STRUCTURAL AND SYNTHETIC STUDIES OF BIOACTIVE NATURAL PRODUCTS

by

Carl E. Heltzel

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David G.I. Kingston

D.G.I. Kingston, Chairman

H. M. Bell

N. Castagnoli

J. S. Merola

L.T. Taylor

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(ABSTRACT)

Bioassay directed fractionation of the methyl ethyl ketone extract of *Crescentia cujete* resulted in the isolation of nine bioactive compounds, and detailed spectroscopic interpretation led to the assignment of their structures as (2*S*,3*S*)-3-hydroxy-5,6-dimethoxy dehydroiso-α-lapachone [**2.10**], (2*R*)-5,6-dimethoxydehydroiso-α-lapachone [**2.11**], (2*R*)-5-methoxy dehydroiso-α-lapachone [**2.12**], 5-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione [**2.13**], 2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione [**2.14**], 2-isopropenyl-naphtho[2,3-b]furan-4,9-dione [**2.15**], 5-hydroxydehydro-iso-α-lapachone [**2.16**], 3-hydroxymethylfuro[3,2-b]naphtho[2,3-d]furan-5,10-dione [**2.17**], and 9-hydroxy-3-hydroxymethylfuro[3,2-b]naphtho[2,3-d]furan-5,10-dione [**2.18**]. Compounds **2.10-2.12** are new, showing selective activity towards DNA repair-deficient yeast mutants. The selective DNA damaging activity of known compounds **2.13-2.16** is reported herein for the first time. Compounds **2.17** and **2.18** also show DNA damaging activity, and possess a novel fused ring system.

The bioactive sterols ergosta-5-24(28)-diene-3β,7α-diol [**3.1**] and 24,28-epoxyergost-5-ene-3β,7α-diol [**3.2**], originally isolated from *Pseudobersama mossambicensis*, have been synthesized from stigmasterol. In addition to these sterols, some of their analogs were prepared, and the bioactivity of all compounds were assessed.

"The Most High hath created Medicines out of the Earth.
And the wise man will not scorn the use of them."
Sirach 38:4

TO AMANDA

ACKNOWLEDGEMENTS

I would like to thank my research advisor, D. G. I. Kingston for his ideas, encouragement and support throughout the completion of this work. His method of gentle guidance made this very difficult time in my life a little less stressful. Special gratitude is reserved for Dr. Leslie Gunatilaka who has served as a role model of scientific excellence and integrity. I wish to express my gratitude to Thomas E. Glass for his ideas and also his patience while teaching me the NMR techniques that were invaluable for the completion of this work. I also need to acknowledge Professor Neal Castagnoli for his special support. Thanks go to Dr. John Rimoldi and Mr. Tony Molinero for their camaraderie throughout the last four years. I am compelled to thank my wife and daughters who have made so many sacrifices in order to make this work a reality. My parents and the rest of my family, supporting me in their characteristic style, deserve more gratitude than can be expressed. I want to thank the National Cancer Institute for funding this work, and the Virginia Department of Rehabilitation for tuition support.

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LIST OF ACRONYMS FOR NMR TECHNIQUES USED

HOMO COSY - Homonuclear Correlation Spectroscopy

DQ COSY - Double Quantum Filtered Correlation Spectroscopy

HETCOR - Heteronuclear Correlation Spectroscopy

HMQC - Heteronuclear Multiple-Quantum Correlation

HMBC - Heteronuclear Multiple-Bond Correlation

SELECTIVE INEPT - Selective Insensitive Nuclei Enhanced by Polarization Transfer

All of the above techniques are described in reference 91

I. GENERAL INTRODUCTION

1.1 PLANT - DERIVED MEDICINES

The use of crude plant derived natural products for relief from illness can be traced back to antiquity as illustrated in documents recorded by ancient Chinese, Indian, and Near East civilizations.[1] In the Rig - Veda (compiled between 4500 and 1600 B.C.), one of the oldest repositories of human learning, there are references to medicinal plants in use.[2] The Ayurveda - a later work supplementary to the great Veda - describes the native remedies in more detail, and constitutes the basis of the ancient Indian system of medicine.

Today, much of the world's population relies almost exclusively on plants and their extracts for drugs. In industrialized nations, 25% of all prescriptions dispensed contained active principles that were derived from higher plants.[3] In 1980, consumers in the United States spent over $8 billion on prescriptions containing active compounds isolated from higher plants.[4]

Some of the well known and important plant-derived drugs are atropine, caffeine, colchicine, digitoxin, morphine, pseudoephedrine, quinine, scopolamine, taxol, vincristine and vinblastine.

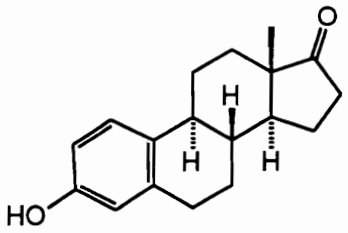
A global survey conducted by Farnsworth *et al.* in 1985 showed that there are about 119 plant-derived chemical compounds of known structure that are currently used as drugs or as biodynamic agents that affect human health.[3] Less than 12 of these were commercially produced by synthesis or chemical modifications of the extracted active compounds, the remainder are extracted and purified directly from plants. These 119 useful drugs are obtained from only

90 species of plants. Conservative estimates are that there are more than 250,000 species of higher plants that exist on this planet. Only a small percentage of these have been investigated phytochemically, and the number subjected to biological or pharmacological screening is even smaller. On the basis of these numbers, it seems highly probable that a continued search for new plant-derived drugs will uncover many new lead compounds. The need to examine the plant kingdom for new pharmacological leads is becoming increasingly pressing considering the rapid deforestation of the tropics and the coexisting loss of biodiversity throughout the entire world.

1.1.1 Natural Products as Anticancer Agents

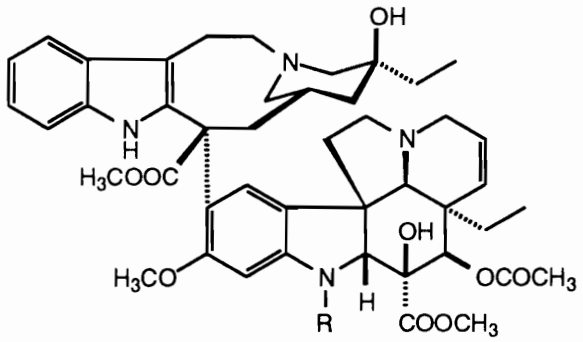
Human cancer, the second leading cause of death in the United States, continues to be characterized by a terrifying number of deaths and results in tremendous costs in terms of human suffering and economic disaster. In 1991, about 1.1 million Americans were diagnosed as having cancer.

Cancer chemotherapy and the use of natural drugs to combat the diseases of cancer has a long history, and it has been reported that there have been over 3000 species of plants that have been used in some form of cancer treatment.[5] The use of pure natural products as anticancer drugs has it's beginnings in 1941, when the first antineoplastic agent, estrone **1.1**, was used in prostate cancer in men.[6]



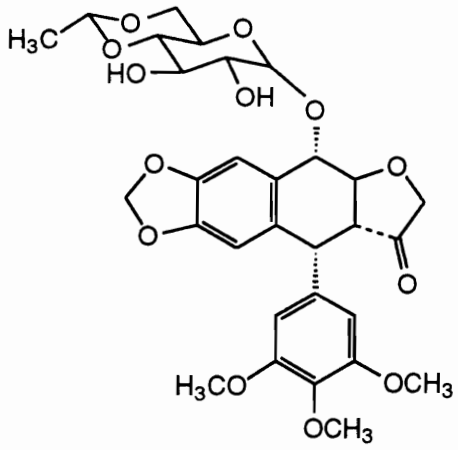
1.1

Only in the last 40 years has there been a rigorous scientific approach to find naturally occuring plant-derived anticancer agents; during this time, levels of interest would rise and fall, but in the last decade interest has been steadily increasing. Between 1957 and 1981 the National Cancer Institue of the United States screened some 35,000 plant species for antitumor activity[7], but with a new *in vitro* primary screen they have employed, samples are now being screened at the rate of about 10,000 extracts *per year*.[8] As a result of this ongoing research a number of clinically useful and market - approved drugs are available, such as the vinca alkaloids vincristine (**1.2**) and vinblastine (**1.3**) from the plant *Catharanthus roseus*,[9,10] the podophyllotoxin derivative etoposide[11] (**1.4**), and of course taxol (**1.5**), isolated from *Taxus brevifolia*.[12]



1.2 R = CHO

1.3 R = CH_3



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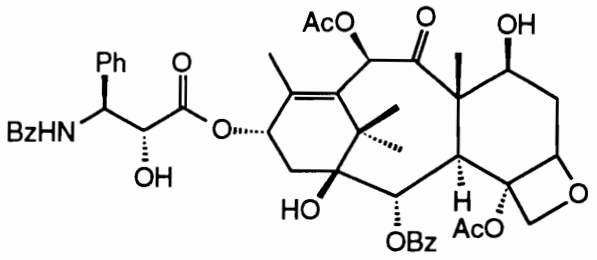


1.5

The available drugs work principally during active cell division through mechanisms of action that include alkylating agents, antimetabolites, DNA binders, tubulin-interactive antimitotics, topoisomerase inhibitors, and mediation of DNA strand breaks.[8]

With regards to the actual selection and collection of plant material for investigation, several approaches are being taken. There is the random method, where complete collections of plants found in a given area are screened. With

this method, large numbers of species can be collected in a short period of time. Another strategy is to target plant families which are known to be rich in biologically active compounds. A third and most fascinating approach is an ethnobotanical method, where a local people's knowledge about the medicinal uses of the indigenous plants is taken into consideration when making plant selections. This is a multidisciplinary area of research involving ethnobotanists and knowledgeable healers in various areas throughout the world.

The screening of plant material and subsequent assaying of fractions during a purification process is described in the following section.

1.2 BIOASSAY-GUIDED FRACTIONATION

1.2.1 General Considerations Concerning Bioassays

In order to have an efficient process for the isolation of novel anticancer natural products, it is essential that the fractionation procedure be guided at each step by assay-detected bioactivity.

There are various types of anticancer agent bioassays available to the natural product chemist. Historically, general cytotoxicity screens have been used for the discovery of anticancer drugs. In 1956 the National Cancer Institute selected the L 1210 mouse leukemia as their main screen and this was used until 1971 when it was replaced by the P388 lymphocytic leukemia assay. This was a more sensitive screen, yet retained characteristics similar to those of L1210. Both screens fared well in the detection of compounds with a broad range of activities. The agents isolated exhibited a broad range of mechanisms of action including

alkylating agents, purine and pyrimidine antimetabolites, antifolates, mitotic inhibitors, DNA interactive compounds including intercalators, alkylators, both single and double strand breakers, minor groove binders, DNA polymerase inhibitors, topoisomerase inhibitors, and inhibitors of protein synthesis working at a variety of steps in that process.[13]

The disadvantage of these screens are that they are complex, time consuming, and often compounds are isolated which are simply too toxic for use. More importantly, when considering the effective leads discovered it is seen that they are limited to rapidly proliferating tumors such as Burkitt's lymphoma, testicular cancer, and non-Hodgkins lymphoma. Treatment of slow growing tumors (mean doubling times of >70 days) such as small cell lung carcinoma, colon adenocarcinoma and advanced breast adenocarcinoma has met with little or no success with antileukemic drugs discovered by use of these assays.[14]

It can be concluded that the L1210 and P388 leukemia assays may detect a wide variety of compounds of various structures and mechanisms, however their effectiveness appears to be limited to rapidly growing tumors, and the reason for this is simply a matter of kinetics. Because rapidly dividing cells will consume more drug than slowly dividing cells, drugs that interfere with cell division have more effect against cells that are rapidly proliferating.

Thus, the answer to the kinetics problem is to find an assay that offers specificity, ideally one which will affect tumor tissue without damaging normal tissue at all. This is the optimal goal of cancer chemotherapy, and because our understanding regarding differentiating cancerous cells vs. normal cells (i.e.. finding a single exploitable qualitative difference to differentiate cancer vs.

normal cells) is still limited, use of a selective bioassay should prove essential to this search.

In addition to the above argument, the use of non-selective cytotoxicity assays would be uneconomical and unproductive considering the requirement to discriminate leads from the vast number of known cytotoxic agents (dereplication of previous work).

Some general considerations for selecting a bioassay are:

1. Sample throughput - the assay should be simple, rapid, reproducible, and inexpensive.
2. Selectivity/Sensitivity - in a crude extract, active constituents are usually present in low concentrations, thus the assay must be sensitive enough to detect them reliably.
3. False positives/false negatives - the test system should be selective enough to keep the number of false positives low. At the same time, it would be ideal if the assay would be insensitive to interferences from plant metabolites such as tannins.

Acknowledging these considerations, one is then faced with two categories of selective bioassays, namely the disease-oriented approach and the mechanism-based approach.

The disease-oriented approach, currently used by the National Cancer Institute, employs a battery of human tumor cell lines.[8] This assay system is proving to be effective, however it is rather complex and time consuming.

With a deeper understanding of cell biology and molecular pharmacology, mechanism-based bioassays have become increasingly important. Due to their selectivity and sensitivity combined with good reproducibility and high sample throughput, this type of assay is given preference for large-scale screening programs in industry or in a collaborative setting.[1]

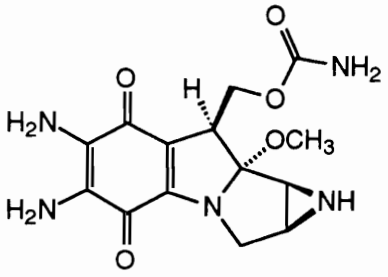
The mechanism-based approach identifies agents that act by a mechanism likely to result in selective antitumor activity. Considering that most of the clinically effective antitumor drugs belong to a small number of mechanistic classes, this approach seems very appropriate. Examples of mechanism-based assays using subcellular structures include inhibition of topoisomerase I and II,[15] tubulin polymerization,[16] and inhibitors of protein kinase C and antagonists of phorbol ester interaction.[17]

Disadvantages of this type of assay may include an inability to detect compounds with unknown mechanisms of action, and the fact that non-specific interactions (i.e.. enzyme inhibitors by tannins) may lead to false positives. But because they are simple yet sophisticated, and sensitive yet carry a high throughput, this type of assay is extremely important in the search for new anticancer agents.

1.2.2 Yeast-Based Bioassay for DNA-Damaging Agents

One selective bioassay, that employed by the Kingston research group, is a DNA-damaging assay. This is a system which simply targets agents that bind to or induce DNA damage. The logic inherent in the selection of such an assay is that the effective anticancer drugs etoposide **1.4** [18] mitomycin C **1.5**,[19] cisplatin

1.6,[20] and adriamycin **1.7**[21] owe their activity to this type of mechanism. Detection of anticancer activity using microbiological assays have been reported in the literature.[22-24]



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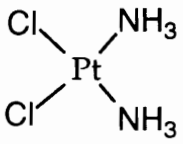

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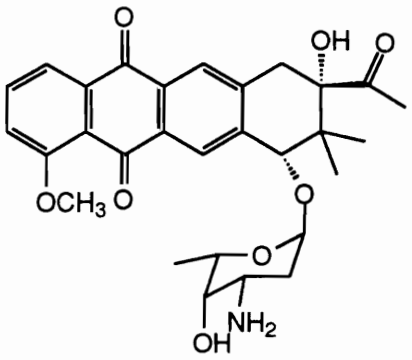


1.7

The screen which we have been using is based on the differential response of DNA repair-deficient and repair-proficient yeast strains to the sample being tested. A yeast screen fares well with our general considerations for selecting an assay as this screen is simple, rapid (48 hour turnaround time), sensitive, and reproducible. In addition, because of the closer genetic and biochemical resemblance of yeast cells to mammalian cells (eukaryotic vs.

prokaryotic), a yeast screen is preferable to a bacterial screen such as one based on *E. coli*.[25]

Investigators studying cellular response to DNA damage have used the yeast *Saccaromyces cerevisiae* for many years.[26] Over 40 years ago a correlation between sensitivity to radiation killing and the level in ploidy in yeast calls was reported, indicating that the genome is a radiation-sensitive target.[27] Radiosensitive yeast mutants were isolated in the late 1960's, and extensive genetic analysis of DNA repair in yeast cells began in the 1970's. Investigations of these supersensitive (to both physical and chemical mutagens) yeast strains have allowed DNA repair pathways to be defined. Three of the major repair pathways defined in yeast are: *rad 3*, *rad 6* and *rad 52*. The *rad 3* pathway is associated with excision repair; *rad 6* pathway is the error prone pathway (similar to *E. coli* post-replication recombination repair); and *rad 52* is associated with repair of double strand breaks and meiotic recombination.[28] Yeast strains lacking each of these specific repair pathways and also having nystatin treated, radiation induced, increased cell membrane permeability are available. In addition, a repair-proficient yeast strain which has increased cell membrane permeability (referred to as wild-type or $rad^{+)}$ is also available.

The assay used in this report is based on *S. cerevisiea* rad 52. The protocol for the assay is to treat both the repair-deficient and the repair-proficient yeast strains with the test sample, and then measure the growth of inhibition of each following a 48 hour incubation period. A yeast strain lacking one of the repair pathways will show a greater growth inhibition than the wild-type strain if the agent it is treated with possesses a mechanism of action which damages DNA predominantly by a method which would normally be repaired by that specific

pathway. In this manner, selective DNA-damaging agents may be detected. Assay results are reported in the form of an IC_{12} (in μg/ml), and in order to consider a compound to be selective it's IC_{12} in rad 52 must be less than three times that of it's IC_{12} in the rad^+ strain. Details of the assay will be further described in the experimental section of the following chapter.

1.3 OVERVIEW OF WORK ACCOMPLISHED

Two chapters follow which describe work completed by the author. The first deals with the application of bioassay-guided fractionation to isolate novel, bioactive natural products. The second chapter then investigates an alternative approach, the synthesis of known natural products and analogs for structure-activity relationship studies and additional testing.

II. FURANONAPHTHOQUINONES FROM *CRESCENTIA CUJETE*

2.1 INTRODUCTION

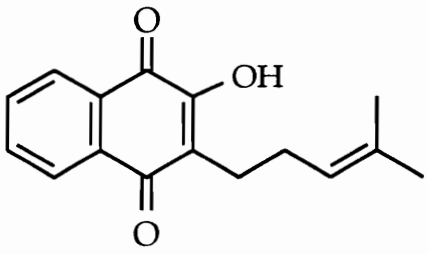
2.1.1 Occurrence of Naphthoquinones

Over 1200 naturally occurring quinones have been reported, and these are widely distributed in both animals and plants as pigments and as intermediates in cellular respiration and photosynthesis.[29] Some of these compounds have shown chemotherapeutic properties against cancer.

The naphthoquinones, with over 300 examples known, comprise the largest group of naturally occurring quinones. Their distribution covers arachnids, slime moulds, and lichens as well as higher plants,[29] and several members have shown cytotoxic activity. Cytotoxic naphthoquinones have been isolated from a variety of families, most notably from the Bignoniaceae family, and some examples of these are given below.

The stem bark of the South-American tree *Tabeuia avellanedae* Lorentz *ex* Griseb., (Bignoniaceae) known in folk medicine as Pau d' Arco, Ipe' Roxo, Taheebo, and Lapacho, has been used in North and South America for many years as an anticancer, antifungal, antibacterial, and antiinflammatory drug.[30,31] The major naphthoquinones of the heartwood of this plant are lapachol (**2.0**) and dehydro-α-lapachone (**2.1**) and these were found to be active against different types of tumors.[32,33] Lapachol, with an ED_{50} value of 4.4μg.ml in the KB cell

culture assay, showed sufficient in vivo activity to reach clinical trials at the National Cancer Institute.[34]



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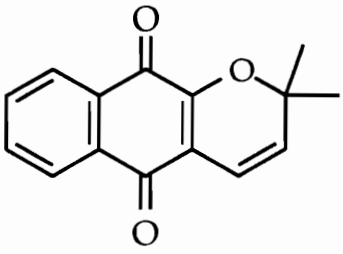
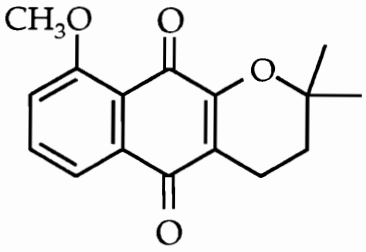
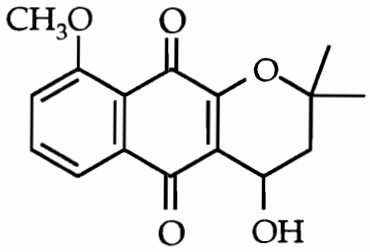


2.1

Mansoa alliacea (Bignoniaceae) has been used in the upper Amazon basin of Peru as a medicinal plant having reported antirheumatic properties. Chromatographic purification guided by cytotoxic activity led to the isolation of the two cytotoxic quinones 9-methoxy-α-lapachone (**2.2**) and 4-hydroxy-9-methoxy-α-lapachone (**2.3**) showing cytotoxic activities against V-79 cells with IC_{50} values of 5.6 and 6.0 μg/ml respectively.[35]



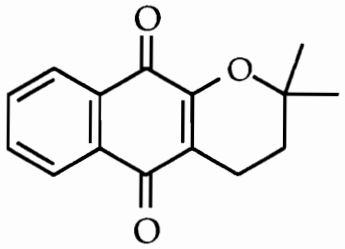
2.2



2.3

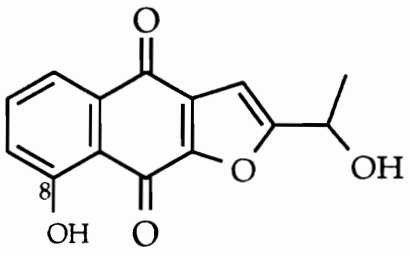
Another cytotoxic naphthoquinone, α–lapachone (**2.4**), was isolated from the wood and callus tissue of *Catalpa ovata*, from the heartwood and roots of

Haplophragma adenophyllum, and from the heartwood of *Tabebuia guayacan, T. pentaphylla,* and *Zehera tuberculosa* (all Bignoniaceae).[29] This well known compound has been reported at one time as being commercially available in Brazil as an antitumor drug,[33] but clinical trials have since proven it to be unsatisfactory due to its serious side effects.[36]



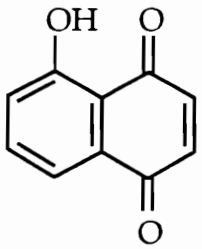
2.4

Kigelia pinnata DC., a bignoniaceous plant native to Africa, bears a fruit that is known to possess a purgative activity and has a bark which has a healing action for ulcers.[37] A cytotoxic furanonaphthoquinone was isolated[38] from the wood of this plant, and identified as 2-(1-hydroxyethyl)-8-hydroxy-naphtho[2,3-b]furano-4,9-dione. This was given the common name kigelinone and is represented by structure **2.5**, although the absolute configuration of the side chain hydroxyl group was not determined due to scarcity of material.



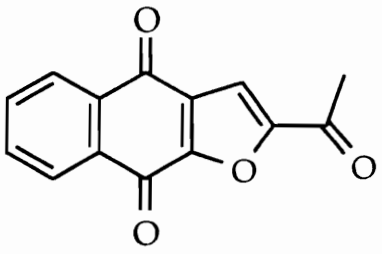
2.5

The aromatic hydroxyl group was assigned to the 8 position based on the chemical shift of it's proton in comparison to a related compound they had isolated. This assignment is also supported by an empirical rule devised by Musgrave *et al* [39] which deals with the effects of substituents on the chemical shift of the *peri* hydroxyl group in juglone-type naphthoquinones.



juglone

An alcoholic extract of the stem bark of *Tabebuia cassinoides* (Bignoniaceae) was investigated due to the crude extract's activity in the P-388 *in vivo* bioassay.[40] Three cytotoxic furanonaphthoquinones were isolated, 2-acetyl-naphtho[2,3-b]furan-4,9-dione (**2.7**), 2-(1-hydroxyethyl)-naphtho[2,3-b]furan-4,9-dione (**2.8**), and 5(or 8)-hydroxy-2-(1-hydroxyethyl)-4H,9H-naphtho[2,3]furan-4,9-dione (**2.5 or 2.6**), showing ED_{50} values in the KB cell culture assay of 1.0, 2.0, and 1.0 μg/ml, respectively. These are significant values when considering the ED_{50} of lapachol in the KB cell culture assay was reported to be 4.4 μg/ml.



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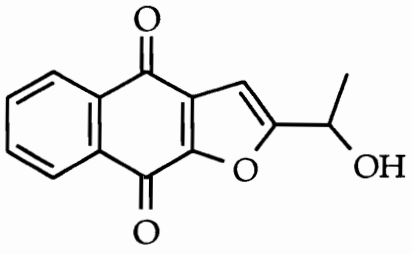
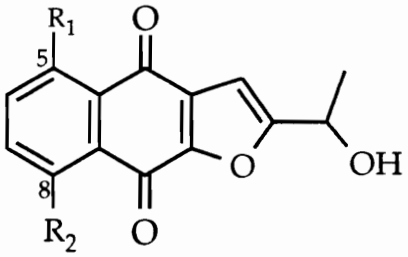


2.8

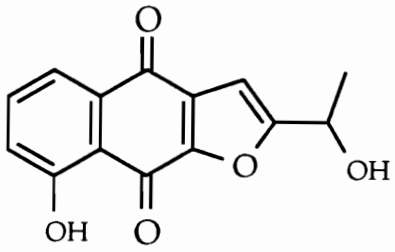
The chiral center in **2.8** was shown to be racemic based on a study using its Mosher's ester. Unambiguous assignment of the aromatic hydroxyl group of **2.5** was not made; however the authors claimed that it was likely to occupy the 8 position (**2.5**) based on biogenetic grounds.



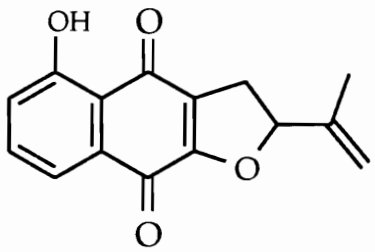
2.5 R_1=H, R_2=OH

2.6 R_1=OH, R_2=H

The only previously cited work carried out on *Crescentia cujete* was reported by Chen[41] in 1983, where two furanonaphthoquinones, 2-(1-hydroxyethyl)-8-hydroxy-furano-1,4-naphthoquinone (**2.5**), and 5-hydroxy-dehydro-iso-α-lapachone (**2.9**) were isolated, with no biological activity reported.



2.5



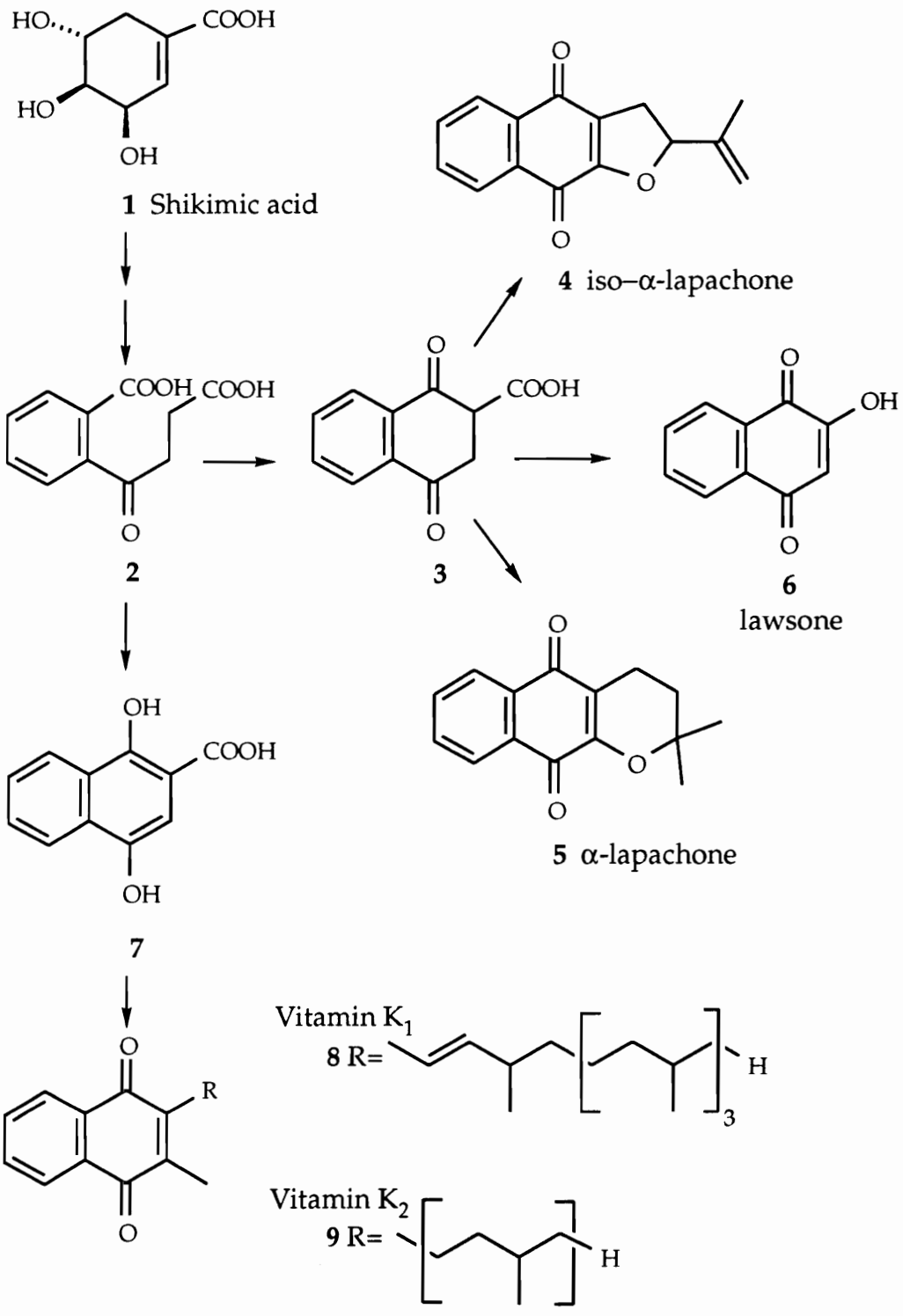
2.9

The absolute configuration at the side chain chiral center was not determined. Placement of the phenolic hydroxyl group was based upon the Musgrave empirical rule already mentioned.

