolated target" approach. This would allow for the confirmation that a compound active in a targeted assay will also be active on the intact cell level.

Isolated targets are the most efficient screen to use when available. There are few good reasons to screen for enzyme inhibitors in living cells when those enzymes are available isolated away from whole cells. Enzymatic assays are far more sensitive and rapid. The targets themselves do not require constant maintenance as do cell cultures and unlike microbial or animal cell assays, totally sterile conditions are usually not necessary.

The following work details the application of three separate assays as preliminary and separatory screens for the isolation of biologically active molecules. Additional assays were utilized to further define the mode of action of the active compounds. Each of the primary assays was selected for their ability to screen for potential anti-neoplastic molecules. The majority of plant species were collected from the southwestern U.S. for investigation because of the commitment of the Southwest Center for Natural Products Research and Commercialization to identify medicinally active local flora. Further details regarding the assays used and the species studied follow.

CHAPTER 2

PLANT COLLECTIONS AND EXTRACTIONS

2.1 Hoffmann Collection

The following plant materials were collected for the use in the laboratory of Dr. Joseph J. Hoffmann. Aerial portions of plants were obtained by multiple collectors and samples were submitted to the University of Arizona Herbarium (ARIZ). Upon identification, the plants were desiccated in a drying room over a period of three days. The dried material was then ground finely in a Wiley mill fitted with a three millimeter sieve. Unextracted biomass and extracted material was stored at 4°C. The collection represents 26 species and 19 families.

Angelica lineariloba Gray (Apiaceae)
SPM5711; 1989, E.Sierra Madres, Mono County, CA

Ceanothus fendleri Grey (Rhamnaceae)
SPM6792; 1998,1992, Marijilda Canyon, Mt. Hopkins, Santa Cruz County, AZ

Chamaesaracha sordida (Dun.) Grey (Solanaceae)
55031; 1990, Highway 80, Cochise County, AZ

Dalea formosa (Torr.) (Fabaceae)
SPM6933; 1995, Santa Rita Mountains, Santa Cruz County, AZ

Dalea pulchra (Torr.) (Fabaceae)
SPM6934; 1995, Santa Rita Mountains, Santa Cruz County, AZ

Datisca glomerata (Presl) Baill. (Datiscaceae)
SPM5688; 1989, Red Bluff, Tehama County, CA

Dyssodia pentachaeta (D.C.) B.L. Rob. (Asteraceae)
55269; 1990, Hwy 54, Culberson County, TX

Eriogonum palmerianum Reveal (Polygonaceae)
SPM6790; 1988, Graham Mountains, Graham County, AZ

Erysimum capitatum (Dougl.) Greene (Brassicaceae)
SPM6848; 1992, Marijilda Canyon, Graham County, AZ

Gaillardia parryi Greene. (Asteraceae)
SPM5930; 1990, Kane County, UT

Heracleum lanatum Michx. (Apiaceae)
SPM4757; 1988, Graham Mountains, Graham County, AZ

Hymenoxys scaposa (D.C.) Parker var. *linearis* (Nutt.) Parker (Asteraceae)
SPM5896; 1990, I40/84, Guadalupe County, NM

Larrea tridentata (DC) Cov. (Zygophyllaceae)
55013; 1990, Hwy. 170, Presidio County, TX

Linanthus nutalli (Grey) Greene ssp. *floribundus* (Grey) Hunz (Polemoniaceae)
SPM5523; 1989, La Posta Indian Reservation, San Diego County, CA

Mahonia wilcoxii Rehder (Berberidaceae)
98012; 1990, Chiricahua Mtns., Cochise County, AZ

Mentzelia multiflora Gray (Loasaceae)
SPM5884; 1990, Navajo County, AZ

Mertensia franciscana Heller (Boraginaceae)
SPM4758; 1988, Graham Mountains, Graham County, AZ

Polanasia dodecandra (L.) D.C. (Capparidaceae)
SPM4738; 1988, Graham Mountains, Graham County, AZ

Proboscidia altheifolia (Benth.) Decne (Martyniaceae)
SPM4802; 1988, Buenos Aires N.W.R., Pima County, AZ

Psorothamnus emoryii Gray (Rydb.) (Fabaceae)
SPM6935; 1995, Algodones, Imperial County, CA

Psorothamnus thompsoniae (Vail) Welsh and Atwood var. ***thompsoniae*** (Fabaceae)
SPM6939; 1990, Garfield County, UT

Pycnanthemum incanum (L.) Michx. (Lamiaceae)
55156; 1990, Highway 72, Roane County, TN

Rotala ramosoir (L.) Koehne (Lythraceae)
55198; 1990, Salines County, AR

Salvia sonomensis Greene. (Lamiaceae)
SPM5526; 1989, Laguna Mtns., Orange County, CA

Saponaria officinalis L. (Caryophyllaceae)
SPM5698; 1989, Trinity River Canyon, Trinity County, CA

Sassafras albidum (Nutt.) Ness. (Lauraceae)
55166; 1990, Chilhowee Mtns., Blount County, TN

2.2 Gunatilaka Collection

The following plants materials were collected for the use in the laboratory of Dr. A. A. Leslie Gunatilaka. Above-ground portions of plants were obtained by Dr. Stephen McLaughlin and myself from southern Arizona and samples of all plants have been submitted to the University of Arizona Herbarium (ARIZ). Upon identification, the plants were desiccated in a drying room over a period of three days. The dried material was then ground finely in a Wiley mill fitted with a three millimeter sieve. Unextracted material was stored at 4°C. The collection represents 118 species and 43 families. The most represented family was Asteraceae with 43 species (36% of collection). Twenty-eight families were represented by only one species.

Acalypha lindheimeri Muell.-Arg. (Euphorbiaceae)
SPM7742; 1998, Huachuca Mountains, Cochise County

Acalypha neomexicana Muell.-Arg. (Euphorbiaceae)
SPM7823; 1998, Santa Catalina Mountains, Pima County

Acourtia thurberi (A. Gray) Reveal & King (Asteraceae)
SPM7673, SPM8123; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Ageratina herbacea (A. Gray) King & H.E. Robins. (Asteraceae)
SPM7722; 1998, Huachuca Mountains, Cochise County

Aloysia wrightii Heller *ex* Abrams (Verbenaceae)
SPM7816; 1998, Santa Catalina Mountains, Pima County

Amorpha fruticosa L. (Fabaceae)
SPM7827; 1998, Santa Catalina Mountains, Pima County

Aralia humilis Cav. (Araliaceae)
SPM7668; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Arctostaphylos pungens Kunth (Ericaceae)
SPM7733; 1998, Huachuca Mountains, Cochise County

Artemisia dracunculus L. (Asteraceae)
SPM7833; 1998, Santa Catalina Mountains, Pima County

Artemisia ludoviciana Nutt. (Asteraceae)
SPM7684; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Astrolepis sinuata (Lag. *ex* Sw.) Benham & Windham (Adiantaceae)
SPM7817; 1998, Santa Catalina Mountains, Pima County

Bahia dissecta (A. Gray) Britt. (Asteraceae)
SPM7741; 1998, Huachuca Mountains, Cochise County

Bidens leptocephala Sherff (Asteraceae)
SPM7653; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Boerhavia coccinea P. Mill. (Nyctaginaceae)
SPM7664; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Brickellia californica (Torrey & A. Gray) A. Gray (Asteraceae)
SPM7721; 1998, Huachuca Mountains, Cochise County

Brickellia grandifolia (Hook.) Nutt. (Asteraceae)
SPM7836; 1998, Santa Catalina Mountains, Pima County

Calyophus toumeyi (Small) Towner (Onagraceae)
SPM7720; 1998, Huachuca Mountains, Cochise County

Carminatia tenuiflora DC. (Asteraceae)
SPM7688; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Castelleja patriotica Fern. (Scrophulariaceae)
SPM7726; 1998, Huachuca Mountains, Cochise County

Centaurea rothrockii Greenm. (Asteraceae)
SPM7740; 1998, Huachuca Mountains, Cochise County

Chamaecrista nictitans (L.) Moench (Fabaceae)
SPM7828; 1998, Santa Catalina Mountains, Pima County

Chenopodium neomexicanum Standl. (Chenopodiaceae)
SPM7714; 1998, Huachuca Mountains, Cochise County

Cocculus diversifolis DC. (Menispermaceae)
SPM7651; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Cordylanthus wrightii Heller *ex* Abrams (Scrophulariaceae)
SPM7818; 1998, Santa Catalina Mountains, Pima County

Cosmos parviflorus (Jacq.) Pers. (Asteraceae)
SPM7746; 1998, Huachuca Mountains, Cochise County

Crotalaria pumila Ortega (Fabiaceae)
SPM7666; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Cuphea wrightii A. Gary (Lythraceae)
SPM7748; 1998, Huachuca Mountains, Cochise County

Cyperus fendlerianus Boeckl. (Cyperaceae)
SPM7841; 1998, Santa Catalina Mountains, Pima County

Cyperus mutisi (H.B.K.) Griseb. (Cyperaceae)
SPM7716; 1998, Huachuca Mountains, Cochise County

Dalea albiflora A. Gray (Fabaceae)
SPM7675; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Datura wrightii Regel (Solanaceae)
SPM7659; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Delphinium andesicola Ewan (Ranunculaceae)
SPM7755; 1998, Huachuca Mountains, Cochise County

Desmodium cinerascens A. Gray (Fabaceae)
SPM7678; 1998, Santa Rita Mountains; Mt. Hopkins Road

Dicliptera resupinata (Vahl) Juss. (Acanthaceae)
SPM7821; 1998, Santa Catalina Mountains, Pima County

Epilobium canum (Greene) Raven ssp. ***latifolium*** (Hook.) Raven (Onagraceae)
SPM7663; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Erigeron arizonicus A. Gray (Asteraceae)
SPM7840; 1998, Santa Catalina Mountains, Pima County

Erigeron platyphyllus Greene (Asteraceae)
SPM7723; 1998, Huachuca Mountains, Cochise County

Eriogonum abertianum Torrey (Polygonaceae)
SPM7655; 1998, Santa Rita Mountains; Mt. Hopkins Road

Eriogonum polycladon Benth. (Polygonaceae)
SPM7814; 1998, Santa Catalina Mountains, Pima County

Eryngium lemmonii Coult. & Rose (Apiaceae)
SPM7730; 1998, Huachuca Mountains, Cochise County

Fraxinus velutina Torrey (Oleaceae)
SPM7658; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Froelichia arizonica Thornb. Ex Standl (Amaranthaceae)
SPM7713; 1998, Huachuca Mountains, Cochise County

Gaillardia pinnatifida Torrey (Asteraceae)
SPM7754; 1998, Huachuca Mountains, Cochise County

Galium wrightii A. Gray (Rubiaceae)
SPM7722B; 1998, Huachuca Mountains, Cochise County

Garrya wrightii Torrey (Garryaceae)
SPM7819; 1998, Santa Catalina Mountains, Pima County

Gaura hexandra Ortega ssp. ***gracilis*** (Woot. & Standl.) Raven & Gregory (Onagraceae)
SPM7718; 1998, Huachuca Mountains, Cochise County

Gentianella microcalyx (J.G. Lemmon) J. Gillett (Gentianceae)
SPM7732; 1998, Huachuca Mountains, Cochise County

Geranium caespitosum James (Geraniaceae)
SPM7725; 1998, Huachuca Mountains, Cochise County

Gnaphalium canescens DC. (Asteraceae)
SPM7686; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Gnaphalium leucocephalum A. Gray (Asteraceae)
SPM7756; 1998, Huachuca Mountains, Cochise County

Gnaphalium viscosum Kunth (Asteraceae)
SPM7736; 1998, Huachuca Mountains, Cochise County

Gossypium thurberi Todaro (Malvaceae)
SPM7654; 1998, Santa Rita Mountains; Mt. Hopkins Road

Guardiola platyphylla A. Gray (Asteraceae)
SPM7676; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Gutierrezia sarothrae (Pursh) Britt. & Rusby (Asteraceae)
SPM7743; 1998, Huachuca Mountains, Cochise County

Gutierrezia wrightii A. Gray (Asteraceae)
SPM7727; 1998, Huachuca Mountains, Cochise County

Gymnosperma glutinosum (Spreng.) Less. (Asteraceae)
SPM7829; 1998, Santa Catalina Mountains, Pima County

Hedeoma dentatum Torrey (Lamiaceae)
SPM7685; 1998, Santa Rita Mountains; Mt. Hopkins Road

Hedeoma hyssopifolia A. Gray (Lamiaceae)
SPM7739; 1998, Huachuca Mountains, Cochise County

Helianthella quinquenervis Rydb. (Asteraceae)
SPM731; 1998, Huachuca Mountains, Cochise County

Heliopsis parvifolia A. Gray (Asteraceae)
SPM7674; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Heterosperma pinnatum Cav. (Asteraceae)
SPM7689; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Heterotheca subaxillaris (Lam.) Britt. & Rusby (Asteraceae)
SPM7757; 1998, Huachuca Mountains, Cochise County

Heuchera sanguinea Engelm. (Saxifragaceae)
SPM7669; 1998, Santa Rita Mountains; Mt. Hopkins Road

Hymenoxys quinquesquamata Rydb. (Asteraceae)
SPM7729; 1998, Huachuca Mountains, Cochise County

Hymenoxys wrightii A. Gray (Asteraceae)
SPM7832; 1998, Santa Catalina Mountains, Pima County

Ipomopsis thurberi (Torrey *ex*. A. Gray) V. Grant (Polemoniaceae)
SPM7677; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Juniperus deppeana Steud. (Cupressaceae)
SPM7719; 1998, Huachuca Mountains, Cochise County

Laennecia schiedeana (Less.) Nesom (Asteraceae)
SPM7843; 1998, Santa Catalina Mountains, Pima County

Laennecia sophiifolia (Kunth) Nesom (Asteraceae)
SPM7715; 1998, Huachuca Mountains, Cochise County

Lathryus graminifolius (S. Watson) White (Fabaceae)
SPM7843; 1998, Santa Catalina Mountains, Pima County

Lonicera albiflora Torrey & A. Gray var. ***dumosa*** (A. Gray) Rehder (Caprifoliaceae)
SPM7744; 1998, Huachuca Mountains, Cochise County

Lotus oroboides (H.B.K.) Ottley (Fabaceae)
SPM7679; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Lupinus palmeri S. Watson (Fabaceae)
SPM7834; 1998, Santa Catalina Mountains, Pima County

Machaeranthera canescens (Pursh) A. Gray var. ***incana*** (Lindley) A. Gray (Asteraceae)
SPM7822; 1998, Santa Catalina Mountains

Macroptilium gibbosifolium (Ortega) A. Delgado (Fabaceae)
SPM7680; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Marabilis multiflora (Torrey) A. Gray (Nyctaginaceae)
SPM7813; 1998, Santa Catalina Mountains, Pima County

Maurandya antirrhiniflora (Humb. & Bonpl. *ex* Willd.) Rothm (Schrophulariaceae)
SPM7660; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Melampodium longicorne A. Gray (Asteraceae)
SPM7670; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Mirabilis longiflora L. (Nyctaginaceae)
SPM7687; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Monarda citriodora Cerv. *ex*. Lag. ssp. ***austromontana*** (Epling) Scora (Lamiaceae)
SPM7691; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Monarda fistulosa L ssp. ***fistulosa*** var. ***menthifolia*** (Graham) Fern. (Lamiaceae)
SPM7825; 1998, Santa Catalina Mountains, Pima County

Monnima wrightii A. Gray (Polygalaceae)
SPM7747; 1998, Huachuca Mountains, Cochise County

Parthenice mollis A. Gray (Asteraceae)
SPM7672; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Phaseolus acutifolius A. Gray (Fabiaceae)
SPM7667; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Phoradendron californicum Nutt. (Viscaceae) from [***Cercidium microphyllum*** (Torrey) Rose & I.M. Johnson]
1998 Jct. 79/77, Pinal County

Phoradendron californicum Nutt. (Viscaceae) from [***Prosopis velutina*** Woof.]
1998 Jct. 79/77, Pinal County

Phoradendron juniperinum Engelm. (Viscaceae) from [***Juniperus deppeana*** Steud.]
TRF001; 1998, Santa Catalina Mountains, Pima County

Phoradendron villosum (Nutt.) Nutt. (Viscaceae) from [***Quercus oblongifolia*** Torrey]
SPM7830; 1998, Santa Catalina Mountains, Pima County

Plumbago scandens L. (Plumbaginaceae)
SPM7665; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Poinsettia heterophylla (L.) Klotzsch & Garcke (Euphorbiaceae)
SPM7662; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Psoralidium tenuiflorum (Pursh) Rydb. (Fabaceae)
SPM7820; 1998, Santa Catalina Mountains, Pima County

Pteridium aquilinum (L.) Greene (Dennstaedtiaceae)
SPM7724; 1998, Huachuca Mountains, Cochise County

Quercus oblongifolia Torrey (Fagaceae)
SPM7831; 1998, Santa Catalina Mountains, Pima County

Rhamnus californica Esch. (Rhamnaceae)
SPM7826; 1998, Santa Catalina Mountains, Pima County

Rhus virens Lindh. ex. A. Gray var. ***choriophylla*** (Woot. & Standl.) L. Benson (Anacardiaceae)
SPM7652; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Rivina humilis L. (Phytolaccaceae)
SPM7661; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Salvia lemmonii A. Gray (Lamiaceae)
SPM7749; 1998, Huachuca Mountains, Cochise County

Sambucus mexicana Presl (Caprifoliaceae)
SPM7681; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Schoencrambe linearifolia (A. Grey) Rollins (Brassicaceae)
SPM7717; 1998, Huachuca Mountains, Cochise County

Scrophularia parviflora Woot. & Standl. (Scrophulariaceae)
SPM7837; 1998, Santa Catalina Mountains, Pima County

Senecio bigelovia A. Gray (Asteraceae)
SPM7838; 1998, Santa Catalina Mountains, Pima County

Silene laciniata Cav. (Caryophyllaceae)
SPM7737; 1998, Huachuca Mountains, Cochise County

Silene scouleri Hooker (Caryophyllaceae)
SPM7839; 1998, Santa Catalina Mountains, Pima County

Solidago velutina DC. (Asteraceae)
SPM7671; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Solidago wrightii A. Gray (Asteraceae)
SPM7734; 1998, Huachuca Mountains, Cochise County

Sphaeralcea fendleri A. Gray (Malvaceae)
SPM7745; 1998, Huachuca Mountains, Cochise County

Stachys coccinea Jacq. (Lamiaceae)
SPM7683; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Stevia micrantha Lag. (Asteraceae)
SPM7690; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Stevia plummerae A. Gray (Asteraceae)
SPM7728; 1998, Huachuca Mountains, Cochise County

Tagetes lemmonii A. Gray (Asteraceae)
SPM7824; 1998, Santa Catalina Mountains, Pima County

Tecoma stans (L.) Juss ex Kunth (Bignoniaceae)
SPM7815; 1998, Santa Catalina Mountains, Pima County

Tetramerium nervosum Nees (Acanthaceae)
SPM7657; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Thalictrum fendleri Engelm. ex A. Gray (Ranunculaceae)
SPM7835; 1998, Santa Catalina Mountains, Pima County

Valeriana sorbifolia H.B.K. (Valerianaceae)
SPM7750; 1998, Huachuca Mountains, Cochise County

Vicia pulchella Kunth (Fabaceae)
SPM7753; 1998, Huachuca Mountains, Cochise County

Viguiera dentata (Cav.) Spreng var. ***dentata*** (Asteraceae)
SPM7682; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Viguiera dentata A. Gray var ***lancifolia*** Blake (Asteraceae)
SPM7656; 1998, Santa Rita Mountains; Mt. Hopkins Road, Santa Cruz County

Xanthocephalum gymnospermoides (A. Gray) Benth & Hook. f. (Asteraceae)
SPM7750; 1998, Huachuca Mountains, Cochise County

2.3 Extraction Protocols

Each collection was solvent extracted in a specific manner. For initial screening purposes, the plants of the Hoffmann collection were extracted to produce two extracts. Ground plant material was soaked for 24 hours in dichloromethane (DCM). The solvent was then removed and the plant material washed with a 50:50 DCM:methanol solution. Another 24 hour soak with methanol followed. The methanol extract was collected and pooled with the DCM:methanol wash. The solvent solution was filtered and the solvent removed *in vacuo* through rotary evaporation. The resulting material was termed the solvent or "S" fraction. The solvent extracted material was then soaked in deionized (DI) water for 24 hours. The aqueous solution was filtered and freeze-dried. The resulting material was termed the aqueous or "W" fraction. Selected plants were also extracted using 24 hour soaks as previously mentioned but the solvents were rotary evaporated *in vacuo* and kept separately. All extracts were sealed and stored in darkness at 4°C.

The plants of the Gunatilaka collection underwent a three solvent extraction. Ground plant was exposed to solvents for two 24 hour periods. The first solvent, hexane, was used to separate non-polar substances such as waxes from the rest of the plant material. Methylethylketone (MEK) was next used to extract compounds of intermediate polarity. Lastly, methanol (MeOH) removed the more polar compounds while leaving most sugars and tannins behind. A water extraction was not performed due the belief that the most polar compounds are historically of low medicinal interest, the potential complications caused by sugars in a yeast-based assay, and the levels of labor involved in preparing 118 water extracts.

CHAPTER 3

DNA SCISSION ASSAY PRESCREEN

3.1 DNA Scission Assay Background

As a purely mechanistic assay, the DNA scission assay is useful on two fronts. This assay allows for the screening of extracts for compounds with the ability to cause single stranded nicks in DNA. The anticancer drug, bleomycin (Fig. 3-1), is an example of a compound testing positive in this assay. Additionally, due to the presence of copper(II) in the assay buffer system, the screen also identifies compounds with antioxidant potential. These antioxidants, particularly those bearing benzenediol or benzenetriol groups provide numerous false positives when screening specifically for true cleavage agents.

This type of assay has been used to isolate compounds from numerous plants (Fig. 3-2). These include petalostemumol, petalopurpurenol [(+)-3,4-dihydroxy-8,9-methylene dioxypterocarpan] from *Petalostemon purpureus* (Wall *et al.*, 1995,1996), hamiltrone from *Uvaria hamiltonii* (Kinghorn *et al.*, 1998), and 1,3-dihydroxy-5-pentadec-*cis*-8'-enylbenzene from *Hakea trifurcata* (Hecht *et al.*, 1988b). Previously discovered compounds (Fig.3-3) also demonstrate activity, such as (+) and (-) epicatechin (Hecht *et al.*, 1988a) and curcumin (Hadi and Ahsan, 1998).

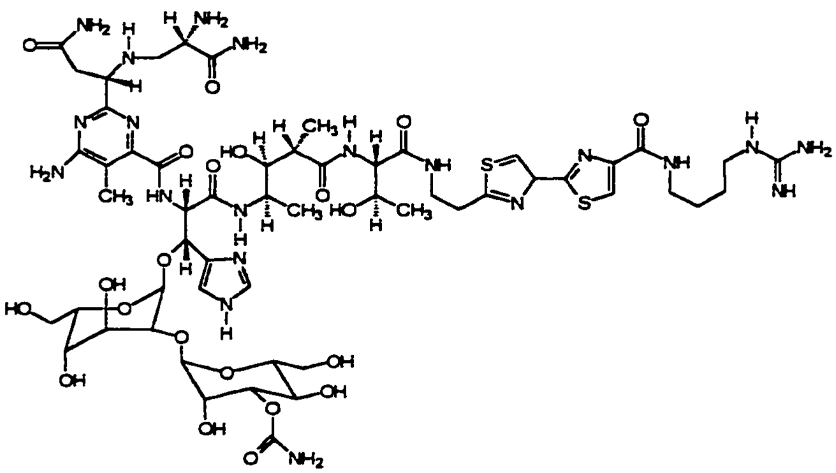


Figure 3-1. Structure of Bleomycin B_2.

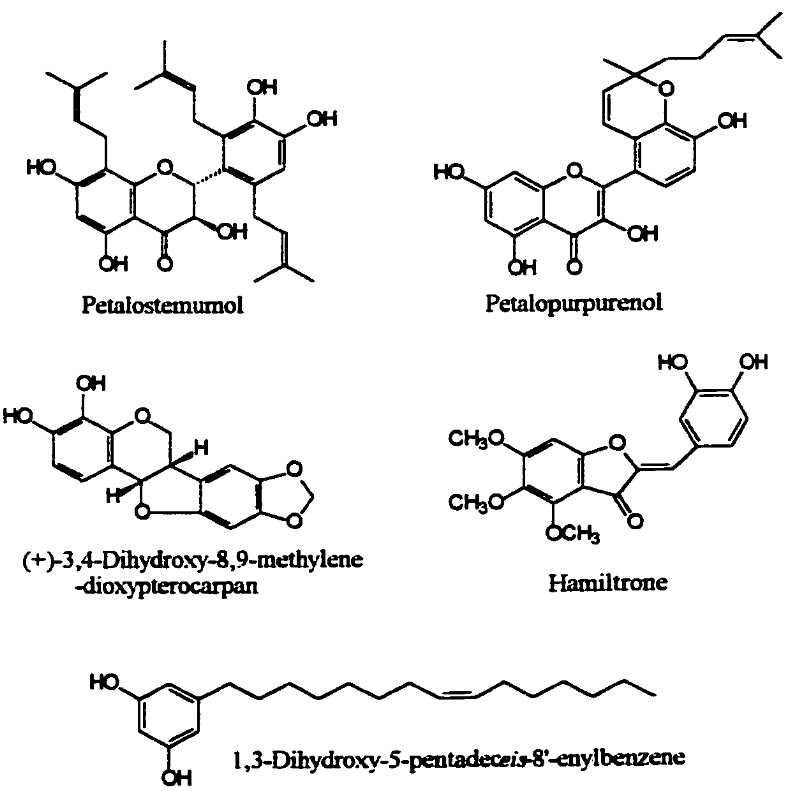
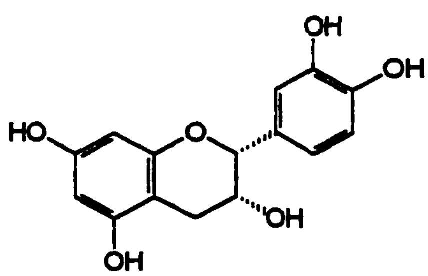


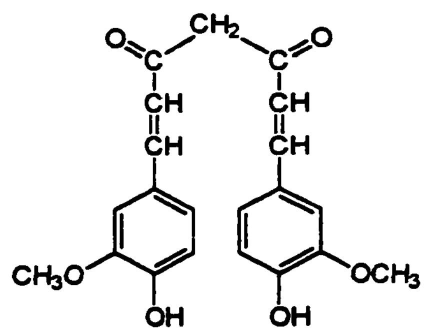
Figure 3-2. Novel Compounds Isolated with a DNA Scission Assay.

3.2 Assay Procedure

The scission assay was performed using the Kinghorn (1995) procedure with minor modifications. Briefly, the assay was conducted in a buffer containing 25 mM cacodylate buffer (pH 7.0), 0.3 mM $CuCl_2$, and 500 ng plasmid. The plasmid pBR322 was used for the initial extract screening. Due to it being a low copy number plasmid and therefore being difficult to isolate in large quantities from *Escherichia coli*, it was replaced in this assay with the high copy number plasmid pBluescript KS+. Samples were tested at a concentration of 25 µg/mL. (-)-Epicatechin was used as the positive control. Reaction mixtures were incubated in the dark at room temperature for 30 minutes. Samples can be removed from TLC plates through extraction with DMSO. These may be assayed but require that sensitivity be increased through incubation at 37°C. Samples are run on a 1 % agarose gel with a TBE buffer at 75 V for 2 hours. Gels are stained post-run with ethidium bromide and visualized on a transilluminator. Due to the compact nature of supercoiled



(-)-Epicatechin



Curcumin

Figure 3-3. Known Compounds with Scission Activity.

DNA, undamaged plasmid moves rapidly through the gel. Cleavage was observed through the appearance of slower migrating bands of plasmid DNA (open circular and linear). The more a plasmid is nicked, the possibility of two nicks in the same location increases. This produces the linear DNA.

Due to the nature of hexane extracts (poor aqueous solubility and high wax content) only MEK and methanol extracts were screened. Screening results are seen in Table 3-1.

Species	Extract Code[a]	Scission Result[b]
Cocculus diversifolis	LG-6-7-1	X
	LG-6-7-2	I
	LG-6-7-3	I
Rhus virens	LG-6-8-1	X
	LG-6-8-2	I
	LG-6-8-3	A/A
Bidens leptocephala	LG-6-9-1	X
	LG-6-9-2	I
	LG-6-9-3	I
Gossypium thurberi	LG-6-10-1	X
	LG-6-10-2	I
	LG-6-10-3	A/A
Eriogonum abertianum	LG-6-11-1	X
	LG-6-11-2	I
	LG-6-11-3	A/A
Viguiera dentata	LG-6-12-1	X
	LG-6-12-2	I
	LG-6-12-3	I
Tetramerium nervosum	LG-6-13-1	X
	LG-6-13-2	I
	LG-6-13-3	I
Fraxinus velutina	LG-6-14-1	X
	LG-6-14-2	I
	LG-6-14-3	I
Datura wrightii	LG-6-15-1	X
	LG-6-15-2	I
	LG-6-15-3	I
Maurandya antirrhiniflora	LG-6-16-1	X
	LG-6-16-2	I
	LG-6-16-3	I
Rivina humilis	LG-6-17-1	X
	LG-6-17-2	I
	LG-6-17-3	I
Poinsettia heterophylla	LG-6-18-1	X
	LG-6-18-2	I
	LG-6-18-3	I
Epilobium canum	LG-6-19-1	X
	LG-6-19-2	I
	LG-6-19-3	I

Table 3-1. Plant Extract Scission Assay Results.