2.1.2 Biosynthesis of Naphthoquinones

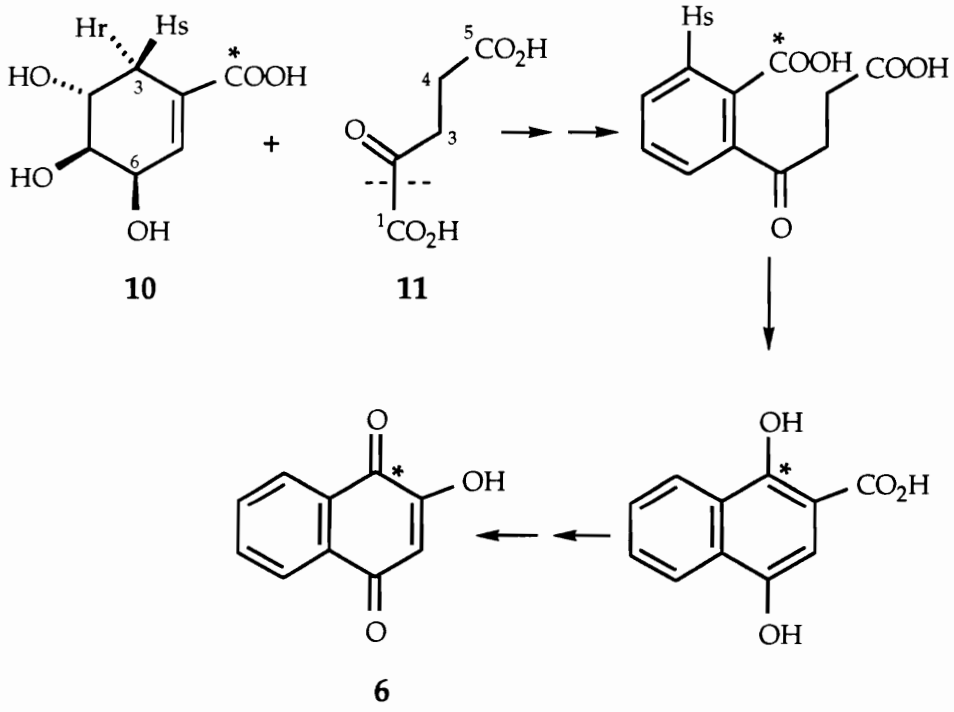
Many naphthoquinones are formed through the shikimate pathway, and they have been organized into three groups according to their pathway after shikimate; (1) 4-(2'-carboxyphenyl)-4-oxobutyrate-derived quinones, (2) 4-hydroxybenzoate-mevalonate-derived quinones, and (3) homogentisate-mevalonate-derived quinones.[42]

This first group has been well studied and classes of compounds that are derived along this pathway are exemplified in bacteria by the menaquinones such as vitamin K_1 (**8**), and in higher plants by vitamin K_2 (**9**), lawsone (**6**), α-lapachones (**5**), and the iso-α-lapachones (**4**). An outline of the biosynthetic pathway is shown in Scheme 1.[43]



Scheme 1. Outline of Biogenesis of Naphthoquinones

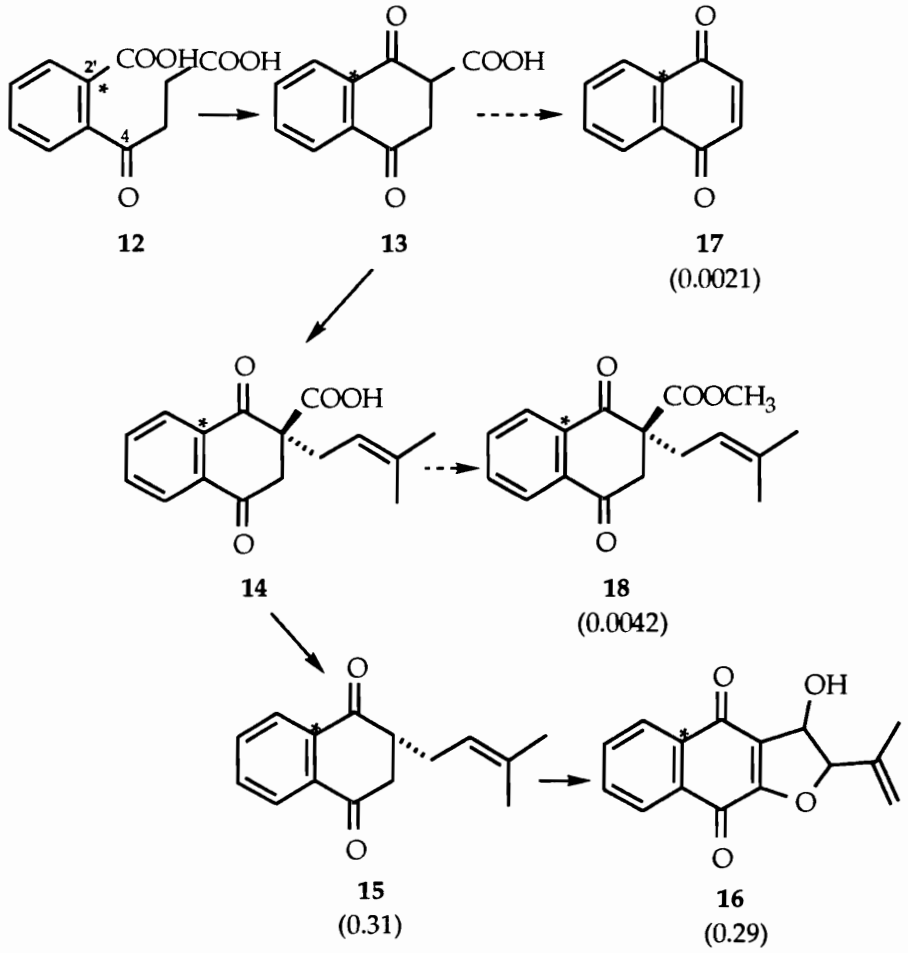
Experiments with [1,6-$^{14}C_2$] and [3-^{3}H] shikimic acid (**10**) have shown that lawsone (**6**), from the plant *Impatiens balsamina,* is derived from shikimate with C-1 and C-2 appearing at the naphthoquinone ring junction (Scheme 2). The carboxy-group of shikimic acid is retained on naphthoquinone formation, (indicated by * in Scheme 2), and this accounts for seven of the ten carbon atoms in **6**. Glutamic acid or its transamination product 2-ketoglutaric acid (**11**) provides the three remaining carbons, incorporating C-2, C-3, and C-4. The 6-*pro R* hydrogen is lost, while the 6-*pro S* hydrogen is retained.[44]



Scheme 2. ^{14}C and ^{3}H - Labeled Shikimic Acid to Lawsone

The incorporation of [2'-^{14}C-carboxyl]-4-(2'-carboxyphenyl)-4-oxobutanoic acid (**12**) into iso-α-lapachones in *Catalpa ovata* has been reported[45], including an elegant study establishing the intermediacy of 2-carboxy-4-oxo-α-tetralone (COT)

13, and 2-prenyl-COT (**14**) for the biosynthesis of these types of compounds. Because of the instability of these compounds, **13** was isolated as it's decarboxylation product, and **14** was isolated in the form of it's methyl ester. The percent incorporation of **12** into the substances examined are shown in parentheses under under their structures in Scheme 3. From these values it clearly follows that **12** is incorporated into 3-hydroxy-dehydro-iso-α-lapachone **16**, while incorporation of the radioactivity of **12** into **13**, **14**, and **15** suggested that COT (**13**) and prenyl-COT (**14**) are on the biosynthetic route to naphthoquinone congeners.



Scheme 3. ^{14}C Incorporation into Iso-α-lapchones in *Catalpa ovata*

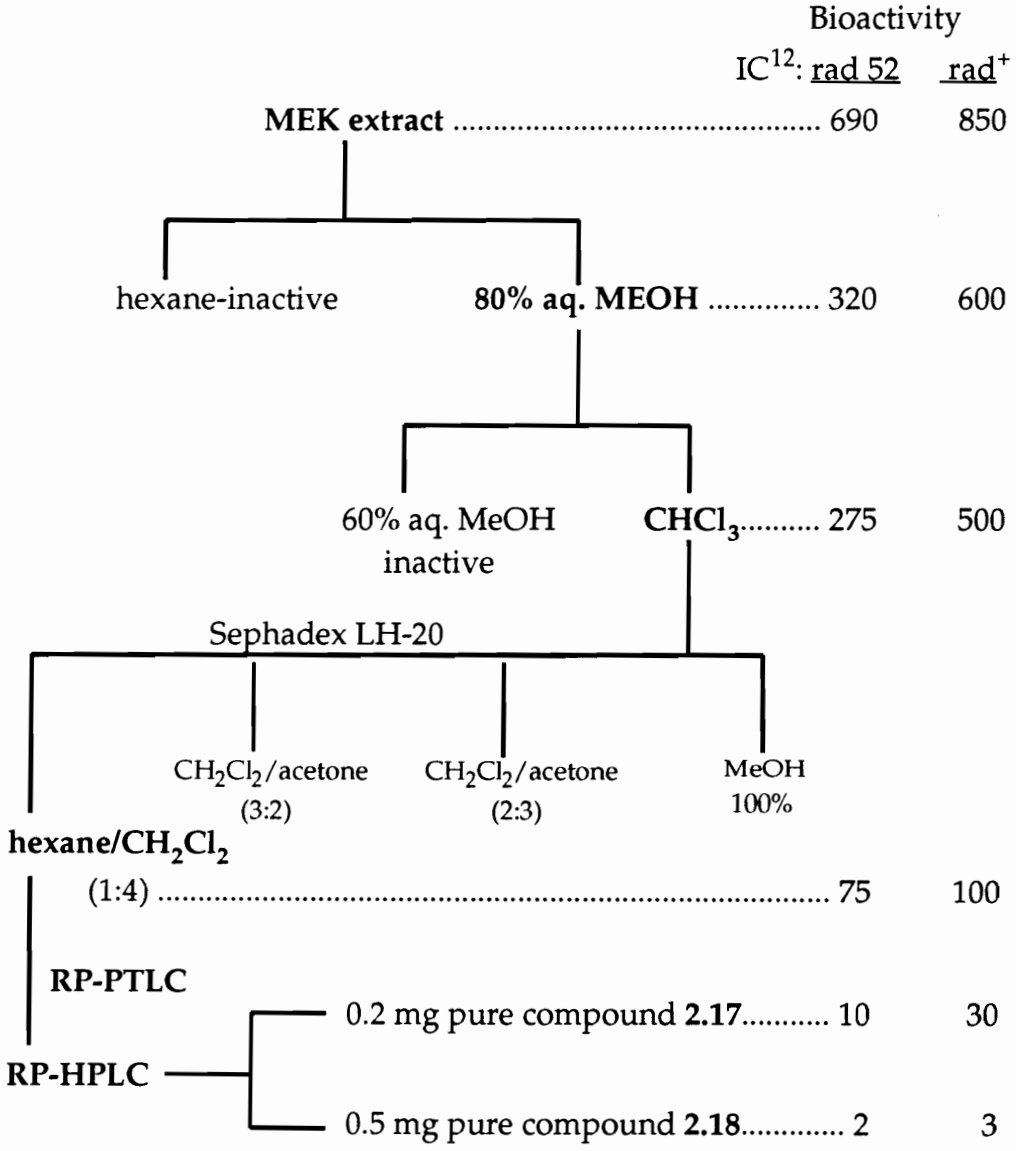
2.2 RESULTS AND DISCUSSION

2.2.1 Isolation of Furanonaphthoquinones from *Crescentia cujete*

As part of a systematic survey of plants for anticancer activity, the wood of *Crescentia cujete* L. was found to show reproducible bioactivity in our yeast-based DNA damaging assay. This plant was thus selected for detailed investigation. Wood chips of the stem of *Crescentia cujete* were pulverized and extracted sequentially with at room temperature with hexanes, methyl ethyl ketone (MEK), methanol, and water. Activity in the rad 52 yeast strain assay was limited to the MEK extract, which gave IC_{12} values of 690 against rad 52 and 840 against the wild type (rad^+). Although these values did not indicate a strongly selective activity, investigation of this plant was initiated with the expectation that selectivity would develop as compounds were purified.

From 3.3 kg of dried plant material, 12 g of crude MEK extract was obtained, and were subjected to solvent-solvent partitioning with hexane-80% aqueous methanol. A small amount of the aqueous MeOH fraction was evaporated, dried, and bioassayed, revealing increased bioactivity and selectivity (IC_{12} rad52= 320, IC_{12} rad^+= 600). The hexane fraction proved to be inactive. The 80% aq. MeOH fraction was then diluted to 60% aqueous MeOH and extracted thoroughly with $CHCl_3$ to give a $CHCl_3$ fraction in which the activity was concentrated. Small scale isolation procedures were then investigated in order to determine the stability of the active compounds. One procedure was to attempt separation by use of gel filtration chromatography, utilizing Sephadex LH-20 and eluants of increasing polarity from hexane:CH_2Cl_2(1:4), CH_2Cl_2:acetone(3:2),

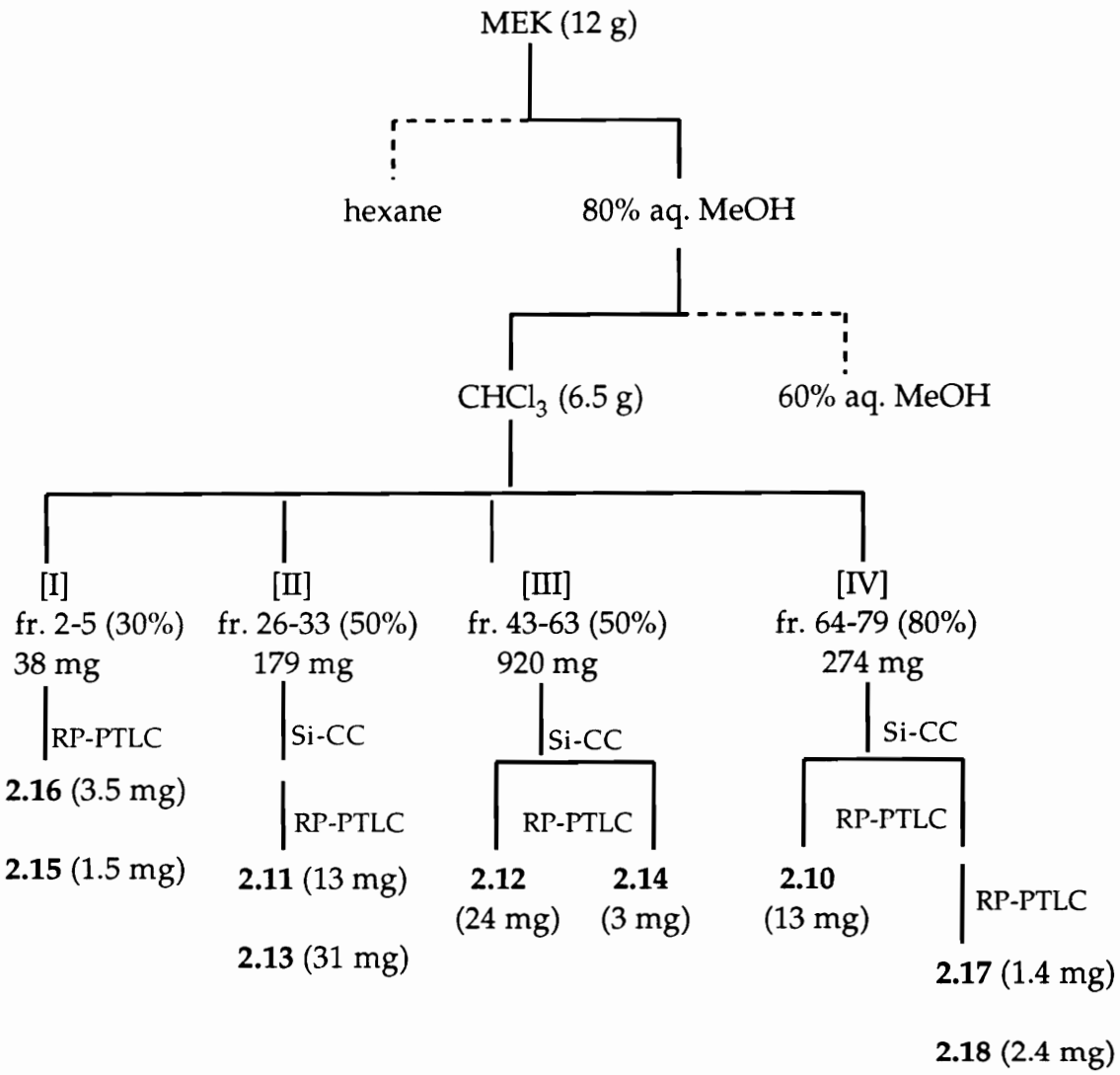
CH_2Cl_2:acetone(1:4), and finally methanol[46]. As a result of this procedure, activity was retained and isolated in the hexane:CH_2Cl_2(1:4) fraction (IC_{12} rad 52 = 75, IC_{12} rad$^+$ = 100). From this fraction, reversed - phase HPLC was used in order to isolate two active constituents, with IC_{12} values in rad 52 of 10 and 2 (Scheme 5).



Scheme 4. *C. cujete* Initial Isolation Procedure

Although the above described procedure allowed the isolation of constituents without loss of activity, it was very time consuming. Thus an alternative method was devised using silica gel chromatography immediately following the solvent - solvent partitioning. After a small scale trial, this method proved suitable as activity was retained, and large - scale isolation proceeded as follows.

Silica gel flash chromatography of the dried $CHCl_3$ extract yielded four active fractions. These active fractions were further purified by additional cc, normal and reversed phase ptlc, and reversed phase HPLC (activity was monitored by single dose response testing in rad 52 only), yielding in addition to the two originally sought constituents [**2.17**, **2.18**], a total of seven more bioactive compounds [**2.10-2.16**] (Scheme 5). All of the compounds were obtained as brightly colored solids, and UV, IR, and ^1H NMR spectra suggested they were furanonaphthoquinones with various functionalities.



Scheme 5. Modified Isolation Scheme for *C. cujete*

2.2.2 Structure Elucidation of Novel Furanonaphthoquinones

2.2.2.1 Naphthoquinone **2.10**

The high resolution electron impact mass spectrum (HREIMS) of **2.10** indicated that it had the molecular formula $C_{17}H_{16}O_6$, and possible

fragmentation is shown in scheme 7. Its UV and IR spectra were consistent with those for other naphthoquinones[29], and comparison of the ^{1}H NMR spectrum of **2.10** with those of known isolapachones[35-41] indicated that **2.10** belonged to this class.

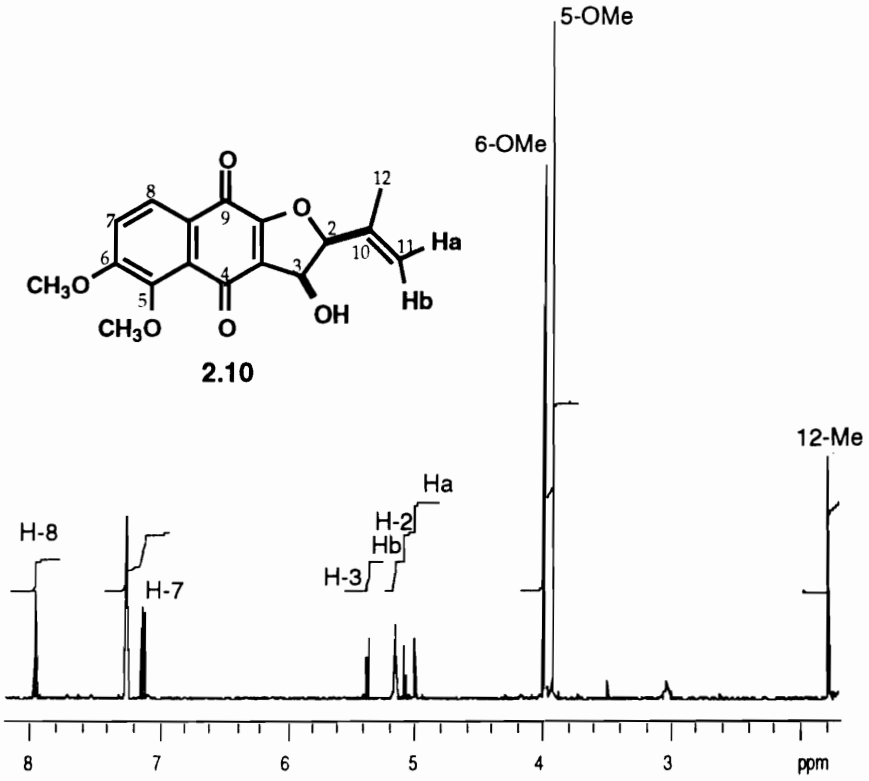


Figure 1. ^{1}H nmr spectrum of **2.10**

Two 3H singlets at δ 3.92 and 3.99 implied the presence of two MeO groups, and two *ortho*-coupled doublets for aromatic protons at δ 7.12 and 7.96 indicated that the MeO groups must be assigned to the 5,6 or to the 7,8 positions. The remaining proton signals of **2.10** could be assigned to an isopropenyl group and an OH group, which must be located at the 2- and 3-positions, respectively,

on the basis of the chemical shifts of the 2- and 3-protons[47] and HMBC (multiple bond C-H) NMR correlations. Conflicting ^{1}H-NMR assignments for the terminal olefin protons (H-11a and H-11b) in the literature[47,48] prompted the use of nOe difference NMR experiments to settle the discrepancy. Irradiation of the signal at δ 1.79 due to the Me group enhanced the signal of H-11a at δ 5.00, and H-11b at δ 5.15 was unaffected, while an nOe on the Me group was seen upon irradiation of H-11a, whereas irradiation of H-11b showed no significant enhancement of the Me group signal.

In addition, irradiation of H-2 at δ 5.08 enhanced the Me group signal, but had no effect on H-3, and likewise irradiation of H-3 at δ 5.08 showed enhancement of the Me group signals with no effect on H-2, suggesting that the C-2 isopropenyl and the C-3 hydroxyl group are *cis* with respect to each other. Relevant nOe spectra are shown in Figure 2.

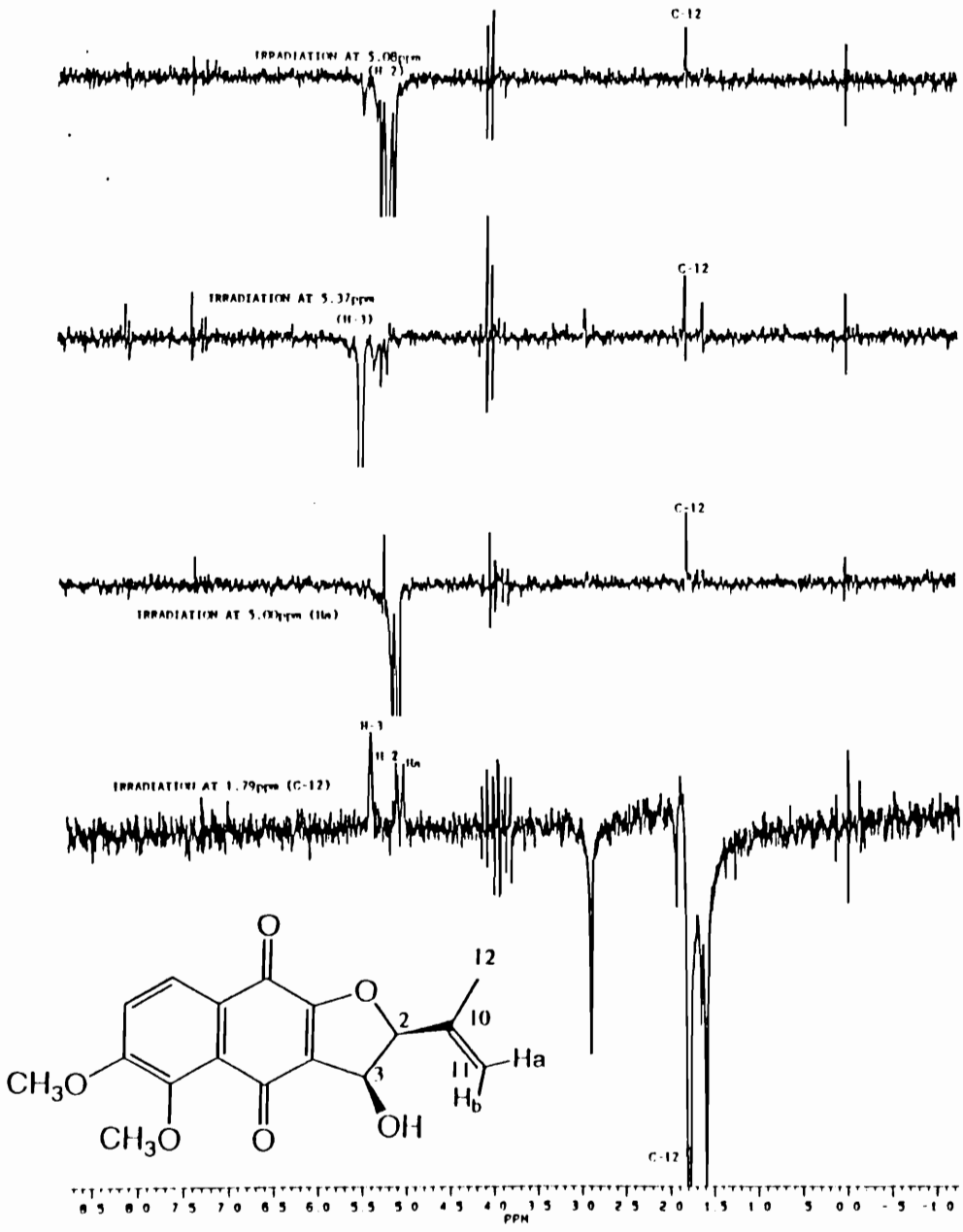
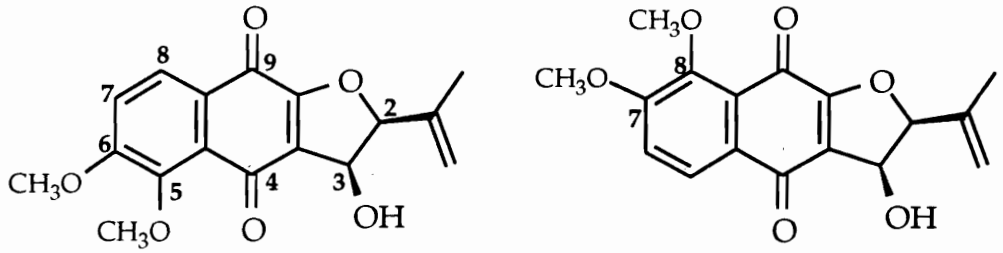


Figure 2. NOe Experiments on **2.10**

These data indicated that compound **2.10** had the structure **2.10** or the alternate structure **2.10-B** in which the methoxy groups were at the 7- and 8-positions on the aromatic ring. Assignment of the structure as **2.10** was achieved by the observation of long-range C-H couplings. In structure **2.10**, the aromatic proton (H-8) would show long-range coupling to the C-9 carbonyl group, and the H-3 proton would show a similar long-range coupling to a *different* (C-4) carbonyl group. On the other hand, the aromatic proton (now H-5) and H-3 in the alternate structure **2.10-B** would both show long range coupling to the *same* C-4 carbonyl group.



2.10-B

Because of the low three bond C-4 to H-3 coupling constant ^3J (C,H)=1.5 Hz, this correlation was not seen in a typical HMBC nmr experiment, and selective INEPT methods[48,49] were used to locate the MeO groups. Thus, irradiation of H-8 at δ 7.96 ppm under selective INEPT conditions (4 Hz) enhanced the signal for C-9 at δ 177.0 ppm, while irradiation of H-3 at δ 5.37 ppm (1.5 Hz) enhanced the signal of C-4 at δ 182.6 ppm (Figure 3); these data clearly showed that the MeO groups are at the 5,6 positions.

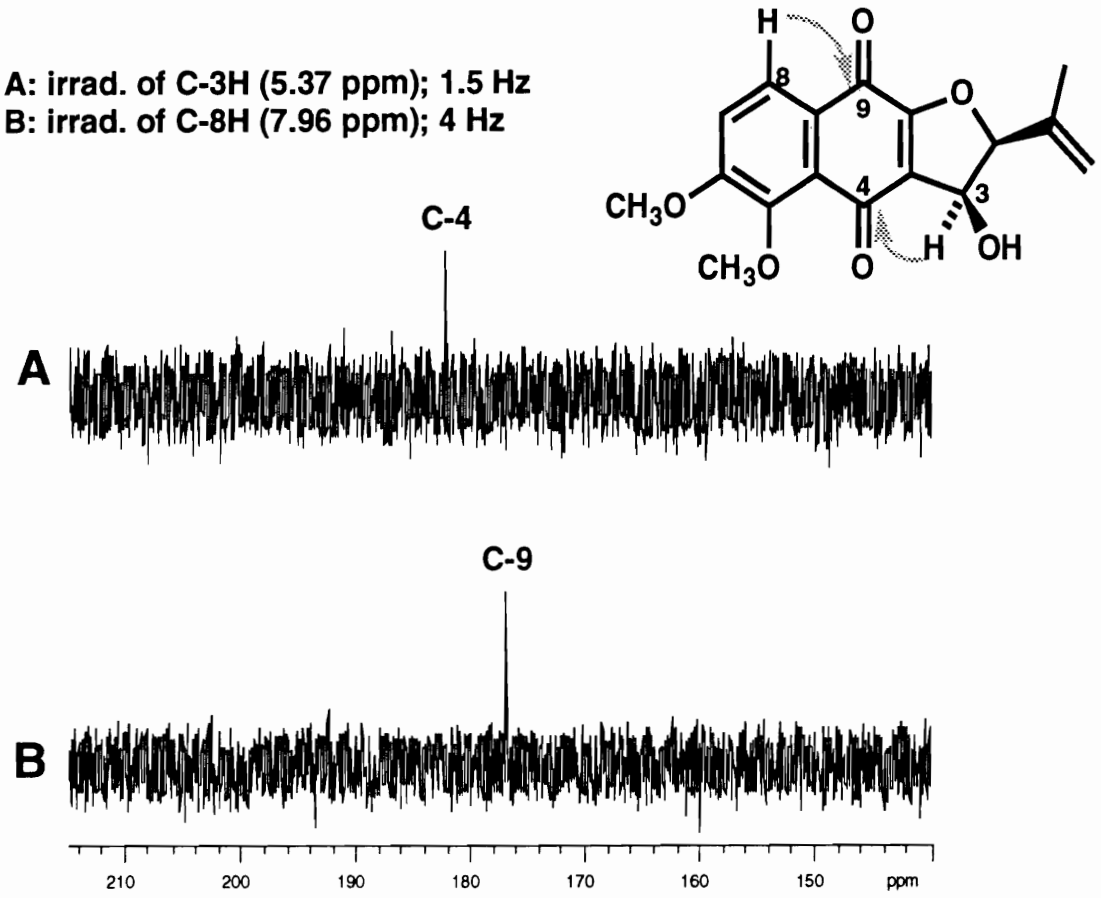
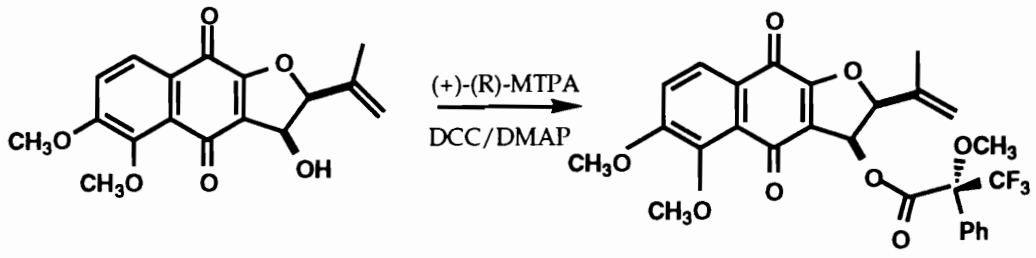


Figure 3. Selective INEPT Spectra for Compound **2.10**

The relative stereochemistry of the isopropenyl and OH groups was determined to be *cis* not only on the basis of the nOe studies, but also because of the observed $J_{2,3}$ value of 4.5 Hz.[47]

Compounds similar to **2.10** have been isolated as mixtures of enantiomers on some occasions.[40,48] In order to determine if both enantiomers of **2.10** were present, its (+) α-methoxy-α-trifluoromethylphenylacetate (MTPA) ester derivative was prepared as shown in scheme 6.[50]



2.10 **2.19**

Scheme 6. Formation of Mosher Ester of **2.10**

The ^{1}H NMR spectrum of this ester showed the C-3 proton doublet shifted downfield from δ 5.37 to 6.60, and it appeared as a single resonance (figure 6). The new MeO group (from the MTPA ester) appeared as a single resonance at δ 3.54. The fact that both of these signals appeared as single resonances indicated the presence of a single enantiomer.