Species	Extract Code[a]	Scission Result[b]
Boerhavia coccinea	LG-6-20-1	X
	LG-6-20-2	I
	LG-6-20-3	I
Plumbago scandens	LG-6-21-1	X
	LG-6-21-2	I
	LG-6-21-3	I
Crotalaria pumila	LG-6-22-1	X
	LG-6-22-2	I
	LG-6-22-3	I
Phaseolus acutifolius	LG-6-23-1	X
	LG-6-23-2	I
	LG-6-23-3	I
Aralia humilis	LG-6-24-1	X
	LG-6-24-2	I
	LG-6-24-3	I
Heuchera sanguinea	LG-6-25-1	X
	LG-6-25-2	A/A
	LG-6-25-3	A/A
Melampodium longicorne	LG-6-26-1	X
	LG-6-26-2	I
	LG-6-26-3	I
Solidago velutina	LG-6-27-1	X
	LG-6-27-2	I
	LG-6-27-3	I
Parthenice mollis	LG-6-28-1	X
	LG-6-28-2	I
	LG-6-28-3	I
Acourtia thurberi	LG-6-29-1	X
	LG-6-29-2	I
	LG-6-29-3	I
Heliopsis parvifolia	LG-6-30-1	X
	LG-6-30-2	I
	LG-6-30-3	I
Dalea albiflora	LG-6-31-1	X
	LG-6-31-2	I
	LG-6-31-3	I
Guardiola platyphylla	LG-6-32-1	X
	LG-6-32-2	I
	LG-6-32-3	I

Table 3-1. Plant Extract Scission Assay Results - continued

Species	Extract Code[a]	Scission Result[b]
Ipomopsis thurberi	LG-6-33-1	X
	LG-6-33-2	I
	LG-6-33-3	I
Desmodium cinerascens	LG-6-34-1	X
	LG-6-34-2	I
	LG-6-34-3	I
Lotus oroboides	LG-6-35-1	X
	LG-6-35-2	I
	LG-6-35-3	I
Macroptilium gibbosifolium	LG-6-36-1	X
	LG-6-36-2	I
	LG-6-36-3	I
Sambucus mexicana	LG-6-37-1	X
	LG-6-37-2	I
	LG-6-37-3	I
Viguiera dentata	LG-6-38-1	X
	LG-6-38-2	I
	LG-6-38-3	I
Stachys coccinea	LG-6-39-1	X
	LG-6-39-2	I
	LG-6-39-3	I
Artemisia ludoviciana	LG-6-40-1	X
	LG-6-40-2	I
	LG-6-40-3	I
Hedeoma dentatum	LG-6-41-1	X
	LG-6-41-2	I
	LG-6-41-3	I
Gnaphalium canescens	LG-6-42-1	X
	LG-6-42-2	I
	LG-6-42-3	I
Mirabilis longiflora	LG-6-43-1	X
	LG-6-43-2	I
	LG-6-43-3	I
Carminatia tenuiflora	LG-6-44-1	X
	LG-6-44-2	I
	LG-6-44-3	I
Heterosperma pinnatum	LG-6-45-1	X
	LG-6-45-2	I
	LG-6-45-3	I

Table 3-1. Plant Extract Scission Assay Results - continued

Species	Extract Code[a]	Scission Result[b]
Stevia micrantha	LG-6-46-1	X
	LG-6-46-2	I
	LG-6-46-3	I
Monarda citriodora	LG-6-47-1	X
	LG-6-47-2	I
	LG-6-47-3	I
Phoradendron californicum 1	LG-6-49-1	X
	LG-6-49-2	I
	LG-6-49-3	I
Phoradendron californicum 2	LG-6-50-1	X
	LG-6-50-2	I
	LG-6-50-3	I
Froelichia arizonica	LG-6-53-1	X
	LG-6-53-2	I
	LG-6-53-3	I
Chenopodium neomexicanum	LG-6-54-1	X
	LG-6-54-2	I
	LG-6-54-3	I
Laennecia sophiifolia	LG-6-55-1	X
	LG-6-55-2	I
	LG-6-55-3	I
Cyperus mutisi	LG-6-56-1	X
	LG-6-56-2	I
	LG-6-56-3	I
Schoencrambe linearifolia	LG-6-57-1	X
	LG-6-57-2	I
	LG-6-57-3	I
Gaura hexandra	LG-6-58-1	X
	LG-6-58-2	I
	LG-6-58-3	I
Juniperus deppeana	LG-6-59-1	X
	LG-6-59-2	I
	LG-6-59-3	I
Calyophus toumeyi	LG-6-60-1	X
	LG-6-60-2	I
	LG-6-60-3	I
Brickellia californica	LG-6-61-1	X
	LG-6-61-2	I
	LG-6-61-3	I

Table 3-1. Plant Extract Scission Assay Results - continued

Species	Extract Code[a]	Scission Result[b]
Ageratina herbacea	LG-6-62-1	X
	LG-6-62-2	I
	LG-6-62-3	I
Galium wrightii	LG-6-63-1	X
	LG-6-63-2	I
	LG-6-63-3	I
Erigeron platyphyllus	LG-6-64-1	X
	LG-6-64-2	I
	LG-6-64-3	I
Pteridium aquilinum	LG-6-65-1	X
	LG-6-65-2	I
	LG-6-65-3	A/A
Geranium caespitosum	LG-6-66-1	X
	LG-6-66-2	I
	LG-6-66-3	I
Castelleja patriotica	LG-6-67-1	X
	LG-6-67-2	I
	LG-6-67-3	I
Gutierrezia wrightii	LG-6-68-1	X
	LG-6-68-2	I
	LG-6-68-3	I
Stevia plummerae	LG-6-69-1	X
	LG-6-69-2	I
	LG-6-69-3	I
Hymenoxys quinquesquamata	LG-6-70-1	X
	LG-6-70-2	I
	LG-6-70-3	I
Eryngium lemmonii	LG-6-71-1	X
	LG-6-71-2	I
	LG-6-71-3	I
Helianthella quinquenervis	LG-6-72-1	X
	LG-6-72-2	I
	LG-6-72-3	I
Gentianella microcalyx	LG-6-73-1	X
	LG-6-73-2	A/A
	LG-6-73-3	I
Arctostaphylos pungens	LG-6-74-1	X
	LG-6-74-2	I
	LG-6-74-3	I

Table 3-1. Plant Extract Scission Assay Results - continued

Species	Extract Code[a]	Scission Result[b]
Solidago wrightii	LG-6-75-1	X
	LG-6-75-2	I
	LG-6-75-3	I
Gnaphalium viscosum	LG-6-76-1	X
	LG-6-76-2	I
	LG-6-76-3	I
Silene laciniata	LG-6-77-1	X
	LG-6-77-2	I
	LG-6-77-3	I
Hedeoma hyssopifolia	LG-6-78-1	X
	LG-6-78-2	I
	LG-6-78-3	I
Centaurea rothrockii	LG-6-79-1	X
	LG-6-79-2	I
	LG-6-79-3	I
Bahia dissecta	LG-6-80-1	X
	LG-6-80-2	I
	LG-6-80-3	I
Acalypha lindheimeri	LG-6-81-1	X
	LG-6-81-2	I
	LG-6-81-3	I
Gutierrezia sarothrae	LG-6-82-1	X
	LG-6-82-2	I
	LG-6-82-3	I
Lonicera albiflora	LG-6-83-1	X
	LG-6-83-2	I
	LG-6-83-3	I
Sphaeralcea fendleri	LG-6-84-1	X
	LG-6-84-2	I
	LG-6-84-3	I
Cosmos parviflorus	LG-6-85-1	X
	LG-6-85-2	I
	LG-6-85-3	I
Monnima wrightii	LG-6-86-1	X
	LG-6-86-2	I
	LG-6-86-3	I
Cuphea wrightii	LG-6-87-1	X
	LG-6-87-2	I
	LG-6-87-3	I

Table 3-1. Plant Extract Scission Assay Results - continued

Species	Extract Code[a]	Scission Result[b]
Salvia lemmonii	LG-6-88-1	X
	LG-6-88-2	I
	LG-6-88-3	I
Valeriana sorbifolia	LG-6-89-1	X
	LG-6-89-2	I
	LG-6-89-3	I
Xanthocephalum gymnospermoides	LG-6-90-1	X
	LG-6-90-2	I
	LG-6-90-3	I
Vicia pulchella	LG-6-91-1	X
	LG-6-91-2	I
	LG-6-91-3	I
Gaillardia pinnatifida	LG-6-92-1	X
	LG-6-92-2	I
	LG-6-92-3	I
Delphinium andesicola	LG-6-93-1	X
	LG-6-93-2	I
	LG-6-93-3	I
Gnaphalium leucocephalum	LG-6-94-1	X
	LG-6-94-2	I
	LG-6-94-3	I
Heterotheca subaxillaris	LG-6-95-1	X
	LG-6-95-2	I
	LG-6-95-3	I
Marabilis multiflora	LG-6-98-1	X
	LG-6-98-2	I
	LG-6-98-3	I
Eriogonum polycladon	LG-6-99-1	X
	LG-6-99-2	I
	LG-6-99-3	I
Tecoma stans	LG-6-100-1	X
	LG-6-100-2	I
	LG-6-100-3	I
Aloysia wrightii	LG-6-101-1	X
	LG-6-101-2	I
	LG-6-101-3	I
Astrolepis sinuata	LG-6-102-1	X
	LG-6-102-2	I
	LG-6-102-3	I

Table 3-1. Plant Extract Scission Assay Results - continued

Species	Extract Code[a]	Scission Result[b]
Cordylanthus wrightii	LG-6-103-1	X
	LG-6-103-2	I
	LG-6-103-3	I
Garrya wrightii	LG-6-104-1	X
	LG-6-104-2	I
	LG-6-104-3	I
Psoralidium tenuiflorum	LG-6-105-1	X
	LG-6-105-2	I
	LG-6-105-3	I
Dicliptera resupinata	LG-6-106-1	X
	LG-6-106-2	I
	LG-6-106-3	I
Machaeranthera canescens	LG-6-107-1	X
	LG-6-107-2	I
	LG-6-107-3	I
Acalypha neomexicana	LG-6-108-1	X
	LG-6-108-2	I
	LG-6-108-3	I
Tagetes lemmonii	LG-6-109-1	X
	LG-6-109-2	I
	LG-6-109-3	I
Monarda fistulosa	LG-6-110-1	X
	LG-6-110-2	I
	LG-6-110-3	I
Rhamnus californica	LG-6-111-1	X
	LG-6-111-2	I
	LG-6-111-3	I
Amorpha fruticosa	LG-6-112-1	X
	LG-6-112-2	I
	LG-6-112-3	I
Chamaecrista nictitans	LG-6-113-1	X
	LG-6-113-2	A/A
	LG-6-113-3	I
Gymnosperma glutinosum	LG-6-114-1	X
	LG-6-114-2	I
	LG-6-114-3	I
Phoradendron villosum	LG-6-115-1	X
	LG-6-115-2	I
	LG-6-115-3	I

Table 3-1. Plant Extract Scission Assay Results - continued

Species	Extract Code[a]	Scission Result[b]
Quercus oblongifolia	LG-6-116-1	X
	LG-6-116-2	I
	LG-6-116-3	I
Hymenoxys wrightii	LG-6-117-1	X
	LG-6-117-2	I
	LG-6-117-3	I
Artemisia dracunculus	LG-6-118-1	X
	LG-6-118-2	I
	LG-6-118-3	I
Lupinus palmeri	LG-6-119-1	X
	LG-6-119-2	I
	LG-6-119-3	I
Thalictrum fendleri	LG-6-120-1	X
	LG-6-120-2	I
	LG-6-120-3	I
Brickellia grandifolia	LG-6-121-1	X
	LG-6-121-2	I
	LG-6-121-3	I
Scrophularia parviflora	LG-6-122-1	X
	LG-6-122-2	I
	LG-6-122-3	I
Senecio bigelovia	LG-6-123-1	X
	LG-6-123-2	I
	LG-6-123-3	I
Silene scouleri	LG-6-124-1	X
	LG-6-124-2	I
	LG-6-124-3	I
Erigeron arizonicus	LG-6-125-1	X
	LG-6-125-2	I
	LG-6-125-3	I
Cyperus fendlerianus	LG-6-126-1	X
	LG-6-126-2	I
	LG-6-126-3	I
Lathryus gramnifolius	LG-6-127-1	X
	LG-6-127-2	I
	LG-6-127-3	I
Laennecia schiedeana	LG-6-128-1	X
	LG-6-128-2	I
	LG-6-128-3	I

Table 3-1. Plant Extract Scission Assay Results - continued

Species	Extract Code[a]	Scission Result[b]
Phoradendron juniperinum	LG-6-129-1	X
	LG-6-129-2	A/A
	LG-6-129-3	A/A

[a]Extract Code: LG-6-xxx-1 = hexane extract
LG-6-xxx-2 = MEK extract
LG-6-xxx-3 = methanol extract

[b]Scission Result: I = inactive (no activity shown in this assay)
X = assay not performed
A/A = Activity confirmed

Table 3-1. Plant Extract Scission Assay Results - continued

3.3 Modification with MADGE

The assay, while not labor-intensive, is time consuming. Each sample is typically prepared in its own vial and transferred to another for assay. Time is a factor when preparing samples for this assay. If too many samples are assayed then the first vial has incubated for several minutes before the last sample has been prepared. MADGE is an attempt to solve this problem and while increasing throughput. In MADGE (Microplate Array Diagonal Gel Electrophoresis), a special casting die produces a gel with wells that match the dimensions of a 96-well microplate. This casting system is only recently available in the United States. Usage of this apparatus has primarily been restricted to PCR analysis (Day *et al.*, 1999). The array is set on an angle so when run each lane does not overlap any of the others (Figure 3-4.) Much time and materials are saved through the use of microplates and multiwell pipetters. Because the gel is only a few millimeters thick and only slightly longer than a conventional microplate, running and development times are rapid. The MADGE system only needs 70 minutes for 96 samples from assay to destain, while a horizontal assay would require at least 3 gels each taking 4 hours.

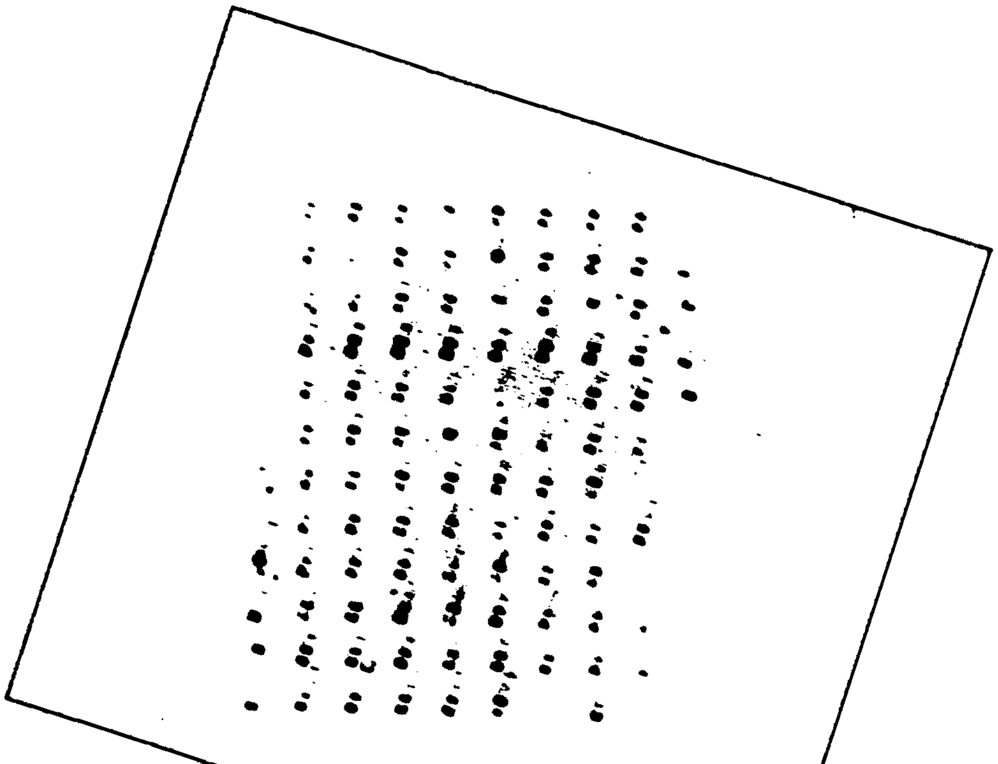


Figure 3-4. MADGE of 96 Plant Extracts.

3.4 Discussion of Scission Assay Results

An extract was considered active if it converted at least 50% of its supercoiled form to relaxed or linear. The following eight species demonstrated reproducible scission activity: *Rhus virens*, *Gossypium thurberi*, *Eriogonum abertianum*, *Heuchera sanguinea*, *Pteridium aquilinum*, *Gentianella microcalyx*, *Chamaecrista nictitans*, and *Phoradenron juniperinum*. *Rhus virens*, *Gossypium thurberi*, *Eriogonum abertianum, and Pteridium aquilinum* are active in the methanol extract only. Priority was given to those plants that had activity in both MEK and methanol extracts. *Phoradendron juniperinum* was selected for continued study because of the dearth of chemical studies available on both the family (Viscaceae) and species.

It should be noted that the other species of *Phoradendron* tested, *P. villosum* and *P. californicum* were inactive in the assay. This genus contains parasitic plants that live on numerous host plants. *P. juniperinum* is a parasite of *Juniperus deppeana*. The extracts of the host tree, *J. deppeana* were found to be inactive in our DNA scission assay.

CHAPTER 4

ISOLATION OF 5-CAFFEOYL-*epi*-QUINIC ACID FROM *PHORADENDRON JUNIPERINUM*

4.1 Introduction

Phoradendron juniperinum belongs to the Viscaceae, a family comprised of parasitic plants. Viscaceae contains only five other genera besides *Phoradendron*: *Viscum* (European mistletoe), *Arceuthobium*, *Dendrophthora*, *Karthasella*, and *Notothixos*. *Phoradendron* is a New World genus of approximately 100 species. *P. juniperinum* is restricted to the southwestern United States and is a general parasite of numerous species of juniper. In Arizona, four species (*Juniperus osteosperma*, *J. deppeana*, *J. monosperma*, and *J. scopulorum*) are the primary hosts (Hawksworth and Mathiasen, 1978).

Phoradendron juniperinum is properly classified as a dioecious hemiparasite. The seeds of the plant are deposited on tree limbs after having been passed through the digestive tracts of birds. These seeds germinate affixed to the limbs by a specialized holdfast structure called a haustoria. The 'root' penetrates the host tissue and directly taps the xylem tissue. The mistletoe can actively photosynthesize and therefore needs little from the host, only those substances it cannot obtain from the soil. *Phoradendron* draws water, minerals, free amino acids (for nitrogen), and if necessary, the simple carbon products of host photosynthesis. Significant carbon thievery from the host only appears to occur when both plants are experiencing conditions of stress. Gehring and Whitman (1992) demonstrated that the levels of mycorrhizae, which associate with the root system of *Juniperus monosperma*, are stable under stressful conditions when no parasite is present, but when *P. juniperinum* is on the limbs of a stressed juniper, mycorrhizae levels drop 27-38%. Therefore, unlike true parasites, hemiparasites being only partially heterotrophic, do not harm their hosts under normal growing conditions.

Medicinal usage of American mistletoe varies with culture (Kay, 1996). While Phoradendron mistletoes are found throughout the United States and Mexico, North American ethnobotanical usage seems restricted to the Native cultures of the United States. The Tohono O'odham use *P. californicum* as a treatment for stomach and menstrual cramps, while the Seri use the plant for "illness inside the body." Cherokee used *P. villosum* to treat epilepsy. The Seri also use the stems and leaves of *P. diguetianum* to treat women in childbirth (hastening the expulsion of the afterbirth) and to reduce diarrhea. The Tarahumara use the wood to make a tea to treat a variety of illnesses from colds to venereal disease. *P. juniperinum* is reported to be used by the Hopi, Zuni, and Navajo (Kay, 1996).

For a plant that has a long medicinal history, little phytochemical analysis has been conducted. *P. serotinum* possesses tyramine and β-phenylethylamine. *P. tomentosum* has had some of its flavonoids charaterized: apigenin, apigenin 4'-*O*-glucoside, vitexin, schaftoside, and isoschaftoside (Dossaji *et al.*, 1983). *P. juniperinum* was found to contain oleanolic acid, a compound with anti-HIV activity (Kashiwada *et al.*, 1998). Recently, the novel cytotoxic compounds, moronic acid and 3,4-*seco*-olean-18-ene-3,28-

dioic acid have been isolated from *P. reichenbachianum* along with numerous common triterpenoids and sterols such as β-sitosterol, squalene, and lupeol (Rios *et al.*, 2001).

More significantly, *Phoradendron* contains a family of proteins called phoratoxins. Like viscumin and viscotoxin from *Viscum album*, phoratoxins are lectins. Phoratoxin can cause bradycardia, hypotension, and peripheral vasoconstriction (Samuelsson and Ekblad, 1967) and has been responsible for several cases of accidental poisoning (Spiller *et al.*, 1996).

4.2 Extraction and Isolation

Large-scale recollection of *Phoradendron juniperinum* (mistletoe) was performed on September 2, 1999 on Prison Camp Road in the Coronado National Forest, north of Tucson. Plant material that penetrated the host limbs could not be isolated from the juniper and were not extracted. Care was taken to prevent juniper material from contaminating the mistletoe. The general isolation protocol is diagrammed in Figure 3-1. Extraction of *P. juniperinum* was conducted on the total aerial biomass. Hexane, MEK and methanol extracts were prepared as outlined in Section 2.2 with minor modifications. Due to the large level of biomass being extracted, four 24 hour washes of each solvent were performed. Solvent was removed *in vacuo* through rotary evaporation. Extraction of 6.03 kg of dried material resulted in 71.3 g of hexane extract, 220.4 g of MEK, and 1192.1 g of methanol extract. The methanol extract represented approximately 20% of the total dry weight.

Since the MEK extract was active, 50 mg of the material was subjected to separatory funnel partitioning. The scission assay was used to guide fractionation. Partitioning began with hexane (5 mL) and 80% methanol in H_2O (5 mL). Activity and most of the biomass was found in the 80% MeOH fraction (43.4 mg). This fraction was diluted with water to make a solution of 60% MeOH. An equal volume of chloroform was added for further partitioning. This separation provided 8.9 mg of chloroform fraction, an interface layer of 7.6 mg and a scission active 60% methanol fraction of 26.9 mg. At each step of partitioning, the solvent layers were each washed three times with a 10% volume of the solvent it was partitioned against. These washes were added back to their equivalent solvent fraction. The 60% methanol fraction contained mostly polar materials and was found to be very similar in composition to the 1.1 kg methanol fraction using thin layer chromatography [C-18; 50:10:40 (methanol: acetonitrile:water)] and 0.1% vanillin in H_2SO_4 as the visualization agent. Testing of the bulk methanol extract demonstrated scission activity so the decision was made to isolate an active compound from this more abundant extract.

An aliquot of the methanol extract (105 g) was separated into n-butanol and water fractions with 2 liters of each solvent. The water and butanol extracts isolated were 40 g and 65 g, respectively. The butanol extract, like the MEK extract, was further separated with 2 liters each of hexane and 80% methanol, resulting in an insoluble interface that contained 3.1 g and a 5.0 g hexane extract. The 80% methanol fraction (56.9 g) was diluted to 60% methanol and partitioned with an equal volume of chloroform (4.4 g). The 60% methanol fraction (52 g) was the most active in the scission assay.

One gram of the 60% MeOH fraction was run through a C-18 (40 μm; J.T. Baker) column (100 g; 4.2 cm x 31 cm) under low pressure. Material was eluted from the column with an isocratic solvent system (HPLC-grade MeOH:H_2O; 80:20). A major scission-active band of interest eluted after 550 mL. The active fraction (165 mL) was rotary evaporated *in vacuo* to yield a pale yellow solid (168 mg) and an aliquot (45 mg) was applied to two C-18 PTLC plates and separated with CH_3CN:H_2O(90:10). The major band (R_f = ~0.4) was removed while still wet and washed through a sintered glass funnel with HPLC-grade acetonitrile. After removing the solvents, 20 mg of a white powder was obtained. This material appeared to be pure by TLC and NMR.

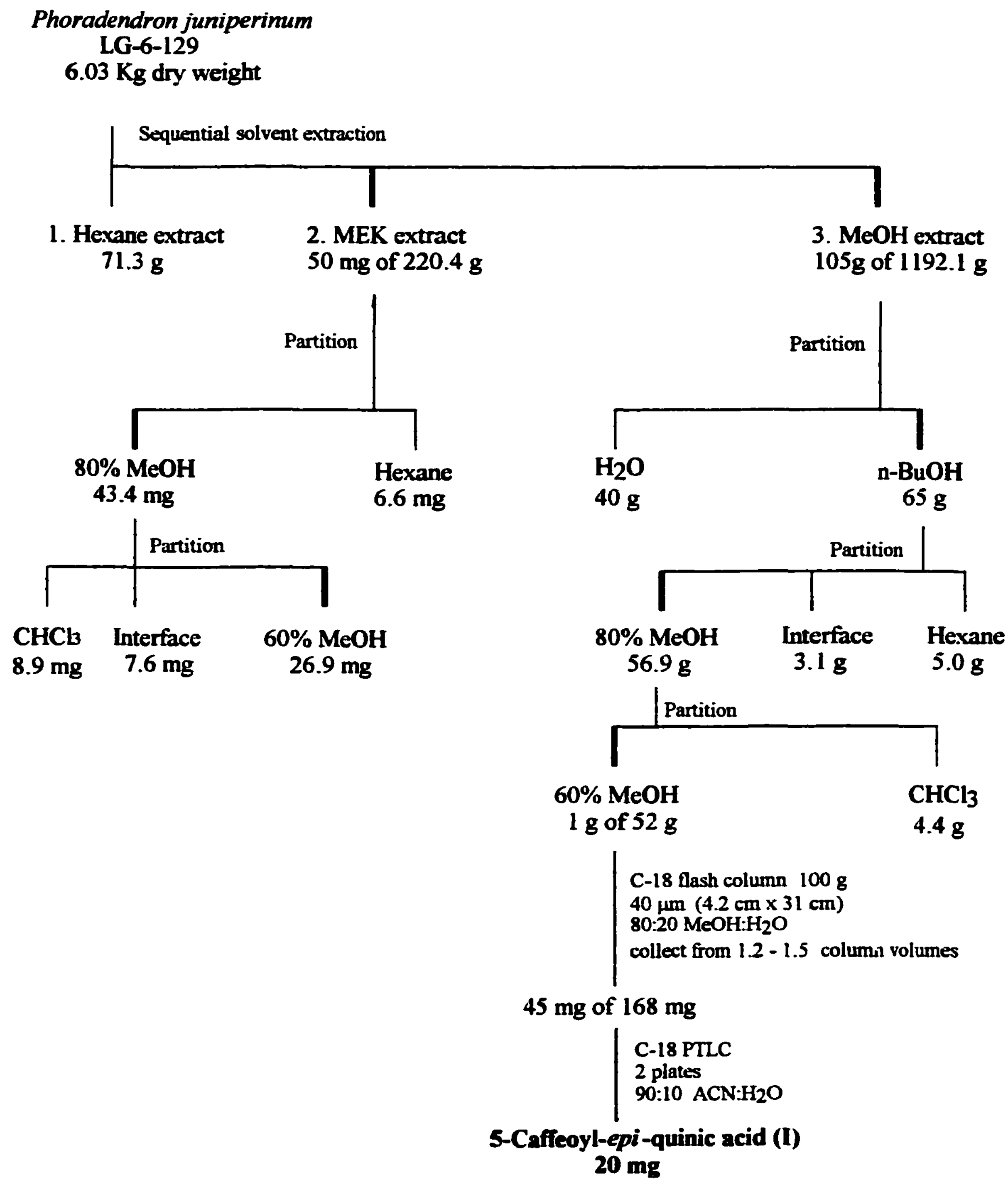


Figure 4-1. Isolation scheme for *Phoradendron juniperinum*.

4.3 Characterization of 5-Caffeoyl-*epi*-quinic acid (I)

Compound I was isolated as a white powder that became a yellow solution when dissolved. This polar compound is poorly soluble in water or methanol, but is readily soluble in dimethylsulfoxide (DMSO).

Mass spectroscopy was run on a Shimadzu LCMS QP8000α using the LCMS Solution software for data analysis. EIMS was run in both APCI +ve and APCI -ve modes. Figure 4-1 shows a base peak at *m/z* 355.05 $[M+1]^+$ with the positive probe and an *m/z* of 353.20 $[M-1]^-$ with the negative probe. The only other significant peak along with m/z 355 was a peak at m/z 337, most likely representing a loss of water $[M+1-18]^+$. Therefore, this compound was assigned a molecular weight of 354.

Infrared spectroscopy was conducted on a Shimadzu FTIR 8300 with data analysis using Hyper IR v1.57 software. Approximately 1 mg was pressed into a KBr pellet and scanned. Results are seen in Figure 4-3. The spectra revealed a significant O-H stretch at 3411.8 cm^{-1}. Peaks at 1700.0 cm^{-1}, 1274.9 cm^{-1}, and 1114.8 cm^{-1} were consistent with a conjugated ester. There was an aromatic C-H stretch at 2925.8 cm^{-1} and a carboxylic acid group with peaks at 1689.5 cm^{-1} and 1602.7 cm^{-1}.

^{13}C NMR was performed on a Bruker 500 MHz NMR at a concentration of 20 mg/mL. The spectrum is seen in Figure 4-4. The DMSO-d_6 solvent septet is clearly seen centered at δ 39.5. An acetonitrile contaminant is found with a peak at δ 118.2 and a septet at δ 1.3. There were sixteen peaks associated with this compound. Carbons at δ 176.4 and δ 165.9 suggest carbonyls. Four carbons at δ 68.3, δ 70.6, δ 70.9, and δ 73.6

appear to be non-aromatic carbons each bound to an oxygen atom. Natural products with the aforementioned traits that are polar and have a molecular weight of 354 are few. With sixteen carbons, the chlorogenic acids (chlorogenic, neochlorogenic and cryptochlorogenic acid) were likely candidates. The utilization of NMR techniques for the characterization of chlorogenic acids is challenging for two reasons: 1.) HPLC analysis is often chosen over NMR for the identification of chlorogenic acids and 2.) the NMR analysis that is performed becomes complicated due to a confusion in nomenclature. There exist two systems of numbering for the quinic acid ring carbons: the IUPAC nomenclature and a pre-existing system. Because of this problem, neochlorogenic and chlorogenic acid are trivial names that are often mistakenly interchanged. Therefore, few studies with reliable and complete NMR data are available. ^{13}C NMR comparisons of neochlorogenic (3-caffeoylquinic acid (Azuma *et al.*, 1999)) and chlorogenic acid (5-caffeoylquinic acid (Nakatani *et al.*, 2000) with the isolated compound were made (Table 4-1). Unlike the other compounds, the NMR spectrum of neochlorogenic acid (3-caffeoylquinic acid) was originally run at 125 MHz in CD_3OD and the variation between its results and the others, run in DMSO-d_6, make it unsuitable for direct comparison. When comparing chlorogenic acid to the *P. juniperinum* compound it is noted that while components of the caffeoyl unit correlate precisely, there are differences between their quinic acid rings. Specifically, these differences are seen in the C-1, C-5, C-6, and C-7 carbons.

The ^{1}H NMR was initially run in CD_3OD with water added for solublization at 300 MHz on a Varian Unity 300. The peak due to water (δ 4.85) was found to be large and may have masked important signals. The ^{1}H NMR was then performed on a Bruker

500 MHz NMR in DMSO-d_6 (Figure 4-5). Values are listed on Table 4-2. The water peak, though less obvious, was still present at δ 3.39. Eight of the NMR signals integrated as one proton each. Two clusters upfield of δ 2.0 integrated for two protons each. These two peaks appear to be alkane CH_2s. No methyl proton groups were apparent. Three other peaks (δ 9.55, δ 9.18, and δ 4.86) integrated each as one proton. The location and broadness of the aforementioned peaks suggest hydroxyl protons. All observed peaks corresponded to those that would be expected for a chlorogenic acid. Coupling was observed for all peaks (except the hydroxyls) and their corresponding J values were calculated (Table 4-2). The NMR run in CD_3OD was used to calculate the coupling constants for what appears to be the H-4 quinic acid component (Figure 4-6). With observed J of 3 and 8 Hz, respectively, the H-4 proton is likely to be axial to one proton and equatorial to another. The proton (H-3) axial to the H-4 is itself equatorial and axial to two others (J = 3,8,8 Hz). The H-5 quinic proton (J = 3,3,8 Hz) is axial to the proton of the quinic C-6. This combination of couplings can best be accounted for by a skewed boat conformation for the quinic acid moiety. The boat conformation has been well documented in the study of 3-caffeoyl-*muco*-quinic acid (Haribal *et al.*, 1998). *muco*-Quinic acid has its C-1 carboxyl group and C-4 hydroxyl group nearly axial to one another and this is the favored configuration. Thus, I would be best depicted to have be *epi*-quinic acid instead of quinic acid. In the caffeoyl component, the C-7' and C-8' protons have a coupling component of J = 15 Hz. This value is consistent with a *trans* configuration. Coupling in the aromatic ring is typical of a catechol group.

A DQF-COSY NMR spectrum run on a Varian Unity 300 MHz NMR helped confirm the assignments by showing coupling between neighbors (Figure 4-7). Connectivity between the caffeoyl H-7' to H-8' and the caffeoyl H-5' to H-6' is seen. The protons comprising the *epi*-quinic ring can be followed from H-2 to H-6. The hydroxyl group (δ 4.86) attached to the H-4 is apparent. The predicted structure is depicted at the bottom of Table 4-3.

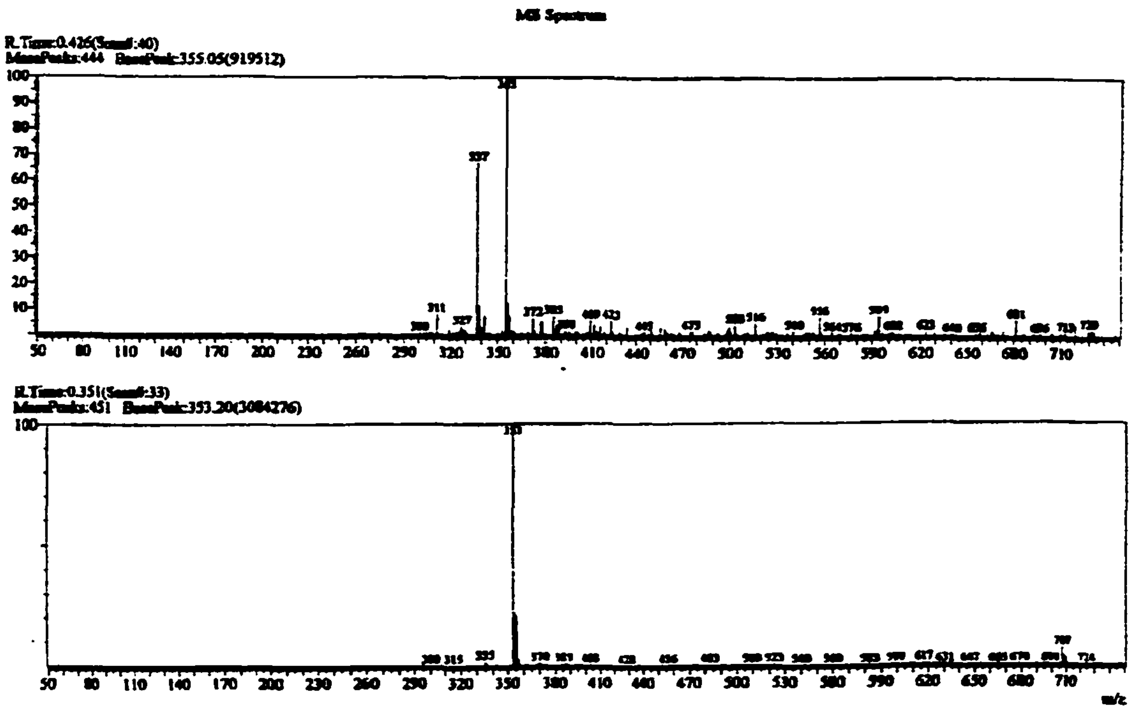
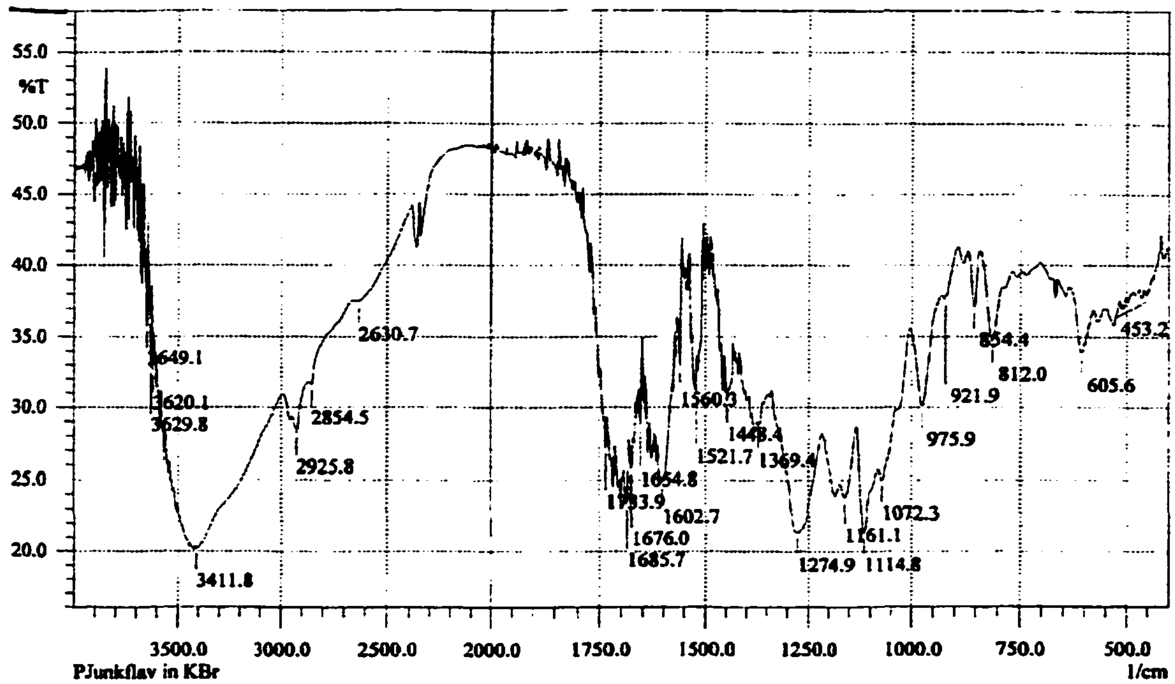


Figure 4-2. LCMS of 5-Caffeoyl-*epi*-quinic acid.



TFPJUNKF.IRS: PJunkflav in KBr

Date:	07/10/2001	Time:	11:15:19	NScans:	25		
Type:	HYPER IR	User:	UNIV_OF_ARIZONA			Detector:	standard
Abscissa:	1/cm	Ordinate:	%T	Apodization:	Happ		
Min:	401.17	Max:	3998.16	Range:	1/cm		
Ndp:	1866	Data Interval:	1.92868	Resolution:	4.0		
Gain:	auto	Aperture:	auto	Mirror Speed:	2.8(low)		

Figure 4-3. FTIR Spectrum of 5-Caffeoyl-*epi*-quinic acid.

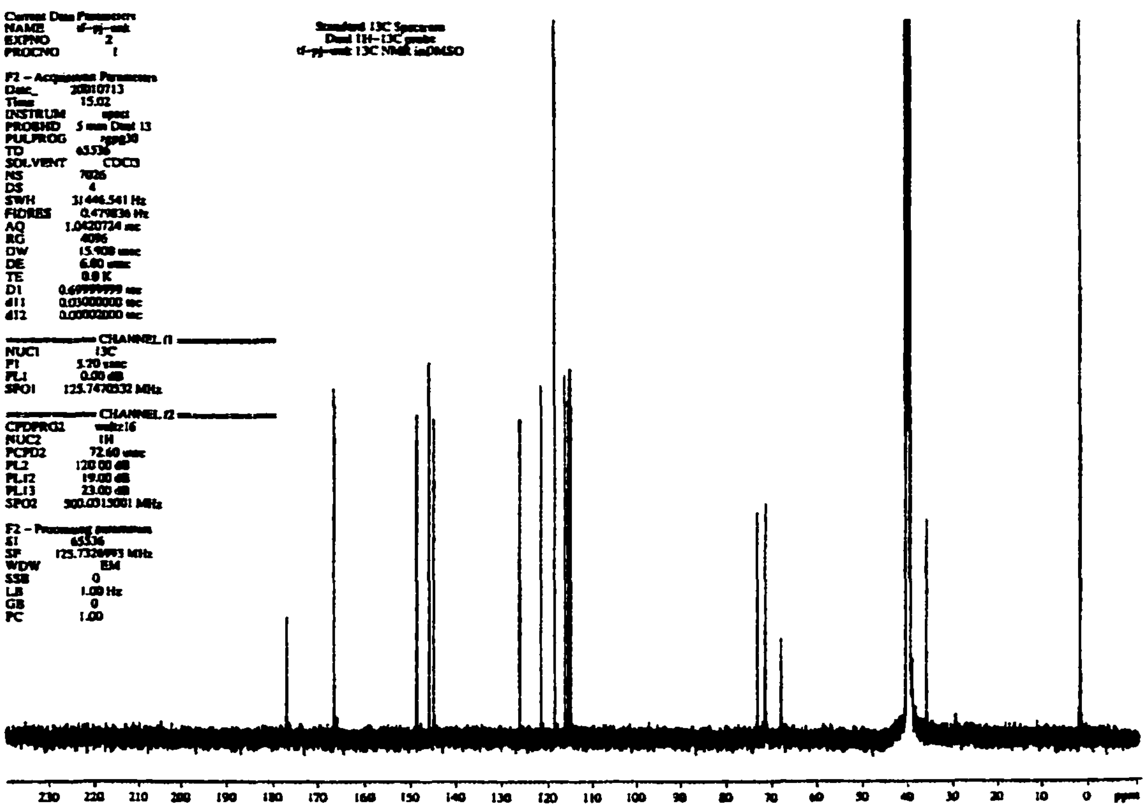


Figure 4-4. **^{13}C NMR Spectrum of 5-Caffeoyl-*epi*-quinic acid.**

	3-Caffeoylquinic acid Neochlorogenic acid* (Nakatani *et al.*, 2000)	5-Caffeoylquinic acid Chlorogenic acid** (Azuma *et al.*, 1999)	5-Caffeoyl-epi-quinic acid from *P. juniperinum****
1'	127.9	125.6	125.5
2'	115.1	114.7	114.8
3'	146.79	145.5	145.4
4'	149.4	148.3	148.0
5'	116.4	115.7	115.6
6'	122.9	121.2	120.9
7'	146.80	144.8	144.3
8'	115.8	114.3	114.4
9'	169.0	165.7	165.9
1	75.4	73.6	72.7
2	36.7	36.5	35.0
3	73.0	70.9	70.8
4	74.8	70.6	70.7
5	68.3	68.3	67.4
6	41.5	37.2	38.4
7	178.3	174.9	176.4

* in CD_3OD; 125 MHz

** in DMSO-d_6; 125 MHz

*** in DMSO-d_6; 125 MHz

Table 4-1. **^{13}C NMR Spectrum Assignments of 5-Caffeoyl-*epi*-quinic acid and Two Other Caffeoylquinic acids.**

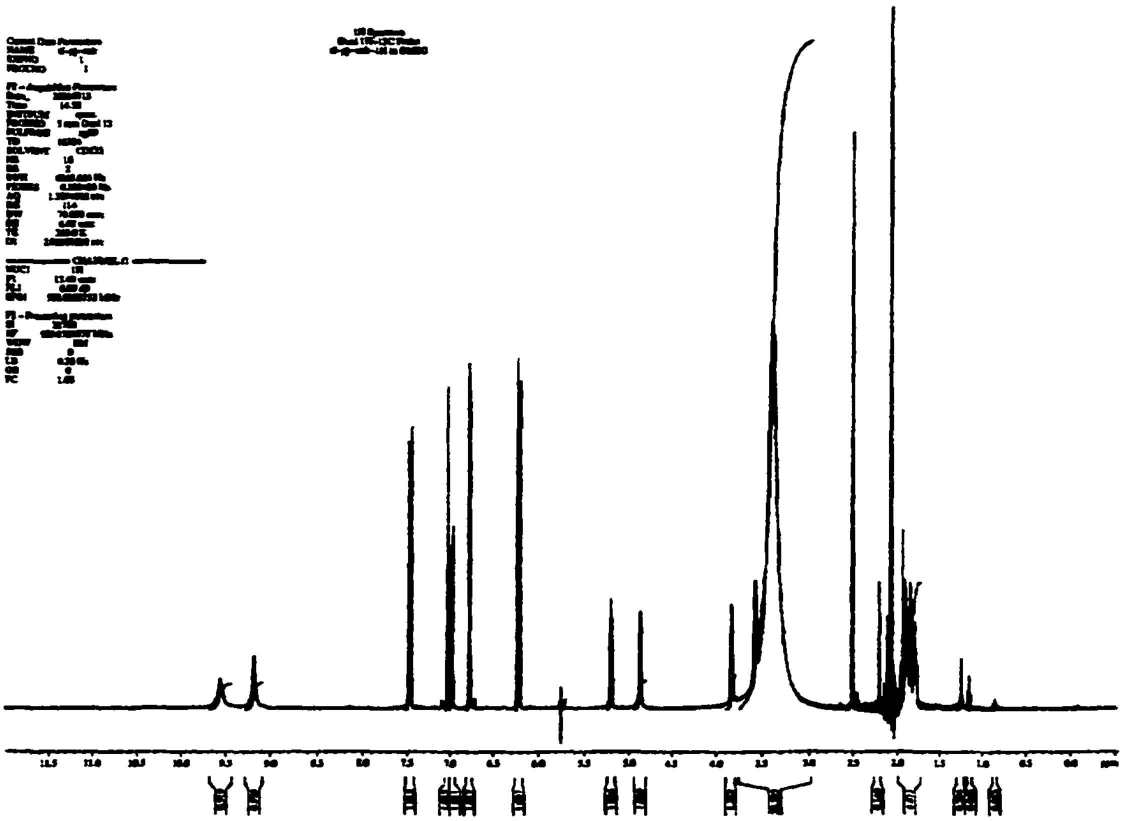


Figure 4-5. ^{1}**H NMR Spectrum of 5-Caffeoyl-*epi*-quinic acid.**

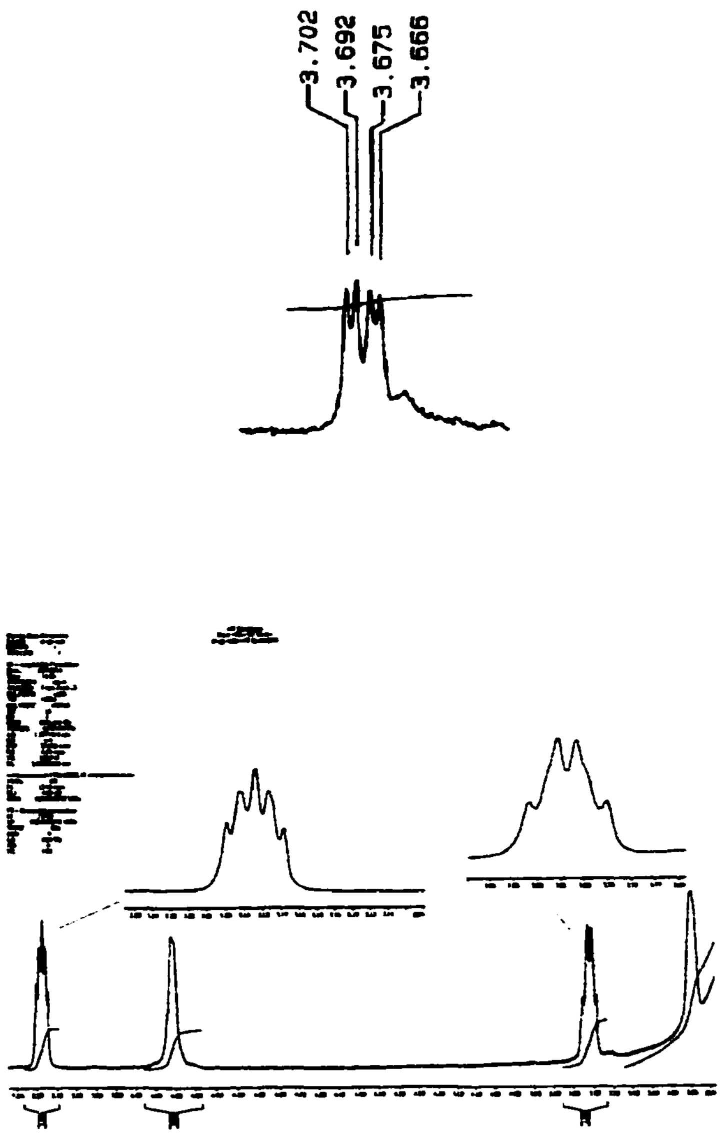


Figure 4-6. Expansion of Selected 5-Caffeoyl-*epi*-quinic acid Proton Peaks.

	3-Caffeoylquinic acid Chlorogenic acid* (Nakatani et al., 2000)	5-Caffeoylquinic acid Neochlorogenic acid** (Azuma et al., 1999)	5-Caffeoyl-epi-quinic acid from P. juniperinum***
2'	7.04 d (J = 2)	7.03 d (J = 2)	7.00 d (J = 2)
5'	6.76 d (J = 8)	6.77 d (J = 8)	6.74 d (J = 8)
6'	6.93 dd (J = 2,8)	6.98 dd (J = 2,8)	6.95 dd (J = 2, 8)
7'	7.58 d (J = 16)	7.42 d (J = 16)	7.44 d (J = 16)
8'	6.30 d (J = 16)	6.15 d (J = 16)	6.19 d (J = 16)
2	2.20 dd, 2.13m	1.98 br d (J = 5)	1.79 m
3	5.34 ddd (J = 3,3,4)	3.94 br s	3.80 ddd (J = 3,8,8)
4	3.63 dd (J = 3,9)	3.55 br d (J = 4)	3.53 dd (J = 3,8)
5	4.14 ddd (J = 3,9,9)	5.08 br d (J = 5)	5.17 ddd (J = 3,3,8)
6	1.95 dd, 2.13m	1.7-2.1	1.90 m

* in CD_3OD; 500 MHz
** in DMSO-d_6; 270 MHz
*** in DMSO-d_6; 500 MHz

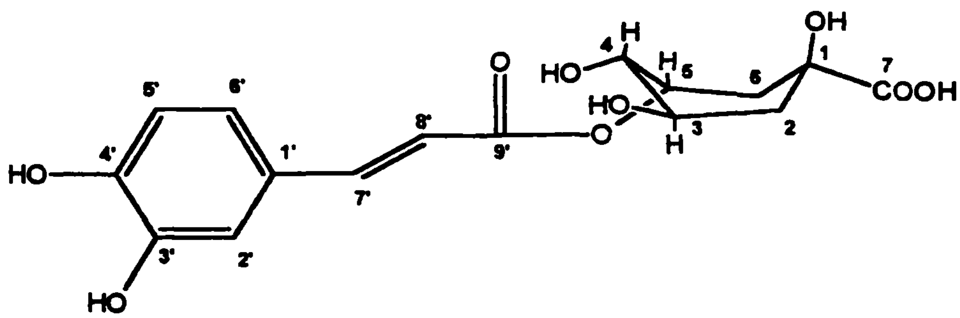


Table 4-2. **[1]H NMR Spectrum Assignments of 5-Caffeoyl-*epi*-quinic acid and Two Other Caffeoylquinic acids and Projected Structure.**

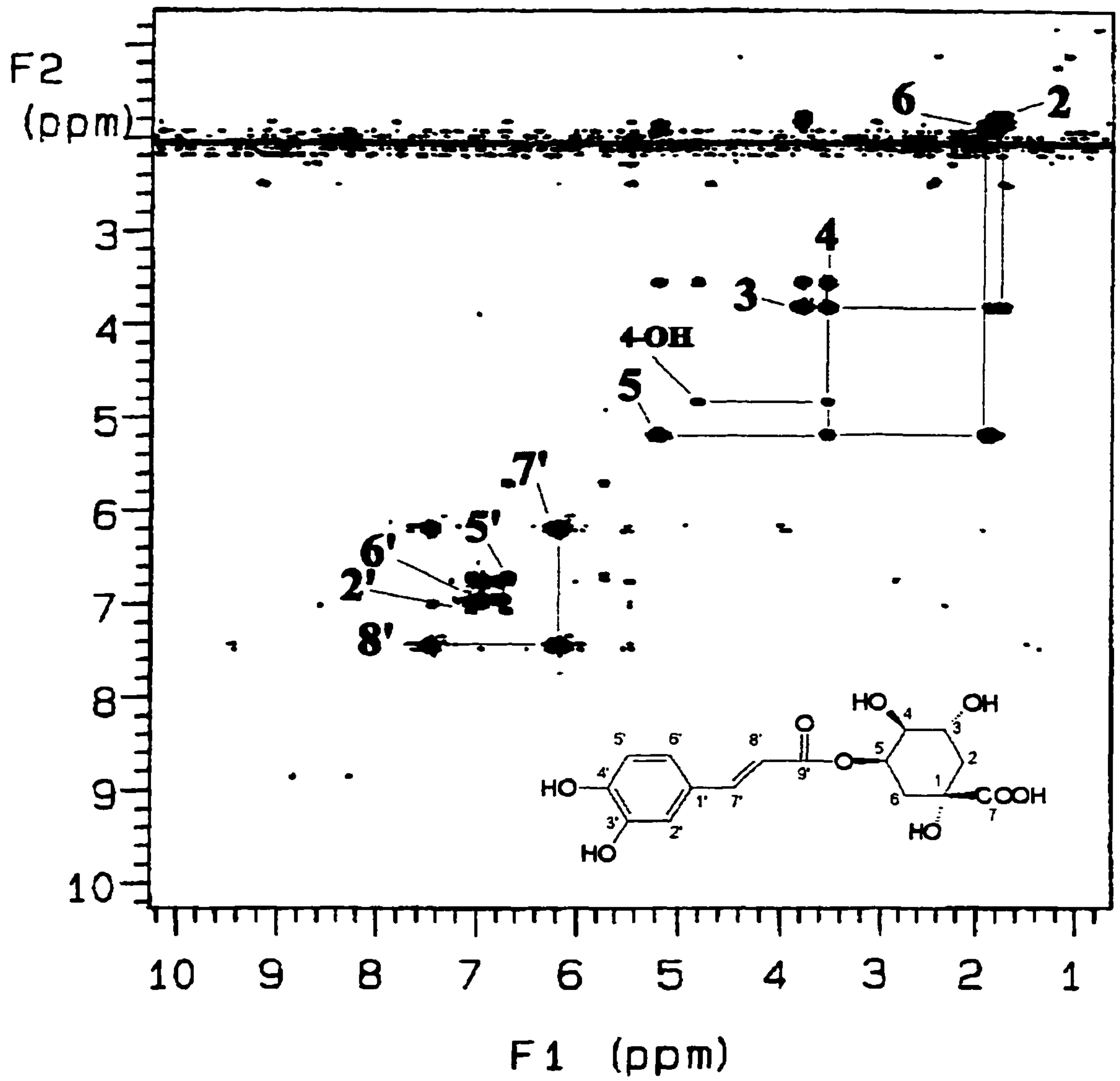
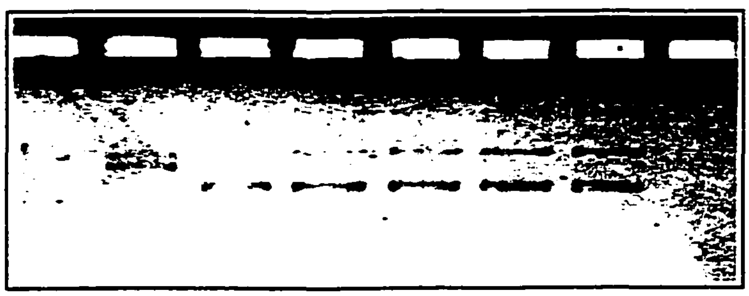


Figure 4-7. DQF-COSY NMR Spectrum of 5-Caffeoyl-*epi*-quinic acid.

4.4 Assays

The purified compound was assayed to determine the concentration necessary to reduce supercoiled pBluescript plasmid levels or to produce linear and open circular DNA by half. The compound was assayed at concentrations of 0 μg/mL(lane 2), 1 μg/mL(lane 3), 5 μg/mL(lane 4), 10 μg/mL(lane 5) and 25 μg/mL(lane 6) following the protocols outlined in Section 3.2, page 32. The stained gel is seen in Figure 4-8. (-)Epicatechin was used as a positive control(lane 1) at a concentration of 50 μg/mL for the purpose of inducing total cleavage. The 5-caffeoyl-*epi*-quinic acid produces 50% cleavage at 76 μM.

The compound was tested in a cancer cell line assay against non-small cell lung cancer (NCI-H460) and CNS glioma (SF-268). The compound was inactive at 100 μM and below.



Lane 1 2 3 4 5 6

Figure 4-8. Scission assay of 5-caffeoyl-*epi*-quinic acid.

CHAPTER 5

POTATO DISK ASSAY PRESCREEN

5.1 Potato Disk Assay Background

The potato disk assay is a simple, inexpensive test designed as a predictor of extracts and compounds that may possess P388 (*in vivo*, murine leukemia) inhibitory activity. *Agrobacterium tumefaciens* transforms a plant cell using its Ti plasmid, resulting in uncontrollable cell growth. In theory, many of the physiological changes that lead to tumor formation in plants are similar to those occurring in animals. So compounds that inhibit gall formation on the potato disks may halt animal cancer growth. Indeed, the potato disk assay and the P388 cell line screens demonstrated high correlation (Anderson *et al.*, 1992). Thus, Ferrigni *et al.* (1982) suggested using the potato disk assay as a prescreen for murine leukemia. These same compounds, also, may be active against other cancers. Figure 5-1 lists of a few compounds that have been tested and/or isolated using the potato disk assay. The variety of activities (brine shrimp, potato disk, 3PS, etc.) of the compounds in Figure 5-1 are summarized in the review by McLaughlin (1991).

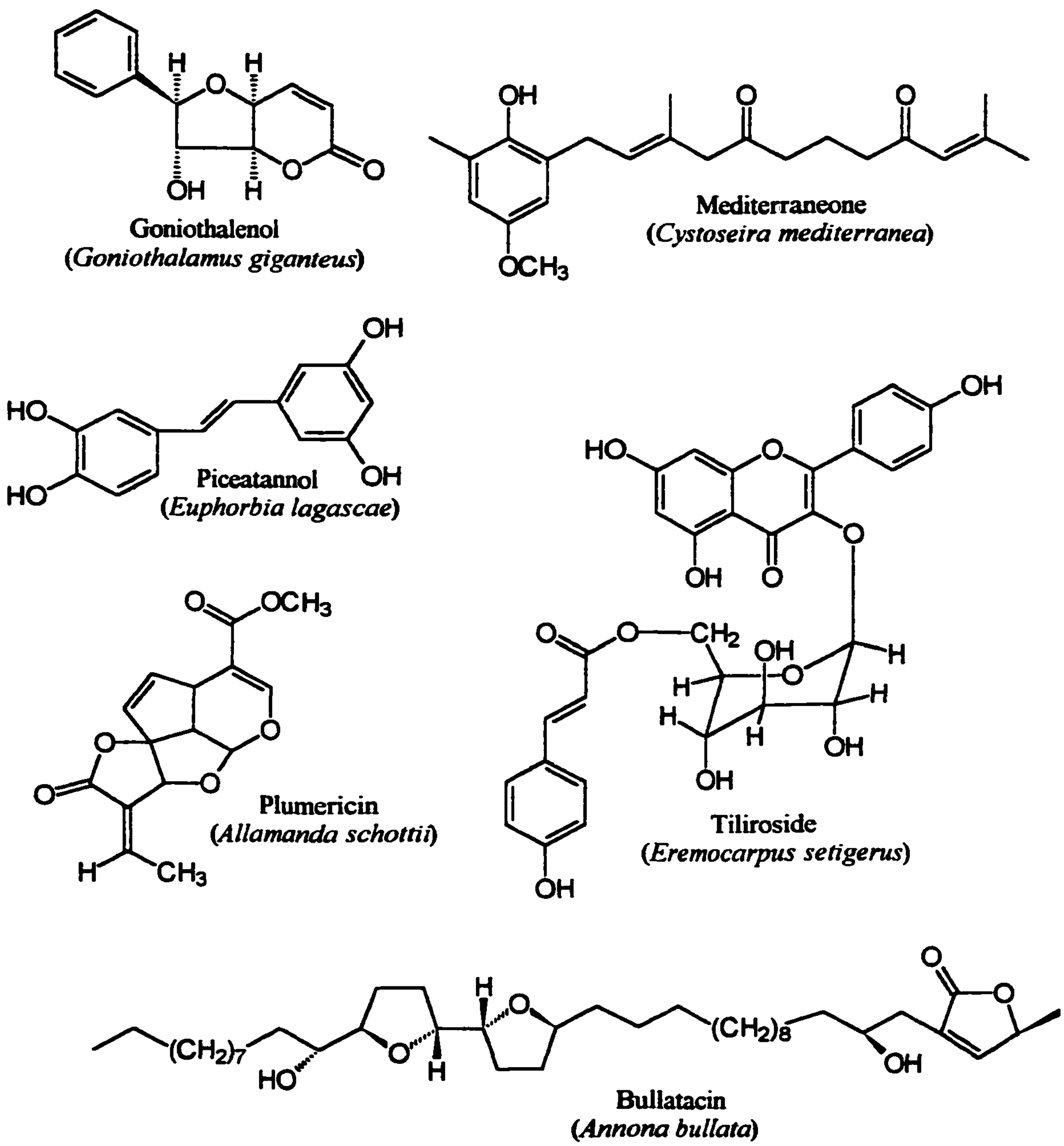


Figure 5-1. Compounds Isolated From or Active in the Potato Disk Assay.