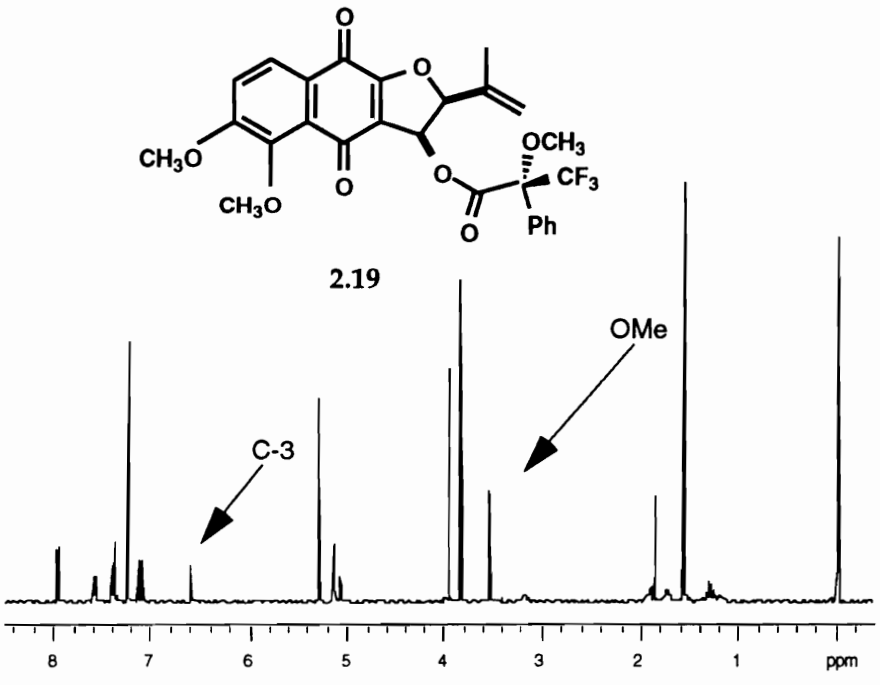


Figure 4. ^{1}H NMR spectrum of **2.19**

Determination of the absolute stereochemistry was made by comparison of the CD spectrum of **2.10** with those of known compounds **2.20**[51] and **2.21**.[47] The CD spectrum of **2.10** appears to be the mirror image of those reported for **2.20** and **2.21**. Since the configurations at C-2 and 3 have been established as *R,R* for **2.20** and **2.21**, it follows that the opposite configurations exist in **2.10**; this is supported by the fact that the specific rotations of **2.20** and **2.21** are opposite in sign to that for **2.10** (see Figure 7). Hence compound **2.10** is assigned as (2*S*,3*S*)-3-hydroxy-5,6-dimethoxydehydroiso-α-lapachone. This is a novel structure, and complete ^{1}H- and ^{13}C-nmr assignments are given in Tables 2 and 3, respectively. HMBC[91] and HMQC[91] correlations (Figure 8 and Table 1) used to make complete ^{13}C assignments follow Figure 7. Complete NMR assignments were made in order to facilitate the structure elucidation of similar compounds which may be isolated in the future.

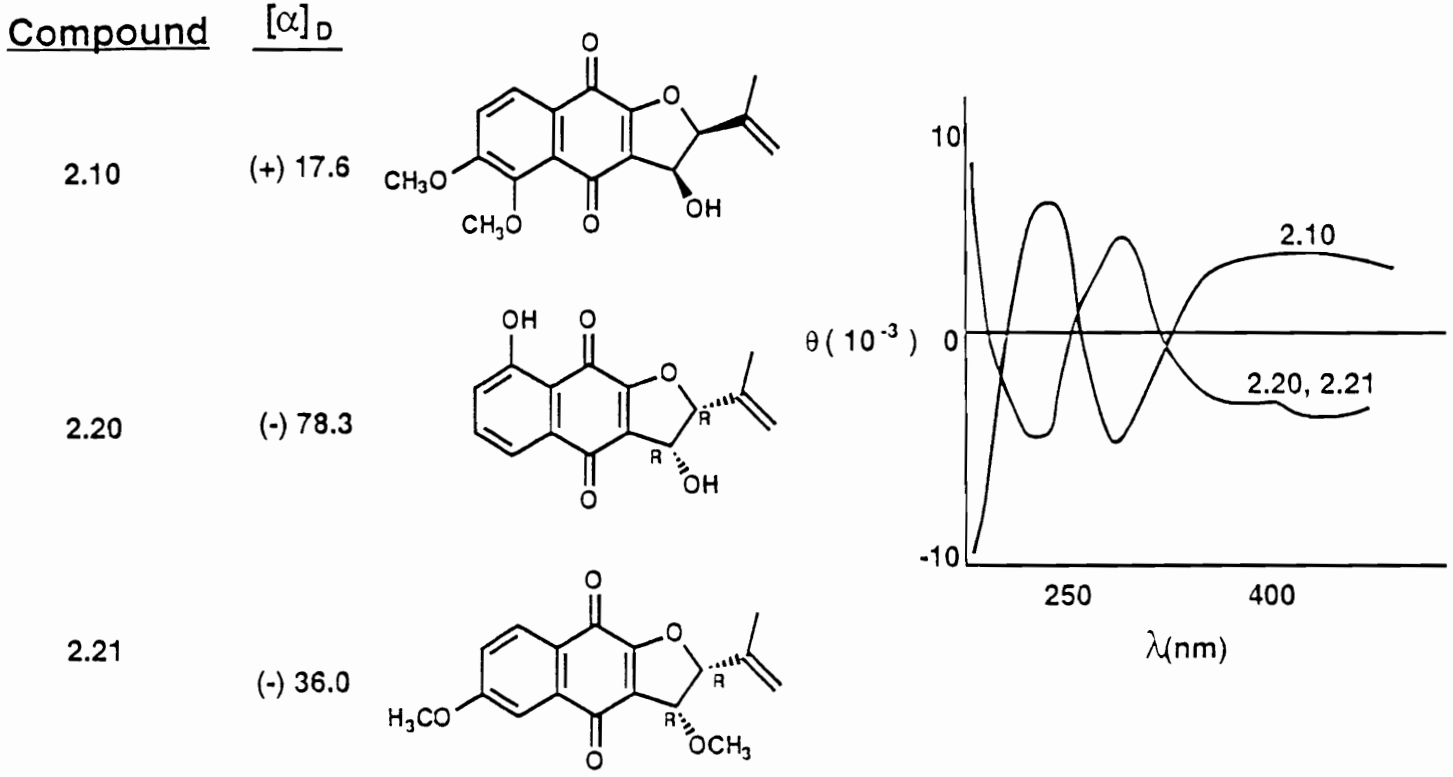


Figure 5. CD Study for Stereochemical Determination

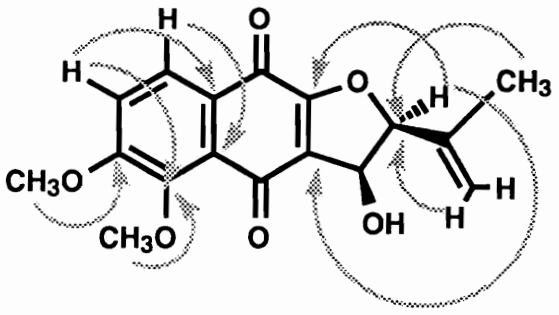
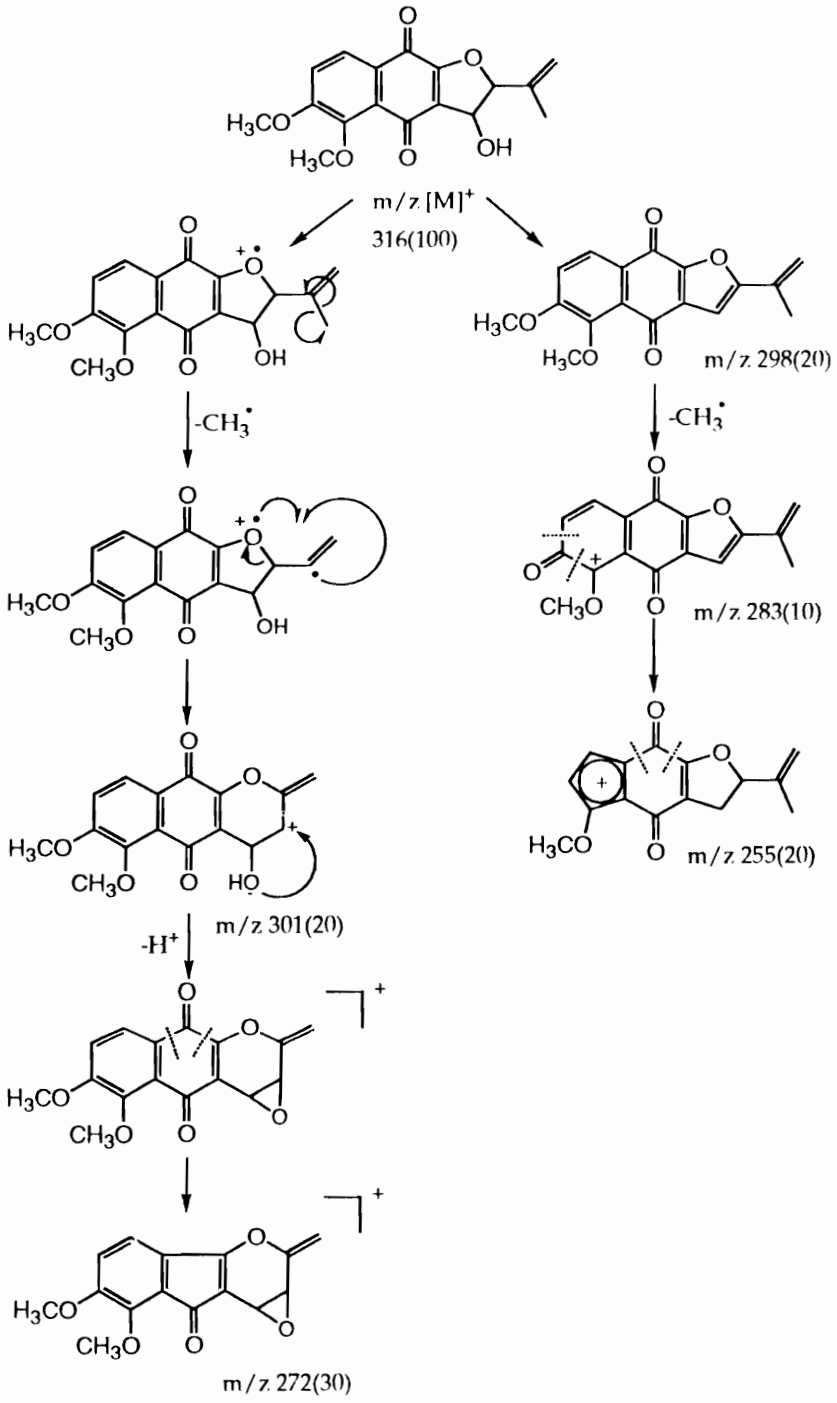


Figure 6. HMBC Correlations for Compound **2.10**

Table 1. NMR Techniques Used to Assign ^{13}C Shifts for **2.10**

POSITION	ASSIGNMENTS BASED ON
2	HMQC, HMBC from H_3-12, H-11
3	HMQC
3a	HMBC from H-3
4	Selective INEPT
4a	HMBC from H-8
5	HMBC from 5-OCH_3, H-7
6	HMBC from 6-OCH_3, H-8
7	HMQC
8	HMQC
8a	HMBC from H-7, H-8
9	HMBC from H-8, selective INEPT
9a	HMBC from H-2, H-3
10	HMBC from H-3
11	HMQC
12	HMQC
5-OCH_3	HMQC, HMBC (OCH_3-C-5)
6-OCH_3	HMQC, HMBC (OCH_3-C-6)



Scheme 7. Mass Spectral Fragmentation of **2.10**

2.2.2.2 Naphthoquinone **2.11**

Compound **2.11** was assigned the composition $C_{17}H_{16}O_5$ by HREIMS, and its IR spectrum, while similar to that of compound **2.11**, showed no signal for a hydroxyl group. The ^1H NMR spectrum of **2.11** indicated the presence of two MeO groups and an isopropenyl group. Its ^1H spectrum also contained the two ortho coupled aromatic doublets as seen for compound **2.10**.

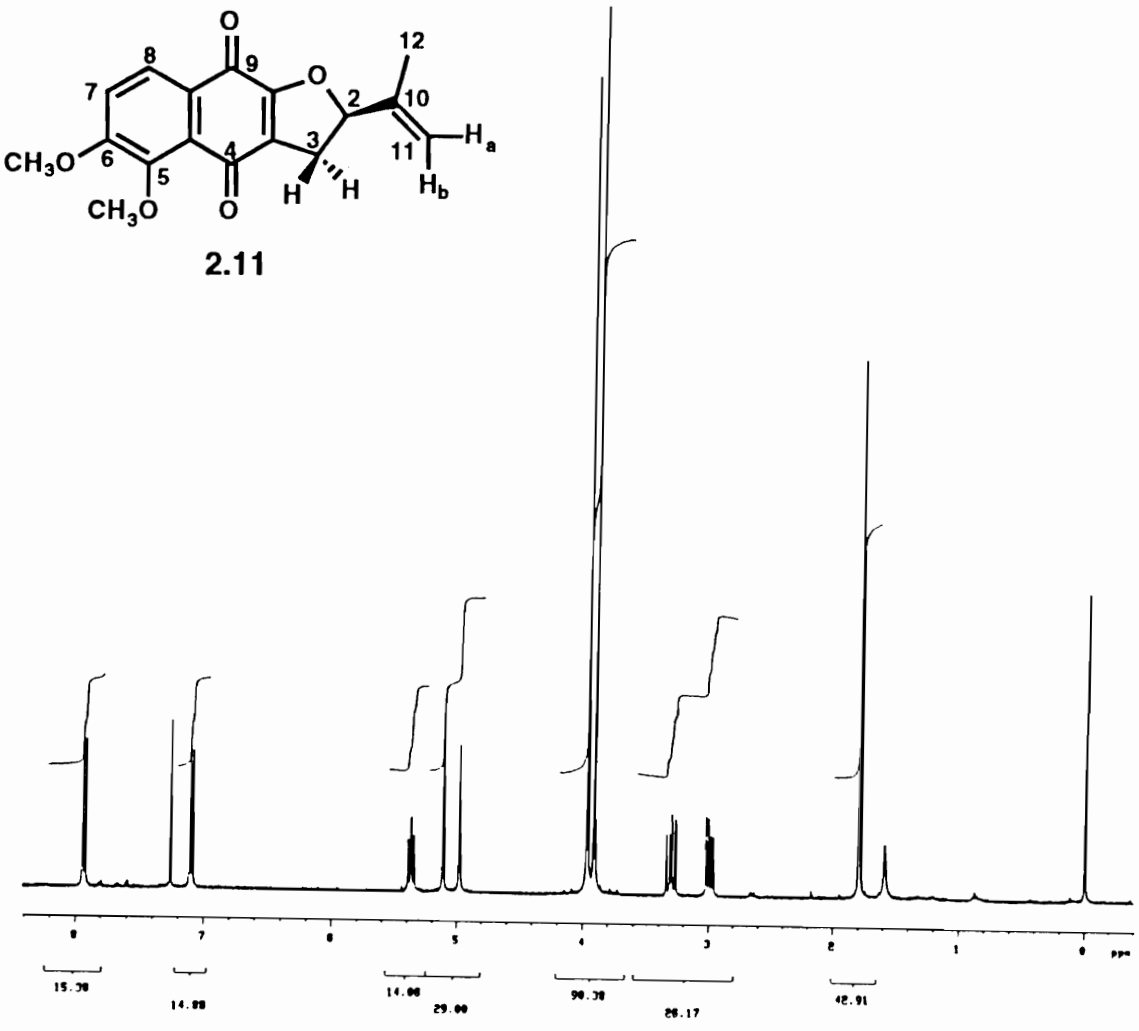


Figure 7. ^1H NMR Spectrum of Compound **2.11**

The major difference between the ^{1}H-nmr spectra of **2.10** and **2.11** was at C-3, where **2.11** showed two doublets of doublets at δ 2.99 and 3.30, consistent with the presence of a 2,3-dihydrofuran ring. Assignments for protons H-11a and H-11b were based on nOe difference nmr experiments, as for compound **2.10**. Irradiation of H-11a at δ 4.98 enhanced the Me group signal, and had no effect on the C-3 protons (Figure 10). Irradiation of H-11b at δ 5.11 also enhanced the Me group signal, but to a lesser degree than when H-11a was irradiated (Figure 11). More importantly, irradiation at H-11b showed enhancement of both C-3 protons at δ 2.99 and 3.30. Irradiation of the Me group protons at δ 1.79 showed enhancement of H-11a and had no effect on H-11b (Figure 12). Irradiation of the signal at δ 1.79 also showed enhancement of the H-3 signal at δ 2.79, defining it as the β proton. These experiments clarify the discrepancy of the ^{1}H NMR literature values for the C-11 protons and also add definitive assignments for the C-3 α and β protons which have not been previously reported.

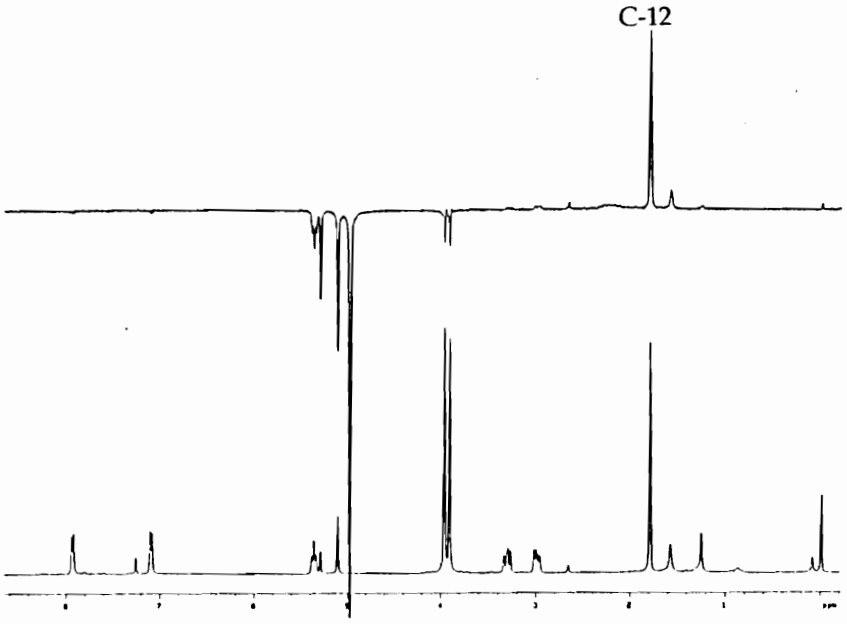
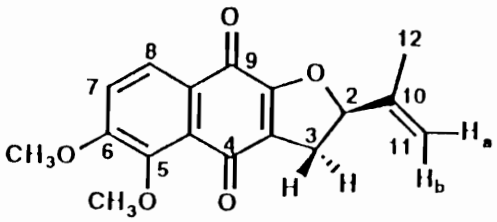


Figure 8. Irradiation of Ha (4.98 ppm)



2.11

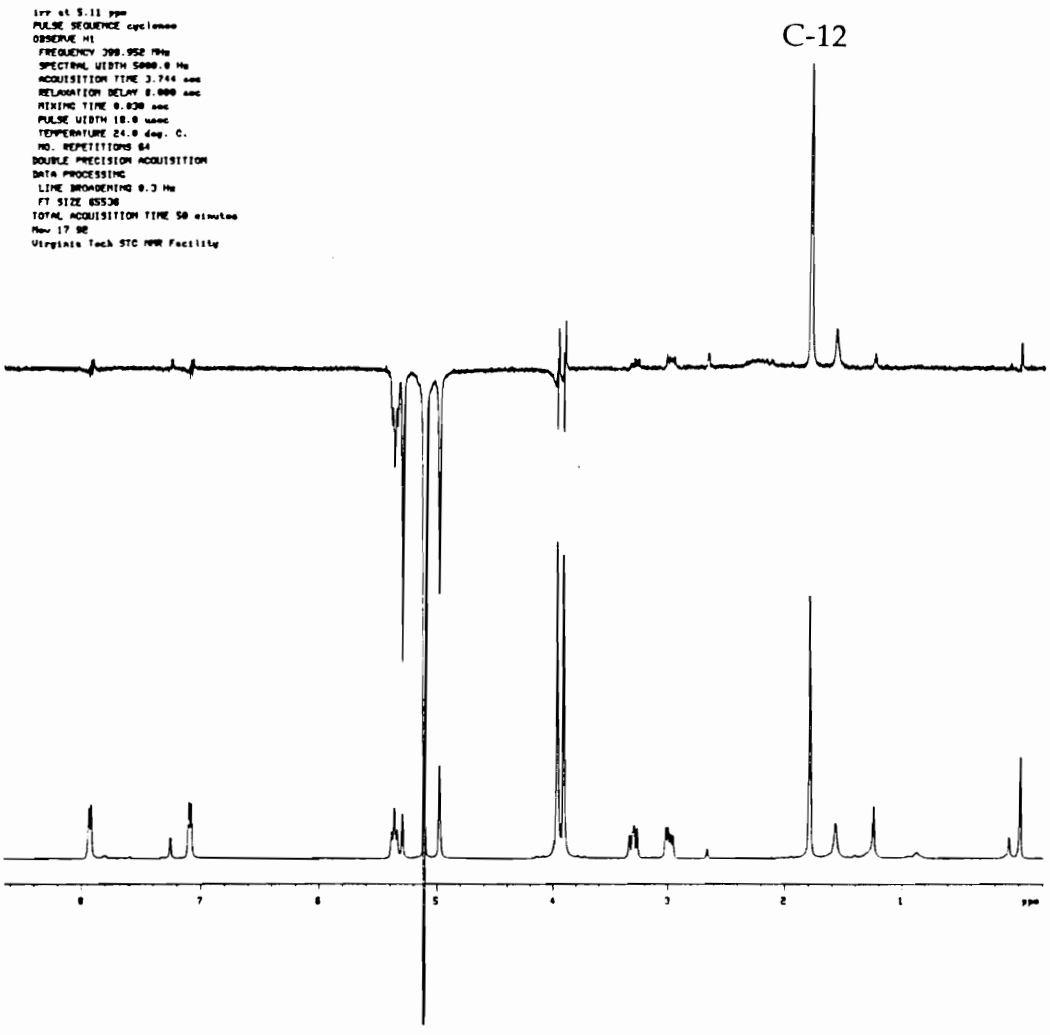


Figure 9. Irradiation of Hb (5.11 ppm)

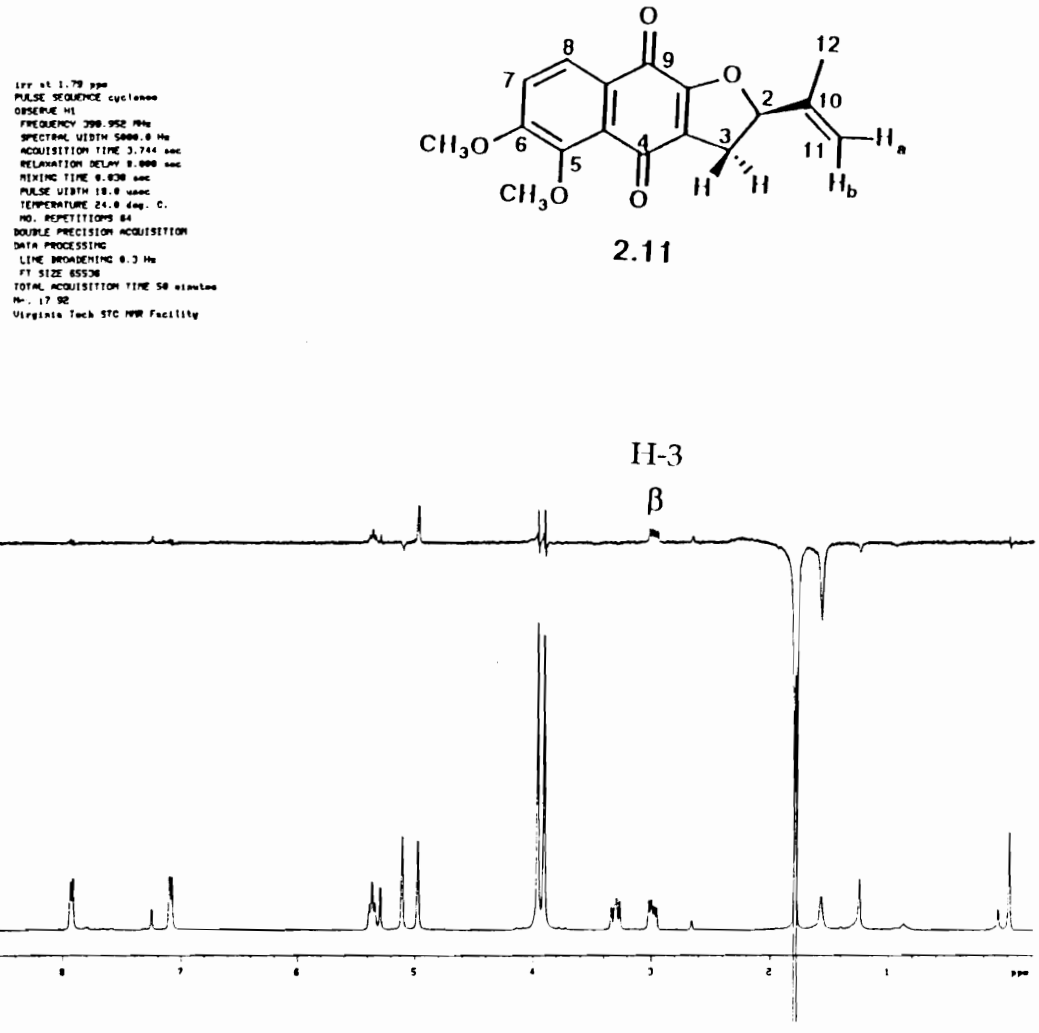


Figure 10. Irradiation of C-12 Me (1.79 ppm)

Assignment of the MeO groups to the C-5 and C-6 positions was again achieved by selective INEPT experiments. Irradiation of H-8 at δ 7.94 (4 Hz) enhanced C-9 at δ 176.8, while irradiation of H-3α at d 2.99 (1.5 Hz) enhanced the signal for C-4 at δ 182.1. It is interesting to note that irradiation of H-3β under these conditions (1.5Hz) did not show enhancement of C-4.

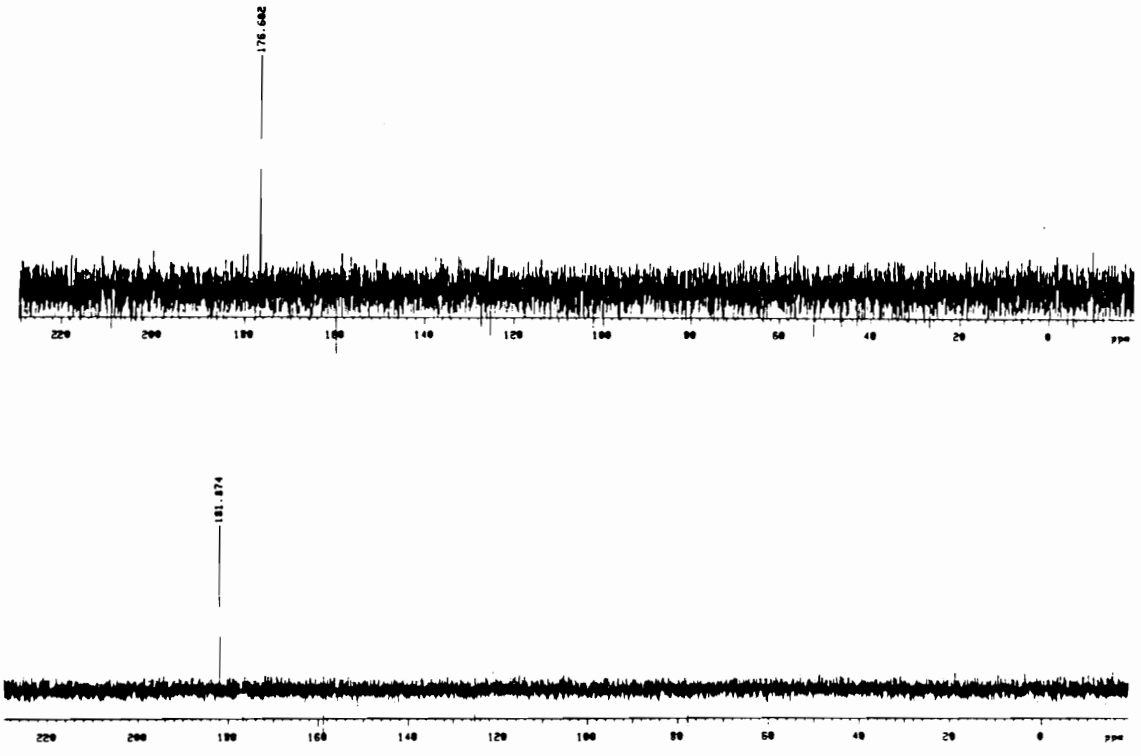
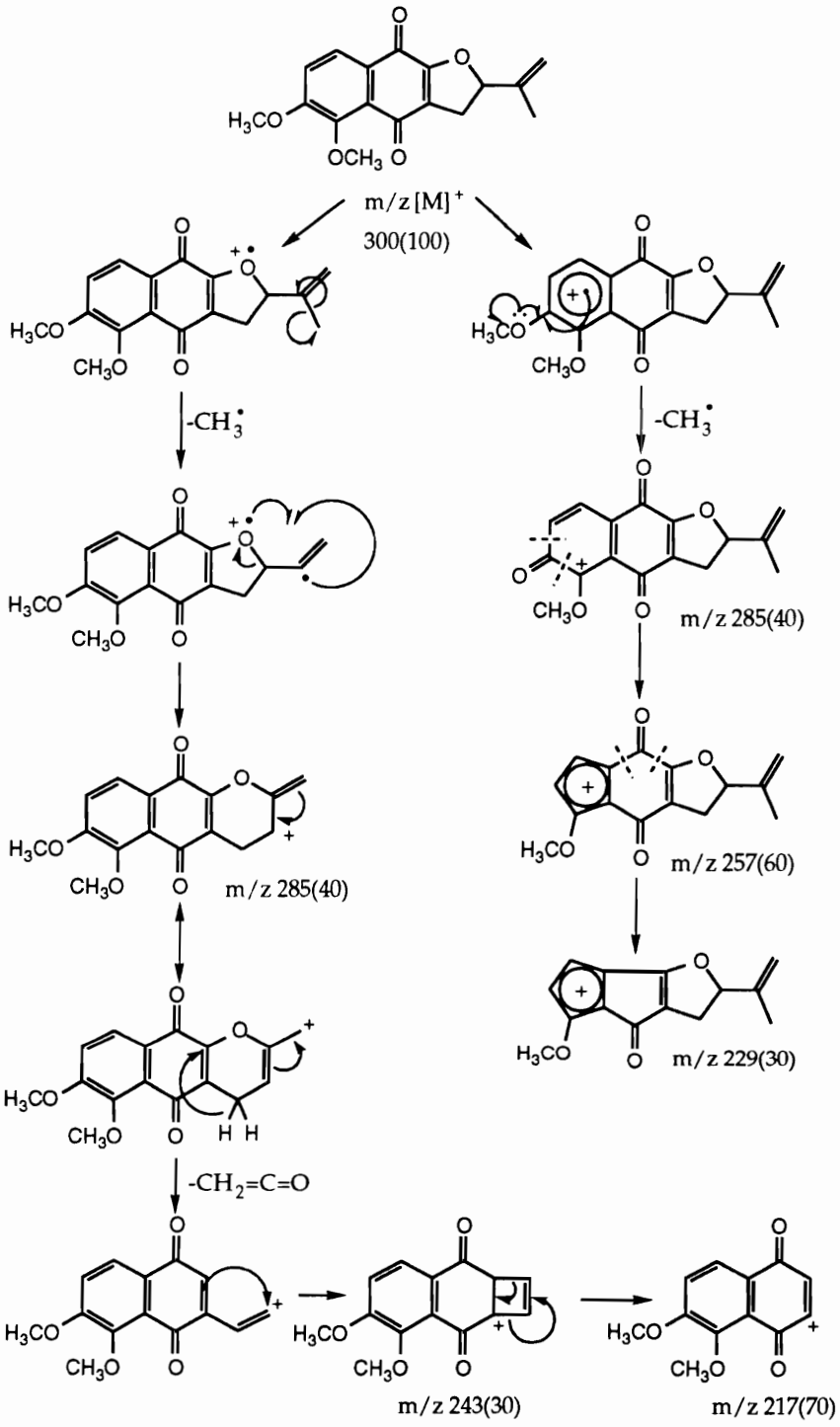


Figure 11. Selective INEPT NMR Experiment on **2.11**

HMBC experiments at 6 and 9 Hz allowed complete ^{13}C NMR assignments and these are given in Table 3. Complete ^{1}H NMR assignments are given in Table 2.

The absolute stereochemistry of **2.11** at C-2 was determined by comparison of its CD spectrum with that for compound **2.23**.[51] The cd spectrum of **2.11**, although shifted slightly to a longer wavelength, is nearly identical to that for **2.23**, each characterized by a negative first Cotton effect. CD data is listed in the experimental section. Thus, it was concluded that **2.11** is (2*R*)-5,6-dimethoxydehydroiso–α–lapachone. A literature search revealed that this is a new compound.



Scheme 8 MS Fragmentation of **2.11**

2.2.2.3 Naphthoquinone **2.12**

The HREIMS of **2.12** indicated that it had the molecular formula $C_{16}H_{14}O_4$, and it showed a fragmentation pattern similar to **2.11**. Its UV and IR spectra were also similar to those for **2.11.**

It's ^1H NMR spectrum showed the presence of an isopropenyl group as well as the multiplets for the C-3 protons (δ 2.99 and 3.32) of the furan moiety as seen for compound **2.11**. The ^1H NMR spectrum indicated the presence of a single MeO group; an ABX pattern and the coupling constants for the aromatic proton signals (δ 7.66, 7.62, and 7.71) suggested that it be placed at either C-5 or C-8.

Selective INEPT experiments assigned the MeO group to C-5: irradiation of H-8 at δ 7.66 (4 Hz) enhanced C-9 at δ 177.7, while irradiation of H-3α at δ 2.99 (1.5 Hz) enhanced the signal for C-4 at δ 182.5 ppm. Complete ^1H and ^{13}C-nmr assignments are given in Tables 2 and 3, respectively. Its ^1H NMR spectrum may be found in the appendix.

The determination of the absolute stereochemistry of **2.12** was based on the fact that its CD spectrum is nearly identical to that for compound **2.11**; the same reasoning used for **2.11** applies here as well. Thus, **2.12** is (2*R*)-5-methoxydehydroiso-α-lapachone, another new structure.

Complete NMR data for the new compounds **2.10 - 2.12** are listed in Tables 2 and 3.

2.2.2.4 ^{1}H and ^{13}C NMR Data for Compounds **2.10-2.12**

Table 2: ^{1}H NMR Chemical Shifts (400 mHz) of Isolapachones **2.10-2.12** in $CDCl_3$.[a]

Proton	COMPOUND		
	2.10	**2.11**	**2.12**
2	5.08 (d,4.6)	5.38 (dd,10.6, 9.2)	5.37 (dd,10.4,9.1)
3 a	5.37 (d,4.6)	3.30 (dd,17.4,10.3)	3.32 (dd,17.5,6.7)
b		2.99 (dd,17.4,8.7)	2.99 (dd,17.5,8.8)
6	--	--	7.31 (dd,8.4,0.98)
7	7.12 (d,8.6)	7.10 (d,8.6)	7.62 (dd,8.5,7.5)
8	7.96 (d,8.6)	7.94 (d,8.6)	7.66 (dd,7.7,1.1)
11H$_a$	5.00 (d,0.5)	4.98 (d,0.9)	4.98 (d,0.9)
11H$_b$	5.15 (d,0.9)	5.11 (d,0.9)	5.11 (d,0.9)
12	1.79 (s)	1.79 (s)	1.79 (s)
5-OCH$_3$	3.92 (s)	3.91 (s)	3.99 (s)
6-OCH$_3$	3.99 (s)	3.97 (s)	--

[a] Chemical shifts (relative to TMS) are in ppm and observed peak spacings (in parenthesis) in Hz

Table 3: ^{13}C-nmr Chemical Shifts (100.57 mHz) of Isolapachones **2.10-2.12** in $CDCl_3$.[a,b]

Carbon	COMPOUND		
	2.10	**2.11**	**2.12**
C-2	95.1	88.5	88.3
C-3	75.4	32.4	32.5
C-3a	125.0	125.5	126.0
C-4	182.5	182.1	182.5
C-4a	124.1	126.1	134.24
C-5	149.5	149.5	159.7
C-6	160.1	160.0	119.2
C-7	114.5	114.3	134.18
C-8	125.2	124.8	119.6
C-8a	125.9	125.8	120.0
C-9	177.0	176.8	177.7
C-9a	159.7	159.1	157.9
C-10	139.4	141.9	142.0
C-11	113.9	113.9	113.8
C-12	17.2	17.0	17.0
C-5 OCH_3	56.4	56.4	56.7
C-6 OCH_3	61.3	61.4	--

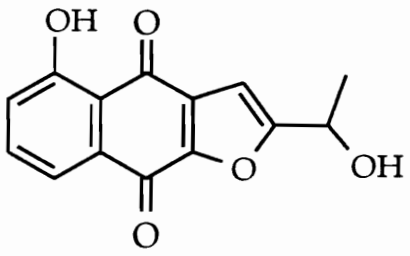
[a]In ppm from TMS

[b]Assignments based on HMQC (140 Hz), and HMBC (3 and 8 Hz) correlations

2.2.3 Identification of Known Furanonaphthoquinones

2.2.3.1 Naphthoquinone **2.13**

Compound **2.13** was isolated as a yellow-orange powder and it's MP, mass spectrum, ^{1}H and ^{13}C NMR spectra indicated it to be a known compound, namely, 5-hydroxy-2-(1'-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione[48].



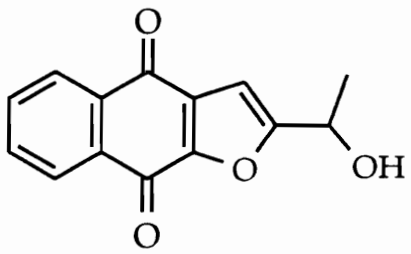
2.13

This compound has an interesting history in the literature. The positional isomer of **2.13** (**2.8**), has been reported from *Kigelia pinnata*[38] , *Tabebuia cassinoides*[40], and from *Crescentia cujete.*[41]. Compound **2.8** has been named[38] "Kigelinone", but each of these references have proposed and supported this structure without convincing spectral or chemical evidence (Rao and Kingston[40] offered both isomers as possible structures). From *Tabebuia avellanedae*, Wagner *et al* .[48] isolated a compound that has chemical properties identical to those reported for "Kigelinone", and using NMR techniques including selective INEPT assigned it the same structure as **2.13**. They have suggested that, due to identical ^{1}H NMR shifts, "Kigelinone" may in fact be the 5-OH positional isomer **2.13**. In their report they called upon the scientific community to reinvestigate

"Kigelinone" isolated from these sources, and in the case of *C. Cujete*, this has been accomplished herein by thorough examination of **2.13**. ^{1}H and ^{13}C NMR data are identical to those reported by Wagner, and the ^{1}H NMR of **2.13** is identical to that reported for "Kigelinone" previously isolated from *C. cujete* . Selective INEPT experiments under the conditions reported by Wagner have supported the placement of the aromatic OH at position 5 for compound **2.13**, thus establishing the correct structure. Complete ^{1}H and ^{13}C NMR shifts and selective INEPT conditions are listed in the experimental section.

2.2.3.2 Naphthoquinone **2.14**

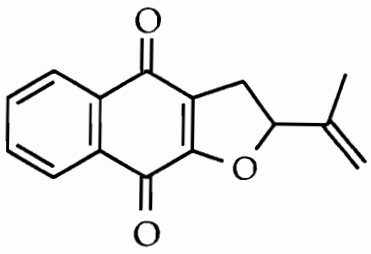
The mass spectrum and ^{1}H NMR of **2.14** indicated it was the known[40] compound 2-(1-hydroxyethyl)-naphtho[2,3-b]furan-4,9-dione. Due to lack of material, the absolute configuration of the chiral center on the side chain was not determined, but it may be noted that the compound isolated from *T. cassinoides* was obtained as a racemate.[40] Proton NMR and mass spectral data are included in the experimental section.



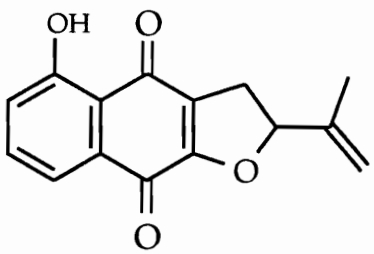
2.14

2.2.3.3 Naphthoquinones **2.15** and **2.16**

Two more known furanonaphthoquinones were isolated in small amounts, and from their MS and ^1H NMR spectra, they were asigned as 2-isopropenylnaphtho[2,3-b]furan-4,9-dione (**2.15**)[52] and 5-hydroxydehydroiso-α-lapachone (**2.16**).[47]



2.15



2.16

Due to scarcity of material, and because these are known compounds, no investigations into the stereochemistry of the C -2 position were carried out. Regarding **2.16**, no definitive experiments to confirm the placement of the phenolic OH were undertaken, however, it was assigned to the 5- postion based on its chemical shift being identical to that reported.[47] Proton NMR and mass spectral data may be found in the experimental section.

2.2.3 Biological Evaluation of Compounds **2.10-2.16**

The biological activity data for compounds **2.10-2.16** in our mechanism-based yeast mutant bioassays, and the cytotoxicity data for selected compounds are given in Table 4. All seven compounds showed moderate but selective

activity against the repair-deficient rad 52 yeast strain, indicating that they act as DNA-damaging agents. Compounds **2.10 - 2.14** were also tested for cytotoxicity against Vero cells; the highest potencies were shown by compounds **2.13** and **2.14**, both of which have an OH group in their side chain. Considering compounds **2.10**, **2.11** and **2.12**, it appears that having an OH group at position 3 increases selectivity without a cost in cytotoxicity. Compounds containing a phenolic OH group appear to be the most cytotoxic. The activity of **2.14** against Vero cells is about an order of magnitude greater than its previously reported cytotoxicity to KB cells.[40]

Table 4: Bioactivity of Furanonaphthoquinones **2.10-2.16**

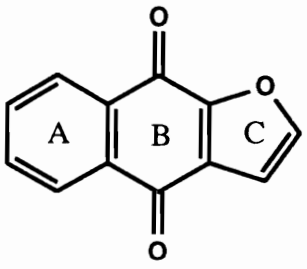
Compound	YEAST STRAIN		CYTOTOXICITY TO VERO CELLS
	(rad 52) [a]	(RAD +) [a]	IC_{50}, mg/ml
2.10	47	>1000	3.7
2.11	33	120	4.7
2.12	48	280	4.3
2.13	3	10	0.21
2.14	14	180	0.41
2.15	60	130	NT [c]
2.16	80	140	NT [c]
Camptothecin[b]	0.6	110	0.054

[a]Results are expressed as IC_{12} values in μg/ml (concentration required to produce an inhibition zone of 12 mm around a 100 μl well in the yeast strain).
[b]Standard reference compound.
[c]Not tested.

2.2.4 Structure Elucidation of Furofuranonaphthoquinones

2.2.5.1 Naphthoquinone **2.17**

There was a total of only 1.4 mg of pure **2.17** isolated, and full characterization was carried out with this small amount. This compound had IC_{12} values in rad 52 and rad^+ of 10 and 30, respectively, indicating selective DNA damaging activity. It had the composition $C_{15}H_{18}O_5$ as determined by HREIMS. Its IR and UV spectra, together with the co-occurrence of isolapachones already described, suggested that **2.17** possessed the furanonaphthoquinone substructure **I.**



I

The presence of an unsubstituted A ring in **2.17** was indicated by two overlapping multiplets of two protons each centered at δ 7.90 and 8.12 in its ^1H NMR spectrum.

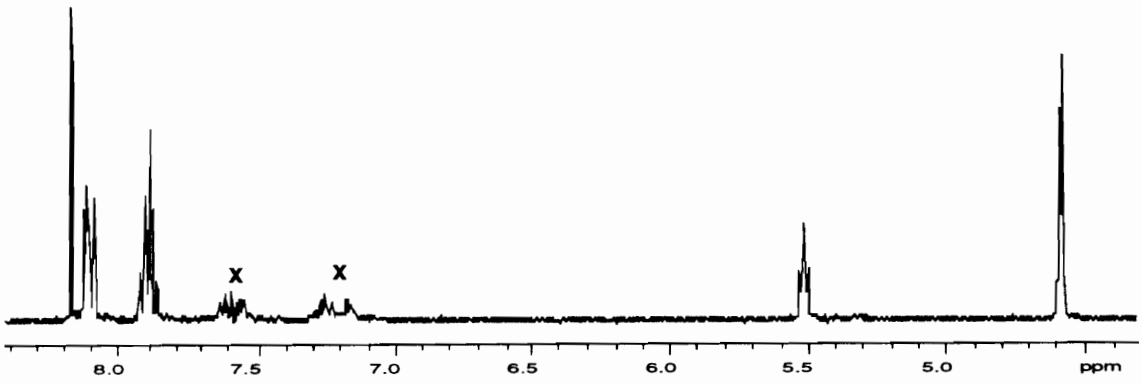
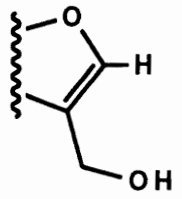


Figure 12. ^{1}H NMR Spectrum of **2.17** in DMSO

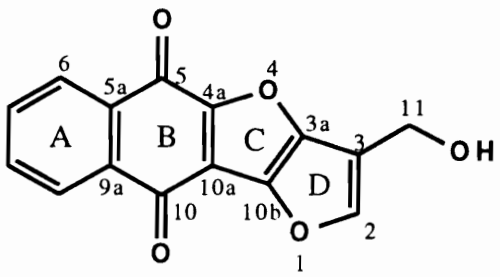
The high-field region of the ^{1}H NMR spectrum of **2.17** showed a two-proton doublet at δ 4.59 (J=5.2 Hz) coupled to a one-proton triplet at δ 5.51 (J=5.2 Hz); this latter signal was lost when the spectrum was obtained in the presence of added D_2O. These data indicated the presence of an isolated-CH_2OH group, and this was supported by a ^{13}C NMR signal for a CH_2 group at δ 54.5.

The substructure **I** and the CH_2OH group account for 13 of the 15 carbons and 4 of the 5 oxygens present in **2.17**. The remaining carbons appear in the ^{13}C-NMR spectrum as a methine carbon at δ 150.8 and a fully substituted olefinic carbon at δ 116.5, and the remaining proton appears as a broad singlet at δ 8.18.

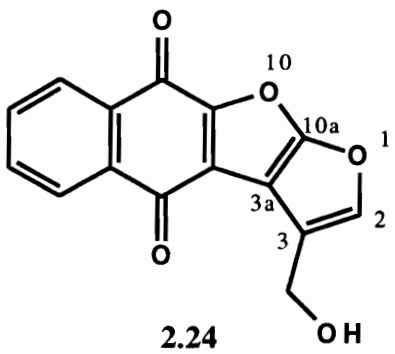


II

These data lead inevitably to the assignment of substructure **II** to the remaining atoms, and combining substructures **I** and **II** leads to the formulation of the new compound either as **2.17** or as **2.24**.



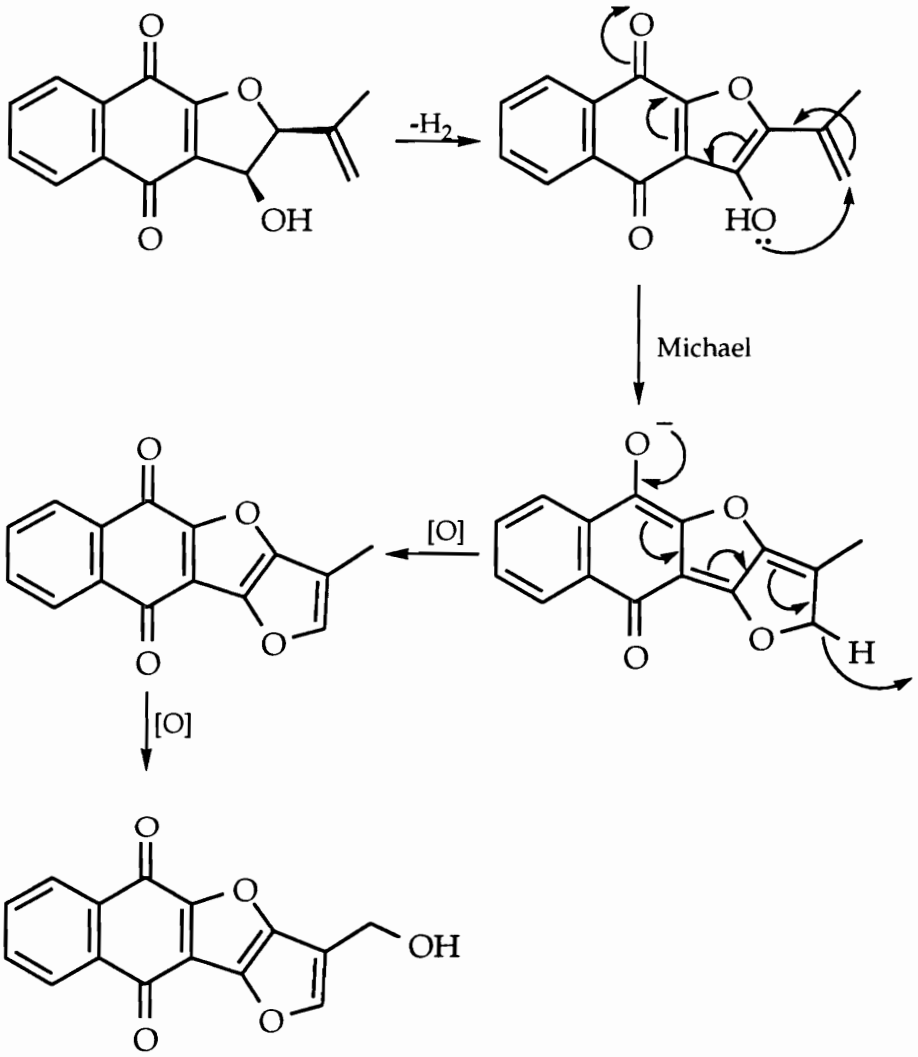
2.17



2.24

Structure **2.17** was indicated as the correct structure for several reasons. In the first place, the chemical shift of the C-2 proton at δ 8.18 was so far downfield that it must be deshielded by being in conjugation with a quinone carbonyl group, since the C-2 protons of simple furans resonate at about δ 7.0.[54] This arrangement can only be achieved in structure **2.17**. Secondly, the chemical shifts of carbons 3a and 10b in **2.17** are very similar (δ 151.9 and 141.2 respectively). The corresponding carbons in the alternate structure **2.24** would be expected to show very different chemical shifts, with carbon 10a appearing much further downfield (typically δ 161-163).[55,56] Finally, a proposed biogenetic

scheme starting with a similar compound (co-metabolite **2.10** minus the C-5 and C-6 methoxy groups) offers a possible pathway for the formation of the new ring skeleton (Scheme 9).



2.17

Scheme 9. Possible Biosynthetic Pathway for **2.17**

A ^{1}H-^{1}H - COSY nmr experiment established a long - range correlation between the C-11 methylene protons and the C-2 methine proton (Figure 13).

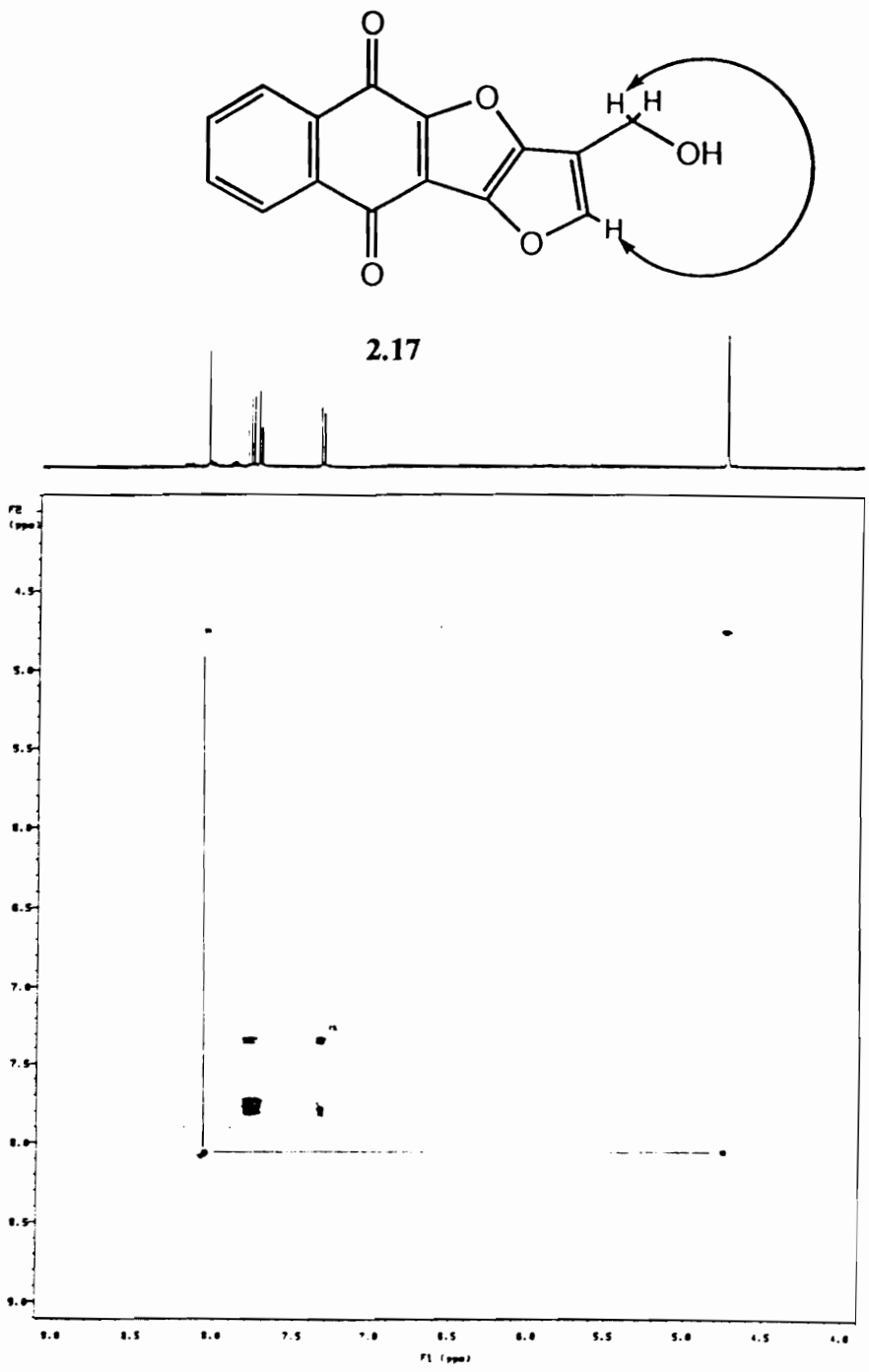


Figure 13. ^{1}H-^{1}H COSY NMR Spectrum of **2.17**

Assignments of the carbons of the furofuran ring system in **2.17** were established by HMBC experiments at 6 and 8 Hz, which showed the following correlations (Figure 17): H-11 (δ 4.59) to C-3a (δ 151.9), C-3 (δ 116.5), and C-2 (δ 150.8); H-2 (δ 8.18) to C-3a (δ 151.9) and C-10b (δ 141.2).

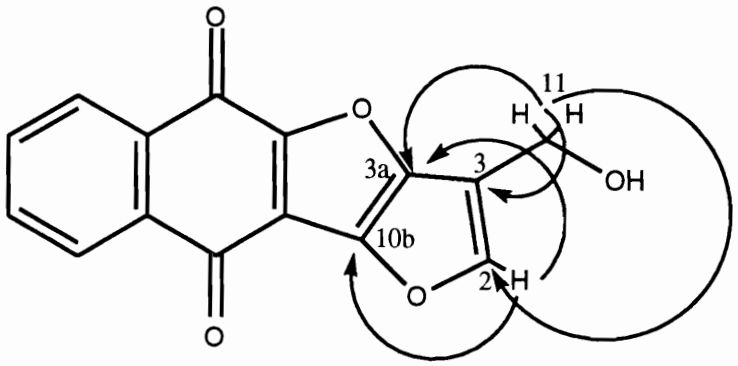


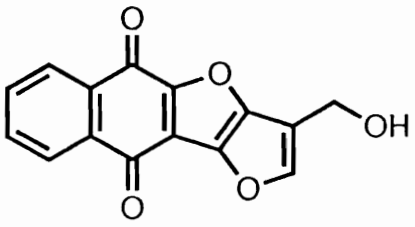
Figure 14. Some Selected HMBC correlations of **2.17**.

Complete NMR assignments for **2.17** were made by a combination of HMBC, HMQC, HETCOR, and DQF-COSY and DEPT experiments. It should be noted that because of the minute amount of material that was isolated and the large number of quaternary carbons in **2.17**, carefully optimized conditions were required in order to procure a usable spectrum. This involved the use of DMSO as a solvent in order to increase solvent - substrate interactions to help reduce tumbling, and the use of a 30° tip angle. Under these conditions, and using a 400 MHz NMR spectrometer, it still required a 24 hour run to obtain an acceptable signal to noise ratio. Because of the increased sensitivity inherent in inverse - detected 2-D experiments, the lengths of the HMBC and HMQC experiments were shorter than the simple ^{13}C run, but they still required overnight runs.

The carbonyl carbons were first assigned on the basis of literature data which shows that the carbonyl carbon of a furanonaphthoquinone *ortho* to the

furan oxygen resonates upfield of that *meta* to the furan oxygen.[57] The Δδ (*meta* carbon minus *ortho* carbon) values range from 0.1 to 6 ppm, but the relative relationship remains constant; this result can be explained on the basis of resonance and inductive effects on the electron density at these carbons. In compound **2.17** the carbonyl carbons appeared at δ179.1 and 173.0, and the signal at δ 173.0 was thus assigned to C-5.

The protons at positions 6-9 were observed as overlapping multiplets which could not be assigned by simple HETCOR or HMBC experiments. A highly resolved DQ-COSY experiment, however, showed that proton signals centered at δ 8.09 and 7.88 were mutually coupled, as were signals centered at δ 8.11 and 7.87 (figure 15).