5.2 Potato Disk Assay

The potato disk assay was conducted as follows (Ferrigni *et al.*, 1982). Red potatoes were purchased from a local market and surface sterilized with sodium hypochlorite (Clorox). In a laminar flow hood, cores of potato tissue were removed with a sterile cork borer (1.5 cm diameter). Each end of the core (2 cm.) was removed with a sterile scalpel and discarded. Disks (0.5 cm height) were cut from the cores and placed onto 1.5% agar plates (5 disks/100 mm diameter dish).

Agrobacterium tumefaciens B_6 was cultured in Sabouraud-maltose liquid media and diluted to 5 x 10^9 cells/mL with water. The sample to be tested was dissolved in DMSO to a concentration of 4 mg/mL and sterilized by passage through a 0.22 μm Millipore filter. The assay mixture was prepared by adding 1.5 mL sterile water to 0.5 mL of the DMSO sample. This was further diluted with the addition of 2 mL of the *A. tumefaciens* broth, resulting in a final sample concentration of 500 μg/mL. Podophyllotoxin (Sigma) and the absence of sample were the positive and neutral controls, respectively.

Fifty microliters of a sample was pipetted to the surface of each of 15 –25 disks. The plates were sealed with Parafilm and stored at room temperature in darkness. After 12 days, each disk was stained with Lugol's Solution and rinsed in deionized water. Tumors lack starch and remain unstained. Inhibition was represented by disks with fewer tumors than the DMSO control.

Inhibition values greater than 40% were considered worthy of further investigation. Fungal contamination of potatoes was not unusual, with as many as one in four contaminated. A plate was re-run if any disk turned black from fungus. Potatoes also varied in water content, density and starch levels. These factors resulted in a great deal of variability in tumor inhibition levels. Podophylotoxin produced tumor inhibition levels between 60 - 90% at 250 μM. The results were meant as a guide for bioassay-directed fractionation and not as quantitative data. Another observation was that the coefficient of variance grew dramatically as the average number of tumors decreased. The number of tumors on the negative control disks were kept above 25, and if the number of tumors were less than 25, higher concentrations of *A. tumefaciens* were used.

% Inhibition[a]	Species	ID#[b]	L1210[c]
86.0	*Larrea tridentata*	363S	450
80.0	*Pycananthum incanum*	101S	460
78.0	*Angelica lineariloba*	325S	460
78.0	*Marah gilensis*	427W	-
72.9	*Heracleum lanatum*	179W	60
71.2	*Mertensia fransciscana*	180W	0
69.0	*Datisca glomerata*	303S	460
62.5	*Salvia sonomensis*	270S	320
61.8	*Psorothamnus thompsoniae*	355S	450
56.1	*Saponaria officianalis*	313W	210
45.9	*Phlox austromontana*	36W	160
44.7	*Mahonia wilcoxii*	135S	650
43.8	*Eriogonum palmerianum*	172W	20
42.5	*Erysimum capitatum*	754W	300
40.9	*Linanthus nutalli*	267S	210
39.7	*Chamaesaracha sordida*	69S	300
36.6	*Saponaria officianalis*	313S	80
36.0	*Rotala ramisoir*	586S	-
28.6	*Heracleum lanatum*	761W	0
27.0	*Linanthus floribundus*	267W	190
24.2	*Eriogonum palmerianum*	172S	180
23.4	*Eriogonum polycladon*	753W	210
17.7	*Cynodon dactylon*	465S	160
8.8	*Polanasia dodecandra*	171W	320
4.4	*Ceanothus fendleri*	163W	200
4.4	*Sassafras albidum*	106S	60
0.0	*Gaillardia parryi*	362S	630
-4.2	*Hymenoxys scaposa*	498W	50
-17.3	*Proboscidea altheifolia*	197W	230
-20.0	*Ceanothus fendleri*	752W	-
-23.7	*Mentzelia multiflora*	331W	400
-38.0	*Pycananthum incanum*	101W	320
-40.0	*Dyssodia penachaeta*	768S	160
-100.0	*Dyssodia penachaeta*	768W	0

[a] % Inhibition: 100 -[Avg. tumors(test compound)/Avg. tumors (negative control)*100]

[b] ID#: Extract code

[c] L1210: Zonal differential (mm) between the L1210 culture and fibroblast cultures

Table 5-1. Potato Disk Inhibition and L1210 Differential Results.

5.3 Discussion of Potato Disk Assay Results

The plant extracts tested in the potato disk assay were initially selected due to their demonstrating solid tumor selectivity vs. fibroblasts in human or mouse cancer screens. The extracts tested in the potato disk assay were also screened for L1210 activity. The L1210 line is a mouse lymphocytic leukemia culture. If the disk assay is a predictor of compounds that may be active against the P388 line, it may be active against the L1210 line as well. The mouse lymphocytic leukemia screens were performed at Wayne State University School of Medicine in the laboratory of Dr. Tom Corbett. Protocols and descriptions of the L1210 assay are detailed in Corbett et al. (1995).

The results (Table 5-1) show that a large number of extracts achieved our 40% suppression level required for further consideration. Extracts above 40% tumor suppression represented 44% (15/34) of the total. Moving this selection level to 60% suppression reduced the number of active extracts to 26% (9 extracts). Seven non-suppressing extracts had the ability to stimulate tumor growth above those of the control from 4.2% - 100%. Six of these are water extracts and may just be supplying nutrients to the *A. tumefaciens* that are enhancing their growth thereby leading to more tumors.

With three exceptions (*Marah gilensis*, *Rotala ramisoir*, and *Ceanothus fendleri*) all extracts were tested against L1210. The figures in Table 5-1 represent the zonal differential between the L1210 culture and fibroblast cultures (representing normal cells). The extracts with activities above 40% inhibition had an average zonal differential of 300. Only four of these extracts had activities below 200. Those below 40% had an average of 205. It appeared that the potato disk assay detected L1210 activity with few false negatives, at least within this limited data set, but it appeared to miss detecting some extracts with major activity, such as *Polanasia dodecandra* (8.8%/320), *Gaillardia parryi* (0%/630), *Mentzelia multiflora*(-23.7%/400), and *Pycananthum incanum* (-38%/320). Due to *Psorothamnus thompsoniae* having activity in both assays (61.8%/450) and lacking extensive phytochemical study, *P. thompsoniae* was chosen for fractionation directed by the potato disk assay.

CHAPTER 6

ISOLATION OF DALRUBONE AND 5-METHOXYDALRUBONE FROM *PSOROTHAMNUS*

6.1 Introduction

The genus *Psorothamnus* (Fabaceae: Amorphae) is composed of over 150 species of New World small trees and shrubs. Species of *Psorothamnus* have previously been known by the genus names *Dalea*, *Parosela*, and *Psorodendron* (Shreve and Wiggins, 1964). Natives of the southwestern United States and Mexico have traditionally used *Psorothamnus* and *Dalea* for multiple applications. *Dalea polyadenia* was utilized as a general cure-all for treating illnesses as diverse as influenza, pneumonia, tuberculosis, smallpox, rheumatism, toothache, and neuralgia (Balls, 1975). On the other hand, the indigo bush *Psorothamnus* (*Dalea*) *emoryi* was utilized for the non-medicinal purpose of dyeing baskets a bright yellow, as well as, the seed having been used as a source of food (Balls, 1975).

6.2 Extraction and Isolation

The solvent fraction [4.9 g extract (DCM/MeOH + MeOH) from 51.1 g dried biomass] of *Psorothamnus thompsoniae* was found to possess potato disk tumor inhibitory activity of 61.8%. Extraction was performed as described in Section 2.3. The solvent extract (2 g) was fractionated on a DCM-equilibrated silica column (40-60μm)(Fig. 6-1) and eluted with DCM. Activity appeared following elution with 410 mL of DCM. A small volume (90 mg; 150 mL) contained inhibitory activity of 88.8%. The ground plant material (275 g) was then extracted with DCM only and 8.5 g of the resulting 9.0 g were likewise run (Fig 6-1) through a silica column (60 – 200 μm; 550g silica)(Fig. 6-2). A more nonpolar gradient was used to more efficiently separate the active fraction. The gradient began with 2 L of hexane, followed by 2 L each of 50:50, 40:60, 30:70, 20:80, and 10:90 (hexane:DCM). Fractionation proceeded with increasing volumes of DCM in hexane: 5:95 (5 L), 2.5:97.5 (2 L), and finally 100% DCM (2 L). The active fraction (500 mg; 86.2% tumor suppression) was eluted with hexane/DCM (5:95). The component responsible for inhibition was further purified with a C-18 column on a Waters C-4000 HPLC. An aliquot of the active sample (50 mg) was applied to the column and washed with 450 mL of 35% ACN before applying a 35% - 50% gradient at 1%/5 mL. The active fraction was eluted with ACN:H_2O (46:64) to yield 22 mg of material that inhibited potato tumors to a level of 86.2% at 250 μg/mL. This active fraction was separated on a NP-PTLC plate and eluted with 50:48:2 (hexane:DCM:MeOH). The activity (90.2%) was concentrated into a single band. This band was one compound and was determined through NMR and other spectroscopic techniques be dalrubone (II)(Dreyer et al., 1975).

A new collection of *Psorothamnus thompsoniae* and other related species were extracted and tested to determine if activity was present only in *Psorothamnus thompsoniae* or if this potato tumor inhibiting ability was widespread. *Linanthus floribundus* was tested to include an unrelated species with low expected inhibition. The procedure for testing is described in Section 5.2. Activity was found within both *Psorothamnus* species. In fact, *Psorothamnus emoryi* had higher activities in both the DCM and DCM/MeOH extracts. *Dalea formosa* demonstrated significant inhibition as well.

Species	DCM	DCM/MeOH	MeOH	MeOH/H_2O	H_2O
Dalea formosa	44.0	59.3	47.0	-71.0(0)	-39.0(0)
Dalea pulchra	24.7	-19.5(0)	32.5	-	-
Psorothamnus emoryi	63.9	23.7	-25.3	-	-
P. thompsoniae	56.9	-3.7(0)	1.2	-	-
Linanthus floribundus	34.5	23.3	1.4	-	-

Table 6-1. Percent Potato Tumor Inhibition of Additional Plant Extracts.

Psorothamnus emoryi was selected for fractionation for the dual purposes of obtaining larger quantities of dalrubone and possibly isolating additional related compounds. With dalrubone identified, additional bioasssay-guided fractionation proved unnecessary. Using the previously isolated dalrubone as a guide, TLC was the method used for tracking the purification of the compound. The isolation protocol is outlined in Fig. 6-3. Eleven kilograms of above-ground material were soaked in DCM:MeOH to produce 1.19 kg of extract. The crude extract was further separated into hexane soluble (206 g) and insoluble (984 g) fractions. The hexane soluble fraction (56 g) was partitioned between hexane and ACN:H_2O resulting in fractions of 35 g and 21 g respectively. The total ACN:H_2O crude extract was applied to an excess of C-18 in a Büchner funnel and washed consecutively with 25% ACN, 75% ACN, 100% MeOH, and DCM:MeOH. Dalrubone appeared to elute with the 75% ACN (12.0 g) wash. The process was repeated with the 75% ACN fraction using washes of 45% ACN, 100% ACN and 100% MeOH. The band of interest (9.08 g) eluted with the 100% ACN wash. Eight grams of this fraction were bound to an MPLC reverse phase column with a methanol step gradient. The material was bound with 30% methanol in water and consecutively washed in steps of 40%, 45%, 50%, 55%, 60%, 70%, and 100%. Each solvent step was run until no additional compounds eluted. The primary peak, late in the 60% methanol step was dalrubone (2.842 g). Cancer cell line assays run by Wayne State University reported that in this final isolation of dalrubone, another region of anti-leukemic activity against cell line L1210 was present (Hoffmann, unpublished data.) An additional significant band (648 g) eluted towards the end of the 70% MeOH wash. This mixture was further fractionated with a C-18 column (20 cm x 3.3 cm; 80 g, 40 μm) utilizing a 80:20 (MeOH:H_2O) isocratic system. The primary band eluted after 550 mL of solvent. The next 570 mL of solvent contained 239 mg of nearly pure compound. The entire fraction underwent recrystallization in 15 mL hexane with ethyl acetate. The resulting crystals (157 mg) were determined through NMR analysis, spectroscopic studies, and previously published data (Dreyer *et al.*, 1975) to be 5-methoxydalrubone (III).

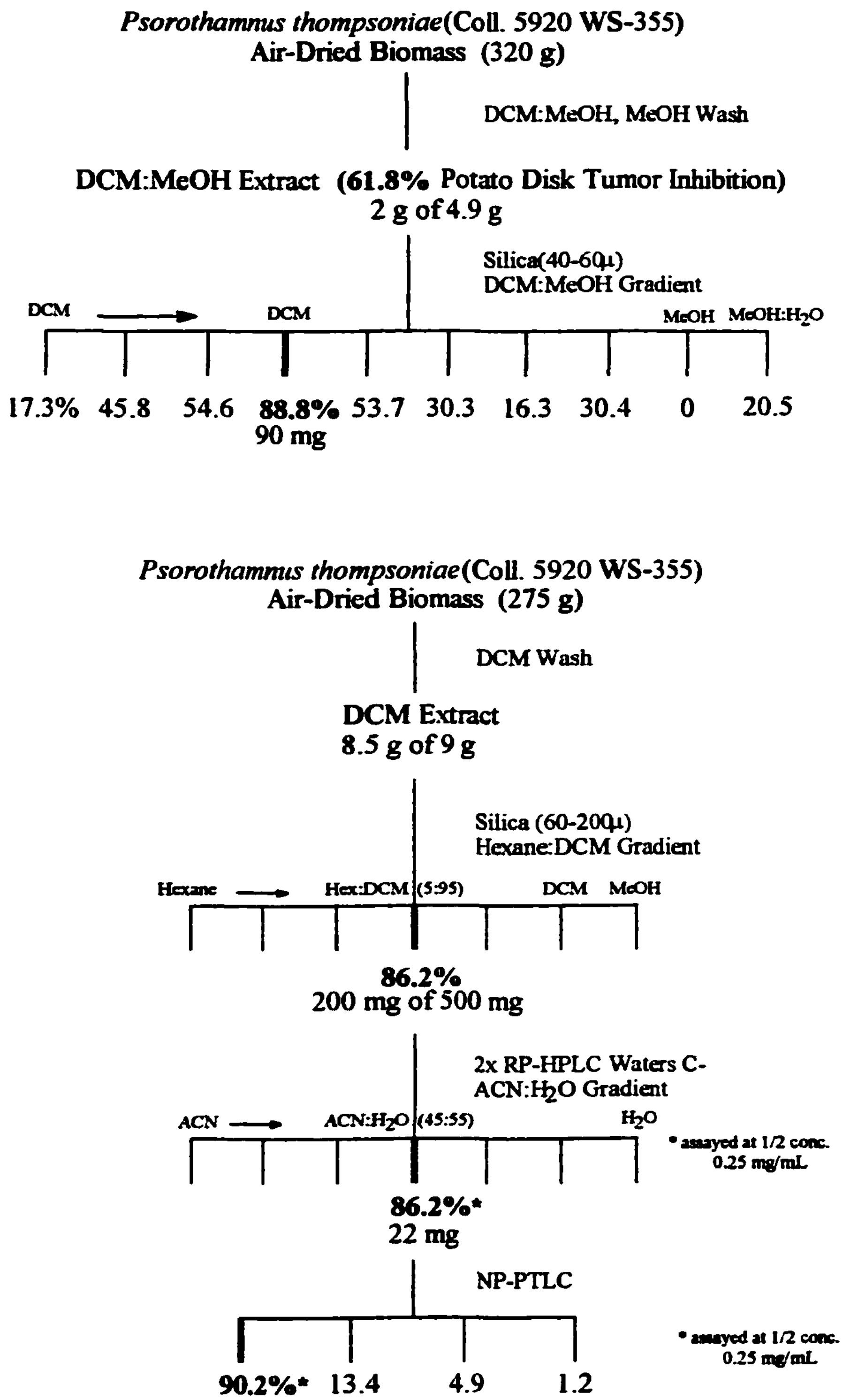


Figure 6-1 Isolation Scheme for *Psorothamnus thompsoniae*.

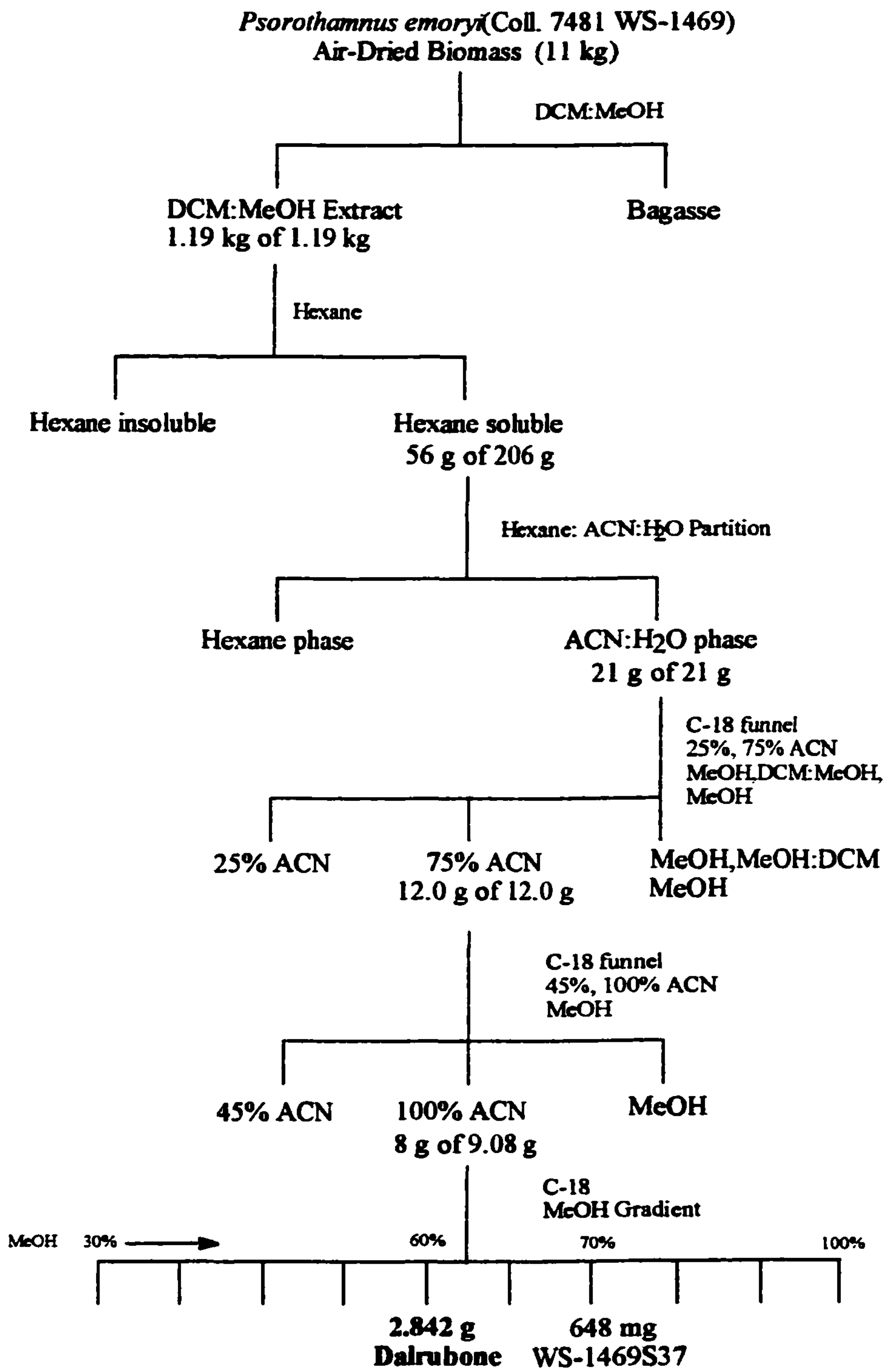


Figure 6-2 **Isolation Scheme for *Psorothamnus emoryi*.**

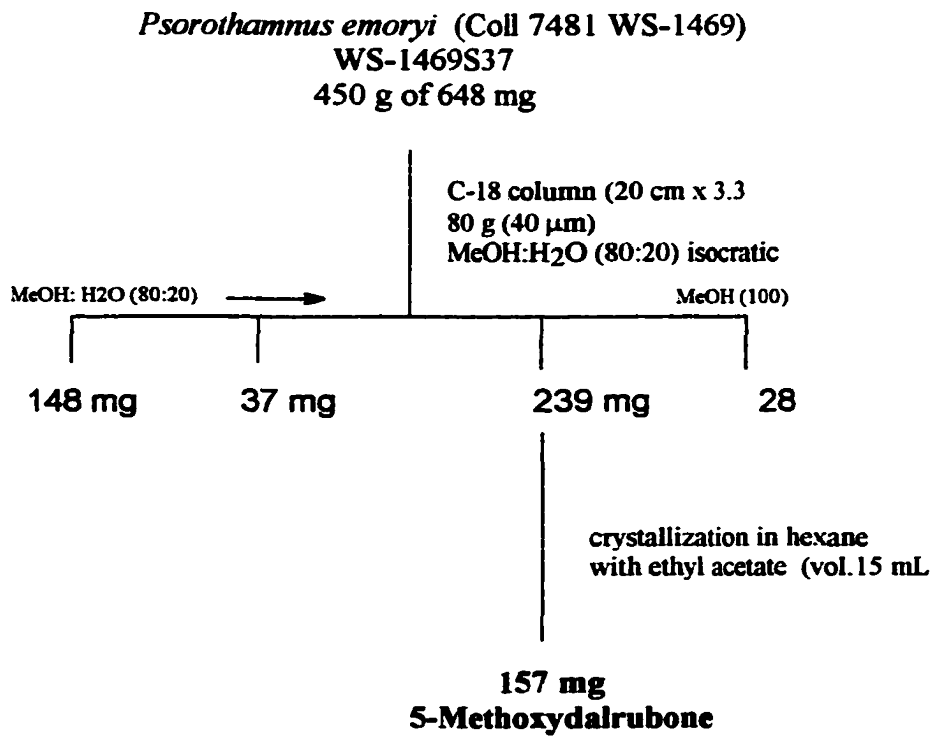


Figure 6-3 Isolation Scheme for *Psorothamnus emoryi*.

6.3 Characterization of Dalrubone

The isolated compound was deep red in color and volatile. Contact with dissolved material resulted in skin stained orange/yellow. The compound (II) was insoluble in hexane and water but soluble in medium-polarity solvents, such as acetone and ethyl acetate. The compound was believed to be dalrubone, a previously reported compound from *Psorothamnus* and *Dalea* (Dreyer *et al.*, 1975). Comparisons were made between experimental spectra and published data to confirm this suspicion.

Infrared spectroscopy was conducted on a Shimadzu FTIR 8300 with data analysis using Hyper IR v1.57 software. Approximately 1 mg was pressed into a KBr pellet and scanned. Results are seen in Figure 6-4. The significant spectral peaks at 1624.0(s), 1560.3(s), 1577.7(s), and 1527.5(s) match those reported by Zhang *et al.* (2000).

^1H NMR was performed on a Varian Unity 300 MHz NMR in $CDCl_3$ (Figure 6-5). The doublets at δ 7.59 and δ 7.08 were protons on the C-3 and C-4 alkene carbons with splitting indicative of *cis* coupling. Peaks at δ 7.32, δ 7.12, δ, 7.35, and δ 7.22 were the hydrogens on the aromatic C-5 through C-8 protons, respectively (Zhang *et al.*, 2000). The methyl protons of the C-7', attached to C-5' alkene possessed a singlet peak at δ 1.85. The remaining two sets of protons for the C-8' and C-9' were both located at δ 1.25. This data precisely matched those of Dreyer *et al.*(1974) (Table 6-2.).

^{13}C NMR was also performed on a Varian Unity 300 MHz NMR in $CDCl_3$ (Figure 6-6). The C-2' and C-4' carbonyl peaks were at δ 202.03 and δ 199.38 respectively. The peaks at δ 166.28 (C-2), δ 156.47 (C-6'), and δ 152.55 (C-9) were quaternary carbons possessing both an alkene bond and a C-O bond. The eight peaks from δ 127.24 - δ 116.12 were the remaining aromatic carbons. The C-5' peak was located at δ 105.36. The δ 57.50 and δ 55.9 peaks were the C-3' and C-6'-methoxy respectively. The remaining methyl groups were found at δ 22.95 (C-8' and C-9') and at δ 9.02 (C-7'). The data again matches Dreyer *et al.* (1974) (Table 6-3) suggesting that the compound is dalrubone.

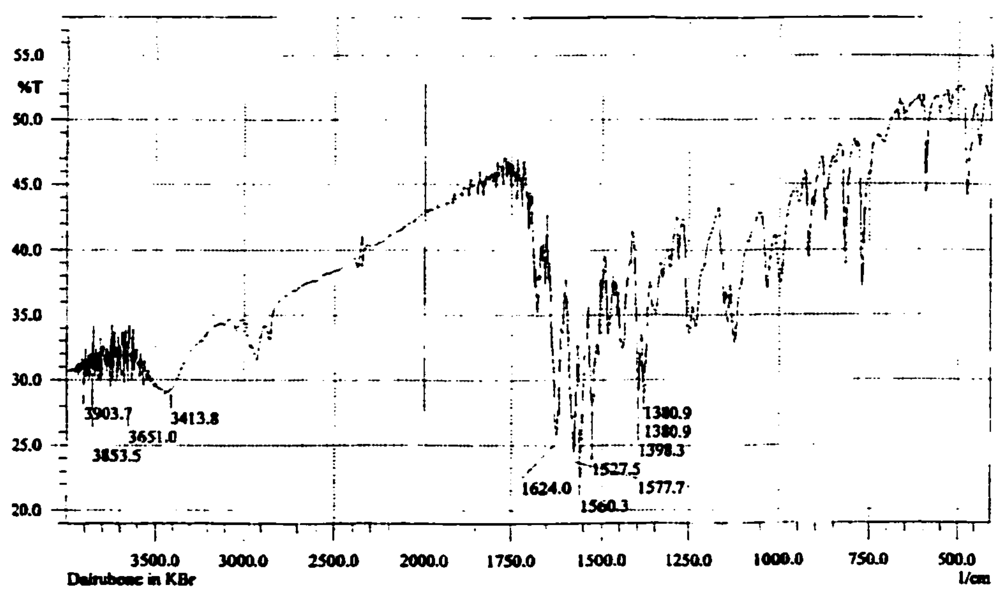


Figure 6-4 **FTIR Spectrum of Dalrubone.**

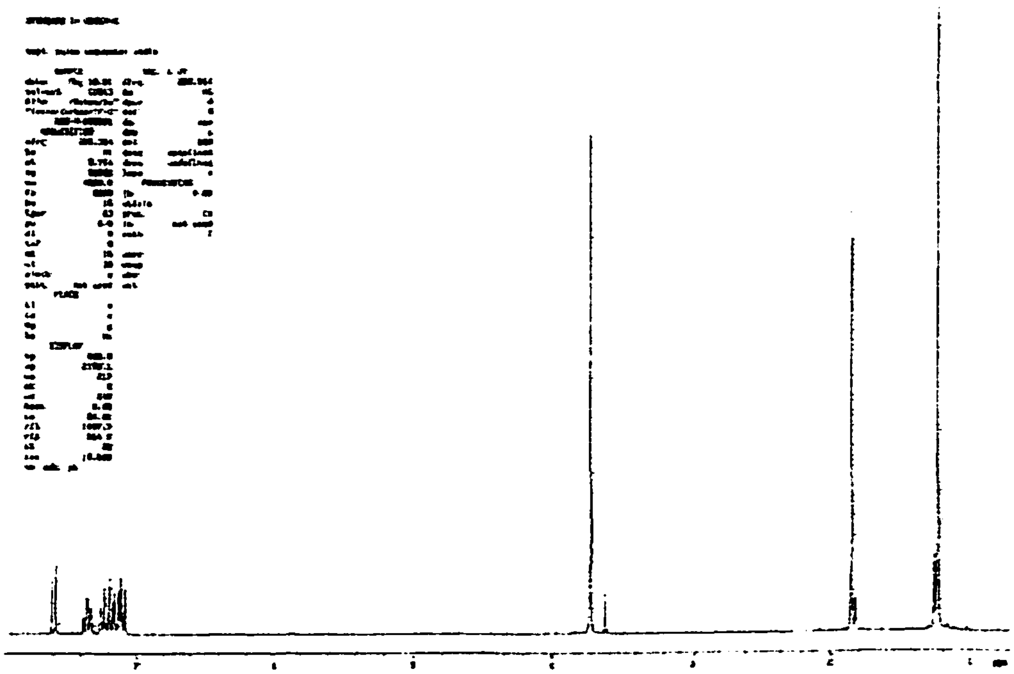
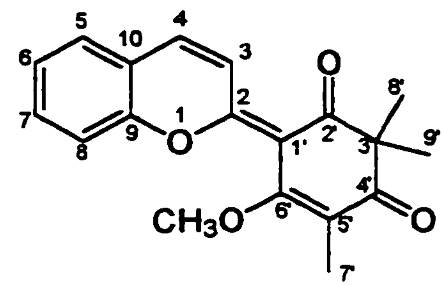


Figure 6-5 ¹H NMR Spectrum of Dalrubone.