2.17

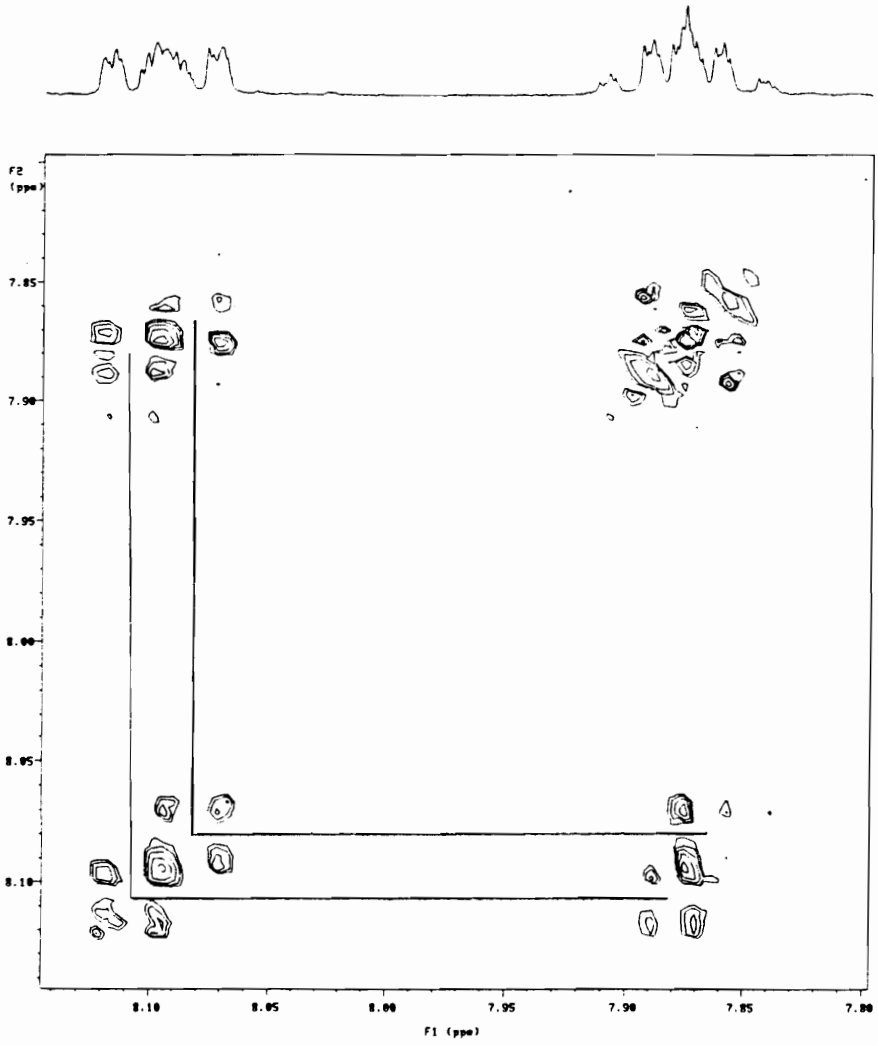


Figure 15. Highly Resolved DQ-COSY Experiment on **2.17**

HMBC data showed three-bond ^{1}H-^{13}C coupling of the proton signal centered at δ 8.09 to the C-10 carbonyl at δ 179.1, and between the signal centered at δ 8.11 and the C-5 carbonyl at 173.0, defining the protons as those at C-9 and C-6 respectively. The proton signals at δ 7.88 and 7.87 could then be assigned to protons on C-8 and C-7 respectively. HMQC and HETCOR experiments then allowed the assignment of carbons 6-9, and HMBC data enabled the assignments of carbons 5a and 9a to be made.

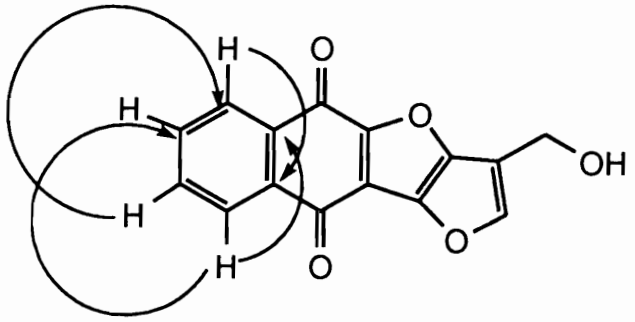
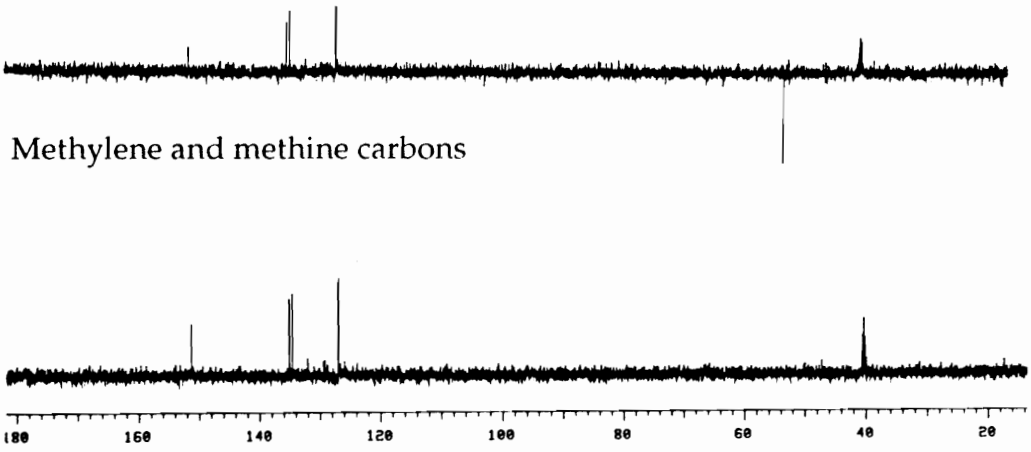


Figure 16. Additional HMBC Correlation's for **2.17**

Multiplicities of the carbon resonances were verified by use of an edited DEPT experiment (figure 20). Complete ^{1}H and ^{13}C assignments for **2.17** are given in Table 5.



Methylene carbons negatively phased
Methine carbons positively phased

Figure 17. DEPT NMR Experiment for **2.17**

Table 5. ^{1}H and ^{13}C NMR Data of Compound **2.17** [a]

Position	δH [b]	δC [c]	HMBC [d]
2	8.18 br s	150.8 d	H-11
3		116.5 s	H-2, H-11, CH_2OH
3a		151.9 s	H-2, H-11
4a		153.3 s	
5		173.0 s	H-6
5a		132.0 s	H-9, H-7
6	8.11 m	126.42 d [b]	
7	7.87 m	134.0 d [b]	
8	7.88 m	134.5 d [b]	
9	8.09 m	126.35 d [b]	
9a		132.2 s	H-8, H-6
10		179.1 s	H-9
10a		117.9 s	
10b		141.2 s	H-2
11	4.59 d (5.20)	54.5 t	H-2
11-OH	5.51 t (5.20)		

[a] Solvent: DMSO; Chemical shifts (relative to TMS) are in ppm, observed peak spacings in parenthesis. ^{1}H and ^{13}C spectra at 400 and 100.57 MHz, respectively.

[b] Deduced from COSY and HETCOR/HMQC analysis

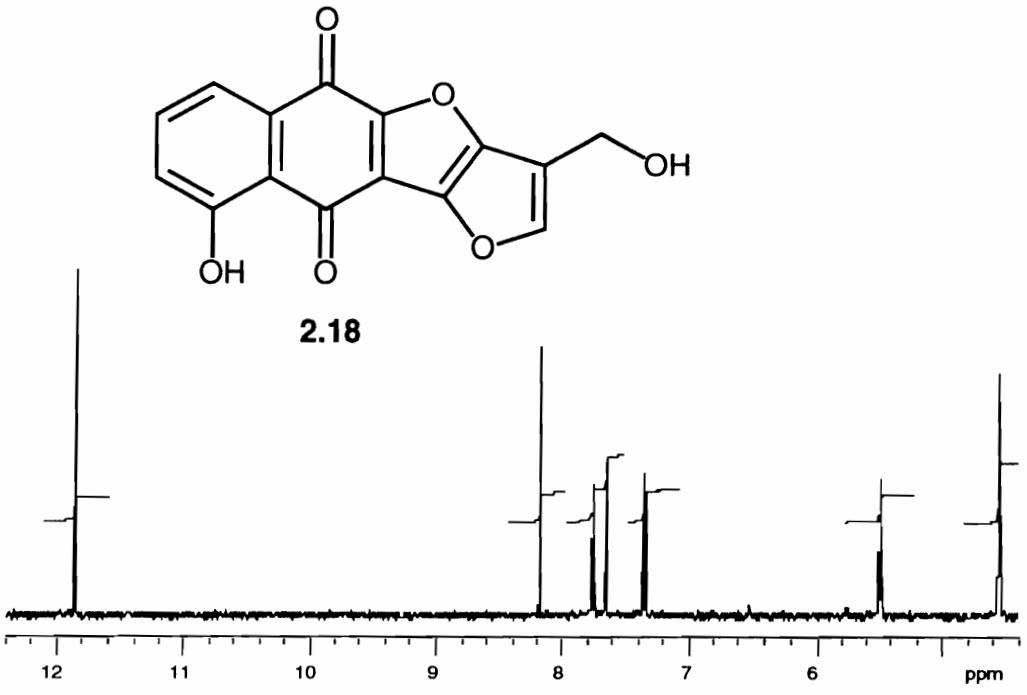
[c] Deduced from DEPT experiment

[d] Protons showing multiple-bond coupling to the indicated carbon (experiments at 6 and 8 Hz)

2.2.5.2 Naphthoquinone **2.18**

Compound **2.18** had the composition $C_{15}H_8O_6$, as determined by HREIMS and ^{13}C-NMR spectroscopy. Its UV and NMR spectra indicated that it was

closely related to compound **2.17**, and this was confirmed by a detailed analysis of the NMR spectra. The ^{1}H NMR spectrum of **2.18** showed three aromatic protons in an ABX pattern and a low-field signal at δ 11.85 (exchangeable with D_2O), suggesting the presence of a chelated hydroxyl group in either the 6- or the 9-position. The presence of a chelated phenolic OH group is also evident as the UV spectrum of **2.18** exhibits a bathochromic shift when treated with NaOH. The signals for the furofuran part of the structure of **2.18** were essentially identical to those for the corresponding protons of **2.17**.



2.18

Figure 18. ^{1}H NMR Spectrum of **2.18**

Proof of the structure of **2.18** as the 9-hydroxyderivative of **2.17** was obtained by analysis of its ^{13}C NMR spectrum. The better resolution of the aromatic protons in **2.18** made ^{13}C NMR assignments possible by HMBC (Figure

19) and HMQC, and the data fully support the assigned structure. Employing the previous argument used for assigning the carbonyl group signals, the lower field δ 184.7 resonance must be attributed to C-10 and the signal at δ 172.1 must be assigned to C-5. Placement of the hydroxyl group at position 9 was supported by a selective INEPT experiment, in which irradiation of the proton at position 6 at δ 7.65 caused enhancement of the C-5 carbonyl group signal at δ 172.1.

Table 6. ^{1}H and ^{13}C NMR Data of Compound **2.18** [a]

Position	dH [b]	dC [c]	HMBC [d]
2	8.18 br s	150.9 d [b]	H-11
3		116.5 s	H-2, H-11, CH_2OH
3a		152.1 s	H-2, H-11
4a		153.1 s	
5		172.1 s	H-6
5a		132.7 s	H-7
6	7.65 dd (8.4, 0.9)	119.3 d [b]	H-8
7	7.77 dd (7.9, 7.9)	136.9 d [b]	
8	7.36 dd (7.5, 0.9)	124.7 d [b]	H-6
9		161.5 s	H-7
9-OH	11.85 s		
9a		114.8 s	H-6, H-8
10		184.7 s	
10a		117.9 s	
10b		141.0 s	H-2
11	4.56 d (5.2)	52.3 t [b]	H-2
11-OH	5.50 t (5.2)		

[a] Solvent: DMSO; Chemical shifts (relative to TMS) are in ppm, observed peak spacings in parenthesis. ^{1}H and ^{13}C spectra at 400 and 100.57 MHz, respectively.

[b] Deduced from COSY and HETCOR/HMQC analysis

[c] Deduced from DEPT experiment

[d] Protons showing multiple-bond coupling to the indicated carbon

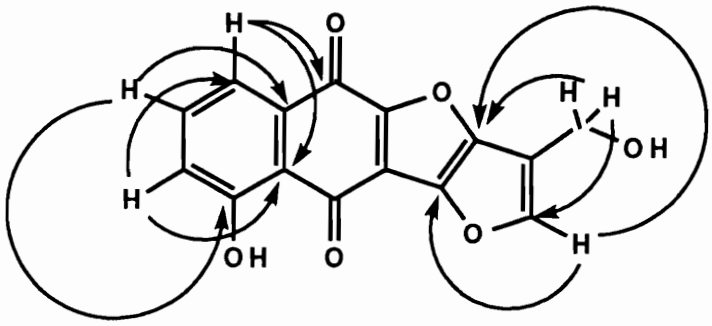


Figure 19. HMBC Correlations for **2.18**

2.2.6 Summary and Biological Activity of Compounds **2.17** and **2.18**

The structures of compounds **2.17** and **2.18** are unique. A search of the Ring Index revealed that this particular ring system has not previously been prepared synthetically, much less isolated as a natural product. Both compounds **2.17** and **2.18** show activity in the yeast-based DNA-damaging assay, but only compound **2.17** showed selective activity. Compound **2.18** was almost as active in the wild-type strain as in the rad 52 repair-deficient strain, and is thus presumably simply a general cytotoxic agent. Compound **2.17**, on the other hand, was three times as active against the repair-deficient strain, indicating a selective mechanism of action as a DNA-damaging agent.

Table 7. Bioactivity of Furofuranonapthoquinones **2.17** and **2.18**

Compound	Yeast Strain		Cytotoxicity to Vero cells
	rad52[a]	rad^{+}[a]	IC_{50}, mg/ml
2.17	10	30	2.3
2.18	2	3	2.9
Camptothecin[b]	0.6	110	.019

a Results are expressed as IC_{12} values in mg/ml (concentration required to produce an inhibition zone of 12 mm around a 100ml well in the yeast strain.

b Standard reference compound.

This work demonstrates the ability of the yeast bioassay to lead to the isolation of compounds which have significant activity in other established bioassays. The fact that the isolated compounds are all planar suggests that intercalation into DNA may be involved in the mechanism of DNA damage.

2.3 EXPERIMENTAL

General Experimental Procedures. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were taken in $CHCl_3$ solution with a Perkin-Elmer Model 241 polarimeter. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 400 spectrometer at 400 and 100.57 MHz, respectively, with TMS as an internal standard. Flash chromatography was performed using silica gel Merck G60 (230-400 mesh), preparative TLC with

silica gel GF_{254} plates (Analtech, 500mm, 20 x 20 cm), and reversed-phase preparative TLC with Whatman PLKC18F linear K reversed-phase (1000, and 250 mm, 20 x 20 cm) plates. HPLC experiments were carried out on a Waters apparatus equipped with a photodiode array detector, using a Whatman Partisil 10 ODS-3 (4.6 mm i.d. x 25 cm) column.

PLANT MATERIAL. - The plant material (PR-43072) was collected in Panama in 1974, and a voucher specimen has been deposited at the Herbarium of the National Arboretum, Agricultural Research Service, USDA, Washington, D.C.

BIOASSAY. - In this report, assay results are reported in the form of an IC_{12} value, where IC_{12} is the concentration required to show a inhibition of growth zone of 12 mm diameter around a 100µl well in the yeast strain used. Five appropriate doses are selected, bracketing the anticipated IC_{12} value. The test sample is applied to the agar filled, yeast strain covered plate in a 50:50 mixture of MeOH-DMSO. After a 48 hour incubation period, the zones of inhibition are measured and a linear regression analysis is run to arrive at the IC_{12} value which is recorded. Camptothecin is used as a positive control in doses of 5 and 200 µl for the rad52 and rad^+, respectively.

ISOLATION OF FURANONAPHTHOQUINONES. - Dried wood chips of *Crescentia cujete* (3.2 kg) were ground into mulch, extracted sequentially at room temperature with 5 liters each of hexanes, MEK, MeOH, and finally water. The bioactive constituents were concentrated in the MEK extract (12 g dried). The crude MEK extract (12 g) was partitioned between hexane and 80% aq. MeOH (2

liters each). Water was added to the aq. MeOH fraction until a 60% aq. MeOH mixture was achieved, and this was extracted thoroughly with $CHCl_3$ (3 liters). The $CHCl_3$ extract was dried under vacuum to yield 6.5 g of active material. This fraction (6.5 g) was loaded onto a silica gel column [15 cm x 50 mm (600 ml column volume)] and compounds were eluted with a gradient of EtOAc in hexane (30-80%), collecting 50 ml fractions.

Fractions 2-5 (I) eluted as a single yellow band in 30% EtOAc; they were combined and dried (38 mg). Fractions 26-33 (II) , eluted with 50% EtOAc, came off as an orange band (179 mg). Fractions 43-63 (III) were combined based on similar tlc, then dried (920 mg). Finally, a large orange band eluted with 80% EtOAc into fractions 64-79 (IV) (274 mg). Each of these four fractions showed bioactivity, and each was further purified as described below.

Combined fraction I (38 mg) was applied to two 1000 mm reversed-phase preparative TLC plates (20 x 20 cm) and eluted with 80% aq. MeOH. The two major yellow bands at R_f 0.35 and 0.45 were collected, extracted with acetone and dried to yield 3.5 mg of **2.16** and 1.5 mg of **2.15** .

Fraction II (179 mg) was loaded onto a 15 cm x 18 mm silica gel column and eluted with a gradient of acetone in dichloromethane (0-5%), collecting 10 ml fractions. Fractions 11-14, eluting with 1% acetone, were combined and dried (28 mg). This was applied to two 1000 mm reversed-phase preparative TLC plates, eluted with 80% aqueous MeOH, and the major yellow band was collected and extracted with acetone. The resulting material was recrystallized

from EtOH to yield 13 mg of pure compound **2.11**. Fractions 17-19 were combined and recrystallized from EtOH to yield 31 mg of the known isolapachone **2.13** .

Combined fraction III (920 mg) was subjected to CC on silica gel (50 g) and eluted with a gradient of acetone in CH_2Cl_2 (0-7%); 50 fractions of 25 ml each were collected. Fractions 3-6 were combined and dried to yield 40 mg of material which was applied to two 1000 mm reversed-phase preparative TLC plates and eluted with 70% aqueous MeOH. The major yellow band at R_f 0.4 was collected, extracted with acetone and dried to yield, after recrystallizing from ethanol, 24 mg of pure **2.12**.

A single orange band eluted into fractions 16-18 which were combined, and after reversed-phase preparative TLC on one 1000 mm plate and final purification by reversed-phase HPLC, 3 mg of the known furanonaphthoquinone **2.14** was obtained.

Combined fraction IV (274 mg) was loaded onto a silica gel column (25 g) and eluted with a gradient of acetone in CH_2Cl_2 (3-10%); 50 fractions of 15 ml each were collected. Fractions 27-28 (eluted with 5% acetone) were combined, dried and then applied to reversed phase PTLC plates which were eluted with 80% aqueous methanol. Two UV active bands at R_f 0.35 and 0.45 were collected and washed with EtOAc. The band at R_f 0.35 yielded, after purification by reversed-phase HPLC, 1.4 mg of pure **2.17**. The band at R_f 0.45 yielded, after reversed-phase HPLC, 2.4 mg of pure **2.18**.

Fractions 30-35 were combined and dried to yield 20 mg of material which was applied to one reversed-phase PTLC plate and eluted with 80% aq. MeOH. The major yellow band at R_f 0.35 was collected, washed with acetone and dried. Recrystallizion from EtOH yielded 13 mg of pure compound **2.10**.

(2*S*,3*S*)-3-HYDROXY-5,6-DIMETHOXYDEHYDROISO-α–LAPACHONE [**2.10**]. - Yellow needles; MP =140-142°; $[\alpha]_D$ = (+) 17.59 (1.86, $CHCl_3$); CD (acetonitrile) θ (nm) -20,000(212), +7000(245), +2000(270), +2200(283), -11,000(302), 0(327), +1200(375), 0(485); UV λ max (log e) in MeOH: 270 (4.10), 300 (3.98), 360 (3.59), 406 (3.32); IR ν max (KBr) 3380, 1680, 1655, 1640, 1570, 1480, 1270, 1200, 1050, 970, 845 cm^{-1}; ^1H NMR: see Table 2; ^{13}C NMR: See Table 3; HREIMS *m/z* (rel. int.) 316.0947 (M^+, $C_{17}H_{16}O_6$ requires 316. 0945), 301(20), 287(18), 272(26), 255(18), 233(20), 160(15), 149(35), 104(22), 76(33), 55(98).

(2*R*)-5,6-DIMETHOXYDEHYDROISO-α-LAPACHONE [**2.11**]. - Yellow-orange powder; MP = 105-106°; $[\alpha]_D$ = (-) 11.97 (0.137, $CHCl_3$); CD (MeOH) θ (nm) -10,700(215), -3829 (245), 0(258), +2780 (270), -2000 (283), -7430 (302), -1560 (327), -840 (485); UV λ max (log e) in MeOH: 245 (4.09), 270 (3.99), 300 (3.79), 350 (3.42); IR ν max (KBr) 1670, 1650, 1630, 1560, 1470, 1270, 1233, 1210, 1060, 975, 940, 895, 840, 750 cm^{-1}; ^1H NMR: see Table 2; ^{13}C NMR: see Table 3; HREIMS *m/z* (rel. int.) 300.1016 (M^+, $C_{17}H_{16}O_5$ requires 300.0998), 285(38), 267(14), 257(55), 245(39), 229(15), 217(60), 165(35), 149(60), 129(40), 115(37), 104(39), 76(41), 65(32), 57(39).

(2*R*)-5-METHOXYDEHYDROISO-α–LAPACHONE [**2.12**]. - Yellow needles; MP = 123°; $[\alpha]_D$ = (-) 17.44 (0.387, $CHCl_3$); CD (methanol) θ (nm) -6200 (224), -2000 (250), $^{+}$1500 (263), 0 (270), -4900 (290), -500 (315), $^{+}$1100 (420), 0 (500); UV λ max (log e) in MeOH: 243 (4.19), 280 (3.98), 324 (3.40), 387 (3.40); IR ν max (KBr) 2910, 1675, 1640, 1580, 1470, 1440, 1270, 1220, 1070, 980, 870, 750 cm^{-1}; ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 3; HREIMS *m/z* (rel. int.) 270.0894 (M^{+}, $C_{16}H_{14}O_4$ requires 270.0892), 255(50), 242(25), 242(20), 227(82), 215(15), 187(30), 163(32), 135(38), 134(36), 115(25), 104(40), 76(92), 55(42).

5-HYDROXY-2-(1'-HYDROXYETHYL)NAPHTHO[2,3-b]FURAN-4,9-DIONE [**2.13**]. Orange-yellow powder, MP=157-158°; ^{1}H NMR ($CDCl_3$) δ 12.17 (1H, s, exchangable with D_2O), 7.76 (1H, dd, J = 7.5,1.0), 7.60 (1H, dd, J = 8.3, 7.6), 7.29 (1H, dd, J = 8.3, 1.0), 6.84 (1H, d, J = 0.7), 5.05 (1H, m), 2.22 (1H, br s,exchangable with D_2O), 1.65 (3H, d,J = 6.5); ^{13}C NMR ($CDCl_3$) δ 187.0, 173.0, 165.5, 162.5, 152.3, 136.4, 133.0, 131.1, 125.5, 120.2, 115.5, 103.5, 64.0, 21.6; selective INEPT conditions: irradiation of H-3 at δ 6.83 showed enhancement of ^{13}C signal at 187. Irradiation of H-8 at δ 7.74 showed enhancement of ^{13}C signal at 173.0; EIMS *m/z* (rel. int.) $[M]^{+}$ 258(60), 243(100), 215(50), 123(40).

2-(1-HYDROXYETHYL)-NAPHTHO[2,3-b]FURAN-4,9-DIONE [**2.14**]. ^{1}H NMR ($CDCl_3$) δ 8.20 (2H, m), 7.75 (2H, m), 6.84 (1H, s), 5.06 (1H, q), 2.30 (1H, br s), 1.69 (3H, d); EIMS *m/z* (rel. int.) $[M]^{+}$ 242(40), 240(75), 227(100), 149(90), 119(60), 91(65), 85(75), 71(80), 57(90).

2-ISOPROPENYLNAPHTHO[2,3-b]FURAN-4,9-DIONE [**2.15**]. ^{1}H NMR ($CDCl_3$) δ 8.08 (2H, m), 7.70 (2H, m), 5.41 (1H, t, *J* = 9), 5.13 (1H, br s), 4.99 (1H, br s), 3.35 (1H, dd, *J* = 17.1,8.8), 3.03 (1H, dd,J = 17.0, 8.7), 1.79 (3H,s); EIMS *m/z* (rel. int.) $[M]^+$ 240(60), 225(50), 212(100), 197(95), 183(20), 169(25), 141(15), 133(30), 104(50), 68(45).

5-HYDROXYDEHYDROISO-α–LAPACHONE [**2.16**]. ^{1}H NMR ($CDCl_3$) δ 12.24(1H, s), 7.64 (1H,dd, *J* = 7.5, 1.0), 7.54 (1H, dd, *J* = 8.0, 7.3), 7.25 (1H, dd, *J* = 7.3, 1.0), 5.40 (1H, t, *J* = 8.8), 5.12 (1H, br s), 4.99 (1H, br s). 3.30 (1H, dd, *J* = 17.4, 8.5), 3.00 (1H, dd, *J* = 17.0, 8.5), 1.79 (3H, s); EIMS *m/z* (rel. int.) $[M]^+$ 256(35), 241(20), 228(20), 213(28), 149(100), 129(70), 113(30), 104(20), 97(15), 83(30), 77(20), 71(65), 57(95).

3-HYDROXYMETHYLFURO[3-2b]NAPTHO[2-3d]FURAN-5,10-DIONE [**2.17**]. Red powder. MP = 217-218°; UV λ max (log e) in MeOH: 272 (3.71), 307 (3.09), 427 (3.03) nm; IR ν max (KBr) 2927, 1667, 1650, 1585, 1548, 1495, 1360, 1324, 1282, 1185, 1152, 1021, 984, 904, 798, 720, 708, 695, 668 cm^{-1}; ^{1}H NMR: see Table 5; ^{13}C-NMR: see Table 5; HREIMS *m/z* 268.0368 ($M^{+\cdot}$, $C_{15}H_8O_5$ requires 268.0372), 251 (2), 239 (7), 212 (2), 183 (10), 173 (9), 128 (15), 104 (10), 76 (15), 55 (10).

9-HYDROXY-3-HYDROXYMETHYLFURO[3-2b]NAPTHO[2-3d]FURAN-5,10-DIONE [**2.18**]. MP = 221-223°; UV λ max (log e) in MeOH: 256 (3.48), 330 (2.72), 425 (2.94) nm. IR ν max (KBr) 3300, 2918, 1640, 1635, 1456, 1322, 1280, 1180, 984, 900, 725, 670 cm^{-1}; ^{1}H NMR: see Table 6; ^{13}C NMR, see Table 6; HREIMS, *m/z*

284.0316 ($M^{+\cdot}$, $C_{15}H_8O_6$ requires 284.0321) 267 (4), 255 (5), 199 (4), 189 (6), 184 (3), 172 (5), 144 (4), 119 (4), 92 (5), 57 (10).

α–METHOXY–α–TRIFLUOROMETHYLPHENYL ACETATE (MTPA) ESTER OF 2.10 [**2.19**]. - COMPOUND **2.10** (2.7 mg, .008mmol) (*R*)-(+) α-methoxy-α-trifluoromethylphenyl acetic acid (9 mg, .038 mmol), DCC (10 mg, .05 mmol) and a cat. amount of DMAP in 1.5 ml dry CH_2Cl_2 were stirred at room temperature for 45 min. The reaction mixture was concentrated, and then passed through a small plug of silica gel, eluting with CH_2Cl_2. The CH_2Cl_2 was dried over Na_2SO_4 and evaporated to yield the MTPA ester **2.19** as a yellow semi-solid in quantitative yield. ^1H NMR ($CDCl_3$): δ 1.86 (3H, s, C-12 Me), 3.54 (3H, d, J=0.8, MTPA OCH_3), 3.85 (3H, s, C-5 OCH_3), 5.02 (1H, br s, H 11a), 5.15 (1H, br s, H 11b), 5.30 (1H, br s, H-2), 6.60 (1H, d, J=3, H-3), 7.12 (1H, d, J=8.4 ,H-7), 7.41 (3H, m) 7.58 (2H, m), 7.98 (1H, d, J=8.5, H-8). EIMS *m/z* (rel int.) $[M]^+$ 533(3), 359(4), 315(3), 295(3), 278(4), 190(100), 189(99), 175(95), 139(60), 119(80), 105(98), 83(90), 69(60), 56(95).

III. SEMI-SYNTHESIS OF BIOACTIVE STEROLS ISOLATED FROM *PSEUDOBERSAMA MOSSAMBICENSIS*

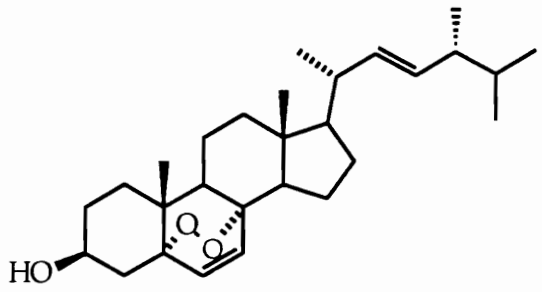
3.1 INTRODUCTION

3.1.1 Known Cytotoxic Sterols

Sterols and triterpenoids containing various oxygen functionalities have demonstrated a wide range of biological activities, including selective cytotoxicity towards human tumor cell lines.[58] Hormonal steroids have been used in the treatment of hormone-dependent tumors and the progestins are used to treat endometrial carcinoma. Steroidal anti-estrogens and aromatase inhibitors are being evaluated in patients with advanced breast cancer.[59] An investigation of traditional drugs used in the Chinese Pharmacopoeia and reported for the treatment of cancer (Bang and Ourisson, 1977)[60,67,68] indicated the successful use of parasitic fungi and animals infected with microorganisms. These drugs have been shown to contain hydroxylated sterols which show toxicity towards HTC (rat hepatoma cells grown *in vitro*) tumor cells but not to normal 3T3 (normal, non cancerous mouse fibroblast strain) cells at the same or higher concentrations.

One of these "animal" drugs studied, known as *Bombyx cum Botryte* consists of silk worms (*Bombyx mori*) killed by infection with the microscopic fungus *Botrytis bassiana* Bals.[61] From this drug, two groups of bioactive natural

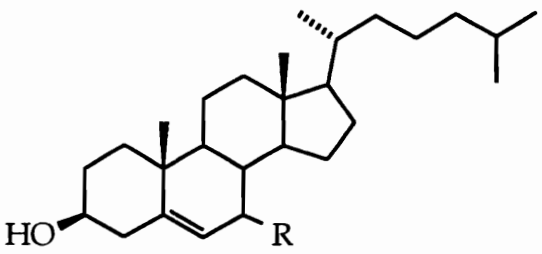
products were isolated;[60] ergosterol peroxide **3.01**, and various 7-hydroxy sterols **3.02-3.07**.



3.01

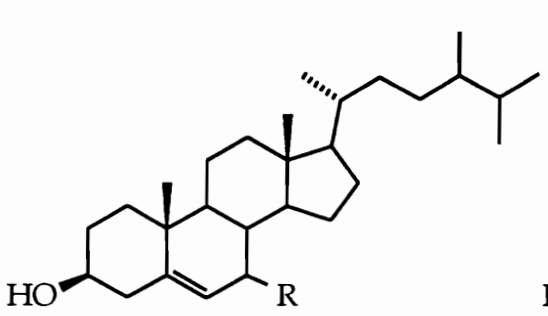
Compound **3.01**, at a dose of 33μg/ml, led to a reduction of viable HTC cells to 80% of their initial number after 3 days. But because it is similarly toxic to normal 3T3 cells it is not considered selective.

On the other hand, some of the 7-hydroxy sterols showed a high degree of selectivity. Specifically, 7β-hydroxycholesterol (7β-OHC **3.02)** was shown to be the most active towards HTC cells (0% viable cells after 3 days at a concentration of 33 μg/ml), but even at 80 μg/ml this compound is not cytotoxic towards the normal fibroblast cell strain 3T3.[61]

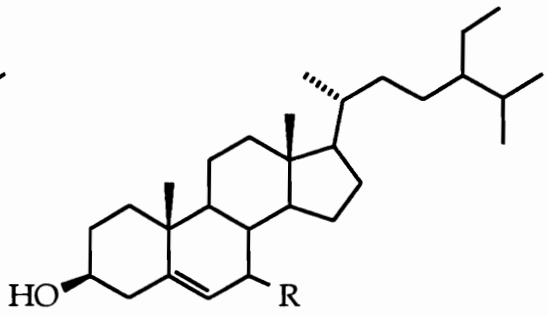


R = 7 β-OH **3.02**
R = 7 α-OH **3.03**

Interestingly, its epimer 7α-hydroxycholesterol **3.03** was shown to be completely inactive. Compounds **3.05**, **3.06**, and **3.07** were also inactive, and 7β-hydroxycampesterol **3.04** showed moderate but selective activity.



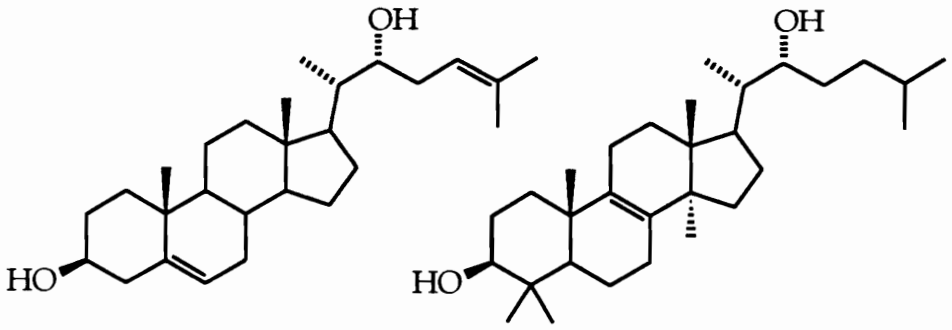
R = 7 β-OH **3.04**
R = 7 α-OH **3.05**



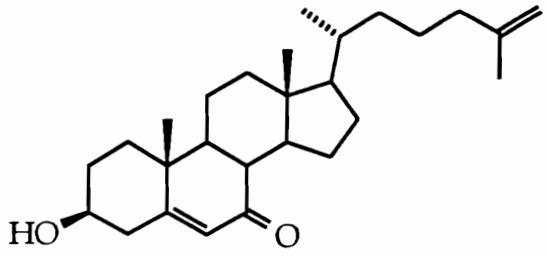
R = 7 β-OH **3.06**
R = 7 α-OH **3.07**

7β-hydroxycholesterol is also found in another Chinese medicine used as an anticancer drug, *Euphorbia fisheriana*, a higher plant of the Euphorbiaceae.[61]

Other interesting cytotoxic hydroxy sterols are the synthetic derivatives - 22*R*-hydroxydesmosterol **3.08**, 3β-hydroxy-5,25(26)-cholestadien-7-one **3.09**, and dihydro-(22*R*)-22-hydroxylanosterol **3.010** (originally isolated from the birch fungus *Inonotus obliquus*, used in the traditional Russian anticancer drug "tchaga").[63]



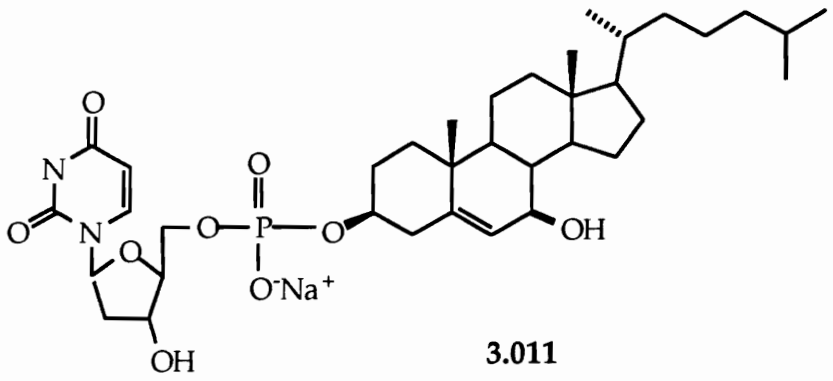
3.08 **3.09**



3.010

All show highly selective activity towards hepatoma cells vs. normal fibroblasts.

Attempts at preparing water soluble derivatives of 7β-OHC such as the sodium bis(hemisuccinate) monophosphoric acid diester[64] **3.11** have met with limited success with respect to increased solubility, however, this work represents continued and recent interest in hydroxylated sterols as anticancer agents.



3.011

3.1.2. Cytotoxic Sterols - Mechanism of Action

In the early 1970's, several hydroxy-sterols had been shown to cause a reduction of the activity of hydroxymethylglutarylcoenzyme A (HMG-CoA) reductase, the enzyme responsible for the production of mevalonic acid, a key step in the biosynthesis of cholesterol.[65] More recently, Brown and Goldstein[66] have summarized thoughts on the control of cholesterol biosynthesis. Specifically, cells obtain cholesterol essential to their membranes by two methods:

1. From exogenous sources, where extracellular cholesterol present in esterified form is transported into the cell by low density lipoprotein. Once in the cell it is then hydrolyzed.

2. From endogenous sources - where it is synthesized by the accepted sequence Acetyl-CoA $\rightarrow$ HMG-CoA $^{+}$ MVA $\rightarrow$ cholesterol. The transformation of HMG-CoA into MVA is a key step in the regulation of cholesterol biosynthesis.[10]

It is also known that the enzyme ACAT (acyl-CoA: cholesteryl acyl transferase) controls a rapid turnover of the cellular cholesterol, excreting cholesterol from the cells.

In normal cells, the exogenous cholesterol acts to block endogenous synthesis by repressing HMG-CoA reductase activity, but in tumorous cells this feedback control appears to be absent. It is possible that this is due to a transport defect whereby exogenously (low density lipoprotein transported) supplied cholesterol esters have a reduced ability to penetrate the tumorous cell.[67] Thus, if the exogenous cholesterol esters are unable to enter the cell to repress HMG-CoA reductase activity, the activity of this enzyme and consequent endogenous cholesterol biosynthesis would be stimulated to maximal level in order to supply the cholesterol needs of the tumorous cell. Then, as experiments by Bang and Ourisson[68] have shown, when the activity of HMG-CoA reductase is sharply reduced by the addition of 7β-hydroxycholesterol, cell growth comes to a halt and the cells die through lack of cholesterol needed for maintenance of their membranes. Concurrently, ACAT is also activated by 7β-OHC, and continues to excrete cholesterol from the cells.

The fact that certain hydroxy sterols strongly reduce HMG-CoA reductase activity and yet are considerably less toxic to normal cells may be explained by suggesting that these sterols which block cholesterol biosynthesis may take the place of cholesterol in the cell membrane.[69-71] Depending on its molecular structure, such compounds may be good or poor substitutes for cholesterol.

3.1.3 Introduction to Sterol Synthesis Project: Original Isolation

In the course of a Natural Product Drug Discovery Group screening program, a methyl ethyl ketone extract (MEK) of the twigs and leaves of *Pseudobersama mossambicensis* (Sim) Verc. (Meliaceae) showed selective activity against the RAD52 yeast strain. A.A. Leslie Gunatilaka and Gamini Samaranayake of the Kingston research group carried out a bioactivity-guided fractionation of this extract, and isolated and characterized three compounds; ergosta-5-24(28)-diene-3β,7α-diol [**3.1**], ξ24,28-epoxyergost-5-ene-3β,7α-diol [**3.2**], and ergost-5-ene3β,7α–ξ,24,28-tetraol [**3.3**][72] (Figure 20)

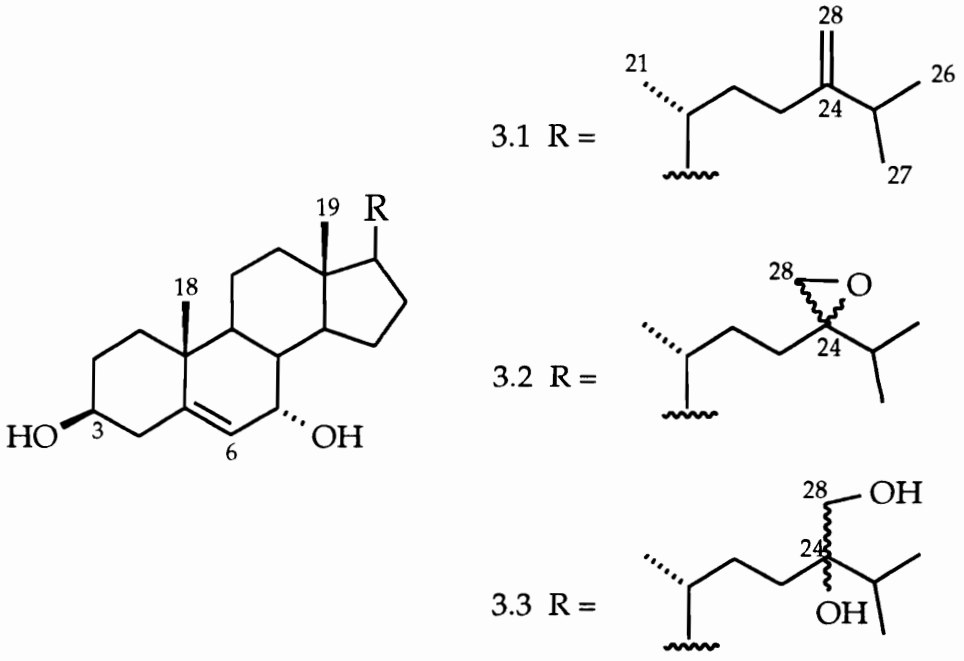


Figure 20. Bioactive Sterols Isolated from *P. mossambicensis*

Each of the three sterols were determined to be DNA damaging agents, as they showed selective activity in the RAD 52 assay (Table 8). Compounds **3.2** and **3.3** were new structures, but careful examination of the ^{13}C data of **3.3** has indicated that it was most probably an artifact of the isolation process. The signals due to C-17, C-22, C-23, C-24, C-26, C-27, and C-28 appeared as doublets, whereas all the other signals appeared as sharp singlets, suggesting that the compound is a mixture of diastereomers differing in stereochemistry at C-24.

Because these sterols showed promising activity in our assay, and because the literature indicated that some hydroxy sterols are cytotoxic, it was decided that further biological studies on compounds **3.1** and **3.2** were in order. But because of the very small amounts of material that were isolated, it was necessary to develop a synthesis in order to have sufficient material for testing.

Table 8. Bioactivity of Sterols **3.1-3.3**[a]

Compound	RAD52	RAD$^+$
3.1	8.0	>1500
3.2	0.4	>50
3.3	1.0	>100
Camptothecin[b]	0.6	110

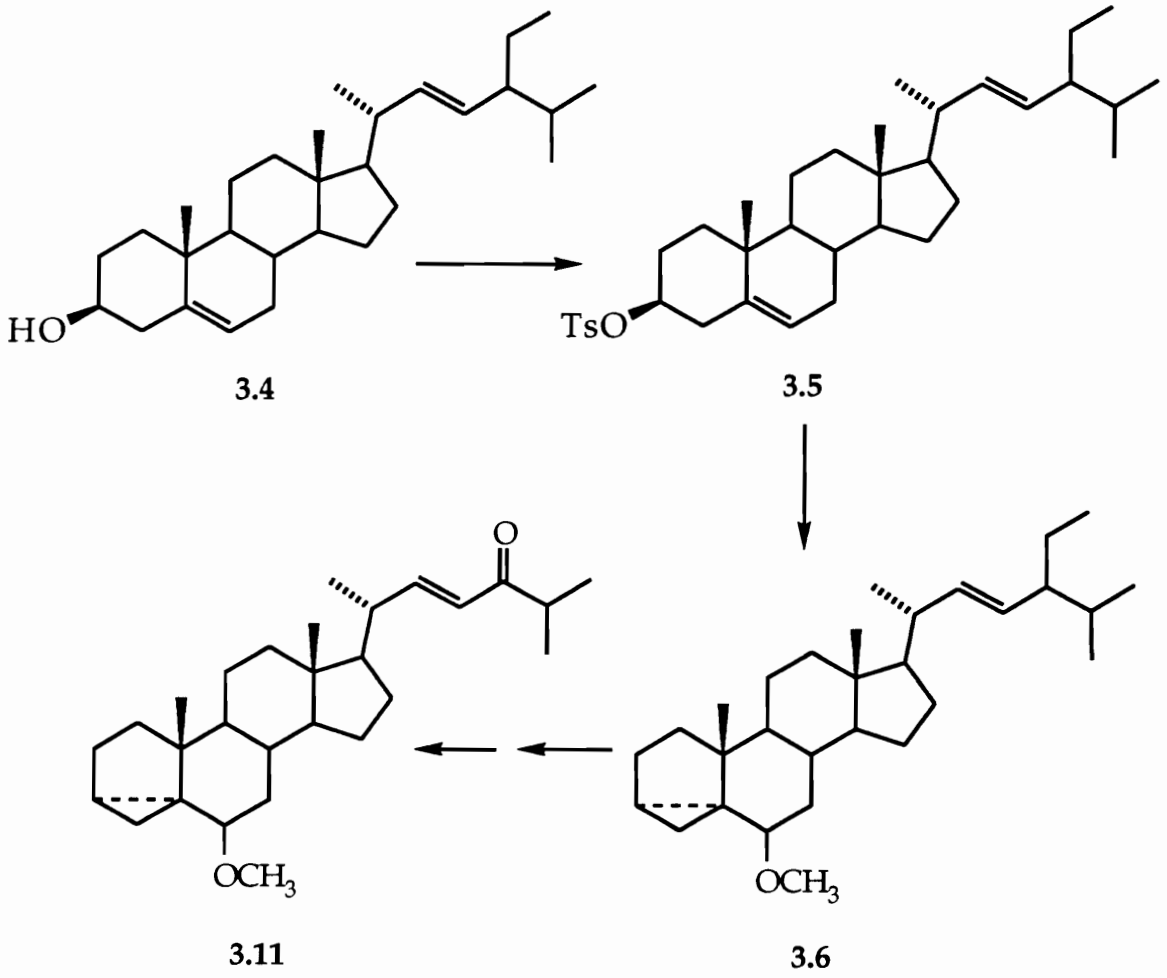
[a]Results are expressed as IC_{12} (μg/ml) values

[b]Standard Reference Compound

3.2 RESULTS AND DISCUSSION

3.2.1 Synthetic Strategy

A semi-synthesis was envisioned (Scheme 10) which started with stigmasterol (**3.4**), and employed Djerassi's methodology[73] to affix an appropriate side chain as shown below.

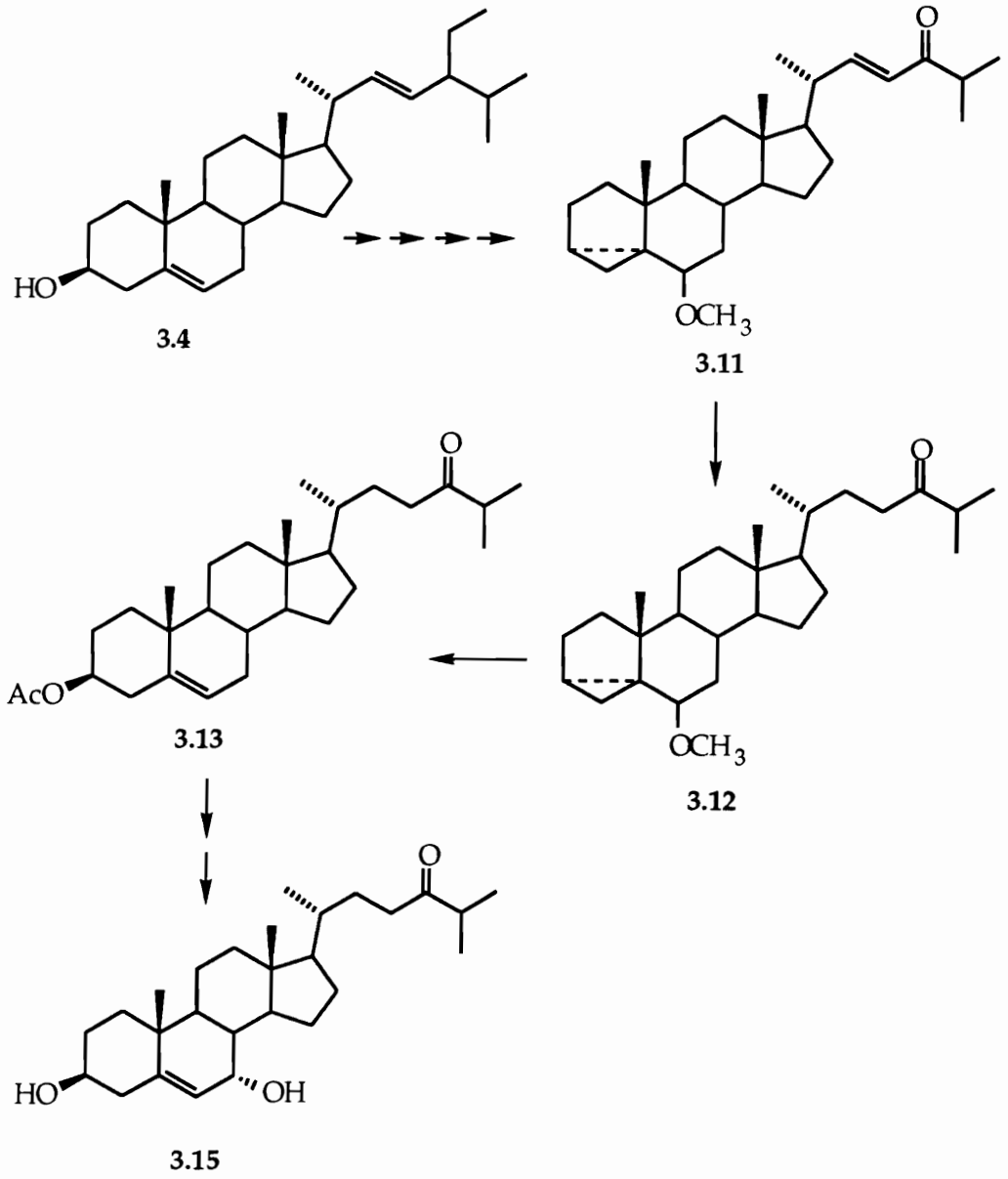


3.4 **3.5**

3.11 **3.6**

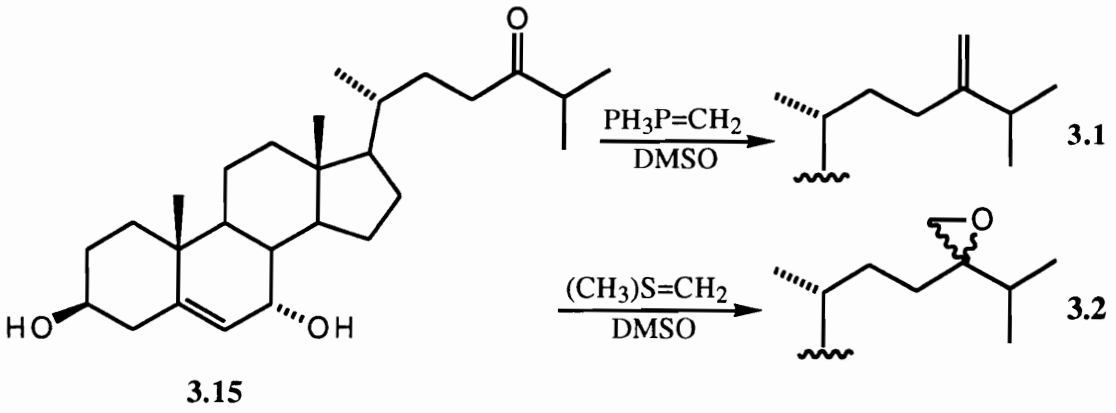
The double bond of the side chain was hydrogenated (**3.12**), and following removal of the Δ^5 3β-hydroxy protection system, the intermediate acetate **3.13** was

realized. The next step was a very critical one whereby oxidation at C-7 took place, and several methods that were used will be discussed.



Scheme 10. Overall Synthetic Strategy

We desired a common precursor to the target compounds, thus a common intermediate (**3.15**) was chosen as an attractive precursor to **3.1** and **3.2**, as the C-24 epoxide and methylene functionalities could be realized by use of appropriate phospho- and sulfur- ylides (Scheme 11).

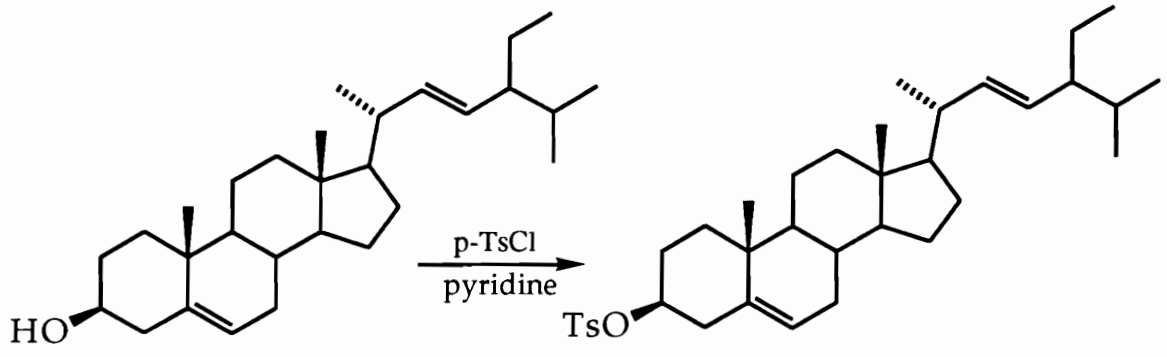


3.15

Scheme 11. Common Precursor to Target Compounds

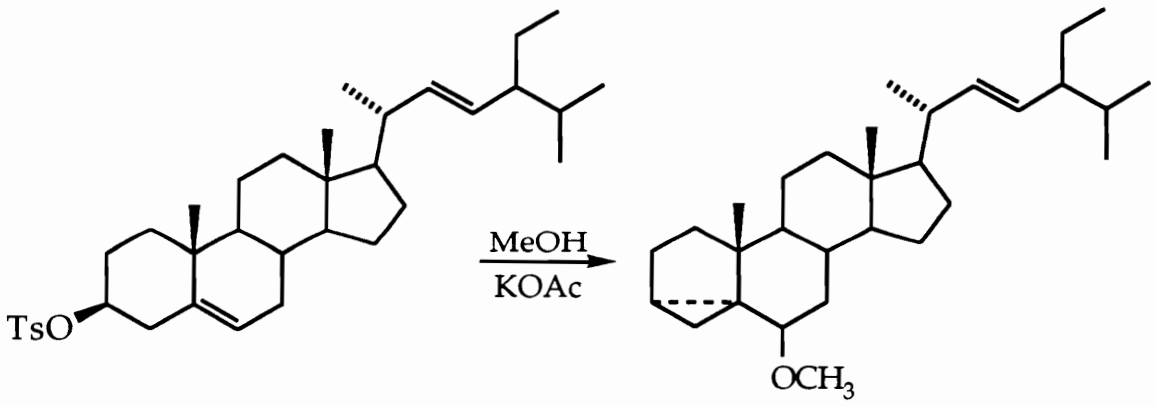
3.2.2 Synthetic Scheme

The synthetic scheme begins with readily available stigmasterol **3.4**, which by Djerassi's methodology[73] was easily manipulated in four steps to the intermediate enone **3.11**. Stigmasterol was first tosylated using p-toluenesulfonyl chloride in pyridine to give **3.5** in 90% yield.



3.4 3.5

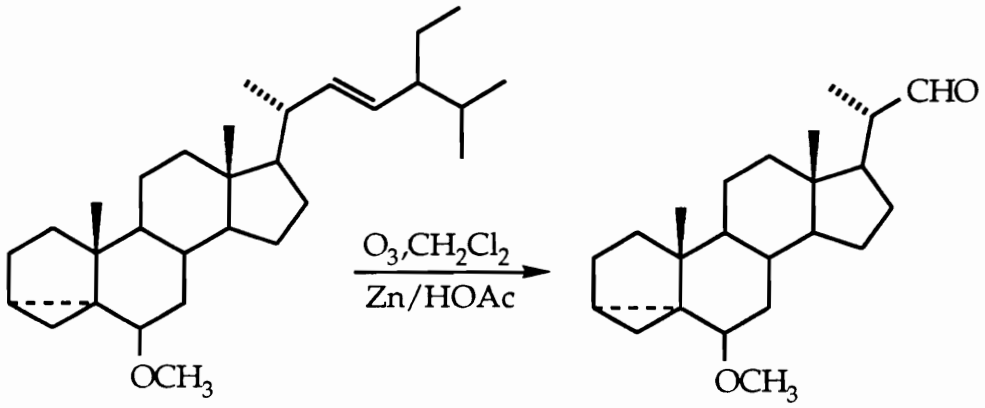
The Δ^5-3β-hydroxy system was protected as it's 3,5-cyclo-6-methylether by methanolysis of **3.5** in the presence of freshly fused potassium acetate[74] to give cyclopropane derivative **3.6** in 78% yield. Comparison of MP, $[\alpha]_D$, and ^1H NMR with literature values indicated the desired compound was obtained.



3.5 3.6

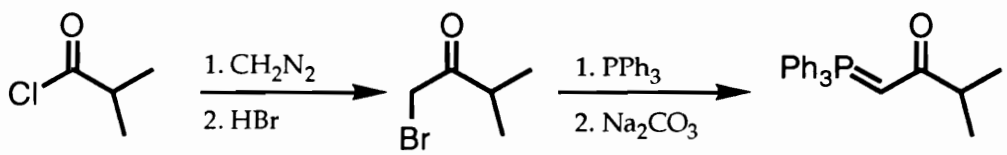
Ozonolysis of **3.6** in methylene chloride containing 1 % pyridine at -78° followed by reductive work-up using zinc and acetic acid yielded the aldehyde **3.7**. A small amount was purified and a ^1H NMR spectrum of this indicated an aldehyde resonance at δ 9.95, and the absence of the olefinic protons. Its IR

spectrum showed a carbonyl stretch at 1720 cm^{-1}. The bulk of this material was not purified due to it's instability, and was reacted immediately in the next step.



3.6 **3.7**

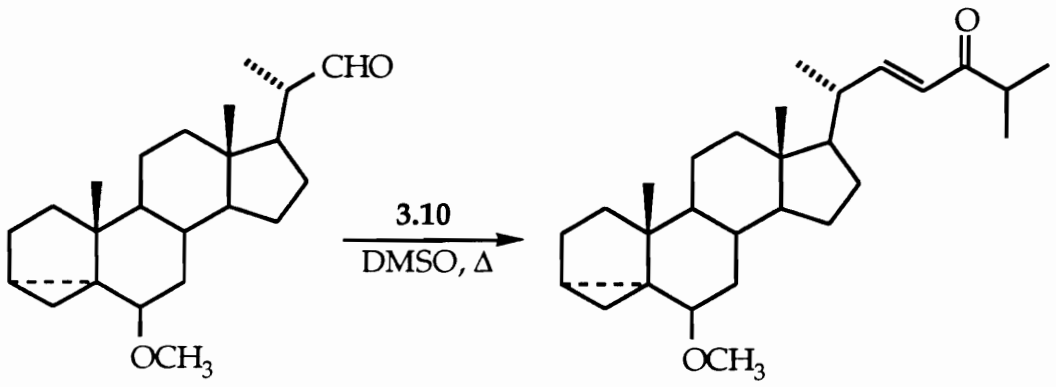
One of the key steps in the synthesis consisted of coupling the aldehyde **3.7** with the ylide **3.10**. The ylide **3.10** was prepared from isobutyryl chloride **3.8** by reaction with freshly prepared diazomethane in ether at 0°, followed by HBr gas to give the α-bromoketone **3.9.** This method achieves selective bromine substitution on the methyl group.[75] Addition of PPh_3 to **3.9** in benzene afforded the phosphonium bromide salt (not shown) which was then treated with Na_2CO_3 in water to give rise to the desired ylide **3.10** which precipitates out of solution overnight[76] (Scheme 12).