	Experimental	*(Dreyer et al. ,1975)*
3	7.59 d (J = 10)	7.57 d (J = 10)
4	7.08 d (J = 10)	7.0 d (J = 10)
5	7.32 m	7.2 m
6	7.12 m	7.2 m
7	7.35 m	7.2 m
8	7.22 m	7.2 m
7'	1.85	1.85
8'	1.25	1.25
9'	1.25	1.25
6'-OMe	3.75	3.72

300 MHz ^{1}H-NMR Data in $CDCl_3$ (Varian Unity)

Table 6-2 **^{1}H NMR Spectral Assignments (Experimental and Literature) of Dalrubone and Projected Structure.**

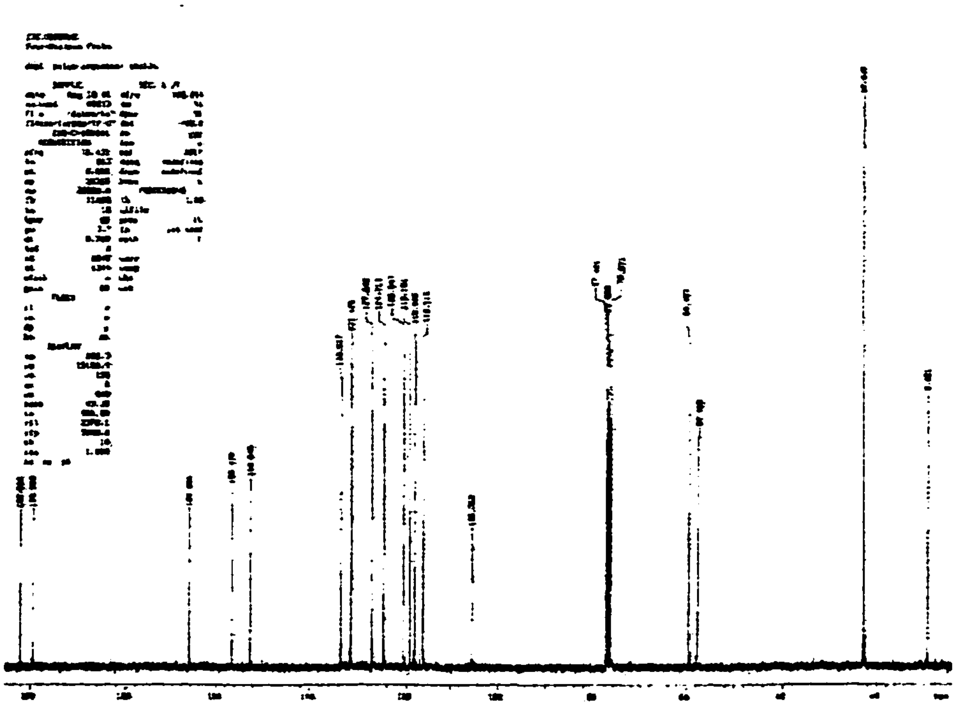


Figure 6-6. ^{13}C **NMR Spectrum of Dalrubone.**

	Experimental	*(Dreyer et al., 1975)*
2	166.3	166.4
3	116.1	116.0
4	133.5	133.7
5	119.2	119.1
6	124.7	124.7
7	127.2	127.2
8	131.4	131.4
9	152.5	152.5
10	118.0	117.8
1'	120.6	120.6
2'	202.0	201.7
3'	57.5	57.4
4'	199.4	199.4
5'	105.3	105.2
6'	156.5	156.6
7'	9.0	9.0
8'	23.0	22.9
9'	23.0	22.9
6'-OMe	55.9	59.4

75 MHz ^{13}C-NMR Data in $CDCl_3$ (Varian Unity 300 MHz)

Table 6-3. **^{13}C NMR Spectral Assignments (Experimental and Literature) of Dalrubone.**

6.4 Characterization of 5-Methoxydalrubone

The isolated compound was deep orange in color with an unpleasant odor. Contact with dissolved material resulted in skin stained orange/yellow. The compound was insoluble in hexane and water but soluble in medium-polarity solvents, such as acetone and ethyl acetate. Initial spectral studies suggested that the compound was 5-methoxydalrubone, a compound detected by Dreyer *et al.* (1975) in *Dalea* (*Psorothamnus*) *emoryi*, so comparisons were made between experimental spectra and published data to confirm its identity.

Infrared spectroscopy was conducted on a Shimadzu FTIR 8300 with data analysis using Hyper IR v1.57 software. Approximately 1 mg was pressed into a KBr pellet and scanned. Results are seen in Figure 6-7. The significant spectral peaks at 1618.2(s), 1560.3(s), 1521.7(s), and 1398.3(s) are similar to those of dalrubone. An additional major peak is seen at 1467.7(s), likely representing the methoxy-substitution of the aromatic A ring.

^{1}H NMR was performed on a Varian Unity 300 MHz NMR in $CDCl_3$ at 75.4 MHz (Figure 6-8). The proton spectra was nearly identical to the Dreyer (1974) data (Table 6-2) The additional methoxy group was visible at δ 3.94. The C-5 proton peak found in dalrubone was absent here due to the carbon now being quaternary. Noise in the spectrum is due to a contaminated NMR probe.

^{13}C NMR was also performed on a Varian Unity 300 MHz NMR in $CDCl_3$ (Figure 6-9; Table 6-5). No carbon data was available in the literature. As with the proton data, the addition of a methoxy group may be seen in the appearance of a peak at δ 59.50 (C-5-

OMe) and the movement of the C-5 signal downfield to δ 155.39. A DEPT experiment (Figure 6-10) showed the addition of the methoxy group and the loss of the CH (C-5) peak clearly. No CH_2 carbons were present, as expected.

With the identical proton data and logical changes to those of the carbon data, the compound is confidently established as 5-methoxydalrubone (III).

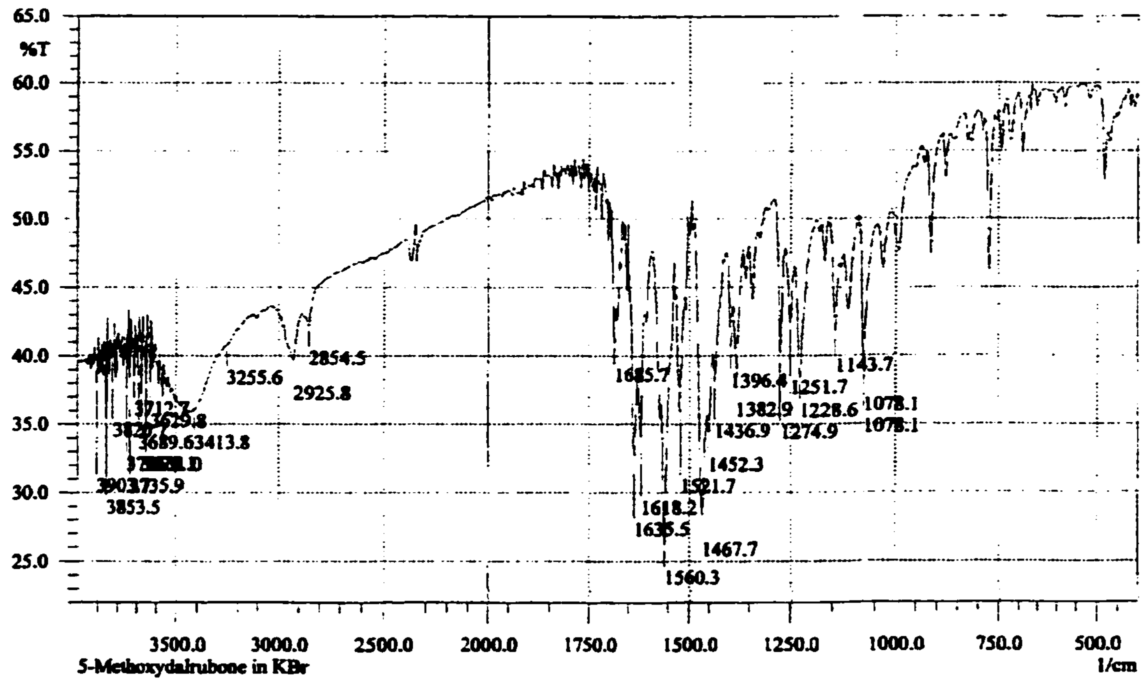


Figure 6-7 FTIR Spectrum of 5-Methoxydalrubone.

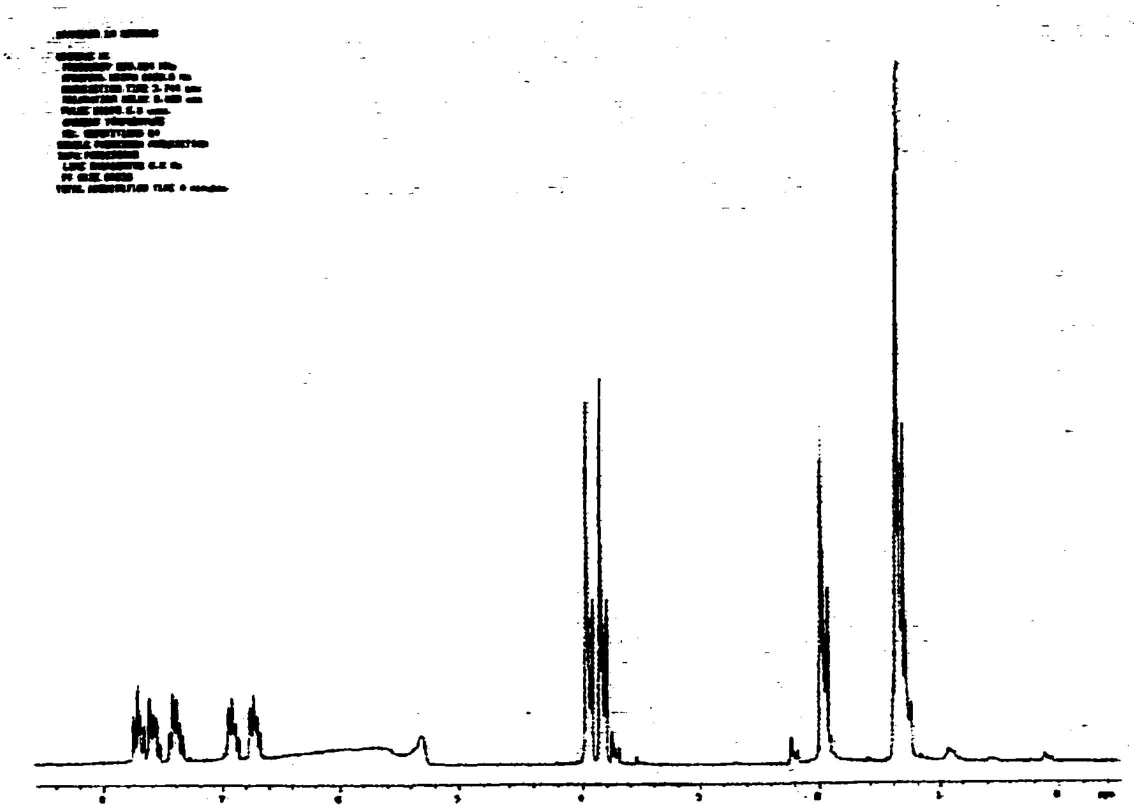
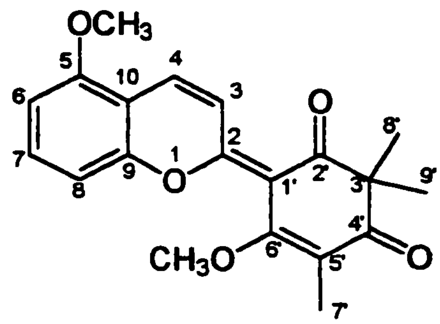


Figure 6-8 ^{1}H NMR Spectrum of 5-Methoxydalrubone.



	Experimental	*(Dreyer et al., 1975)*
3	7.71 d (J = 10)	7.71 d (J = 10)
4	7.61 d (J = 10)	7.65 d (J = 10)
6	6.92 d (J = 8)	6.87 d (J = 8)
7	7.38 m (J = 8)	7.38 t (J = 8)
8	6.73 d (J = 8)	6.69 d (J = 8)
5-OMe	3.96	3.94
7'	1.98	1.96
8'	1.38	1.38
9'	1.38	1.38
6'-OMe	3.85	3.83

300 MHz ^{1}H-NMR Data in $CDCl_3$ (Varian Unity)

Table 6-4 **^{1}H NMR Spectral Assignments (Experimental and Literature) of 5-Methoxydalrubone and Projected Structure.**

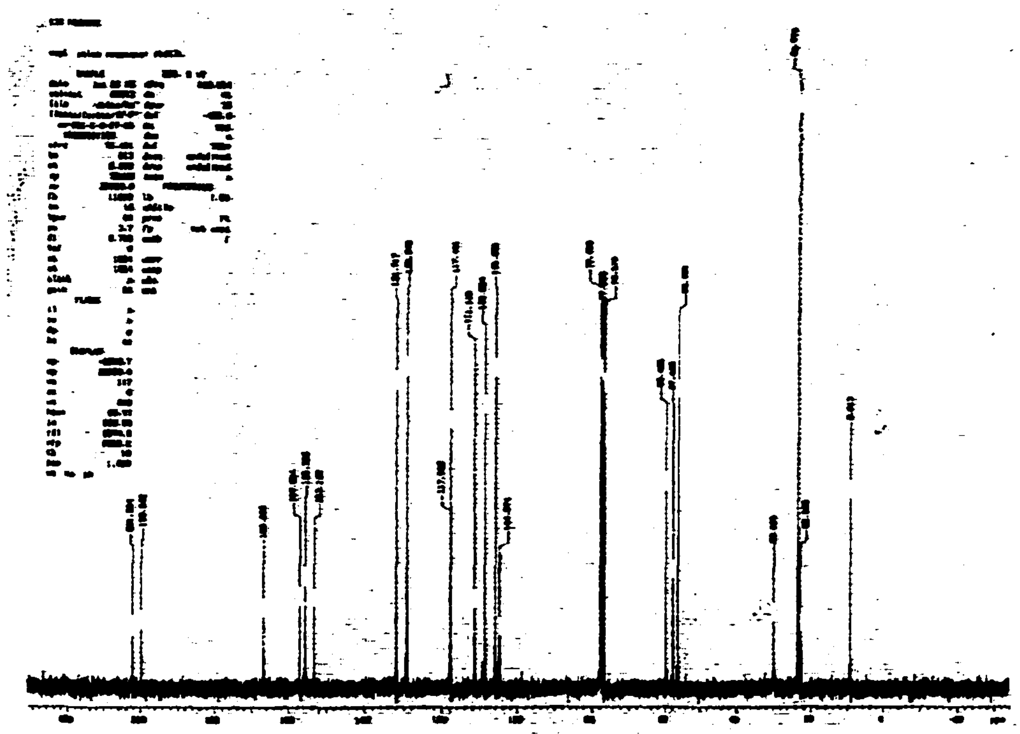


Figure 6-9 ^{13}C NMR Spectrum of 5-Methoxydalrubone.

	Experimental
2	157.0
3	117.5
4	128.9
5	155.4
6	105.6
7	131.7
8	108.5
9	153.2
10	111.2
5-OMe	59.5
1'	117.5
2'	199.5
3'	57.4
4'	202.0
5'	104.6
6'	166.6
7'	9.0
8'	23.07
9'	23.07
6'-OMe	56.0

75 MHz ^{13}C-NMR Data in $CDCl_3$
(Varian Unity 300 MHz)

Table 6-5 ^{13}C NMR Spectral Assignments of 5-Methoxyalrubone.

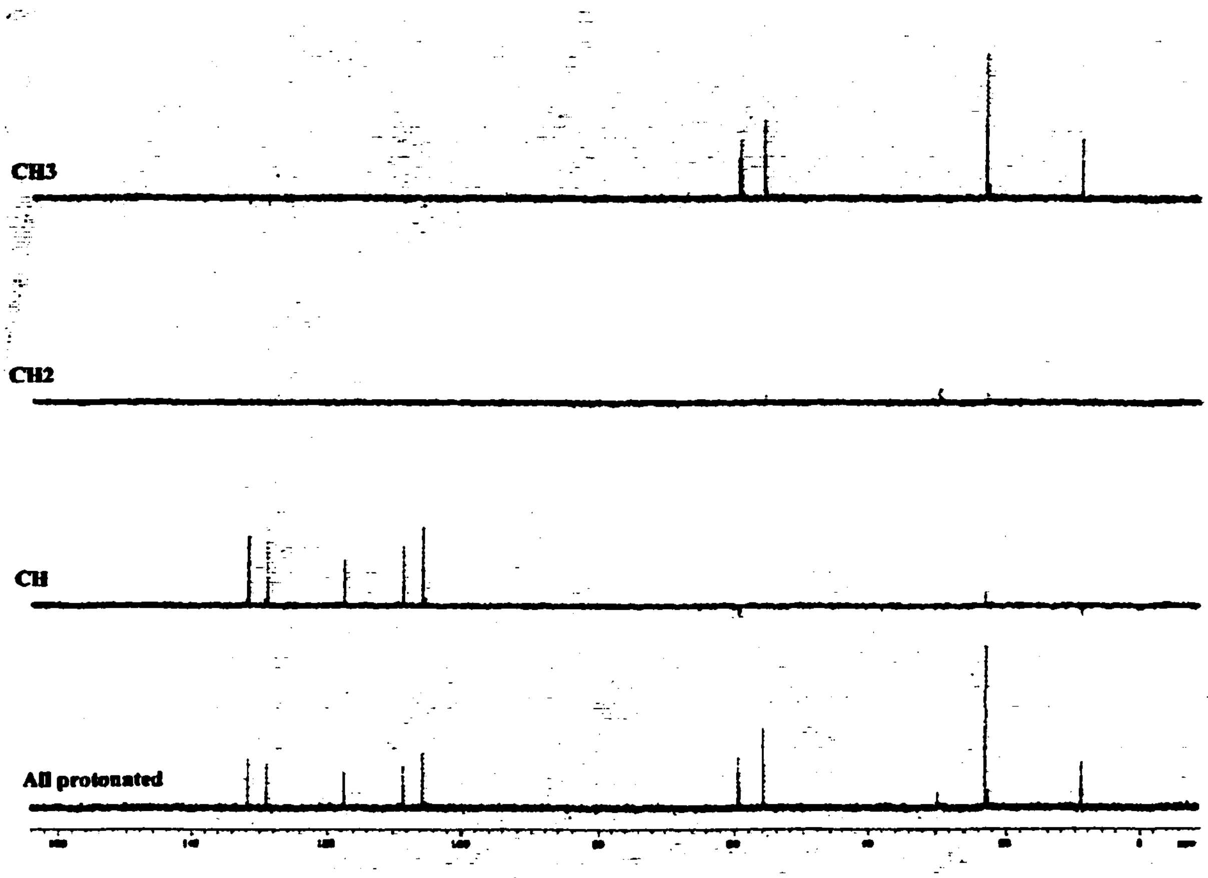
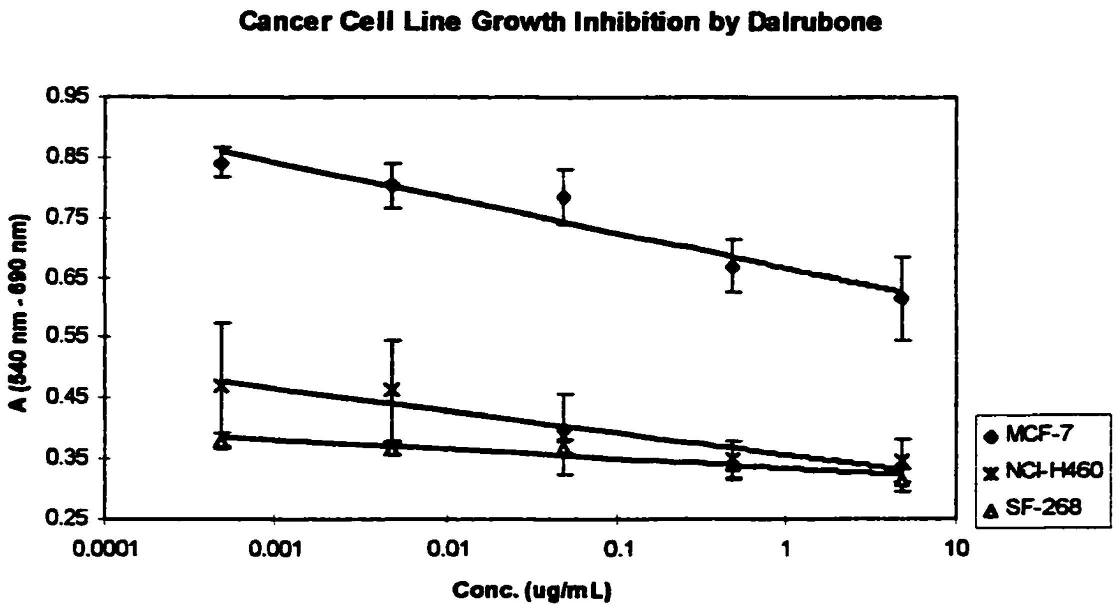


Figure 6-10 DEPT Analysis of 5-Methoxydalrubone

6.5 Assays

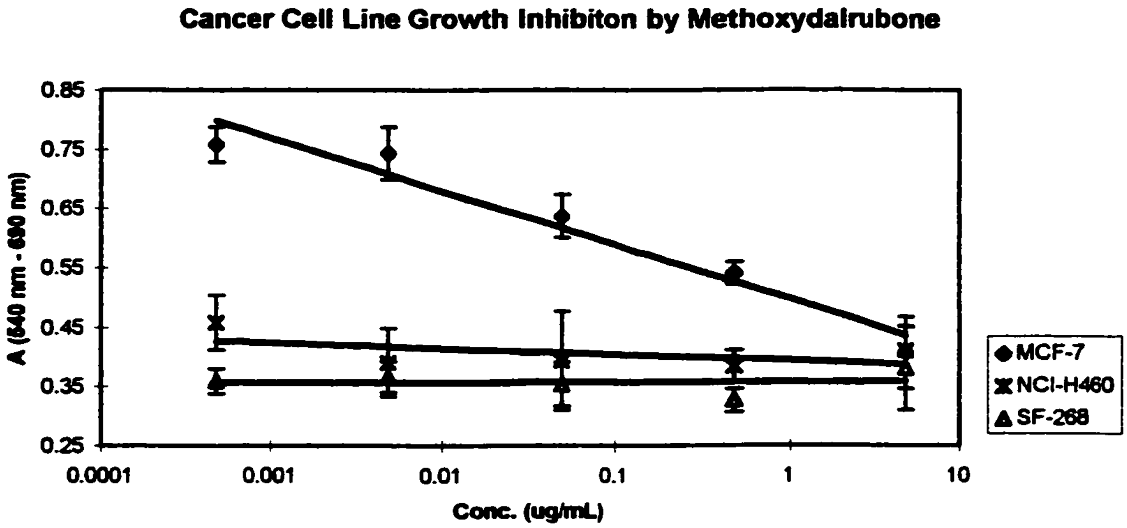
Dalrubone and WS1469S37 (a methoxydalrubone-containing fraction; Fig. 6-2.) were tested for a variety of activities. When suspended in nutrient agar at a concentration of 0.1 mg/mL and inoculated with *Escherichia coli*, *Agrobacterium tumefaciens* and *Staphylococcus aureus*, no anti-microbial activity was present at 48 hours. The lack of activity against *A. tumefaciens* is important, because were it active, the bacteria would not be present to produce the tumors on the potato disks. Inhibition of tumor growth would have been due to antibacterial activity. Both samples were inactive in the SKB mutant yeast assay. This proprietary assay detects compounds with the ability to either produce DNA damage and/or inhibit topoisomerase (I or II). Details of the yeast-based assay are found in the paper by Gunatilaka, Kingston and Johnson (1994) and the specific assay procedure is described in Chapter 7. The negative results of this assay also demonstrate the inability of the compounds to kill *Saccharomyces cerevisiae*. The planar structure of dalrubone required that it be tested for the ability to intercalate DNA. The assay used was the methyl green-DNA displacement assay (Sinicropi *et al.*, 1994). This method has been shown to be a safe and sensitive alternative to the ethidium bromide-DNA displacement assay. When methyl green is displaced from the DNA, it is converted to a colorless carbinol through the addition of water. Dalrubone (100 μg/mL) was exposed to a methyl green-DNA complex and the mixture was monitored with a microplate reader over a period of 4.5 hours. The known intercalating compound daunorubicin (30 μg/mL) was used as a control. Daunorubicin displaced 88% of the dye, dalrubone only 1 %. Therefore, dalrubone does not appear to intercalate to DNA.

Cancer cell line assays against MCF-7 and NCI-H460 point to a very minor cytotoxic activity with dalrubone, with IC_{50}s above 1.3 mM. 5-Methoxydalrubone appears to be breast cancer selective (28.2 μM) with fifty times the activity of dalrubone (Figure 6-11 and Figure 6-12). Neither compound had significant activity against SF-268 (CNS) cancer.



Dalrubone	(IC_{50})
MCF-7	1.3 mM
NCI-H460	1.8 mM
SF-268	NA

Figure 6-11 Cancer Cell Line Growth Inhibition by Dalrubone.



5-Methoxydalrubone	(IC_{50})
MCF-7	28.2 μM
NCI-H460	NA
SF-268	NA

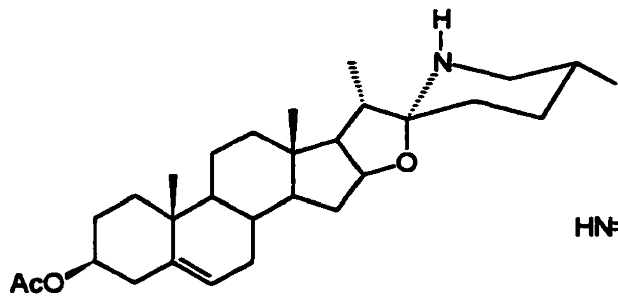
Figure 6-12 Cancer Cell Line Growth Inhibition by 5-Methoxydalrubone.

CHAPTER 7

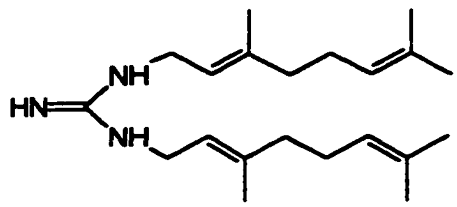
MUTANT *SACCHAROMYCES CEREVISIAE* ASSAY PRESCREEN

7.1 Mutant *Saccharomyces cerevisiae* Assay Background

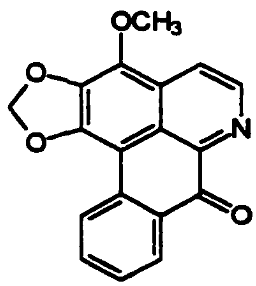
The yeast assay was developed by SmithKline Beecham Pharmaceuticals as an inexpensive method for detecting compounds that are potential DNA damaging agents and/or topoisomerase inhibitors. Specific details regarding the construction of their yeast mutants is proprietary but the general details follow (Nitiss and Wang, 1988). Typical yeast are fairly insensitive to DNA-interacting drugs and have the natural ability to keep these compounds from penetrating the cell membrane. A semi-dominant mutation (ISE2) was selected for that reduced membrane stability and made the yeast membranes 'leaky' to xenobiotics. An additional mutation was introduced that damaged the repair and recombination system of the yeast. This strain is known as *rad52*. The undamaged strain, the wild type, is termed *RAD+*. *Rad52* functions with reduced levels of topoisomerase II. This yeast is killed by high levels of topoisomerase I-inhibiting compounds. *Rad52* was further modified through the deletion of the topoisomerase I gene. These *top1-1* mutants (*RS321* strain) possess only low levels of topoisomerase II. *RS321* is used to detect topoisomerase II inhibitors. Numerous strains of each have been developed. These mutants react in specific and reproducible ways when exposed to topoisomerase drugs. Examples of compounds that have been isolated using the yeast assay are depicted in Figure 7-1. As seen, there are a wide variety of structural variations among these selectively active compounds. Each represents a family of structures isolated and tested with the screen. These include diterpene dibenzoates from *Caesalpinia pulcherrima* (Fabaceae) (Patil *et al.*, 1997), dioxoaporphine alkaloids from *Artabotrys zeylanicus* (Annonaceae) (Wijeratne *et al.*, 1995), guanidine alkaloids from *Pterogyne nitens* (Fabaceae) (Bolzani *et al.*, 1995), steroidal alkaloids from *Solanum umbelliferum* (Solanaceae) (Kim *et al.*, 1996), and rosane diterpenoids from *Vellozia candida* (Velloziaceae) (Valente *et al.*, 1997).



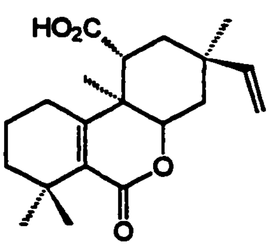
O-Acetylsolasodine
(*Solanum umbelliferum*)
toxic to rad52Y



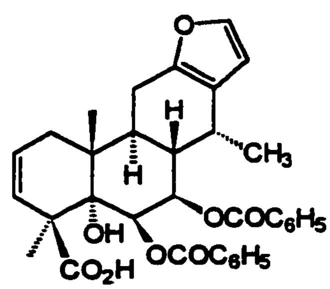
Nitensidine A
(*Pterogyne nitens*)
toxic to RS321N



Atherospermidine
(*Artabotrys zeylanicus*)
toxic to rad52Y



Candidalactone
(*Vellozia candida*)
toxic to rad52Y



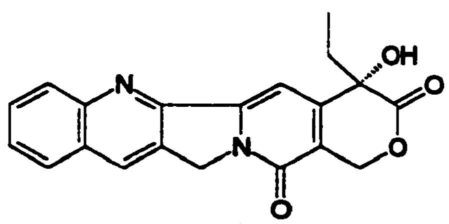
Pulcherrimin B
(*Caesalpinia pulcherrima*)
toxic to RS321N

Figure 7-1. Examples of Compounds Selectively Active in the Yeast Assay.

While this assay is described as mechanism-based, this not entirely true. It is safer to call this assay "semi-mechanistic" as this screen depends on a living organism that may deal with different xenobiotics in unique ways (metabolism, expulsion, non-specific binding, etc.). Extracts may also stimulate growth through the supplementation of the growth media with a variety of phytochemical nutrients. Growth inhibition of all strains is often seen due to an anti-fungal component in the plant extract. Lastly, but most importantly, there is the assay design's assumption that yeast topoisomerases share enough homology with human topoisomerases to react in an identical fashion to potential topoisomerase drugs.

7.2 Assay Procedure

The *RS321N*, *rad52Y*, and *RAD+* strains of *Saccharomyces cerevisiae* were obtained through kind donations to Dr. Leslie Gunatilaka from Dr. David G. I. Kingston (Virginia Polytechnic Institute and State University) and Dr. Randall K. Johnson (SmithKline Beecham Pharmaceuticals). Individual strains of yeast were seeded onto 1% nutrient agar plates. Samples were solublized in DMSO:MeOH (1:1) to a concentration of 2000 μg/mL and placed in agar wells (100 μL) made through the removal of 7 mm plugs from the media. Activity was measured as the zone (mm) surrounding the well where no yeast growth was visible. For active compounds, multiple concentrations were tested and the dose responsible for an inhibition zone of 12 mm was calculated (IC_{12}) through logarithmic regression analysis. Camptothecin (toxic to *rad52Y*) and streptonigrin (toxic to *RS321N*) (Figure 7-2) were used at 5 μg/mL as controls to verify that the yeast being tested behaves typical to the strain.



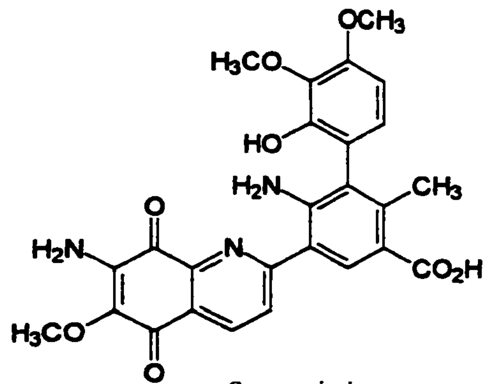


Figure 7-2. Mutant Yeast Assay Positive Control Compounds.

Hexane extracts were not typically tested due to their relative insolubility and a lack of interest in very non-polar compounds. Since differential activity between *Rad52Y* and *RS321N* is of more interest than general antifungal or otherwise cytotoxic activity, extracts were tested against each of these two strains. Differential activity was followed up with the calculation of the IC_{12} and RAD+ testing.

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Cocculus diversifolis	LG-6-7-1	X	X	X
	LG-6-7-2	X	I	I
	LG-6-7-3	X	I	I
Rhus virens	LG-6-8-1	X	X	X
	LG-6-8-2	X	I	I
	LG-6-8-3	I	[8000]	[4490]
Bidens leptocephala	LG-6-9-1	X	X	X
	LG-6-9-2	X	I	I
	LG-6-9-3	X	I	I
Gossypium thurberi	LG-6-10-1	X	X	X
	LG-6-10-2	X	I	I
	LG-6-10-3	X	I	I
Eriogonum abertianum	LG-6-11-1	X	X	X
	LG-6-11-2	X	7mm*	7mm*
	LG-6-11-3	X	I	I
Viguiera dentata	LG-6-12-1	X	X	X
	LG-6-12-2	X	I	I
	LG-6-12-3	X	I	I
Tetramerium nervosum	LG-6-13-1	X	X	X
	LG-6-13-2	X	I	I
	LG-6-13-3	X	I	I
Fraxinus velutina	LG-6-14-1	X	X	X
	LG-6-14-2	X	I	I
	LG-6-14-3	X	I	I
Datura wrightii	LG-6-15-1	X	X	X
	LG-6-15-2	X	I	I
	LG-6-15-3	X	I	I
Maurandya antirrhiniflora	LG-6-16-1	X	X	X
	LG-6-16-2	X	I	I
	LG-6-16-3	X	I	8mm*
Rivina humilis	LG-6-17-1	X	X	X
	LG-6-17-2	X	I	I
	LG-6-17-3	X	I	I
Poinsettia heterophylla	LG-6-18-1	X	X	X
	LG-6-18-2	X	I	I
	LG-6-18-3	X	I	I
Epilobium canum	LG-6-19-1	X	X	X
	LG-6-19-2	X	I	I
	LG-6-19-3	10mm***	16mm***	17mm***

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results.

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Boerhavia coccinea	LG-6-20-1	X	X	X
	LG-6-20-2	X	I	I
	LG-6-20-3	X	I	I
Plumbago scandens	LG-6-21-1	X	X	X
	LG-6-21-2	15mm***	15mm***	15mm***
	LG-6-21-3	13mm***	10mm***	13mm***
Crotalaria pumila	LG-6-22-1	X	X	X
	LG-6-22-2	X	I	I
	LG-6-22-3	X	I	I
Phaseolus acutifolius	LG-6-23-1	X	X	X
	LG-6-23-2	X	I	I
	LG-6-23-3	X	I	I
Aralia humilis	LG-6-24-1	X	X	X
	LG-6-24-2	X	I	8mm*
	LG-6-24-3	X	[8000]	[16,000]
Heuchera sanguinea	LG-6-25-1	X	X	X
	LG-6-25-2	X	I	I
	LG-6-25-3	I	I	[8680]
Melampodium longicorne	LG-6-26-1	X	X	X
	LG-6-26-2	X	I	I
	LG-6-26-3	X	I	I
Solidago velutina	LG-6-27-1	X	X	X
	LG-6-27-2	X	I	I
	LG-6-27-3	X	I	I
Parthenice mollis	LG-6-28-1	X	X	X
	LG-6-28-2	X	I	[4594]
	LG-6-28-3	X	I	15mm**
Acourtia thurberi	LG-6-29-1	I	I	I
	LG-6-29-2	I	I	[4708]
	LG-6-29-3	I	I	I
Heliopsis parvifolia	LG-6-30-1	X	X	X
	LG-6-30-2	X	7mm*	9mm*
	LG-6-30-3	X	I	I
Dalea albiflora	LG-6-31-1	X	X	X
	LG-6-31-2	X	I	I
	LG-6-31-3	X	I	I
Guardiola platyphylla	LG-6-32-1	X	X	X
	LG-6-32-2	[8000]	[6730]	[850]
	LG-6-32-3	X	I	11mm*

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Ipomopsis thurberi	LG-6-33-1	X	X	X
	LG-6-33-2	X	I	I
	LG-6-33-3	X	17mm	17mm
Desmodium cinerascens	LG-6-34-1	X	X	X
	LG-6-34-2	X	I	I
	LG-6-34-3	[2060]	[840]	[1000]
Lotus oroboides	LG-6-35-1	X	X	X
	LG-6-35-2	X	I	I
	LG-6-35-3	X	I	I
Macroptilium gibbosifolium	LG-6-36-1	X	X	X
	LG-6-36-2	X	I	I
	LG-6-36-3	X	I	I
Sambucus meXicana	LG-6-37-1	X	X	X
	LG-6-37-2	X	I	I
	LG-6-37-3	X	I	I
Viguiera dentata	LG-6-38-1	X	X	X
	LG-6-38-2	X	I	I
	LG-6-38-3	X	I	I
Stachys coccinea	LG-6-39-1	X	X	X
	LG-6-39-2	X	I	I
	LG-6-39-3	X	I	I
Artemisia ludoviciana	LG-6-40-1	X	X	X
	LG-6-40-2	X	I	8mm*
	LG-6-40-3	X	I	I
Hedeoma dentatum	LG-6-41-1	X	X	X
	LG-6-41-2	X	I	I
	LG-6-41-3	X	I	I
Gnaphalium canescens	LG-6-42-1	X	X	X
	LG-6-42-2	I	[5440]	[5660]
	LG-6-42-3	X	I	I
Mirabilis longiflora	LG-6-43-1	X	X	X
	LG-6-43-2	X	I	I
	LG-6-43-3	X	I	I
Carminatia tenuiflora	LG-6-44-1	X	X	X
	LG-6-44-2	X	I	I
	LG-6-44-3	X	I	I
Heterosperma pinnatum	LG-6-45-1	X	X	X
	LG-6-45-2	16mm***	31mm***	43mm***
	LG-6-45-3	21mm***	10mm***	19mm***

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Stevia micrantha	LG-6-46-1	X	X	X
	LG-6-46-2	X	I	I
	LG-6-46-3	X	I	I
Monarda citriodora	LG-6-47-1	X	X	X
	LG-6-47-2	X	7mm*	I
	LG-6-47-3	X	I	I
Phoradendron californicum 1	LG-6-49-1	X	X	X
	LG-6-49-2	X	I	I
	LG-6-49-3	X	I	I
Phoradendron californicum 2	LG-6-50-1	X	X	X
	LG-6-50-2	X	I	I
	LG-6-50-3	X	I	I
Froelichia arizonica	LG-6-53-1	X	X	X
	LG-6-53-2	X	I	I
	LG-6-53-3	X	I	I
Chenopodium neomexicanum	LG-6-54-1	X	X	X
	LG-6-54-2	X	I	I
	LG-6-54-3	X	I	I
Laennecia sophiifolia	LG-6-55-1	X	X	X
	LG-6-55-2	X	I	I
	LG-6-55-3	11mm***	10mm***	9mm***
Cyperus mutisi	LG-6-56-1	X	X	X
	LG-6-56-2	X	I	I
	LG-6-56-3	X	I	I
Schoencrambe linearifolia	LG-6-57-1	X	X	X
	LG-6-57-2	X	I	I
	LG-6-57-3	X	I	I
Gaura hexandra	LG-6-58-1	X	X	X
	LG-6-58-2	X	I	I
	LG-6-58-3	X	I	I
Juniperus deppeana	LG-6-59-1	X	X	X
	LG-6-59-2	X	I	8mm*
	LG-6-59-3	X	I	I
Calyophus toumeyi	LG-6-60-1	X	X	X
	LG-6-60-2	X	I	14mm**
	LG-6-60-3	X	I	[1898]
Brickellia californica	LG-6-61-1	X	X	X
	LG-6-61-2	X	9mm*	I
	LG-6-61-3	X	I	I

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Ageratina herbacea	LG-6-62-1	X	X	X
	LG-6-62-2	X	8mm*	I
	LG-6-62-3	X	I	I
Galium wrightii	LG-6-63-1	X	X	X
	LG-6-63-2	X	I	I
	LG-6-63-3	X	I	I
Erigeron platyphyllus	LG-6-64-1	X	X	X
	LG-6-64-2	X	I	I
	LG-6-64-3	X	I	I
Pteridium aquilinum	LG-6-65-1	X	X	X
	LG-6-65-2	X	I	I
	LG-6-65-3	X	I	I
Geranium caespitosum	LG-6-66-1	X	X	X
	LG-6-66-2	X	I	I
	LG-6-66-3	X	I	I
Castelleja patriotica	LG-6-67-1	X	X	X
	LG-6-67-2	I	I	11mm*
	LG-6-67-3	X	I	I
Gutierrezia wrightii	LG-6-68-1	X	X	X
	LG-6-68-2	X	I	I
	LG-6-68-3	X	I	I
Stevia plummerae	LG-6-69-1	X	X	X
	LG-6-69-2	X	I	I
	LG-6-69-3	X	I	I
Hymenoxys quinquesquamata	LG-6-70-1	X	X	X
	LG-6-70-2	X	I	I
	LG-6-70-3	X	I	I
Eryngium lemmonii	LG-6-71-1	X	X	X
	LG-6-71-2	X	I	I
	LG-6-71-3	X	I	I
Helianthella quinquenervis	LG-6-72-1	X	X	X
	LG-6-72-2	X	9mm*	I
	LG-6-72-3	X	I	I
Gentianella microcalyx	LG-6-73-1	X	X	X
	LG-6-73-2	X	I	I
	LG-6-73-3	X	I	I
Arctostaphylos pungens	LG-6-74-1	X	X	X
	LG-6-74-2	X	I	I
	LG-6-74-3	X	I	I

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Solidago wrightii	LG-6-75-1	X	X	X
	LG-6-75-2	X	I	I
	LG-6-75-3	X	I	I
Gnaphalium viscosum	LG-6-76-1	X	X	X
	LG-6-76-2	X	I	I
	LG-6-76-3	X	I	I
Silene laciniata	LG-6-77-1	X	X	X
	LG-6-77-2	X	I	I
	LG-6-77-3	X	I	I
Hedeoma hyssopifolia	LG-6-78-1	X	X	X
	LG-6-78-2	X	I	I
	LG-6-78-3	X	I	I
Centaurea rothrockii	LG-6-79-1	X	X	X
	LG-6-79-2	X	I	I
	LG-6-79-3	X	I	I
Bahia dissecta	LG-6-80-1	X	X	X
	LG-6-80-2	X	I	I
	LG-6-80-3	X	I	I
Acalypha lindheimeri	LG-6-81-1	X	X	X
	LG-6-81-2	X	I	I
	LG-6-81-3	I	10mm*	10mm*
Gutierrezia sarothrae	LG-6-82-1	X	X	X
	LG-6-82-2	X	I	I
	LG-6-82-3	I	10mm*	I
Lonicera albiflora	LG-6-83-1	X	X	X
	LG-6-83-2	X	I	I
	LG-6-83-3	X	I	I
Sphaeralcea fendleri	LG-6-84-1	X	X	X
	LG-6-84-2	X	I	I
	LG-6-84-3	X	I	I
Cosmos parviflorus	LG-6-85-1	X	X	X
	LG-6-85-2	X	I	8mm*
	LG-6-85-3	X	I	I
Monnima wrightii	LG-6-86-1	X	X	X
	LG-6-86-2	X	I	I
	LG-6-86-3	X	I	I
Cuphea wrightii	LG-6-87-1	X	X	X
	LG-6-87-2	X	I	6mm*
	LG-6-87-3	X	I	I

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Salvia lemmonii	LG-6-88-1	X	X	X
	LG-6-88-2	X	9mm*	9mm*
	LG-6-88-3	X	I	I
Valeriana sorbifolia	LG-6-89-1	X	X	X
	LG-6-89-2	X	I	I
	LG-6-89-3	X	I	I
Xanthocephalum gymnospermoides	LG-6-90-1	X	X	X
	LG-6-90-2	X	I	I
	LG-6-90-3	X	I	I
Vicia pulchella	LG-6-91-1	X	X	X
	LG-6-91-2	X	I	I
	LG-6-91-3	X	I	I
Gaillardia pinnatifida	LG-6-92-1	X	X	X
	LG-6-92-2	X	I	9mm*
	LG-6-92-3	X	I	I
Delphinium andesicola	LG-6-93-1	X	X	X
	LG-6-93-2	X	I	I
	LG-6-93-3	X	I	I
Gnaphalium leucocephalum	LG-6-94-1	X	X	X
	LG-6-94-2	X	7mm*	9mm*
	LG-6-94-3	X	I	I
Heterotheca subaxillaris	LG-6-95-1	X	X	X
	LG-6-95-2	X	14mm	11mm*
	LG-6-95-3	X	I	I
Marabilis multiflora	LG-6-98-1	X	X	X
	LG-6-98-2	X	I	I
	LG-6-98-3	X	I	I
Eriogonum polycladon	LG-6-99-1	X	X	X
	LG-6-99-2	X	X	10mm*
	LG-6-99-3	X	X	I
Tecoma stans	LG-6-100-1	X	X	X
	LG-6-100-2	X	I	I
	LG-6-100-3	X	I	I
Aloysia wrightii	LG-6-101-1	X	X	X
	LG-6-101-2	X	I	10mm*
	LG-6-101-3	X	I	I
Astrolepis sinuata	LG-6-102-1	X	X	X
	LG-6-102-2	X	I	I
	LG-6-102-3	X	I	I

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Cordylanthus wrightii	LG-6-103-1	X	X	X
	LG-6-103-2	X	I	I
	LG-6-103-3	X	I	I
Garrya wrightii	LG-6-104-1	X	X	X
	LG-6-104-2	X	6mm*	I
	LG-6-104-3	X	I	I
Psoralidium tenuiflorum	LG-6-105-1	X	X	X
	LG-6-105-2	X	I	I
	LG-6-105-3	X	I	I
Dicliptera resupinata	LG-6-106-1	X	X	X
	LG-6-106-2	X	I	I
	LG-6-106-3	X	I	I
Machaeranthera canescens	LG-6-107-1	X	X	X
	LG-6-107-2	X	I	I
	LG-6-107-3	X	I	I
Acalypha neomexicana	LG-6-108-1	X	X	X
	LG-6-108-2	X	I	I
	LG-6-108-3	X	I	I
Tagetes lemmonii	LG-6-109-1	X	X	X
	LG-6-109-2	X	I	I
	LG-6-109-3	X	I	I
Monarda fistulosa	LG-6-110-1	X	X	X
	LG-6-110-2	X	I	I
	LG-6-110-3	X	I	I
Rhamnus californica	LG-6-111-1	X	X	X
	LG-6-111-2	X	9mm*	I
	LG-6-111-3	X	I	I
Amorpha fruticosa	LG-6-112-1	X	X	X
	LG-6-112-2	X	I	I
	LG-6-112-3	X	I	I
Chamaecrista nictitans	LG-6-113-1	X	X	X
	LG-6-113-2	X	I	I
	LG-6-113-3	X	I	I
Gymnosperma glutinosum	LG-6-114-1	X	X	X
	LG-6-114-2	X	I	I
	LG-6-114-3	X	I	I
Phoradendron villosum	LG-6-115-1	X	X	X
	LG-6-115-2	X	I	I
	LG-6-115-3	X	I	I

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Quercus oblongifolia	LG-6-116-1	X	X	X
	LG-6-116-2	X	I	I
	LG-6-116-3	X	I	9mm*
Hymenoxys wrightii	LG-6-117-1	X	X	X
	LG-6-117-2	X	I	I
	LG-6-117-3	X	I	I
Artemisia dracunculus	LG-6-118-1	X	X	X
	LG-6-118-2	[312]	[1140]	I
	LG-6-118-3	X	11mm*	10mm*
Lupinus palmeri	LG-6-119-1	X	X	X
	LG-6-119-2	X	I	I
	LG-6-119-3	X	I	I
Thalictrum fendleri	LG-6-120-1	X	X	X
	LG-6-120-2	X	I	I
	LG-6-120-3	X	I	I
Brickellia grandifolia	LG-6-121-1	X	X	X
	LG-6-121-2	X	I	I
	LG-6-121-3	X	I	I
Scrophularia parviflora	LG-6-122-1	X	X	X
	LG-6-122-2	X	I	I
	LG-6-122-3	X	I	I
Senecio bigelovia	LG-6-123-1	X	X	X
	LG-6-123-2	X	I	I
	LG-6-123-3	X	I	I
Silene scouleri	LG-6-124-1	X	X	X
	LG-6-124-2	X	I	I
	LG-6-124-3	X	I	I
Erigeron arizonicus	LG-6-125-1	X	X	X
	LG-6-125-2	X	I	I
	LG-6-125-3	X	I	8mm*
Cyperus fendlerianus	LG-6-126-1	X	X	X
	LG-6-126-2	X	I	I
	LG-6-126-3	X	I	I
Lathryus gramnifolius	LG-6-127-1	X	X	X
	LG-6-127-2	X	I	I
	LG-6-127-3	X	I	I
Laennecia schiedeana	LG-6-128-1	X	X	X
	LG-6-128-2	X	I	I
	LG-6-128-3	X	I	I

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

Species	Extract Code[a]	*RAD+*[b]	*Rad52Y*[b]	*RS321N*[b]
Phoradendron juniperinum	LG-6-129-1	X	X	X
	LG-6-129-2	X	I	I
	LG-6-129-3	X	I	I

[a]Extract Code: LG-6-xxx-1= hexane extract
LG-6-xxx-2 = MEK extract
LG-6-xxx-3 = MeOH extract

[b]*Rad+,Rad52Y,RS321N*: I = inactive (no activity shown in this assay)
X = assay not performed
[xxxx] = ug/mL for IC_{12}
* = zone under 12mm, IC_{12} not determined
** = re-run but no activity found
*** = IC_{12} not determined due to probable anti-fungal activity

Table 7-1. Mutant *Saccharomyces cerevisiae* Assay Results. - continued

7.3 Discussion of Mutant *Saccharomyces cerevisiae* Assay Results

An extract was considered active provided it met several conditions. The initial screen needed to provide a zone larger than 12 mm. The IC_{12}, when calculated, should be 8000 μg/mL or below. Selectivity depends upon the IC_{12} of one or two strains being less than a third the IC_{12} value of the other strain(s). If the growth of both *RS321N* and *Rad52Y* are inhibited and the *RAD+* strain is unaffected, then the activity is defined as being topoisomerase nonspecific; it acts by either blocking both forms of topoisomerase or through some other undefined mode of action. When all three strains are killed, then the extract is defined as being anti-fungal or a general cytotoxic.

As an assay, the screen is fairly selective (Table 7-2). Of the four categories of selectivity an extract may be placed in, no category represented more than 5% of the total plants. Interestingly, no plant had two active extracts in more than one category. Nearly half of the plants in Table 7-2 were from the family Asteraceae. Nothing is particularly significant about this when one considers the large representation (36 %) of plants from this family in the collection. Eight of the plants (9 extracts) were in the antifungal/cytotoxic and topo nonspecific categories and were therefore not considered for further fractionation. *Acourtia thurberi* was selected for bioassay-guided fractionation due to it having strain specific activity. No activity at all was seen in the *RAD+* and *Rad52Y* yeasts, which was not true for the remaining three plants in this category. *Acourtia thurberi* also has an ethnobotanical history in the Southwest and Mexico.

Possible Activity	Active Extracts
Antifungal/Cytotoxic	*Artemisia dracunculus* (Asteraceae) MEK *Epilobium canum* (Onagraceae) MeOH *Heterosperma pinnatum* (Asteraceae) MEK, MeOH *Heterotheca subaxillaris* (Asteraceae) MEK *Ipomopsis thurberi* (Polemonaceae) MeOH *Plumbago scandens* (Plumbaginaceae) MeOH
Topoisomerase Nonspecific/ Unknown	*Rhus virens* (Anacardiaceae) MeOH *Gnaphalium canescens* (Asteraceae) MEK
Topoisomerase II Inhibition	*Acourtia thurberi* (Asteraceae) MEK *Calyophus toumeyi* (Onagraceae) MeOH *Guardiola platyphylla* (Asteraceae) MEK *Parthenice mollis* (Asteraceae) MEK

Table 7-2. Summary of Active Plant Extracts and Their Possible Activities.

CHAPTER 8

ISOLATION OF 14,15-DIACETOXY-, 8-HYDROXY-, 3-(3-METHYLBUTANOYL)-14,15-EPOXY-ISOCEDRENE FROM *ACOURTIA THURBERI*

8.1 Introduction

Acourtia thurberi (Gray) Reveal & King (Asteraceae) is a New World plant belonging to the Subtribe Naussauviinae in the Tribe Mutisieae. This genus has approximately forty species. *A. thurberi* and numerous other *Acourtia* species had been previously classified as members of the genus *Perezia* and are often referred to in the literature this way. *Acourtia thurberi* is also known as Thurber's desert peony, yerba de la tarantula, mata gusano, pipichawi, and matarique (Linares and Bye, 1987). This plant ranges from southern Arizona and New Mexico to northern Mexico.

A. thurberi has a history of medicinal usage. Part of a group of plants collectively known as 'matarique' (Linares and Bye, 1987), *Acourtia thurberi* along with *Psacalium sinuatum*, *P. decompositum*, and *P. peltatum* are each used to treat similar ailments. The plants are used to treat diabetes, urinary tract and kidney problems, and sexual ailments such as penile dysfunction and menstrual flow irregularities (Gentry, 1942). *A. thurberi* has been tested in mice and rabbit systems to determine its level of anti-diabetic effect. Treatment with *A. thurberi* was found to be ineffective at reducing blood glucose levels

(Alarcon-Aguilar *et al.*, 1997). *A. thurberi* extracts have been tested and found active against tuberculosis and certain cancer cell lines (J.J Hoffmann, unpublished data.)

Phytochemically, *Acourtia* is a poorly studied genus. Most research has concentrated on perezone, a sesquiterpene quinone with dye and antimicrobial potential (Arellano *et al.*, 1996) and a class of compounds called isocedrenes. An isocedrene is a tetracyclic sesquiterpene likely formed from a perezone precursor. The isocedrenes have no reported biological activity and research on these compounds was conducted in the laboratory of Dr. Ferdinand Bohlmann (Germany) mainly from a structural and chemotaxonomic point of view (Bohlmann *et al.*, 1991; Bohlmann and Zdero, 1979). Cedrenes, a structurally related and more widespread class of compounds, have reportedly demonstrated anti-feedant activities (Wibe *et al.*, 1997.)

8.2 Modification of the Yeast Assay for Bioassay-guided Fractionation.

The yeast assay usually requires 48 hours to provide definitive results and can therefore be very time consuming. With a typical column providing numerous fractions, the time and effort required for drying each fraction and for preparing each sample for assay can be large. A system was developed by the author that accelerates the time required to obtain information regarding fraction activity. Fractions (5 μL) are spotted onto an analytical TLC plate (aluminum-backed) in a long row and labeled with pencil. This strip is inserted perpendicularly into an inoculated yeast plate until each of the spots is half submerged in agar. This plate is covered and incubated overnight at 32°C. Zones of inhibition form around each spot possessing activity. This system may also be applied to samples separated within a TLC system. Two lanes of the same sample are run in the separatory solvent of choice. When complete, the plate is cut down the middle and one lane is sprayed with vanillin for identification purposes while the other is implanted. In this way, the specific band with activity may be localized and targeted for separation. This bioautography accelerates the isolation and identification process significantly.

8.3 Extraction and Isolation

Above ground biomass, weighing 2.6 kilograms (dried), was separated into leaf and stem material (2201.7 g) and seed pods and pale purple flowers (403.6 g). Seeds were retained for possible future study. The dried, ground leaves and stems were sequentially extracted as described in Section 2.2 with four 24 hour washes of hexane, MEK and methanol. The crude extracts weighed 76.1 g, 75.0 g, and 36.1 g respectively. Each extract was tested in the mutant yeast assay as described in Section 7.2. Bioassay-guided fractionation proceeded using the active MEK extract (*RS321N* IC_{12} = 8000 μg/mL) (Figure 8-1). This large-scale extraction produced an extract that, while still selective, was less active than the previous small-scale extraction (Table 7-1). The loss of activity is possibly due to either incomplete extraction of the biomass or compound instability. The MEK fraction was partitioned with hexane (2 L) and 80% methanol in H_2O (2 L). The majority of the biomass migrated to the hexane phase (49.5 g) but the activity was found in the 80%. MeOH fraction (25.5 g). The compound appeared to be unstable because while all activity moved to the aqueous phase, activity had not been significantly concentrated (IC_{12} = 7436 μg/mL). The aqueous methanol fraction was diluted with water to 60% methanol and partitioned with an equal volume of chloroform. All activity and most mass moved to the chloroform (24.4g ; IC_{12} = 6297 μg/mL). This material had its solvent removed *in vacuo* through rotary evaporation and was stored at 4°C until used.

A single gram of the chloroform extract was bound to 4 g of 40 μm SiO_2 and layered onto the top of a DCM equilibrated 50 g (40 μm SiO_2 ; 1.9 cm x 40 cm) flash column. Elution began with 150 mL of 99:1 (DCM:MeOH) and was stepped to 99.5:0.5 (DCM:MeOH). The active fraction (all fractions were spot tested for activity) eluted after 80 mL. The column was repeated and similar fractions by TLC were pooled. An aliquot (6 mg) was retained for future assays and the remaining material (330 mg) was bound to 1.32 g of silica and run on the same column, but with eluted with 100% dichloromethane. The active fraction (130 mg) had an IC_{12} of 2618 μg/mL.

Five NP-PTLC plates were streaked with the active fraction and run in hexane:ethyl acetate (50:50). The primary band (25 mg; quenches black at 254 nm; colors visibly bright red-orange when sprayed with vanillin reagent Rf ~0.6) was removed, extracted (with a sintered glass funnel), and applied to a single RP-PTLC plate and developed with an 80:20 (methanol:water) solvent system. The resulting compound was 17 mg of a pale yellow oil and had an IC_{12} of 340 μg/mL. This material was solublized in $CDCl_3$ and analyzed with NMR.

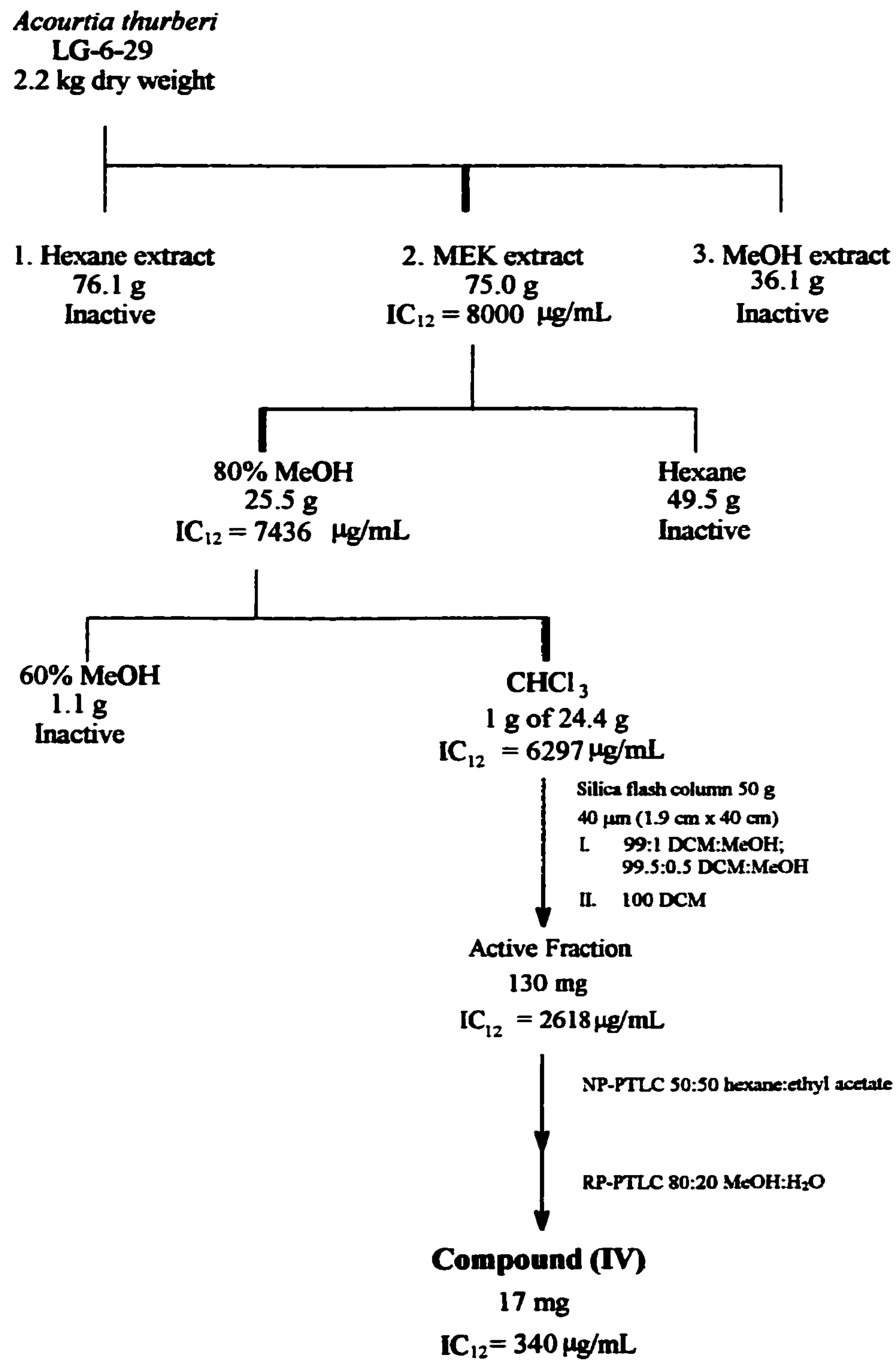


Figure 8-1. Isolation Scheme *for Acourtia thurberi.*

8.4 Characterization of 14,15-Diacetoxy-, 8-Hydroxy-, 3-(3-Methylbutanoyl)-14,15-Epoxy-Isocedrene (IV)

The isolated compound was a pale, yellow oil. The compound was insoluble in water. The material was easily soluble in methanol, acetone, and chloroform.