3.8 **3.9** **3.10**

Scheme 12. Preparation of Phosphorane Ylide

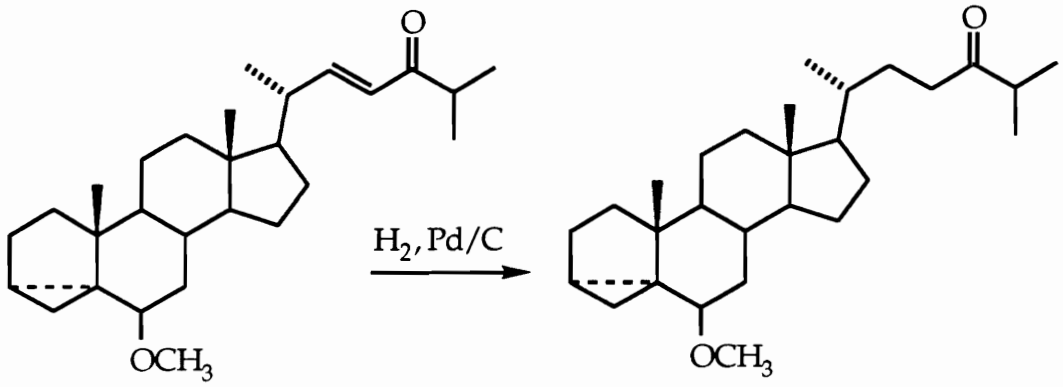
The desired side chain was affixed to **3.7***via* the Wittig reaction[77] using six equivalents of the ylide **3.10** in dry DMSO to afford *trans*-3α,5-cyclo-6β-methoxy-5α-cholesta-22-en-24-one **3.11** (41% yield for the two steps from **3.6**).



3.7 **3.11**

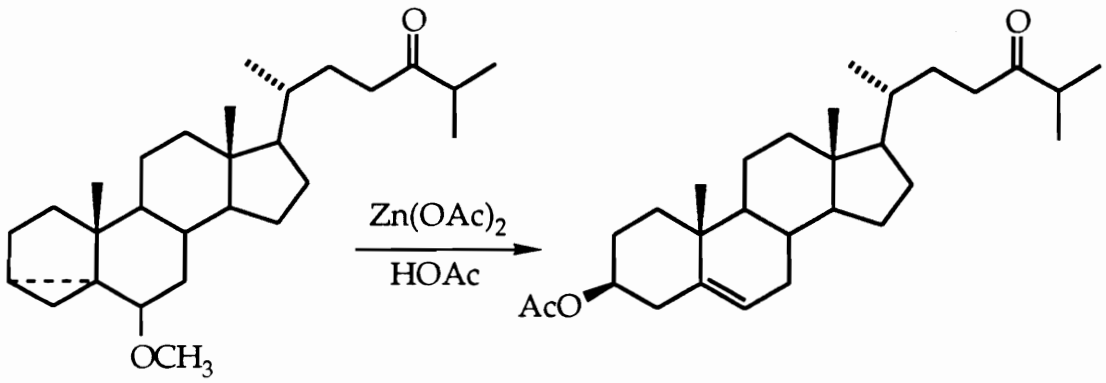
The Δ^{22} double bond was shown to be *trans* by the olefinic region of the ^{1}H-NMR: δ 6.71(1H, doublet of doublets, *J* = 16 and 9 Hz, -CH=C-CO-), and δ 6.05 (1H, doublet of doublets, *J* = 16 and 1 Hz, -C=CHCO-).[73]

At this point, the synthetic scheme diverges from that reported by Djerassi, and where new compounds were made they were adequately characterized. Hydrogenation of **3.11** over palladium on carbon in ethyl acetate gave the ketone **3.12** (91% yield) whose ^{1}H-NMR spectrum revealed the absence of Δ 22(23) olefinic protons. It's ^{13}C spectrum indicated the expected number of carbons, including an isolated carbonyl resonance at δ 215.2. The mass spectrum of **3.12** showed an $[M]^+$ at *m/z* 414.



3.11 **3.12**

In order to open the cyclopropyl methyl ether protection system, **3.12** was treated with zinc acetate in boiling acetic acid[74] to afford the acetate **3.13** in 90% yield.



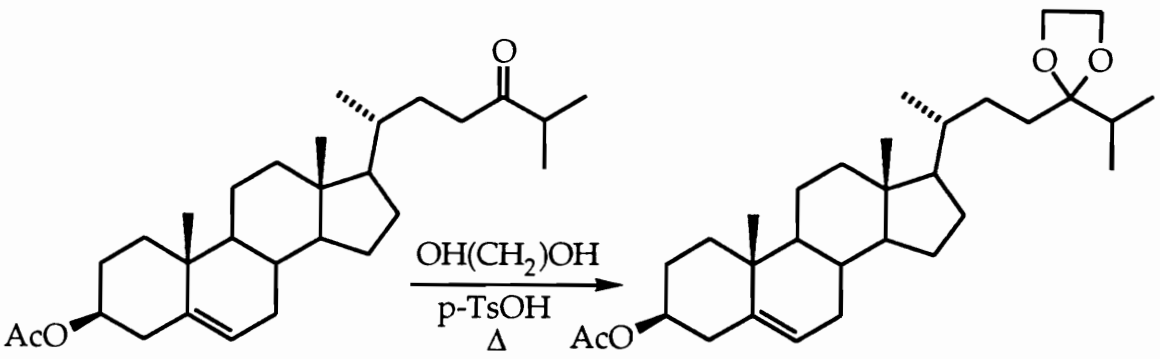
3.12 **3.13**

The ^{1}H-NMR spectrum of **3.13** showed the absence of the three cyclopropyl protons of **3.12** at δ 0.3-0.8, the emergence of a one proton olefinic singlet resonance at δ 5.36 (C-6), and a three proton singlet resonance at δ 2.03

indicating the presence of the C-3 acetate. The mass spectrum of **3.13** is characterized by important ions at *m/z* 399 (M^+- C_3H_7), and 382 (M^+-60).

The most critical step in the synthesis was oxidation at position 7. Initially, selective α-hydroxylation at this position was investigated along the following pathway.

Before oxidation and selective reduction at C-7 could take place, the C-24 carbonyl had to be protected. This was carried out by treating **3.13** with ethylene glycol and a catalytic amount of *p*-toluene sulfonic acid in benzene.[78] Refluxing this mixture overnight yielded the ketal **3.14** in 96 % yield.



3.13 **3.14**

The ^1H NMR of **3.14** showed a 4H singlet at δ 3.93 indicating the presence of the ketal methylene protons. Its IR spectrum lacked a signal for a carbonyl group The CI mass spectrum of **3.14** indicated the expected $[M+1]^+$ at *m/z* 487, and its EI mass spectrum showed significant peaks at *m/z* 443 (M^+-43), 426 (M^+-60), and 115 (Figure 21).

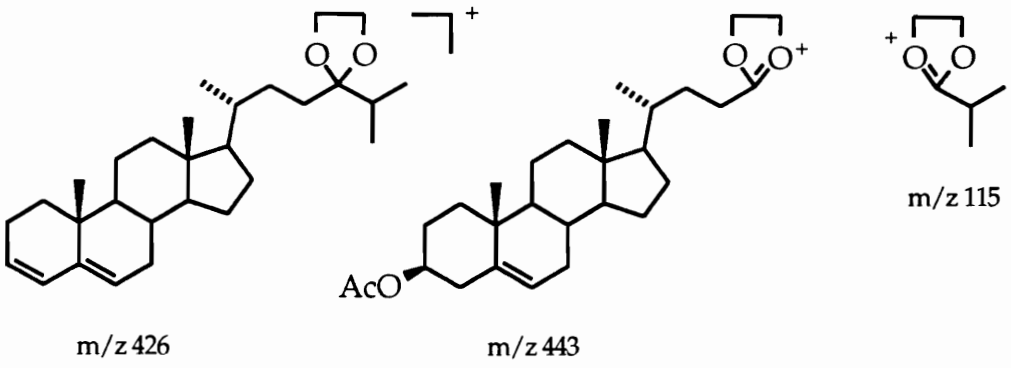
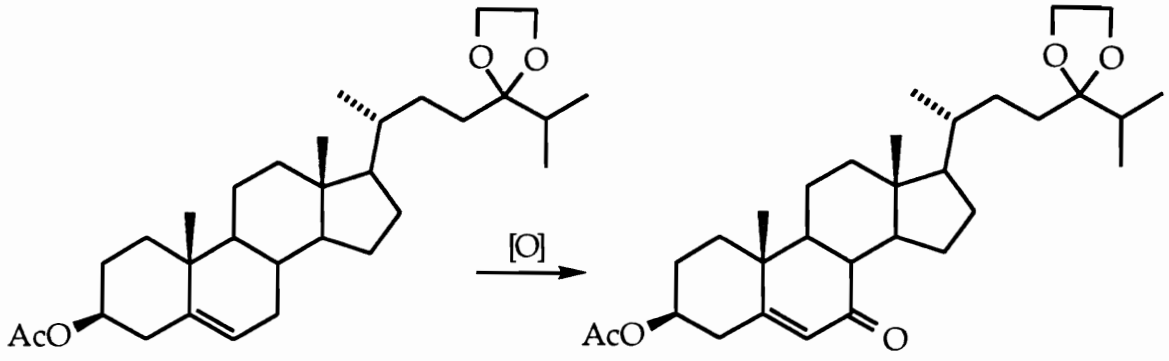


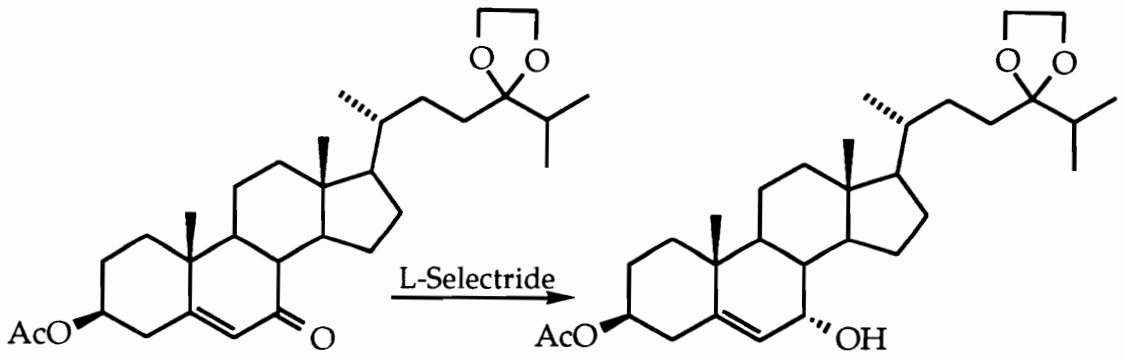
Figure 21. Important MS Fragments of **3.14**

The next step was oxidation at C-7, and a variety of different reagents and conditions were employed. The first method tried was pyridinium chlorochromate in refluxing benzene[79] which gave a 42% yield of C-7 carbonyl product, however; about 1/2 of this had been deprotected at C-24. PDC in refluxing pyridine[79] gave **3.15** in 15% yield. Another method was NBS in water and dioxane under visible irradiation,[80] which gave the desired enone **3.15** in 24% yield. The best method which was investigated was the use of $Cr(CO)_6$ and *tert*-butylhydroperoxide in refluxing CH_3CN [81] which afforded the enone **3.15** in 35% yield. The ^1H NMR of **3.15** showed the H-C7 resonance as a sharp singlet at δ 5.72 where it had been seen as a broad doublet for **3.14**. The CI mass spectrum of 3.14 showed the expected $[M+1]^+$ at *m/z* 501.



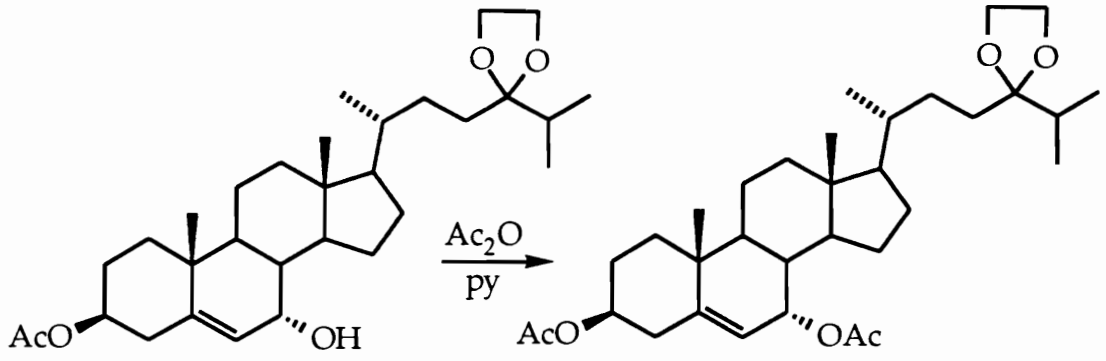
3.14 **3.15**

The next step was selective reduction of the C-7 carbonyl group, and this was carried out by treating **3.15** with L-Selectride in THF at -78°.[82] to afford **3.16** in 50 % yield. The ^{1}H NMR of **3.16** showed H-C6 again as a doublet, and a new multiplet at δ 3.85 was seen, which was expected for the βH-C7.



3.15 **3.16**

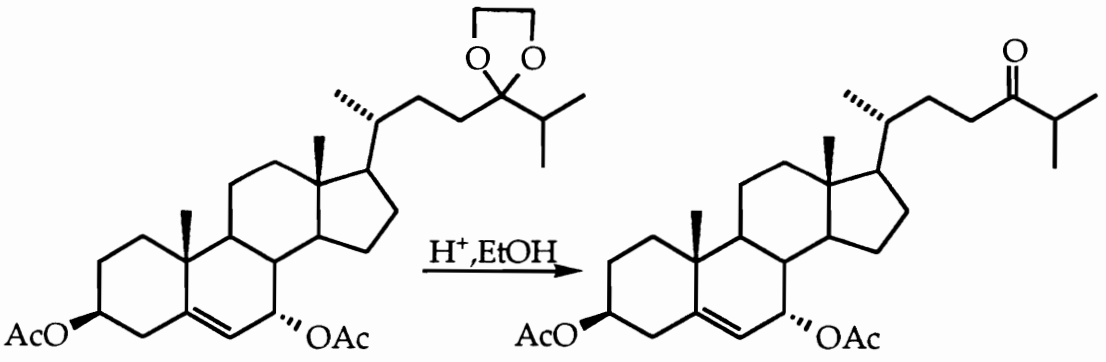
Initial attempts at C-24 deprotection showed that the C-7 hydroxyl group was very labile, and it was decided that this would need to be protected as its acetate prior to ketal removal. This was carried out by treating **3.16** with acetic anhydride in dry pyridine, yielding diacetate **3.17** in 96% yield.



3.16 **3.17**

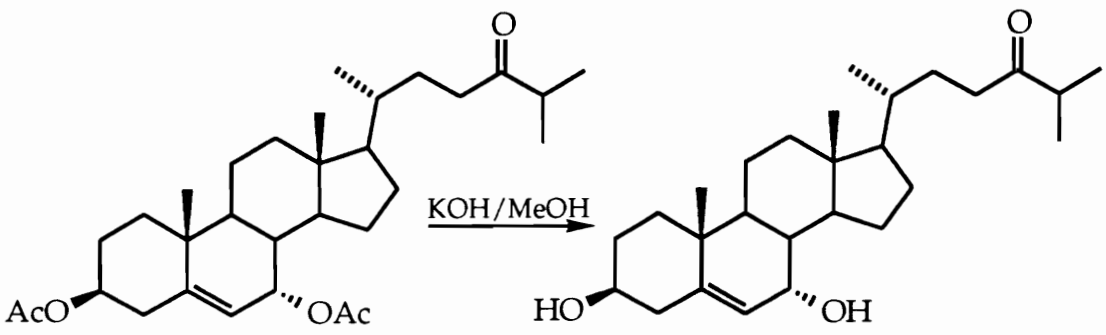
The ^1H NMR spectrum of **3.17** showed the presence of two overlapping acetate groups at δ 2.14.

Deprotection of the C-24 carbonyl was carried out under mild conditions using *p*-TsOH in absolute ethanol.[83] This procedure gave **3.18** in 70% yield, and its ^1H NMR spectrum showed the absence of the ketal methylene protons.



3.17 **3.18**

Hydrolysis of **3.18** using 5% KOH in MeOH yielded **3.19** in 95% yield.



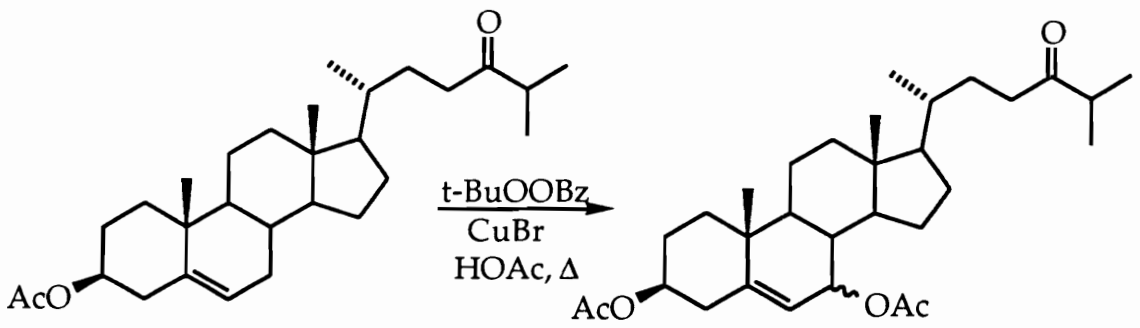
3.18 **3.19**

The IR spectrum of **3.19** exhibited a broad OH stretch at 3380 cm^{-1}, and a carbonyl stretch at 1710 cm^{-1}. The ^1H NMR of **3.19** revealed a 1H doublet of doublets (J = 5.2,1.2) at δ 5.60, corresponding to the vinylic C-6 proton. A H-7α proton was present, represented by a 1H multiplet at δ 3.85[82]. A 1H multiplet at δ 3.60 indicated the H-3α as expected. At δ 2.60, a 1H septet (J = 6.9) was seen, which is typical for H-25 when C-24 bears a carbonyl group[73]. Two overlapping doublets centered at δ 1.08(J = 7.0) were assigned to H_3-26 and H_3-27. Other methyl signals included a singlet at δ 0.92, a doublet(J = 6.4) at δ 0.92, and a singlet at δ 0.68. The ^{13}C NMR spectrum of **3.19** indicated the expected number of carbons, including a carbonyl resonance at δ 215.3. The mass spectrum of **3.19** showed a base peak at *m/z* 398 $[M\text{-}H_2O]^{+\cdot}$ and another significant peak at *m/z* 380 $[M\text{-}2\ H_2O]^+$.

Although the above procedure is a viable pathway to **3.19**, it is a long and tedious route. As an alternative, direct allylic acetoxylation of **3.13** was

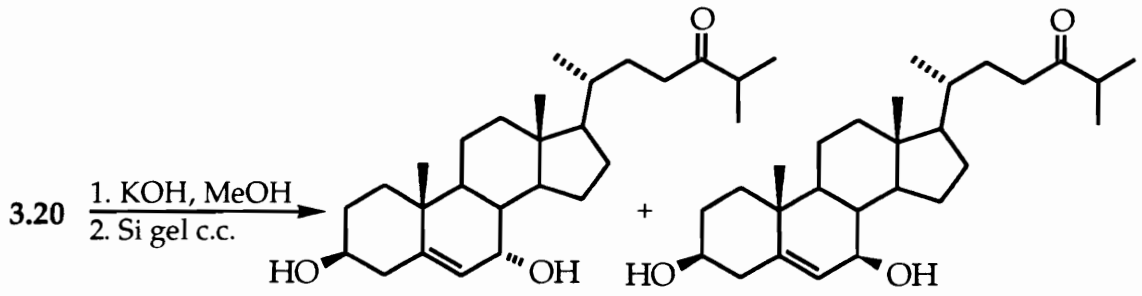
investigated. This was first attempted using $Pd(OAc)_2$ in HOAc with MnO_2 and benzoquinone[84] under various conditions, but yields were rather low (15-25%).

Another method, the one we ultimately chose for the scaled up procedure, was to use *tert*-butylperoxybenzoate in the presence of cuprous bromide in acetic acid[85] which yielded mixture of C-7 acetates. Although the literature reference claims that the α-product is formed exclusively, the compounds obtained in this work were generally a mixture of ca. 1.5:1 (α:β).



3.13 **3.20**

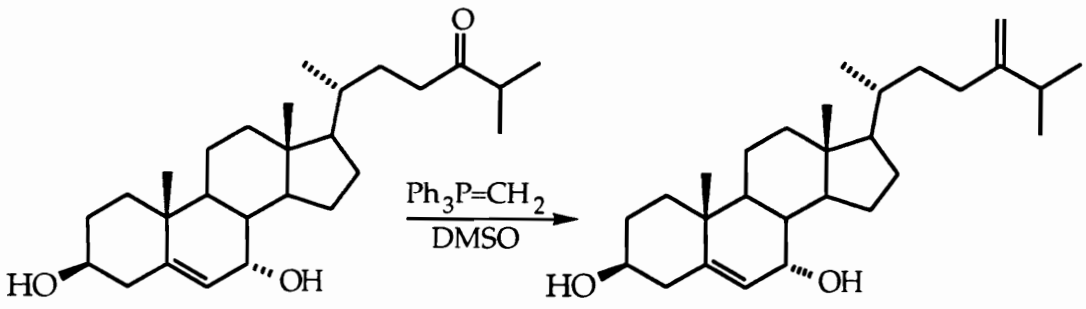
Prior to purification, the mixture of diacetates was subjected to hydrolysis using 5% KOH in methanol to yield the diols **3.21** and **3.22** which could be separated by careful chromatography on a silica gel column.



3.21 **3.22**

The yield of pure intermediate **3.21**, while relatively low (16%) was still better than the overall yield of the more circutious route (11%) described earlier. Direct acetoxylation of **3.13** not only saved steps, but it afforded enough of the 7-β-OH material **3.22**, which was used to generate the β analogs of **3.1** and **3.2**, useful for structure-activity relationship study.

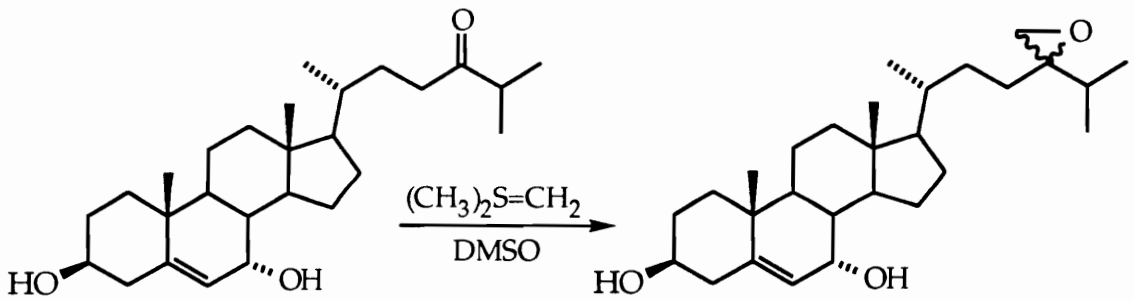
With the diol **3.21** in hand, both **3.1** and **3.2** could then be generated. To prepare the olefin **3.1**, the precursor **3.21** was treated with triphenyl phosphonium methylide[77] (formed *in situ* using Ph_3PCH_3Br and NaH in DMSO) in DMSO (75% yield).



3.21 **3.1**

The IR spectrum of **3.1** showed no carbonyl stretch, and its $[\alpha]_D$ was in good agreement with that for the natural product. It's ^1H NMR showed the presence of two one - proton singlets for the C-28 protons at δ 4.71 and 4.65 which are seen with the natural product. The ^{13}C NMR spectrum of **3.1** is identical to that for the authentic sample. The mass spectrum of **3.1** exhibited a base peak at *m/z* 396 $[M\text{-}H_2O]^+$, and a significant peak at *m/z* 378 $[M\text{-}2H_2O]^+$.

Attempts to form the epoxide **3.2** by treating **3.21** with dimethylsulfoxonium methylide[86] (not shown) were met with sluggish resistance. But success was achieved by treating **3.21** with the more reactive dimethylsulfonium methylide[87] (formed *in situ* using trimethyl sulfonium iodide and NaH in DMSO) in DMSO to yield a mixture of diastereomers in 63% yield.



3.21 **3.2**

Compound **3.2** had the same TLC Rf (with both normal and reversed-phase) in several solvent systems as the authentic sample, and it had the same retention time on a reversed-phase HPLC column. The IR spectrum of **3.2** showed the absence of a carbonyl stretch. Its ^1H-NMR spectrum revealed that

the H(C-25) septet at δ 2.60 was now distorted and shifted upfield, merging with the two proton multiplet seen for the C-23 protons. A new two proton multiplet centered at δ 2.56 was attributed to the C-28 methylene protons. In the naturally occurring product, these protons are represented by a doublet of doublets at δ 2.53 and 2.60. The fact that these signals for the synthetic product appear as a complex multiplet is rationalized on the basis of the presence of diastereomers at C-24. Multiple doublets are also seen for the C-26 and C-27 methyl groups at δ 0.88 - 0.96. The remaining major ^{1}H NMR signals are identical to those of the authentic sample. The ^{13}C NMR spectrum of **3.2** compares well with that for the natural product, the difference being that doublets are seen for carbons 17, 20,22,23,25,26,and 27 as would be expected with a mixture of diastereomers at position 24.[72]

The mass spectrum of **3.2** was similar to that for the authentic sample, showing significant peaks at *m/z* 412 $[M- H_2O]^+$, 394 $[M - H_2O]^+$.

Compound **3.2** was prepared as a mixture of diastereomers, which ran as a single spot on TLC in several solvent systems. Attempts at separation of these diastereomers by HPLC using both reversed phase columns and a normal phase, chiral column were unsuccessful.

3.2.3 NMR Assignments of Sterols **3.1** and **3.2**

NMR assignments were made based on comparison with authentic samples, and are listed in Tables 9 and 10. ^{13}C Attached Proton Test NMR experiments were run on both compounds in order to confirm multiplicities.

Table 9: Selected ^{1}H NMR Chemical Shifts (400 MHz) of Sterols **3.1** and **3.2** in $CDCl_3$.[a]

Proton	Sterol 3.1	Sterol 3.2
H-3	3.59 m	3.59 m
H-6	5.61 dd (5.2,1.6)	5.61 dd (5.2,1.6)
H-7	3.85 m	3.85 m
H_3-18	0.69 s	0.68 s
H_3-19	0.99 s	0.99 s
H-21	0.96 d (6.8)	0.95 d (6.8)
H_3-26	1.03 d (6.8)	0.88-0.95 pair of doublets
H_3-27	1.02 d (6.8)	0.88-0.95 pair of doublets
H_2-28	4.65 s, 4.71 s	2.56 m

[a]Chemical shifts (relative to TMS) are in ppm and observed peak spacings in Hz.

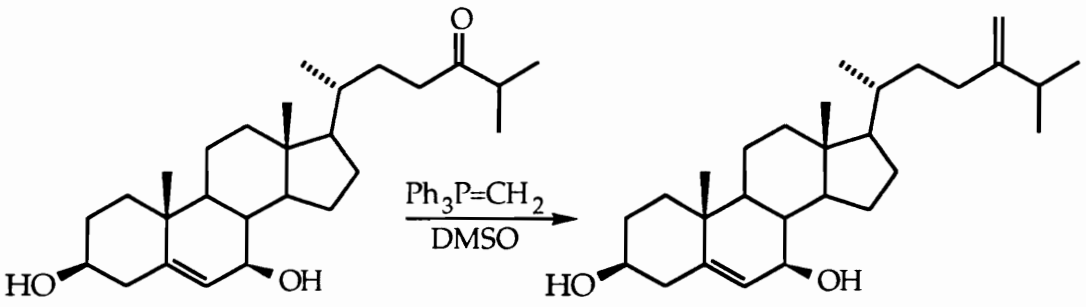
Table 10: ^{13}C NMR Chemical Shifts (100.57 MHz) of Sterols **3.1** and **3.2** in $CDCl_3$.[a]

Carbon	Sterol 3.1	Sterol 3.2	Carbon	Sterol 3.1	Sterol 3.2
1	37.0	37.0	**15**	24.3	24.3
2	31.4	31.4	**16**	28.2	28.2
3	71.3	71.3	**17**	55.6	55.52, 55.48
4	42.2	42.2	**18**	11.6	11.7
5	146.2	146.3	**19**	18.2	18.2
6	123.8	123.8	**20**	35.7	35.8, 35.7
7	65.3	65.3	**21**	18.7	18.7
8	37.4	37.5	**22**	34.6	28.2,28.0
9	42.2	42.3	**23**	30.8	30.1,29.7
10	37.5	37.4	**24**	156.8	62.7
11	20.7	20.7	**25**	33.8	31.6,32.1
12	39.2	39.1	**26**	21.8	17.7,17.9
13	42.0	42.0	**27**	21.9	18.4,18.6
14	49.4	49.4	**28**	105.9	50.5

[a]In ppm from internal TMS.

3.2.4 Analogs of Sterols **3.1** and **3.2** for Structure-Activity Relationship Studies

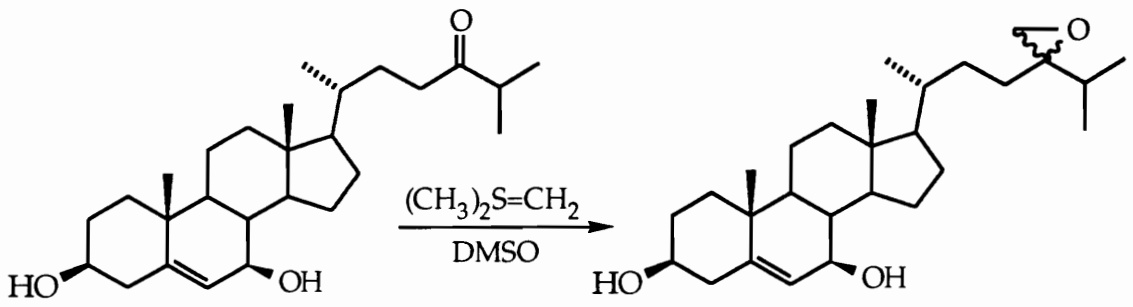
The 7β–hydroxyl analogs of **3.1** and **3.2** were prepared by exactly the same methods as were the target sterols. Thus, treatment of **3.22** with triphenylphosphene methylide in DMSO at 60° yielded the 3β,7β-diol-24(28) olefin **3.23** in 59% yield.



3.22 **3.23**

The IR spectrum of **3.23** showed the absence of a carbonyl stretch, and its ^1H NMR showed the same two 1H singlets at δ 4.71 and 4.65 for the C-28 protons as seen for it's epimer. The only differences in the ^1H NMR spectrum from that of **3.1** (and the natural product) was for the H-C7 and H_3-18 protons. In the β compound, the H-C7 proton resonates at δ 5.29 and appears as a broad singlet, and the H_3-C18 methyl signal has been shifted upfield slightly to δ 1.05. The ^{13}C-NMR spectrum and mass spectrum of **3.23** were similar to those for **3.1**.

The preparation of the 24,28-epoxy-3β,7β diol analog **3.24** was carried out in 63% yield by treating **3.22** with dimethylsulfonium methylide in DMSO at 0°, then allowing the mixture to warm to room temperature.