^{13}C NMR was performed on a Varian Unity 300 NMR at 75 MHz and at a concentration of 15 mg/mL. The spectrum is seen in Figure 8-2. Three carbonyls are clustered downfield at δ 172.7, δ 170.3 and δ 169.6. Two peaks appear to be alkene-bonded carbons at δ 141.1 and δ 120.6. Four additional carbons at δ 75.8, δ 76.1, δ 87.4, and δ 90.9 fall in the region indicative of carbons bound to oxygen atoms. These assignments alone increase the probability that the compound is, in fact, an isocedrene. DEPT analysis was conducted to determine the types of carbon in this system (Figure 8-3). The downfield alkene carbon is quaternary while the other alkene carbon is attached to a single proton. There are six quaternary carbons. If three are carbonyls, then the skeleton must hold the remaining three. The isocedrenes ring system typically has three quaternary carbons. An isocedrene also has two methyl groups. If true, IV must have four additional methyl groups. Acetyl groups have been reported as substituents of isocedrenes. The isocedrene skeleton has fifteen carbons while this *A. thurberi* compound has a total of twenty-six carbons. The remaining nine carbons could be explained by two acetyls and a C_5 unit. An isocedrene, 14α,15β-diacetoxy-, 8β-hydroxy-, 3α-angeloyl-, 14β,15α-epoxy-α-isocedrene isolated from *Prustia pyrifolia* (Bohlmann and Zdero, 1979) is one of few natural isocedrenes with a ^{13}C NMR spectrum reported, so the two are compared in

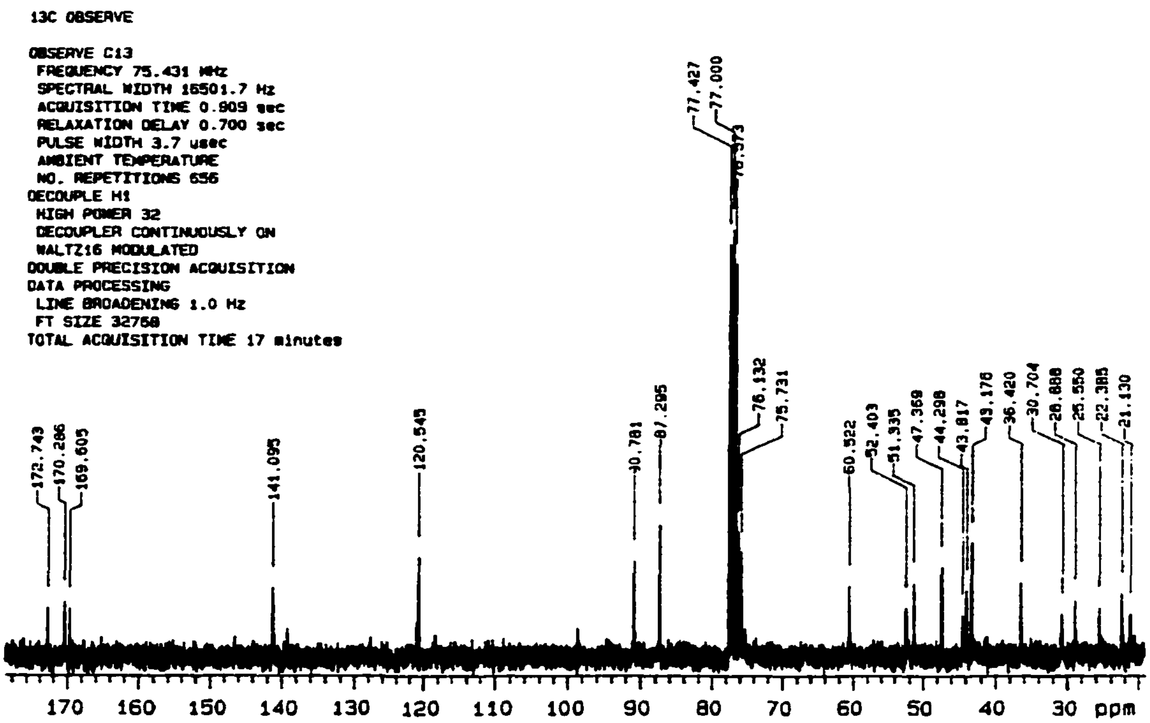


Figure 8-2. ^{13}**C NMR Spectrum of *A. thurberi* Isocedrene.**

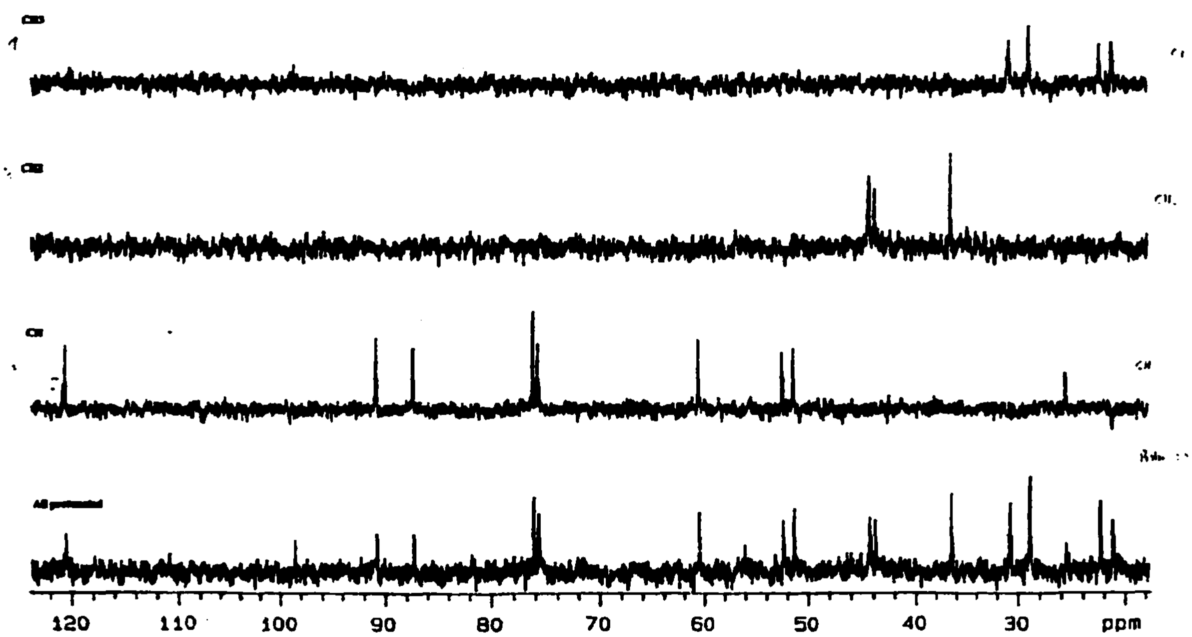


Figure 8-3. **DEPT Spectrum of *A. thurberi* Isocedrene.**

Proustia pyrifolia isocedrene (Bohlmann and Zdero 1979)*	δ	Type of Carbon by DEPT	*A. thurberi* isocedene**	δ	Type of Carbon by DEPT
C-1	44.5	CH_2	C-1	44.3	CH_2
C-2	52.6	CH	C-2	52.4	CH
C-3	75.8	CH	C-3	75.7	CH
C-4	120.7	CH	C-4	120.6	CH
C-5	141.1	C	C-5	141.1	C
C-6	47.5	C	C-6	47.4	C
C-7	51.5	CH	C-7	51.4	CH
C-8	76.1	CH	C-8	76.2	CH
C-9	36.4	CH_2	C-9	36.5	CH_2
C-10	60.6	CH	C-10	60.6	CH
C-11	43.2	C	C-11	43.2	C
C-12	21.2	CH_3	C-12	22.4	CH_3
C-13	29.7	CH_3	C-13	30.7	CH_3
C-14	87.4	CH	C-14	87.3	CH
C-15	90.9	CH	C-15	90.7	CH
15-OAc	170.2	C	15-OAc	170.7	C
	30.9	CH_3		30.9	CH_3
14-OAc	169.6	C	14-OAc	169.5	C
	28.9	CH_3		28.9	CH_3
3-OAngeloyl	167.5	C	O-(C_5)	172.7	C
	127.7	C		43.8	CH_2
	138.9	CH		25.6	CH
	20.7	CH_3		21.2	CH_3
	15.8	CH_3		21.2	CH_3

* in $CDCl_3$; 67.5 MHz
** in $CDCl_3$; 75 MHz

Table 8-1. **^{13}C NMR and DEPT Spectral Assignments for the *A. thurberi* Isocedrene.**

Table 8-1. Carbon assignments are nearly identical with the exception of the five C-3-attached carbons. The DEPT experiment demonstrated that the *A. thurberi* isocedane C5 attachment has two methyls, one CH, one CH_2, and one quaternary. The most likely configuration would be a 3-methylbutanoyl group. This structure allows for the two nearly equivalent methyl groups.

Fast Atom Bombardment mass spectroscopy was conducted at the University of Arizona Mass Spectroscopy Facility in the Department of Chemistry. Results obtained did not show a molecular ion (Figure 8-4). This is not unexpected for a compound that has a secondary alcohol and three ester substituents (Silverstein *et al.*, 1991.). Most fragments seen can be explained by simple cleavages around the carbonyls (Figure 8-5). Fragments larger than m/z 101 and smaller than m/z 231 result from dissolution of the C15 skeleton and are not necessarily a fingerprint for this molecule.

^{1}H NMR was performed on a Varian Unity 300 NMR. Comparisons with other isocedrenes were problematic due to their PMRs being run in other deuterated solvents. Integrations for protons downfield of δ 4.0 indicated five CH groups at δ 4.15, δ 5.25, δ 5.79, δ 6.08, and δ 6.65. The tight cluster upfield to δ 3.0 had what appears to be two acetyl-bound methyls (δ 2.05 and δ 2.04) and four alkane methyls (δ 1.02, δ 2.04, δ 2.06, and δ 2.14). The *Proustia* isocedrene run in (C_6D_6) compares to the Acourtia isocedrene only in respect that each proton is correctly upfield and downfield to its neighbors (Table 8-2). Splitting was difficult to analyze in most proton peaks.

A COSY spectrum was run on the Varian Unity 300 NMR to confirm assignments and identify the difficult to locate protons (Figure 8-7). Correlation between several protons could be seen. The H-14 proton couples to the H-7 which itself is coupled to H-8. H-3 and H-4 demonstrate coupling as well. Intriguingly, allylic coupling is present between the H-15 and the H-4 protons. Expansion of the upfield cluster (Figure 8-8) showed the coupling of the H-9α and H-9β to the H-8 and the H-10. The H-7 proton is not coupled to any proton within this expansion. The methyl protons of the C-12 and C-13 are attached to a quaternary carbon (C-11) and therefore do not correlate to any other peaks on the COSY spectrum. The methylbutanoyl protons are more difficult to identify. The H-4' and H-5' protons both couple to the H-3' CH group. The H-2' is not readily assigned but is probably located in the δ 2.0 - δ 2.2 region.

The compound (IV) (Figure 8-9) was tested in the human topoisomerase IIα enzyme assay at 100 μM and was found to be inactive. While the basic structure of this molecule is new, the stereochemistry has not been determined. Due to the compound's relative instability and its lack of enzyme inhibition, further structure elucidation was discontinued.

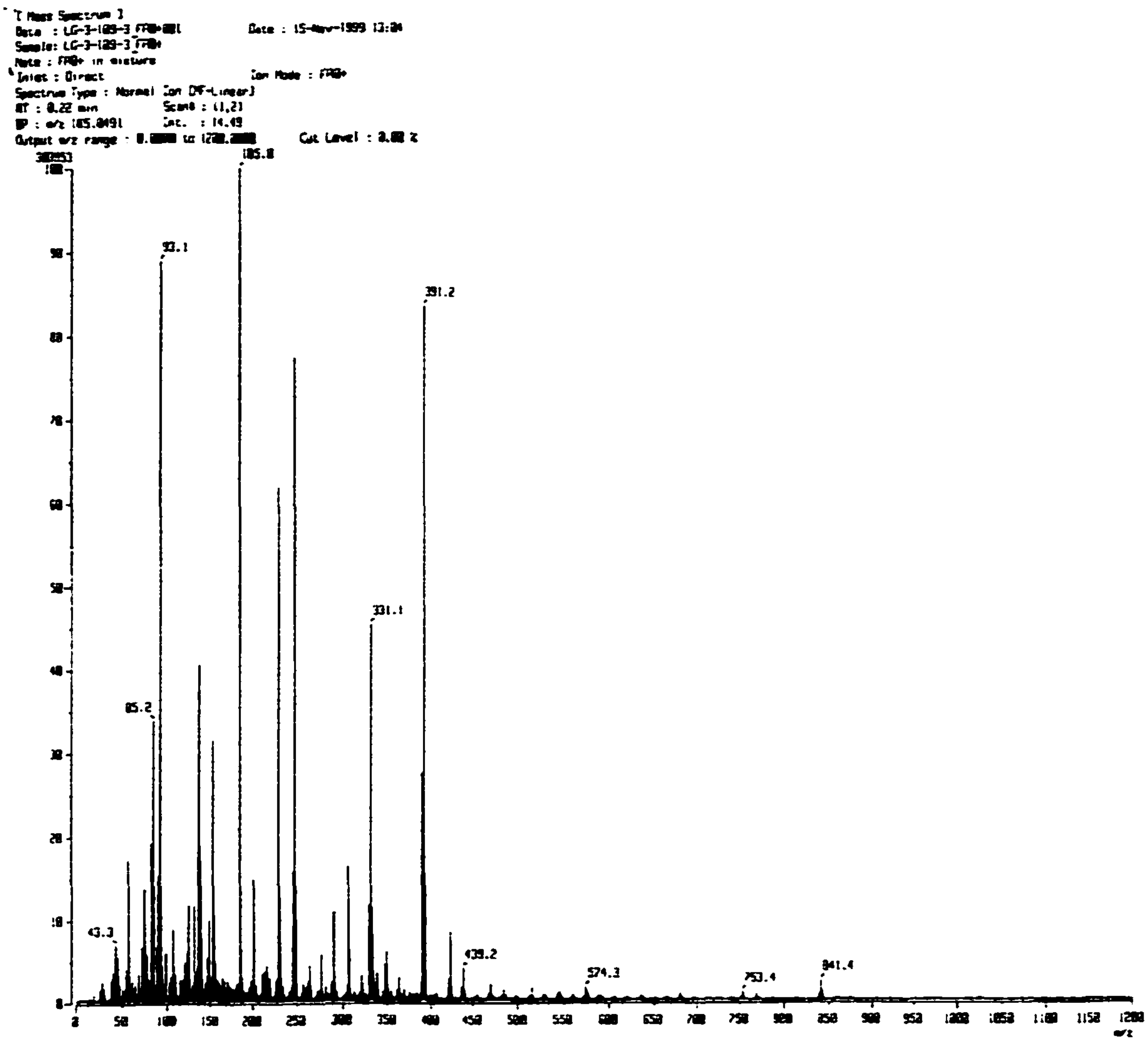


Figure 8-4. **FAB+ MS of *A. thurberi* isocedrene.**

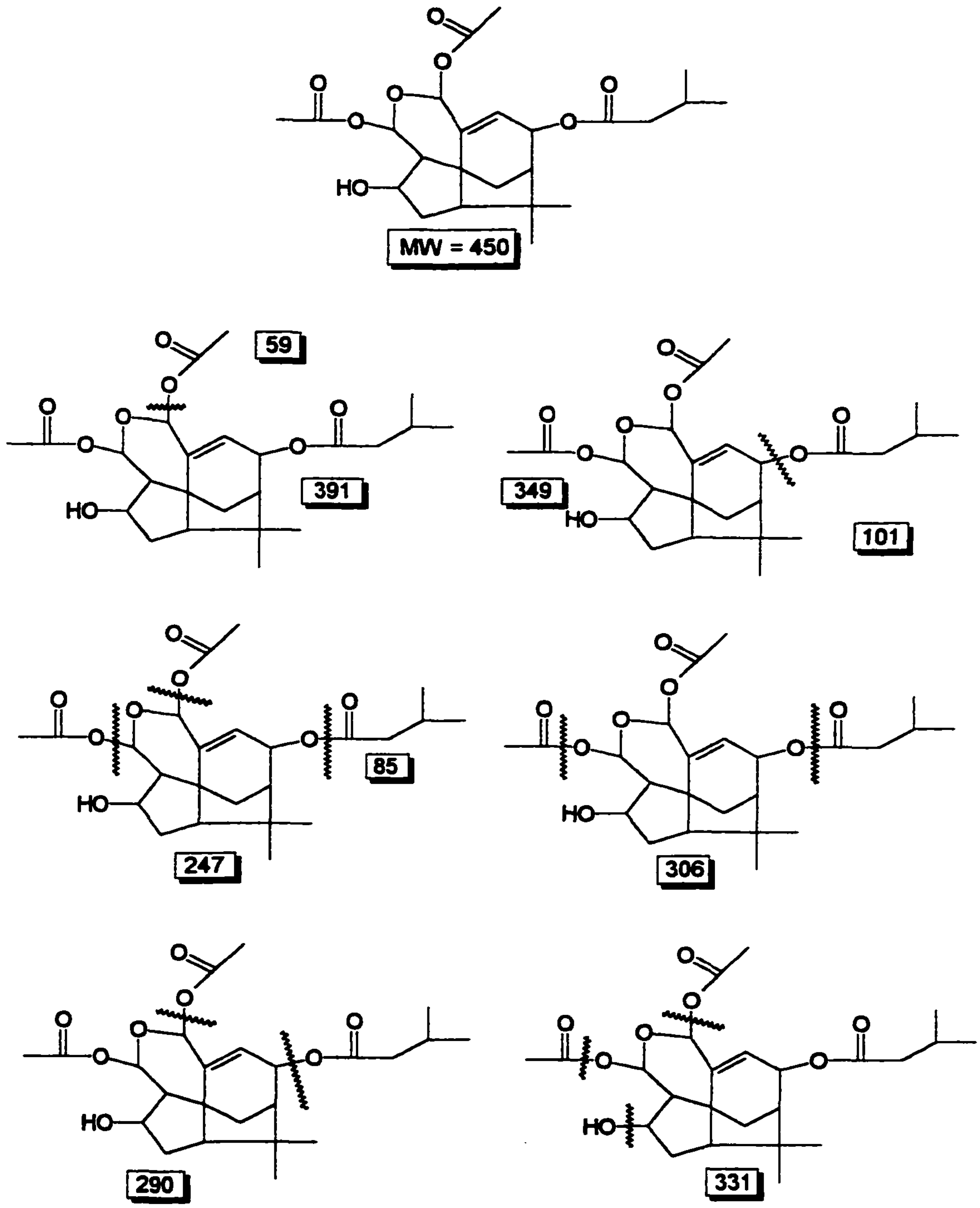


Figure 8-5. MS Fragmentation Possibilities for 14, 15-Diacetoxy, 8-Hydroxy-, 3-(3-Methoxybutanoyl)- 14,15-Epoxy Isocedrene.

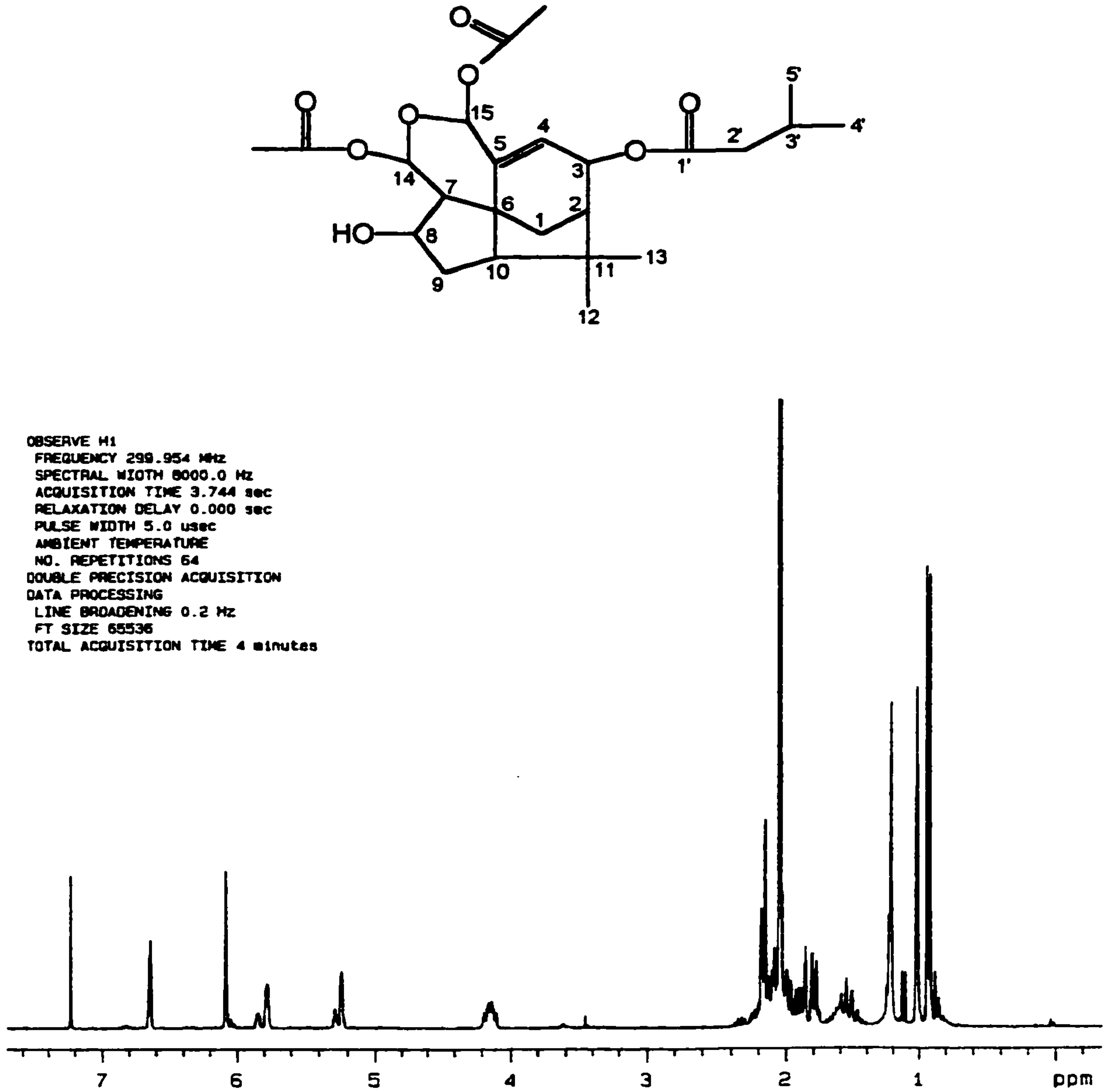


Figure 8-6. ^{1}**H NMR Spectrum of *A. thurberi* Isocedrene.**

Proustia pyrifolia isocedrene (Bohlmann and Zdero 1979)*		*A. thurberi* isocedene**	
H-1α	1.96 br d (J = 12,4.5)	H-1α	2.0 - 2.2
H-1β	1.87 dd (J = 12)	H-1β	2.0 - 2.2
H-2β	2.08 br dd (J = 4.5,4.5)	H-2	2.18
H-3α	5.97 ddd (J = 4.5,2,2)	H-3	5.79
H-4	5.49 ddd (J = 1.5,2,2)	H-4	5.25
H-7α	1.77 dd	H-7	1.80
H-8α	4.00 dddd	H-8	4.15
H-9α	1.62 ddd (J = 5,12)	H-9α	1.86
H-9β	1.37 ddd (J = 11,12)	H-9β	1.56
H-10β	1.85 dd (J = 5,11)	H-10	2.04
H-12	1.27 s	H-12	1.21
H-13	0.84 s	H-13	1.02
H-14	6.39 d (J = 1.5)	H-14	6.08
H-15	6.93 dd (J = 2,2)	H-15	6.65
14-OAc	1.74 s	14-OAc	2.05
15-OAc	1.61 s	15-OAc	2.04
3-OAng	5.73 qq 1.82 dq 1.99 dq	3-O(C_5)	C2' - 2.06 C3' - 2.14 C4' - 0.92 C5' - 0.94

* in C_6D_6; 67.5 MHz; J (Hz) values reported for selected protons.
** in $CDCl_3$; 300 MHz

Table 8-2. [1]H NMR Spectral Assignments for *A. thurberi* Isocedrene.

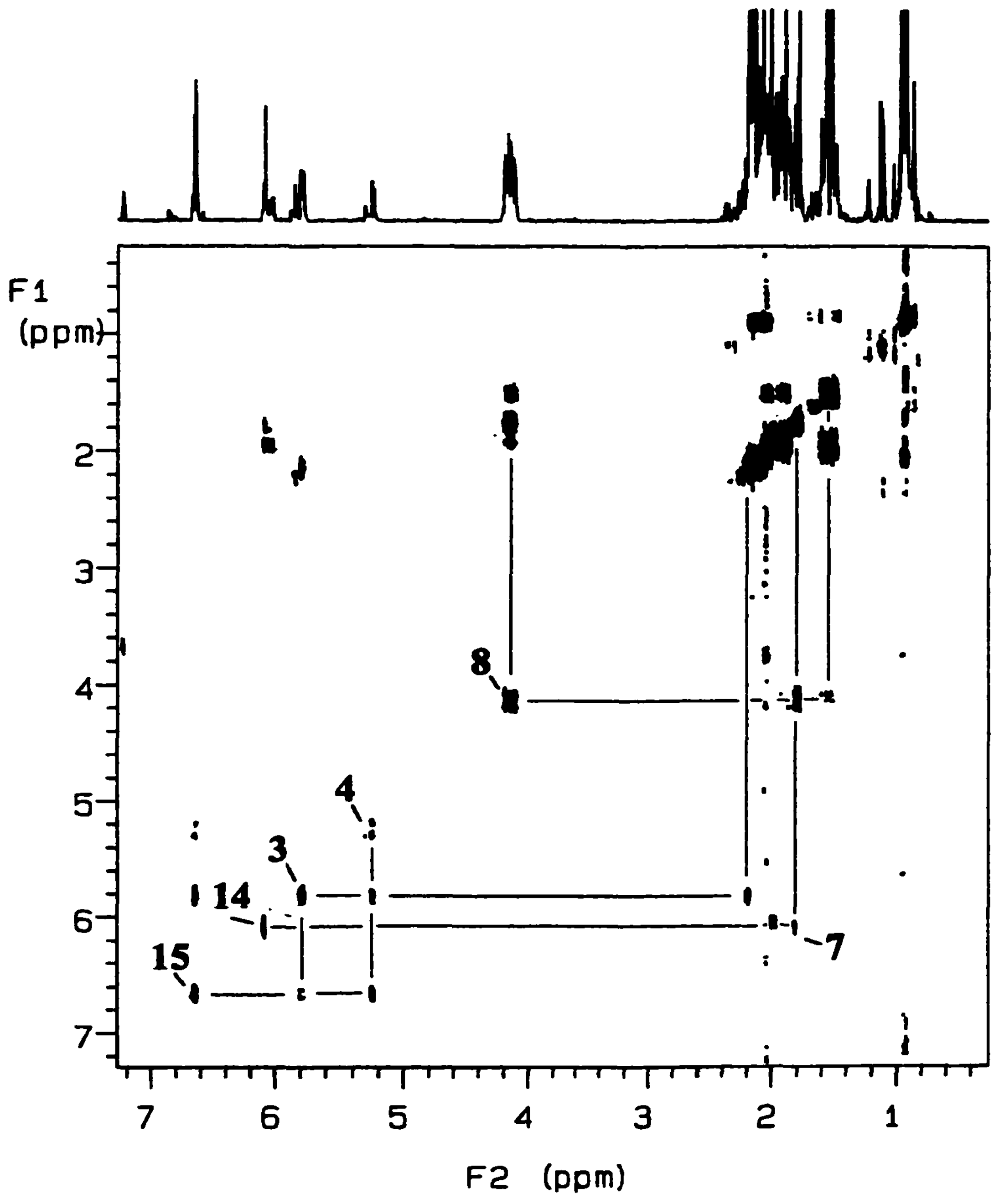


Figure 8-7. COSY NMR Spectrum of *A. thurberi* Isocedrene.

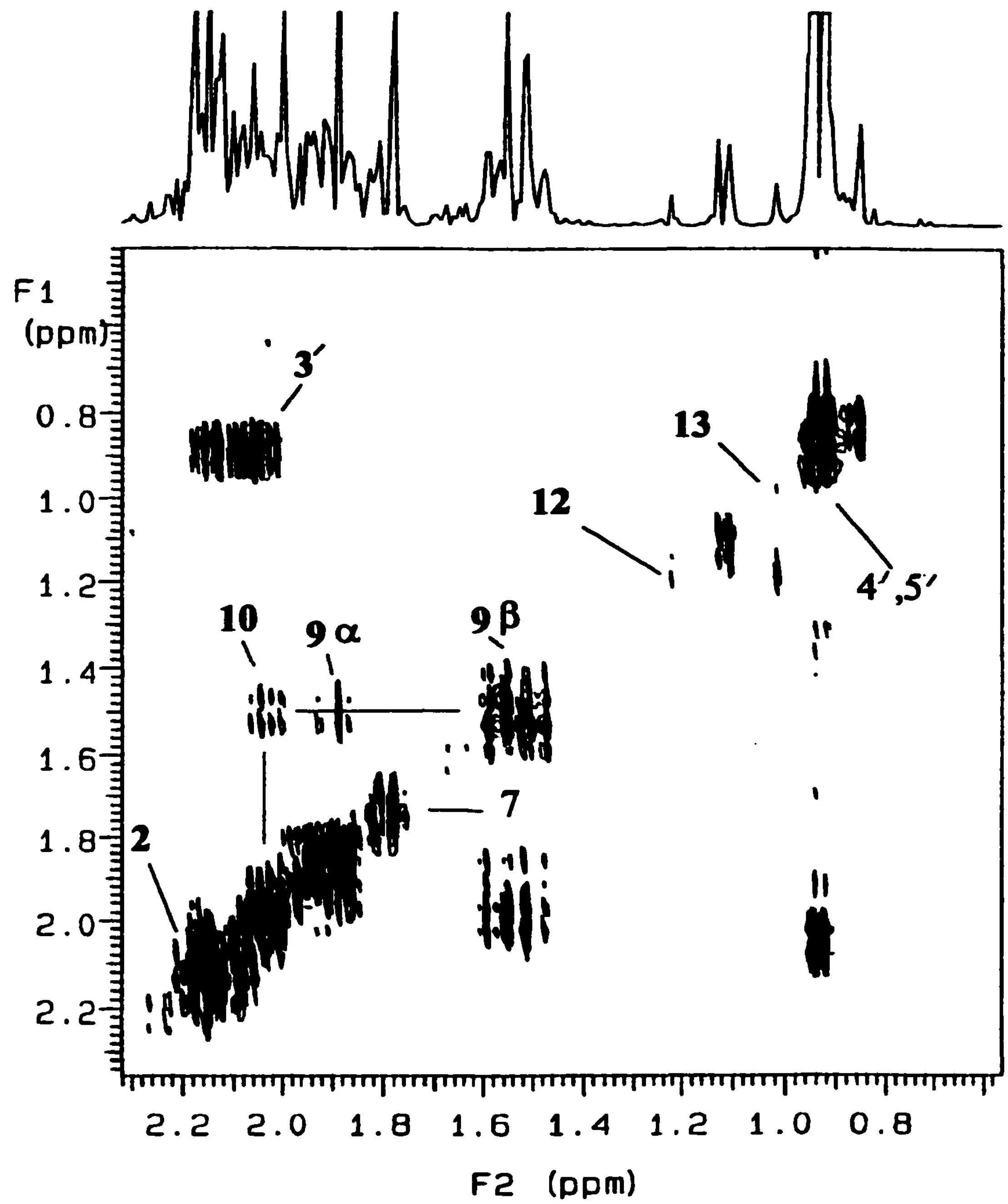


Figure 8-8. **COSY – δ 0.6 - δ 2.3 region expansion.**

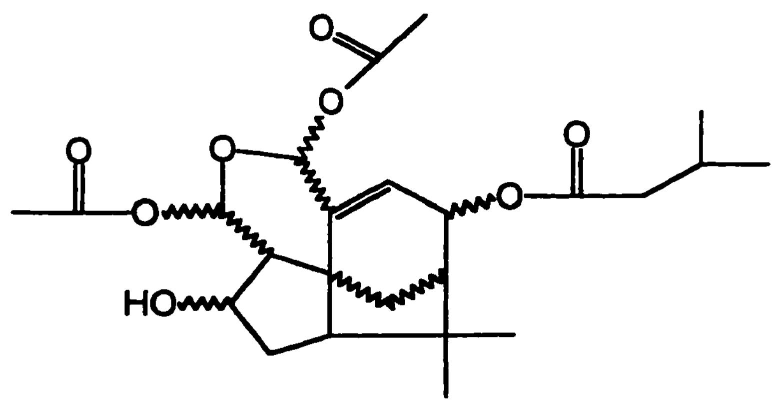


Figure 8-9. 14, 15-Diacetoxy-, 8-Hydroxy-, 3-(3-Methylbutanoyl)-14, 15-Epoxy-Isocedrene.

CHAPTER 9

CATALYTIC INHIBITION OF TOPOISOMERASE IIα BY DEMETHYLZEYLASTERONE, A 6-OXOPHENOLIC TRITERPENOID FROM *KOKOONA ZEYLANICA*

9.1 Introduction

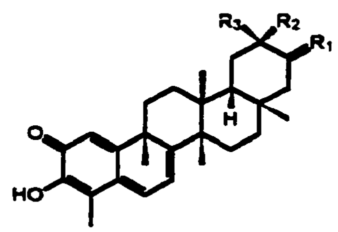
Celastroloids constitute a minor class of unsaturated and oxygenated D:A-*friedo-nor*-oleanane triterpenoids with a wide variety of biological (anticancer, antimicrobial, antimalarial, and spermicidal) activities and found confined to the plant families, Celastraceae and Hippocrateaceae (Gunatilaka, 1996). *In vitro* and *in vivo* anticancer activities shown by a number of celastroloids and their derivatives (Ngassapa *et al.*, 1994) have led to clinical evaluations (De Santa *et al.*, 1971; Melo *et al.*, 1974) of tingenone (**1**) and efforts to understand its possible mechanism of anticancer action. These mechanistic studies have indicated that some celastroloids exert marked inhibitory effects on *in vitro* protein and RNA synthesis (Angeletti and Marini-Bettolo, 1974). Reports of their anticancer activity, together with the availability of a series of celastroloids and structurally related 6-oxophenolic triterpenoids isolated from several Sri Lankan Celastraceae prompted a screen of these and some of their derivatives for topoisomerase II inhibitory activity in genetically engineered yeast and enzyme assays.

The isolation and characterization of tingenone (**1**), 20α-hydroxytingenone (**2**), pristimerin (**4**), and balaenonol (**11**) from *Cassine balae* (Fernando *et al.*, 1989), isoiguesterin (**3**) from *Salacia reticulata* var. *diandra* (Tezuka *et al.*, 1994), and demethylzeylasterone (**5**), zeylasterone (**8**), and zeylasteral (**9**) from *Kokoona zeylanica* (Gamlath *et al.*, 1987), and the semisynthesis of pristimerol (**10**) Gunatilaka and Wimalasiri, 1992), pristimerol diacetate (**6**) (Gunaherath and Gunatilaka, 1983), and pristimerol dimethyl ether (7) (Alley *et al.*, 1988) have been reported elsewhere.

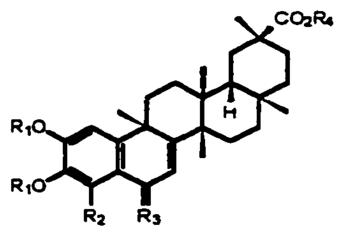
Topoisomerase II (topo II) is an enzyme that has the ability to relieve torsional strain in eukaryotic DNA. The enzyme acts through the cleaving of double-stranded DNA, allowing passage of another helix through the break, and then finally re-ligating the broken DNA strands (Nitiss, 1998). The important role of topoisomerase II in eukaryotic cell viability has made it an effective target for anticancer drug discovery and a number of topo II inhibitors are already in clinical use (e.g. doxorubicin, daunorubicin, etoposide) (Hande, 1998). Topo II inhibitors are generally grouped into one of two classes determined by the stage at which the enzymatic process is blocked. Those compounds that allow the cleavage of the DNA but prevent its re-ligation through the stabilization of the DNA-enzyme complex are collectively known as topo II "poisons." Most of the compounds used clinically fall into this class. Catalytic inhibitors comprise the second class and these inhibitors may stop the initial binding of the topoisomerase enzyme to the DNA (e.g. aclarubicin) (Jensen and Sehested, 1997) halt the cleavage event itself (e.g. novobiocin) (Jensen and Sehested, 1997), or prevent the release of the enzyme from the re-ligated complex (e.g. ICRF-187)(Roca *et al.*, 1994).

9.2 Yeast Assay Prescreen

The prescreen using strains of topoisomerase-deficient *Saccharomyces cerevisiae* (*rad52Y* and *RS321N*) was conducted utilizing an agar well diffusion assay (Gunatilaka *et al.*, 1994). As discussed in Chapter 7, provided there is no inhibition of growth of the wild type (*RAD+*) strain, differential inhibition of growth in any of the mutant strains (*rad52Y* or *RS321N*) acts as an indicator of DNA damaging activity. However, inhibition of the *RS321N* strain in excess of three times that of *rad52Y* indicates potential topoisomerase II inhibitory activity (Gunatilaka *et al.*, 1998). Compounds tested included tingenone (**1**), 20α-hydroxytingenone (**2**), isoiguesterin (**3**), pristimerin (**4**), demethylzeylasterone (**5**), balaenonol (**11**), and the semisynthetic derivatives pristimerol diacetate (**6**), and pristimerol dimethyl ether (**7**) (Figure 9-1.). The yeast assay indicated weak topo II activity in some celastroloids tested (Table 9-1). Three of the celastroloids, tingenone (**1**), 20 α-hydroxytingenone (**2**) and balenonol (**11**) exhibited weak *RS321N* activity (Table 9-1.). Each compound was several hundred-fold weaker than streptonigrin.



1	$R_1 = O$; $R_2 = H$; $R_3 = Me$
2	$R_1 = O$; $R_2 = OH$; $R_3 = Me$
3	$R_1 = H_2$; R_2 ,$R_3 = CH_2$
4	$R_1 = H_2$; $R_2 = CO_2Me$; $R_3 = Me$



5	$R_1 = R_4 = H$; $R_2 = CO_2H$; $R_3 = O$
6	$R_1 = Ac$; $R_2 = R_4 = Me$; $R_3 = H_2$
7	$R_1 = R_2 = R_4 = Me$; $R_3 = H_2$
8	$R_1 = H$; $R_2 = CO_2H$; $R_3 = O$; $R_4 = Me$
9	$R_1 = H$; $R_2 = CHO$; $R_3 = O$; $R_4 = Me$
10	$R_1 = H$; $R_2 = R_4 = Me$; $R_3 = H_2$

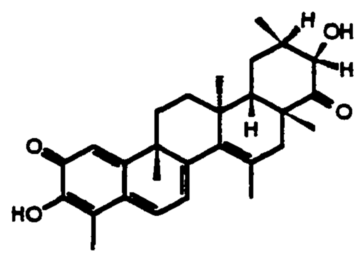


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Figure 9-1. Structures of compounds screened.

Compound	*RAD+*	*rad 52Y*	*RS321N*
Tingenone (1)	NA	NA	325
20α-Hydroxytingenone (2)	NA	NA	185
Isoiguesterin (3)	NA	NA	NA
Pristimerin (4)	NA	NA	NA
Demethylzeylasterone (5)	NA	NA	NA
Pristimerin diacetate (6)	NA	NA	NA
Pristimerin dimethyl ether (7)	NA	NA	NA
Balaenonol (11)	NA	NA	220
Streptonigrin	-	0.4	< 0.4
Camptothecin	110	0.6	> 20

[a] Results expressed as IC_{12} (μg/mL); NA = not active at 200 μg/mL

Table 9-1. Bioactivity Data of Celastroloids, Camptothecin, and Streptonigrin.[a]

9.3 Topoisomerase IIα Enzyme Assay

The supercoiled plasmid pRYG was initially used for the measurement of relaxation through the action of human topoisomerase II. Topoisomerase IIα and etoposide were purchased from TopoGEN Inc. (Columbus, OH). Reaction volumes were 20 μL in an assay buffer (50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol) containing 4 units of human topoisomerase II (p170 form) and approximately 200 ng DNA. Samples were solublized using Cremophor EL (0.5% final concentration). Compounds were tested at 100 μM to identify inhibitory activity. Reaction tubes were incubated for 30 min. at 37°C. Reactions were stopped through the addition of SDS (1%). Enzyme was separated from DNA using proteinase K (50 μg/mL final conc.). Upon addition of loading buffer, samples were clarified of remaining DNA-enzyme complexes through a chloroform:isoamyl alcohol (24:1) partitioning. The aqueous phases were applied to a 1% TAE agarose gel. Agarose gels were run on the BioRad Sub-Cell GT horizontal electrophoresis system. Bands were visualized post-electrophoresis using 0.5 μg/mL ethidium bromide. Gels were visualized using a BioRad Gel Doc 2000 system and DNA bands analyzed with the BioRad Quantity One quantitation software (v.4.1.1.).

While the yeast assay did not suggest a strong topoisomerase II inhibitory activity for any of the compounds, testing these against purified human topoisomerase IIα at a concentration of 100 μM demonstrated strong activity for demethylzeylasterone (5) (Figure 9-2). Unlike the positive control etoposide (100 μM), demethylzeylasterone did not yield any linear DNA. The plasmid stayed in its initial supercoiled form and did not experience any relaxation. Topo II activity inhibited by demethylzeylasterone prompted the testing of some of its structural analogs, zeylasterone (**8**), zeylasteral (**9**), and pristimerol (**10**) in the enzyme assay (Figure 9-3). Interestingly, none of these were found to be active at 100 μM. The inability of the C-29 methyl ester of **5**, namely zeylasterone (**8**) to inhibit topo II enzyme even at a dose of 100 μM suggests the requirement of the C-29 free carboxylic acid group in 6-oxophenolic triterpenoids for topo II inhibitory activity. The absence of DNA that has been cleaved by the enzyme or DNA that has been relaxed suggests that this compound is not acting as a topoisomerase poison but as a catalytic inhibitor (Jensen et al., 1991). The IC_{50} of demethylzeylasterone (**5**) was determined to be 17.6 μM through a dilution series performed in triplicate (Figure 9-4). The experiment was performed with the following ; Lane 1, DNA alone; lane 2, DNA + topo II; lanes 3 - 8, DNA, topo II + 1, 5, 10, 25, 50, 100 μM demethylzeylasterone (**5**); Lane 9, DNA + topo II + 100 μM etoposide. Unlike the previous enzymatic assays, the plasmid pBlueScript +KS was used instead of pRYG. This substitution produced sharper banding and facilitated the quantification of plasmid levels. pBlueScript is a product of Stratagene.

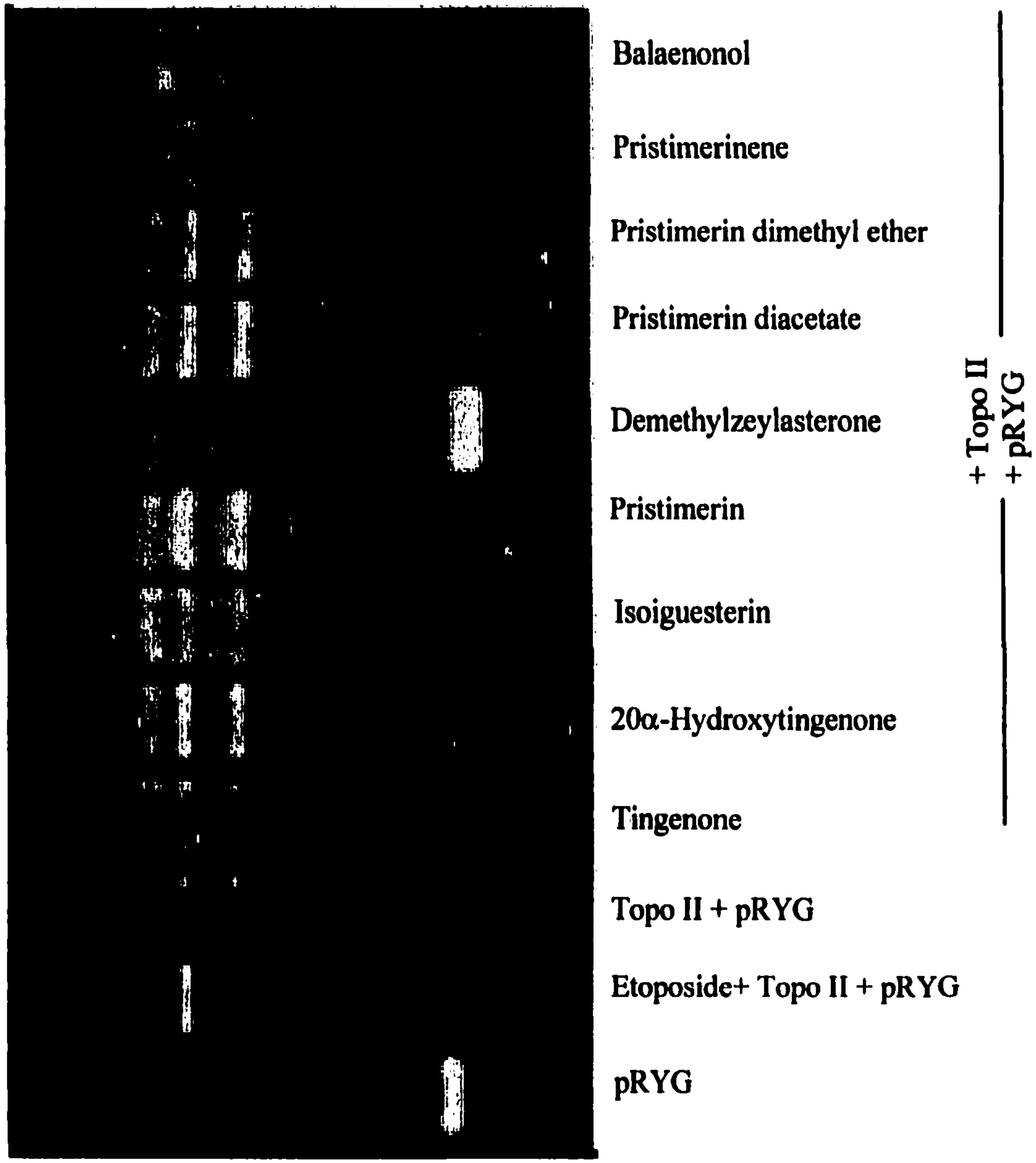

Figure 9-2. Topoisomerase IIα Activity of Various Celastroloids.

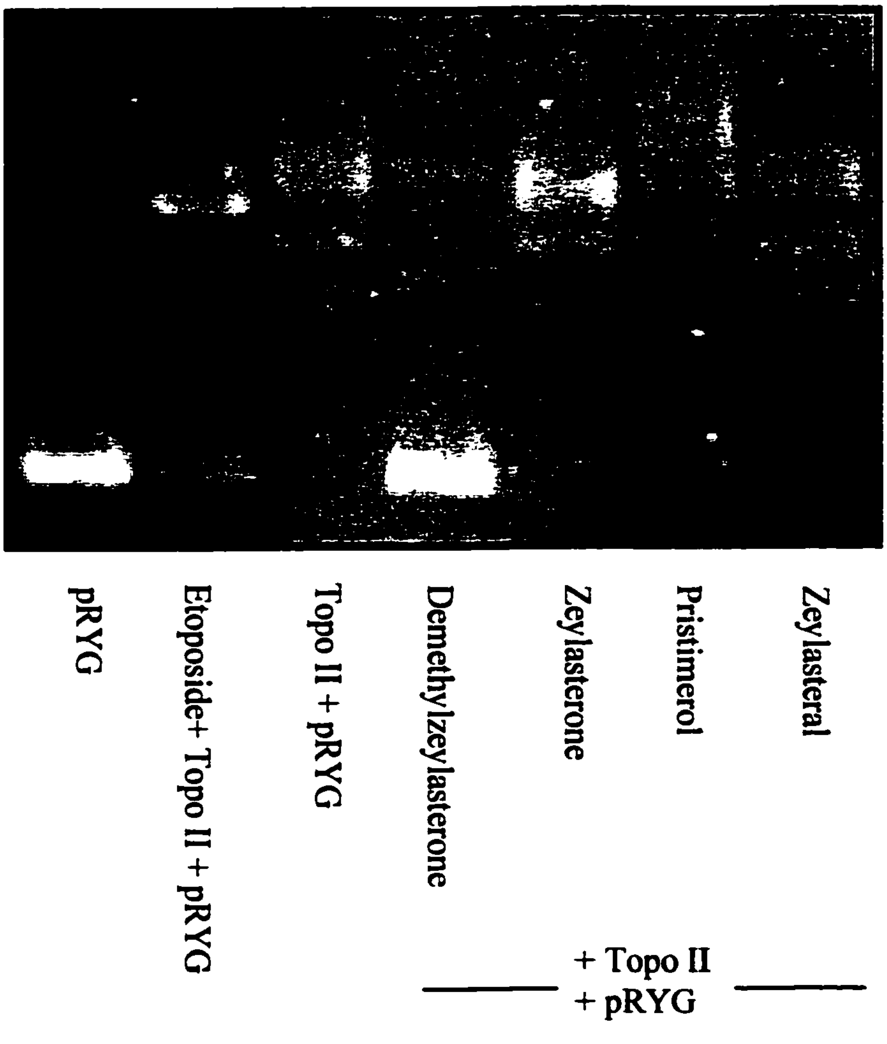

Figure 9-3. Topoisomerase IIα Activity of Various Demethylzeylasterone Analogs.

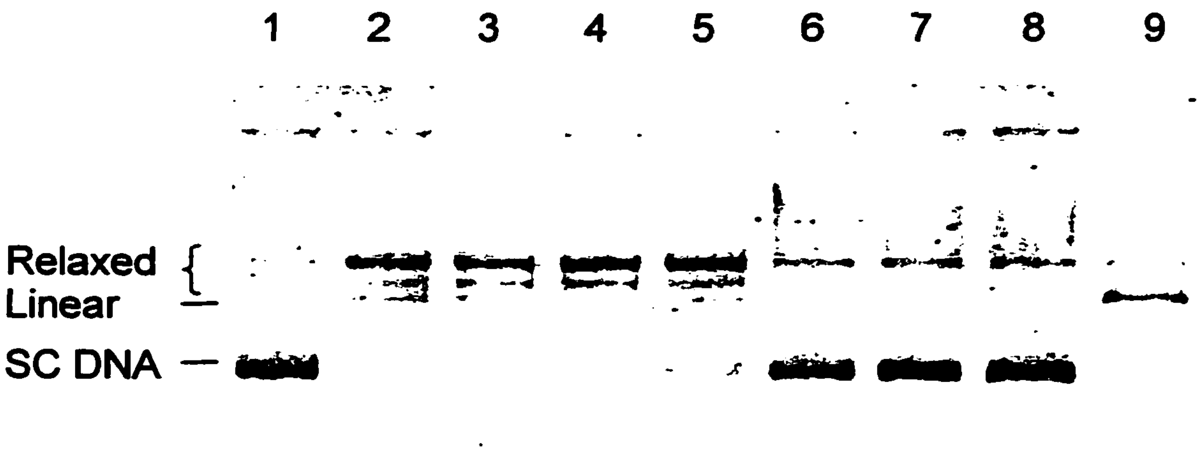

Figure 9-4. The Inhibitory Effects of Demethylzeylasterone (5) on Human p170α Topoisomerase II on pBluescript KS(+) Plasmid DNA.

9.4 Determination of Mode of Action

Having determined that demethylzeylasterone (5) belongs to the catalytic inhibitor class, an attempt was made to determine its mode of topo II inhibition. The methyl green displacement assay, much like ethidium bromide, has been used to identify intercalators of DNA (Burres et al., 1992). The methyl green assay was performed as described by Burres *et al.*(1992). Daunomycin (50 µg/mL) was used as a positive control. A Molecular Devices Thermomax 96-well plate reader with SOFTmax Pro (v.2.4.1) analysis software measured methyl green absorbance.

Since no displacement of methyl green from DNA occurred, it is possible that 5 either binds to the enzyme and prevents DNA from interacting with the topoisomerase II or it allows binding but prevents the cleavage event. The electrophoretic mobility shift assay (EMSA) has the ability to detect protein-DNA interactions (Fortune and Osherhoff, 1998). When run at the ideal pH, the topo II enzyme will retard the movement of the protein-associated DNA through the agarose gel.

The electrophoretic mobility shift assay (EMSA) assay is described in detail elsewhere (Fortune and Osherhoff, 1998; Syrovets *et al.*, 2000). The assay differed from the typical topo II assay in that 6 units of p170 were used in each sample and ATP is absent from the buffer. The pH of the assay buffer, running buffer, and gel was 6.4. The lower pH is designed to improve the degree of shift between free plasmid and topo II-associated plasmid. Reactions were not stopped with SDS and proteinase K but loaded directly onto a TAE gel containing 1.0 µg/mL ethidium bromide.

The control containing only topo II and DNA (Figure 9-5, Lane 2), along with the control containing topo II, DNA, and 50 μM etoposide (Lane 4), exhibited retardation of supercoiled DNA migration. Demethylzeylasterone (5) sample (100μM) (Lane 3) was identical in migration pattern to the supercoiled DNA control (Lane 1). It appears therefore that demethylzeylasterone (5) is preventing the topo II from associating with the DNA. This mode of action is not unique. Another pentacyclic triterpenoid, acetyl-boswellic acid from *Boswellia serrata*, is reported to be a catalytic inhibitor of topoisomerse II as well as acting as an inhibitor of topoisomerase I (Syrovets *et al.*, 2000). This same study had demonstrated weak topo II inhibitory activity in several other pentacyclic triterpenoid acids.

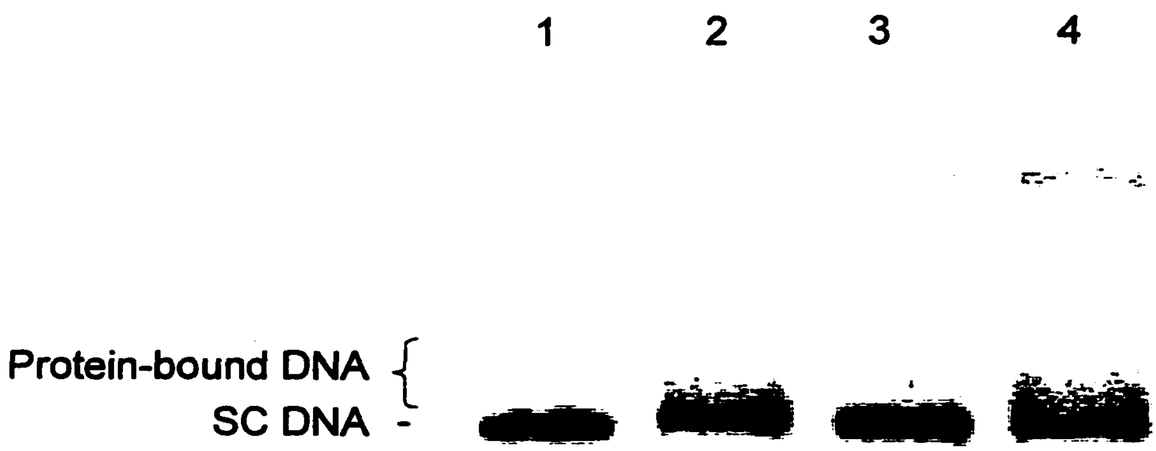

Figure 9-5. Electrophoretic Mobility Shift Assay (EMSA) of pBluescript KS(+) Plasmid DNA against Demethylzeylasterone for the Detection of Enzyme-DNA Interaction.

9.5 Cytotoxic Activity

9.5 Cytotoxic Activity

Demethylzeylasterone (5) was evaluated for cytotoxic activity against several cancer cell lines. The NCI-H460 (non-small cell lung carcinoma), MCF-7 (breast carcinoma), and SF-268 (CNS glioma) cell lines were maintained in RPMI culture medium with 10% fetal bovine serum (FBS). All cell lines were cultured at 37°C in an atmosphere of 5% CO_2 in air (100% humidity). The cells, at log phase of their growth cycle, were treated in triplicate with various concentrations of the test compounds (0 – 10 μM) and incubated for 48 hours at 37°C in a 100% humidified atmosphere of 5% CO_2. Endpoint determinations of growth were made using MTT (Alley et al., 1988). A Molecular Devices Thermomax 96-well plate reader with SOFTmax Pro (v.2.4.1) analysis software measured MTT absorbances.

It is noteworthy that demethylzeylasterone inhibited the growth of breast cancer cell line (MCF-7; IC_{50} = 12.5 μM) but had no effect on non-small cell lung cancer (NCI-H460) and CNS glioma (SF-268) cell lines at concentrations of up to 10 μM.

CHAPTER 10

SUMMARY AND CONCLUSIONS

Over one hundred Southwestern plants were collected and screened for potential antineoplastic activity utilizing assays designed to detect for DNA cleavage, DNA damage and/or topoisomerase inhibition, and potato gall tumor inhibition. For each of the three assays used, one active plant was selected for fractionation. The active compound from each plant was isolated and identified. Of these compounds, two are currently unreported. A third compound has been previously reported but is reported here to demonstrate anti-leukemic activity. Lastly, the screening of some celastroloids in a mutant yeast assay led to the discovery of a potent topoisomerase IIα inhibitor.

Phoradendron juniperinum Engelm. (Viscaceae) was determined to be active in a plasmid DNA scission assay. This assay is designed to detect compounds with the ability to induce nicks in DNA strands. The compound, isolated from the methanol extract was determined through NMR to be 5-caffeoyl-*epi*-quinic acid. While chlorogenic and neochlorogenic acids are widespread, this is the first description of one in the Viscaceae and the first time 5-caffeoyl-*epi*-quinic acid is reported. Inactive in cell line assays, this compound is likely ineffective as an anti-neoplastic agent. The design of the screen allows for the selection of compounds that would ordinarily be considered antioxidants.

Extracts were chosen for testing in the *Agrobacterium tumefaciens*-potato disk gall tumor assay based upon prior activity against murine and human solid tumors.

Psorothamnus thompsoniae (Vail) Welsh and Atwood var. *thompsoniae* (Fabaceae) was selected for fractionation due to their activity in the *Agrobacterium tumefaciens*- potato disk gall tumor assay, an assay that showed a correlation between its activity and that of xenobiotics toxic to murine leukemia (P388). Extracts were also tested against L1210, a murine lymphocytic leukemia. *Psorothamnus thompsoniae* DCM extract was fractionated to obtain dalrubone, the active compound. *Psorothamnus emoryi* Gray (Rydb.) was selected for the isolation of additional dalrubone and the isolation of related compounds. The analog, 5-methoxydalrubone, was purified. With an IC_{50} of 28.2 μM, 5-methoxydalrubone possesses fifty-fold greater activity against MCF-7 (breast cancer) than dalrubone. It has been determined to be active against C38 (human leukemia) and inactive against SF-268 and NCI-H460. The potato disk assay has the disadvantage of being prone to contamination and requiring over two weeks of incubation time. When not contaminated, the assay provided clear and unambiguous results in the testing of *Psorothamnus* sp.

Through the use of a mutant *Saccharomyces cerevisiae* assay developed by SmithKline Beecham, the MEK extract of *Acourtia thurberi* (Gray) Reveal & King (Asteraceae) was fractionated to isolate the novel isocedrene, 14, 15-diacetoxy-, 8-hydroxy-, 3-(3-methylbutanoyl)- 14, 15-epoxy-isocedrene. Due to instability and inactivity in a human topoisomerase IIα enzymatic assay, determination of stereochemistry was not performed.

Eight celastroloids previously isolated from Sri Lankan plants were tested in the yeast assay and three were found to be weakly active; tingenone, 20α-hydroxytingenone, and balaenonol. The celastroloids were then screened with a human topoisomerase IIα enzyme. Demethylzeylasterone was found to be active (IC_{50} = 17.6 μM) against the enzyme. Unlike etoposide, demethylzeylasterone prevented the supercoiled plasmid from being nicked or opened. The demethylzeylasterone acted as a catalytic inhibitor. A methyl green displacement assay showed the compound does not interact with DNA. Electrophoretic mobility shift assays proved that the enzyme is unable to bind to the plasmid in the presence of 100 μM demethylzeylasterone. Structural analogs of demethylzeylasterone were tested against enzyme as well. None of the analogs provided any inhibition at 100 μM. A change as small as methylation of the C-29 carboxylic acid (zeylasterone) completely halts inhibitory activity. Demethylzeylasterone was active against breast cancer (MCF-7) with an IC_{50} of 12.5 μM and inactive against SF-268 and NCI-H460. It must be repeated that the enzyme assay did not detect any of the compounds (isocedrene or celastroloid) reported positive in the yeast assay but did show demethylzeylasterone as being very active.

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