3.22 **3.24**

The IR spectrum of **3.24** indicated the absence of a carbonyl stretch. Its ^1H NMR spectrum was similar to that of its epimer (**3.2**), with the exception of H-C7, where a broad singlet was seen at δ 5.29. Its ^{13}C NMR spectrum and mass spectrum were similar to those for **3.2.**

Table 11. Selected ^1H NMR Chemical Shifts (400 MHz) of Sterols **3.21** and **3.22** in $CDCl_3$.[a]

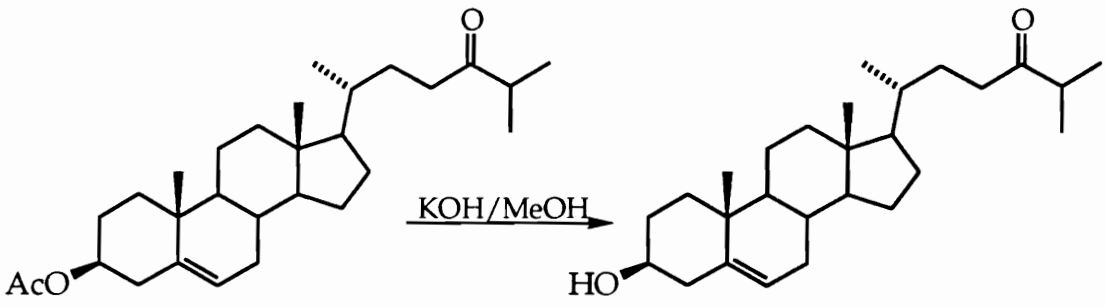
Proton	Sterol 3.21	Sterol 3.22
H-3	3.55 m	3.55 m
H-6	5.29 br s	5.29 br s
H-7	3.84 m	3.84 m
H_3-18	0.70 s	0.69 s
H_3-19	1.05 s	1.05 s
H-21	0.96 d (6.4)	0.95 d (6.8)
H_3-26	1.03 d (6.8)	0.80-0.98 pair of doublets
H_3-27	1.02 d (6.8)	0.80-0.98 pair of doublets
H_2-28	4.65 s, 4.71 s	2.57 m

[a]Chemical shifts (relative to TMS) are in ppm and coupling constants (in parentheses) in Hz.

3.2.5 Preparation of 3β–Hydroxyl Analogs for Structure-Activity Studies

In order to verify that the position C-7 hydroxyl groups were necessary for activity, the 3β-hydroxy C-7 desoxy analogs were prepared as follows.

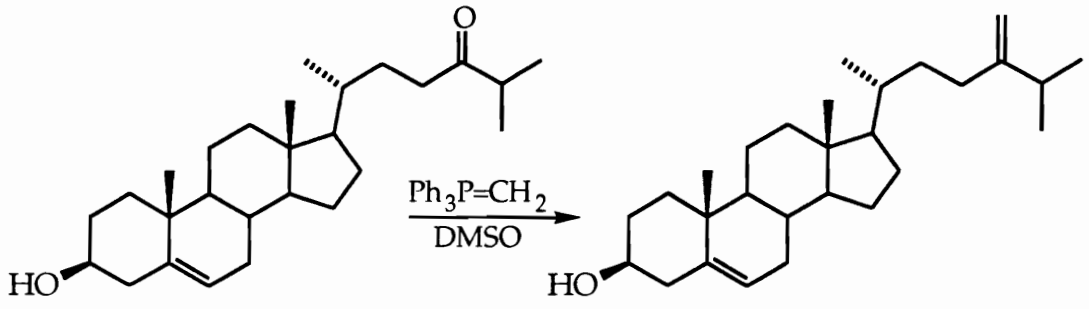
3β-hydroxycholest-5-ene,24-one (**3.25**) was prepared in 98% yield by simple hydrolysis of **3.13** using 5% KOH in MeOH at room temperature.



3.13 **3.25**

The ^1H NMR spectrum of **3.25** showed the absence of an acetate group, and the H-C3 proton was shifted upfield 1.1 ppm to δ 3.53 ppm. The mass spectrum indicated an $[M]^+$ of *m/z* 400, and showed other significant peaks at *m/z* 382 $[M\text{-}H_2O]^+$, and 71 $[C_4H_7O]^+$.

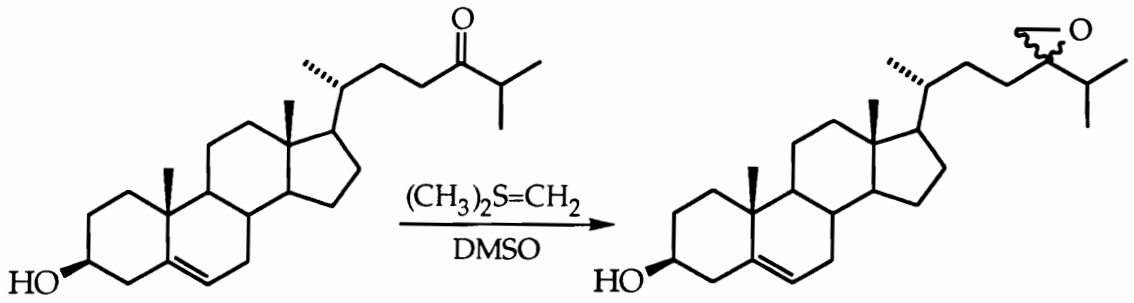
3β-hydroxyergost-5,24(28)-diene **3.26** was prepared from **3.25** as in the previously discussed Wittig olefinations. Thus, **3.25** was treated with an excess of triphenylphosphonium methyide in dry DMSO to yield the desired product **3.26** in 71% yield.



3.25 **3.26**

The IR spectrum of **3.26** shows no carbonyl stretch, and its ^1H NMR shows the same two doublets for the H-28 methlyene protons as seen in the cases of **3.1** and **3.23**. The ^1H NMR also shows no acetate resonance; there was no need for a separate hydrolysis step as the ylide conveniently performs that procedure. The mass spectrum of **3.26** showed the expected parent ion.

The 24,28 epoxide of this series **3.27** was formed with dimethylsulfonium methylide in DMSO (36% yield) as seen for the 7-hydroxy compounds.



3.25 **2.27**

This compound showed no carbonyl stretch in it's IR spectrum, and its ^1H NMR was similar to that of the 7-hydroxy compounds, lacking only the low field C-7 proton. It's mass spectrum indicated the expected parent ion.

3.2.6 Bioactivity of Synthetic Sterols

All of the sterols prepared were assayed in the rad 52 and rad$^+$ DNA-damaging bioassays, and the reults are presented in Table 12. Selected compounds were tested for cytotoxicity to Vero cells at SmithKline and Beecham Pharmaceuticals, and these results are also in Table 12.

The synthetic sterols **3.1** and **3.2** show DNA damaging activity comparable to the authentic natural products. It is noted that with regard to the 7α - OH compounds,there appears to be a trend that indicates increasing activity in the yeast assay is accompanied by decreasing Vero cell culture activity. The 7α - OH sterol which is most active in the DNA-damaging assay is the epoxide **3.2,** with an IC_{12} value of 0.2; in the Vero cell assay, its IC_{50} is > 100, and is therefore considered inactive. The second most active 7α - OH compound in the yeast assay is the olefin **3.1**, and shows an IC_{12} of 7; its IC_{50} in the Vero cell assay is 58, which is considered weakly active. The C-24 ketone **3.21**, the weakest of the three in the DNA damaging assay with an IC_{12} of 14, is the most active in the Vero cell culture with a IC_{50} of 9.9.

The 7β–OH analogs **3.23** and **3.24** which are completely inactive in the yeast assay, show moderate cytotoxicity activity in the Vero cell assay, indicating that they act by a different mechanism of action than their α-counterparts. All of the 3-OH sterols **3.25 - 3.27**, regardless of their side chain funtionalities, are inactive in the yeast assay and were not tested in the Vero cell cultures. The bioassay results of the synthetic sterols are given in Table 12.

Table 12. Bioactivity of Synthetic Sterols

STEROL	DESCRIPTION	IC_{12} (μg/ml) rad 52	IC_{12} (μg/ml) rad$^+$	CYTOTOXICITY to VERO CELLS IC_{50} (μM)
3.1	3β,7α-diol-24(28)-ene	7	NA[a]	58
3.2	3β,7α-diol-24,28-epoxide	0.2	NA	>100
3.19	3β,7α-diol-24-one	14	NA	9.9
3.22	3β,7β-diol-24-one	>500	NA	21
3.23	3β,7β–diol-24(28)-ene	NA	NA	31
3.24	3β,7β-diol-24,28-epoxide	NA	NA	16
3.25	3β-OH-24-one	NA	NA	nt[b]
3.26	3β-OH-24(28)-ene	NA	NA	nt
3.27	3β-OH-24,28-epoxide	NA	NA	nt

[a]Not active

[b]Not tested

3.3 EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.- MPs were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were taken in $CHCl_3$ solution with a Perkin-Elmer Model 241 polarimeter. The ^1H and ^{13}C-NMR spectra were recorded on a Varian Unity 400 spectrometer at 400 and 100.57 MHz, respectively, with TMS as an internal standard. Flash chromatography was performed using silica gel Merck G60 (230-400 mesh), preparative TLC with silica gel GF_{254} plates (Analtech, 500mm, 20 x 20 cm), and reversed-phase preparative TLC with Whatman PLKC18F linear K reversed-phase (1000, and 250 mm, 20 x 20 cm) plates. HPLC experiments were carried out on a Waters apparatus equipped with a photodiode array and refractve index detectors. HPLC columns used were a Whatman Partisil 10 ODS-3 (4.6 mm i.d. x 25 cm), and a Daicel Chemical Chiralcel OD 31-10 (3 mm i.d. x 25 cm).

3β,7α-DIHYDROXYERGOST-5,24(28)-DIENE **[3.1]**. Under argon, 6 mg (0.024 mmol)NaH in 0.5 ml dry DMSO was heated to 70º for 45 min., then cooled to room temperature. Ph_3PCH_3Br (86 mg, 0.24 mmol) in 2 ml DMSO was added to mixture and stirred at room temperature for 10 min. 10 mg (0.024 mmol) of **3.19** in 0.3 ml DMSO was added and the mixture was stirred at 60º for ca. 1 hr. The mixture was cooled, diluted with ethyl ether and washed repeatedly with H_2O. The ether layer was dried over Na_2SO_4, evaporated and after reversed-phase PTLC (eluting with 95% aq. methanol), yielded 7.7 mg (78%) of pure **3.1**. MP=194-195º, $[\alpha]_D$ - 88º(0.21, $CHCl_30$;^1H-NMR: see table 9; ^{13}C-NMR: see table

10.; EIMS *m/z* (rel int.) $[M]^+$ 414.3486 ($C_{28}H_{46}O_2$ requires 414.3498), $[M-H_2O]^+$ 396.3392 ($C_{28}H_{44}O$ requires 396.3392), 378(15), 312(13), 269(10), 143(12), 107(15).

3β-7α-DIHYDROXY-24,28-EPOXYERGOST-5-ENE **3.2**. Under argon, 81mg NaH in 10 ml dry DMSO was heated to 70° for 45 min. This was cooled to room temperature and diluted with 10 ml of dry THF. By syringe, 0.2 ml of this solution was removed and added to an argon filled 2 neck flask. This was cooled to 0° and 7 mg $(CH_3)_3SI$ was added slowly in a minimal volume of DMSO. The resulting mixture was stirred for 1 min. at 0° and 7 mg of **3.19** in THF was added. This was stirred at 0° for ca. 1 min., then allowed to warm to room temperature. Reaction was judged complete in 1 hour and worked up with ether/H_2O. The ether layer was dried over Na_2SO_4, evaporated, and after reversed-phase PTLC (eluting with 95% aq. MeOH), yielded 4.5 mg (63%) of pure **3.2**. MP=172-174°; $[\alpha]_D$-63°(0.15, $CHCl_3$); ^1H NMR - see table 9; ^{13}C NMR - see table 10; EIMS *m/z* (rel. int.) $[M-H_2O]^+$412.3325 ($C_{28}H_{44}O_2$ requires 412.3341), $[M-2H_2O]^+$ 394.3229 ($C_{28}H_{42}O$ requires 394.3235). 380(10), 364(5), 157(40), 143(50), 119(55), 105(55), 91(60), 81(75), 69(70).

STIGMASTEROL TOSYLATE **3.5** To a solution of 5 g of dry stigmasterol(95%) in 70 ml dry pyridine was added 6 g of *p*-toluenesulfonyl chloride . This mixture was stirred overnight in the dark at room temperature, then poured into 300 ml of ice-cold 5% potassium bicarbonate solution. The resulting solid was collected by filtration, washed with water and dried under vacuum at 60°. Recrystallization from acetone gave **3.5** (6.1 g, 90%) as tan crystals. MP=148-

149°(Lit.[74] 147-148°), $[\alpha]_D$ -46° (0.35,$CHCl_3$);^1H NMR ($CDCl_3$) δ 7.78 (2H, m), 7.31 (2H,m), 5.30 (1H,d, *J* =1.3), 5.12 (2H,m), 4.32 (1H,m), 2.45 (3H,s).

i-STIGMASTEROL METHYL ETHER **3.6.** To a refluxing solution of 5.5 g of freshly fused potassium acetate in 270 ml of dry methanol was added 5.5 g of stigmasterol tosylate **3.5**, and the mixture was refluxed for 3 hr. The solvent was removed under vacuum, and the residue was extracted with ether, washed with water, 5% aqueous potassium bicarbonate, and water again, and the ether phase was dried over Na_2SO_4. The solvent was removed under vacuum, leaving 5 g of an oily material which was purified by silica gel c.c. using a gradient of CH_2Cl_2 in hexane (10-30%), yielding 4.2 g (78%) of **3.6** as white crystals. MP= 59-60°(Lit[74] 58-59°); $[\alpha]_D$= +34°(0.27,$CHCl_3$); ^1H NMR δ 5.14 (1H, dd, *J*=15.1, 8.7), 3.32 (3H, s), 2.75(1H, t, *J*= 2.7), 1.02(3H, s), 0.84(3H, d, *J*= 6.4), 0.8(3H, s), 0.73(3H, s), 0.64(1H, m), 0.42(1H, m); ^{13}C NMR δ 138.5, 129.3, 82.5, 56.8, 56.7, 56.3, 51.4, 48.2, 43.5, 42.8, 40.7, 40.3, 35.4, 35.2, 33.5, 32.0, 30.6, 29.1, 25.5, 25.1, 24.4, 22.9, 21.6, 21.3, 21.2, 19.4, 19.1, 13.2, 12.6, 12.4.

ALDEHYDE **3.7.** Sterol **3.6**(2.4g, 5.6 mmol) was dissolved in 125 ml of dry CH_2Cl_2 containing 1% pyridine. The mixture was cooled to -78° and ozone was bubbled slowly in the solution with stirring. The reaction was judged to be complete (blue color) after about 30 min. The reaction vessel was then removed from the cooling bath, and 4 g of zinc dust was added, followed immediately by 10 ml glacial acetic acid. This mixture was stirred at room temperature for 1 hour. The zinc was then filtered off through celite/cotton, and the filtrate was concentrated, diluted, washed with water, and extracted thoroughly with

hexane. The combined organic extracts were washed with saturated sodium bicarbonate and water and then dried over anhydrous sodium sulfate. The solvent was removed, giving 1.98 g of a yellowish oil. 100 mg of this oil was purified by silica gel preparative TLC. IR ν max (KBr) 2933, 2400, 1721,1456, 1380, 1215, 1090, 1020, 925, 753, 667 cm^{-1}; ^1H NMR δ 9.95 (1H, aldehyde doublet), 3.32(3H, s,-OMe); EIMS *m/z* (rel. int.) 344(3), 328(8), 311(20), 289(12), 145(60), 133(50), 121(9), 105(85), 91(100), 79(90), 67(80).

ISOPROPYL BROMOMETHYLKETONE [**3.9**] Under argon, isobutyryl chloride **3.8** (4.3 g, .03 mol) was added slowly to a freshly prepared ethereal solution of diazomethane (.06 mol) at 0°. nitrogen is evolved as the yellow color goes off. After 30 minutes at 0°, dry hydrogen bromide was passed until it appeared that there was excess HBr (brownish color). The solution was kept at 0° for another 30 minutes, and then washed with water and sodium bicarbonate solution. The ether was dried over sodium sulfate and evaporated to yield 5 g of a yellow oil. This material was not characterized, but taken on directly to the next step.

TRIPHENYLPHOSPHINE ISOBUTANONEMETHYLENE[**3.10**]

Isopropylbromomethyl ketone **3.9** (5 g, 0.03 mol) was treated with triphenyl phosphine (10 g, .04 mol) in 30 ml dry benzene and stirred at room temp. for 24 hr. then filtered. The solid was taken up in fresh benzene, filtered, and dried under vacuum to give 14 g of 3-methylbutan-2-one-1-triphenylphosphonium bromide which was recrystallized from EtOAc-hexane. MP= 235-237°(Lit[76] 235-236°); ^1H NMR ($CDCl_3$) δ 7.75-7.89(15H, overlapping multiplets), 5.91(2H, d, *J* =11.7), 3.30(1H, sept., *J* =7.0), 1.13(6H, overlapping doublets, *J* = 7.0). The

phosphonium salt was then added to 50 ml of water and treated with saturated aqueous Na_2CO_3 with stirring at room temp. for 24 hr. The resulting solid was filtered, washed with water and dried under vacuum, then recrystallized from EtOAc-hexane to yield 11 g of **3.10**. MP= 170-172°(Lit[76] 170-171); ^1H NMR ($CDCl_3$) δ 7.4-7.8(15H, m), 3.7(1H, br d), 2.53(1H, sept., *J* = 6.9), 1.18(6H, d, *J* = 6.9).

22-*E*-6β-METHOXY-3α-5-CYCLO-5α-CHOLEST-22-EN-24-ONE [**3.11**]. The crude aldehyde **3.11** (1.98 g) was treated with 12 g of phosphorane **3.10** in 60 ml of anhydrous DMSO under an argon atmosphere and stirred at 95° for 72 hr. Then the reaction mixture was cooled to room temperature, and diluted with 100 ml of cold water. This mixture was extracted thoroughly with hexane, and the combined hexane extracts were washed with 75:25 methanol-water and pure water, and then dried over anhydrous sodium sulfate. Removal of solvent afforded 1.1 g of crude material which was purified by silica gel column chromatography eluting with 4% EtOAc in hexane. This gave 0.95 g (41% for 2 steps) of product. MP=114-115°(Lit[73] 115-116); $[\alpha]_D$ +48°(0.16,$CHCl_3$); ^1H NMR ($CDCl_3$) δ 6.75 (1H, dd, *J* = 16,9), 6.11 (1H, dd, *J* = 16, 1), 3.32(3H, s), 2.80(1H, sept., *J* = 6.9), 2.68(1H, br s), 2.27(1H, m), 1.11(6H, two overlapping doublets, *J* = 7.0), 0.99(3H, s), 0.64(3H,s); ^{13}C NMR ($CDCl_3$) δ 204.6, 152.7, 126.2, 82.4, 56.7, 56.4, 55.2, 48.1, 43.5, 43.3, 40.2, 40.1 38.4, 35.4, 35.2, 33.5, 30.6, 28.4, 25.1, 24.3, 22.9, 21.6, 19.5, 19.4, 18.7, 18.6, 13.2, 12.6.

6β-METHOXY-3α,5-CYCLO-CHOLESTA-24-ONE [**3.12**]. A mixture of 100 mg (0.24 mmol) of enone **3.11**, 15 mg of 10% palladium on carbon in 10 ml of ethyl

acetate was treated with H_2 for 2 hr. The mixture was filtered, the solvent evaporated, and the crude mixture was purified by silica gel column chromatography eluting with 6% ethyl acetate in hexane to yield 90 mg (91%) of pure **3.12**. Mp= 91°. $[\alpha]_D$ + 32°(0.20,$CHCl_3$); IR ν max (KBr) 2930, 2870, 1710, 1470, 1380,1290, 1180, 1100, 1015, 970; ^{1}H-NMR ($CDCl_3$) δ 3.28 (3H, s), 2.76 (1H, t,*J* =2.8), 2.60 (1H, sept.,*J* =6.9), two overlapping doublets at 1.08(*J* =6.9), 1.01 (3H, s),0.90 (3H,d,*J* =5.6), 0.71 (3H, s), 0.64 (1H, m), 0.42 (1H, m); ^{13}C-NMR; δ 215.2, 82.5, 56.7, 56.5, 56.2, 48.1, 43.5, 42.9, 40.9, 40.4, 37.4, 35.6, 35.4, 35.1, 33.5, 30.6, 29.9, 28.4, 25.1, 24.3, 22.9, 21.6, 19.4, 18.6, 18.5, 18.4, 13.2, 12.2; EIMS *m/z* $[M]^+$ 414.3510 ($C_{28}H_{46}O_2$ requires 414.3498), 399(60), 382(65), 359(100), 255(25), 213(30), 107(40).

3β-ACETOXY-5-CHOLESTEN-24-ONE [**3.13**]. A mixture of 150 mg (0.36 mmol) of ketone **3.12**, 1.2 g of freshly fused zinc acetate, and 30 ml of glacial acetic acid was stirred at reflux (120°) for 2 hr. After the mixture was cooled, 40 ml cold water was added and this was extracted with 50:50 hexane:benzene. The organic extracts were combined and washed with water, 5% sodium bicarbonate, and finally brine, then dried over anhydrous sodium sulfate. The solvent was removed, leaving 187 mg of a crude yellow solid. This was subjected to silica gel c.c., eluting with 50:50 hexane:CH_2Cl_2, to yield 145 mg of pure **3.13**. Mp=133°. $[\alpha]_D$ -46°(0.22, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.37(1H, d, *J* = 4.8), 4.60(1H, m), 2.61(sept. *J* = 7.0), 1.08(6H, two overlapping doublets,*J* = 7.0), 1.02 (3H, s), 0.92(3H, d, *J* = 6.4), 0.68(3H, s);^{13}C NMR ($CDCl_3$) δ 215.3, 170.4, 139.5, 122.5, 73.9, 56.6, 55.8, 49.9, 42.3, 40.7, 39.6, 38.0, 37.1, 36.9, 36.5, 35.3, 31.8, 31.7, 29.7, 28.0, 27.7, 24.2, 21.4, 20.9, 19.2, 18.4, 18.3, 18.2, 11.8; EIMS *m/z* (rel int.) $[M-60]^+$ 382.3234

($C_{27}H_{42}O$ requires 382.3235), 367(15), 296(17), 255(17), 213(18), 147(28), 145(26), 107(24).

ETHYLENE DIOXY KETAL OF 3β ACETOXY-5-CHOLESTEN-24-ONE **3.14**. A mixture of 150 mg (.34 mmol) of acetate **3.13**, 350 mg (5.7 mmol) of ethylene glycol, 13 mg (0.07 mmol) of *p*-TsOH in dry benzene was heated to 100° for 24 hr., using a Dean-Stark apparatus to remove water. The rection mixture was cooled, and then washed with dilute $NaHCO_3$ solution. The aqueous wash was back washed with hexane, and the combined organic extracts were dried over Na_2SO_4, and evaporated to yield 171 mg of crude material which was purified by silica gel column chromatography, to yield 160 mg (96%) of pure **3.14**. ^1H NMR ($CDCl_3$) δ 5.38 (1H, br d), 4.62 (1H, m), 3.93 (4H, s), 2.04 (3H,s); EIMS *m/z* (rel. int.) 443(20), 426(5), 383(30),159(20), 145(30), 133(15), 115(100), 105(20), 91(20), 81(25), 73(30), 55(25).

24-ETHYLENE DIOXY KETAL OF 3β-ACETOXY-5-CHOLESTEN-7,24-DIONE **3.15**. The sterol **3.14** (125 mg, 0.25 mmol), tert-butylhydroperoxide (0.78 mmol), $Cr(CO)_6$ (40 mg, .18 mmol), and $NaHCO_3$ (11 mg, 0.13 mmol) in 5 ml of CH_3CN were refluxed at 100° for 72 hours. The mixture was cooled to room temperature, 5 ml of water was added, and this was extracted thoroughly with ether. The ether extract was dried over Na_2SO_4, and the crude mixture was purified by silica gel column chromatography using 20% EtOAc in hexane to yield 45 mg (35%) of pure **3.15.** ^1H NMR ($CDCl_3$) δ 5.72 (1H,s), 4.70 (1H, m), 3.93 (4H,s), 2.04 (3H,s); CIMS *m/z* $[M+1]^+$ 501; EIMS *m/z* (rel. int.) 457(30), 396(35), 311(25), 174(40), 161(35), 134(30), 115(100), 105(30), 91(60), 81(40), 67(30).

ETHYLENE DIOXY KETAL OF 3β-ACETOXY-7-α-HYDROXY-5-CHOLESTEN-24-ONE **3.16**. Under argon, sterol **3.15** (150 mg, 0.3 mmol) in dry THF was cooled to -78°, and L-Selectride was added slowly. After 1 hour the reaction mixture wa treated with 1 ml of water, and the organoborane was oxidized with 1 ml of 6M NaOH and 1 ml of 30% H_2O_2. The THF was removed under vacuum, and the crude product was extracted with CH_2Cl_2 which was dried over Na_2SO_4. The CH_2Cl_2 was removed to yield 157 mg of crude material which was applied to 2 1000 μm PTLC plates and eluted with 30% EtOAc in hexane, to yield 75 mg of pure **3.16**. ^1H NMR ($CDCl_3$) δ 5.62 (1H, br d), 4.65 (1H, m), 3.93 (3H, s), 3.85 (1H, m), 2.04 (3H,s).

ETHYLENE DIOXY KETAL OF 3β,7α-DIACETOXY-5-CHOLESTEN-24-ONE **3.17**. Sterol **3.16** (35 mg, 0.07 mmol), Ac_2O (350μl), and 700μl dry pyridine were sitrred at room temperatue for 24 hours, diluted with CH_2Cl_2, and wshed with 10% HCl. The organic extract was dried over Na_2SO_4, and purified by PTLC eluting with 30% EtOAc in hexane to yield 36 mg (96%) of pure **3.17**. ^1H NMR ($CDCl_3$) δ 5.62 (1H, br d), 4.94 (1H, m), 4.65 (1H, m), 3.93 (4H,s) 2.04 (6H, s).

3β,7α-DIACETOXY-5-CHOLESTEN-24-ONE **3.18**. Sterol **3.17** (30 mg, 0.05 mmol), 1.5 mg (0.008 mmol), *p*-TsOH in absolute ethanol was stirred for 5 hours. Then saturated $NaHCO_3$ was added and this was extracted with $CHCl_3$. The $CHCl_3$ was dried over Na_2SO_4 and purified by PTLC eluting with 5:1 hexane-acetone to give 18 mg (70%) of **3.18**. ^1H NMR ($CDCl_3$) δ 5.62 (1H, br d), 4.94 (1H, m), 4.65 (1H, m), 2.04 (6H,s).

3β,7α-DIHYDROXY-5-CHOLESTEN-24-ONE **3.21**. (Can be obtained in 95% yield by hydrolysis of **3.18** in 5% KOH in MeOH or by the following procedure) A mixture of 80 mg (0.18 mmol) acetate **3.13**, 80 mg CuBr and 156 mg tert-butylperoxybenzoate in 1.0 ml of AcOH under an argon atmosphere was stirred at 120° for 0.5 hr. The mixture was cooled to room temperature, diluted with benzene, filtered, and washed with water, sodium carbonate, and water again. The benzene was evaporated and the crude mixture was subjected to hydrolysis using 2% potassium hydroxide in methanol. Separation of the 7α OH and 7β OH compounds was achieved by silica gel chromatography using 100% ether as eluent. This yielded 12 mg (16%) of pure **3.21**. MP= 120-122°; $[\alpha]_D$ -60°(0.10,$CHCl_3$); IR ν max (KBr) cm^{-1}; 3380, 2930, 1700, 1560, 1470, 1380, 1135, 1060, 950, 825, 790; ^1H NMR ($CDCl_3$) δ 5.60(1H, dd,*J* = 3.8, 1.4), 3.85(1H, br s), 3.58(1H, m), 2.60(1H, sept.,*J* =6.9),2.46(1H,m), 1.08(6H, two overlapping doublets,*J* = 6.9), 0.99(3H, s), 0.92(3H, d, *J* =6.4), 0.68(3H, s); ^{13}C NMR ($CDCl_3$) δ 215.3, 146.2, 123.8, 71.3, 65.3, 55.6, 49.4, 42.2, 42.1, 41.9, 40.8, 39.2, 37.5, 37.4, 37.03, 37.00, 35.3, 31.4, 29.8, 28.1, 24.2, 20.7, 18.5, 18.3, 18.20, 18.26, 11.6; EIMS *m/z* (rel int.) $[M]^+$ 416.3305 ($C_{27}H_{44}O_3$ requires 416.3290), $[M-H_2O]^+$ 398.3179 ($C_{27}H_{42}O_2$ requires 398.3185), $[M-2\ H_2O]^+$ 380.3079 ($C_{27}H_{40}$) requires 380.3079), 271(8), 145(9), 73(62), 55(13).

3β,7β-DIHYDROXY-5-CHOLESTEN-24-ONE **[3.22].** From above procedure, yield was 8 mg (10%) of pure **3.22**. MP= 139°; $[\alpha]_D$= + 10°(0.18,$CHCl_3$);IR ν max (KBr) 3380, 2325, 1705, 1560, 1460, 1380, 1140, 1055, 950, 820, 790; ^1H NMR ($CDCl_3$) δ 5.29(1H, br s), 3.84(1H, m), 3.54(1H, m), 2.60(1H, sept., *J* = 6.9), 2.46(1H,

m), 1.08(6H, two overlapping doublets,J = 6.9), 0.99(3H, s), 0.92(3H, d, J = 6.4), 0.68(3H, s); ^{13}C NMR ($CDCl_3$) δ 215.2, 143.4, 125.5, 73.3, 71.4, 55.9, 55.3, 48.3, 42.9, 41.7, 40.9, 40.8, 39.5, 37.2, 36.9, 36.4, 35.3, 31.6, 29.9, 28.4, 26.3, 21.1, 19.1, 18.5, 18.31, 18.26, 11.8.

3β,7β-DIHYDROXYERGOST-5,24(28)DIENE **3.23**. Under argon, 4.3 mg (0.18 mmol) NaH in 0.3 ml dry DMSO was heated to 70° for 45 min., then cooled to room temperature. Ph_3PCH_3Br (64 mg, 0.18 mmol) in 1.5 ml DMSO was added to mixture and stirred at room temperature for 10 min. 7 mg (.018 mmol) of **3.22** in 0.2 ml was added and the mixture was stirred at 60° for ca. 1 hr. The mixture was cooled, diluted with ether and washed repeatedly with H_2O. The ether layer was dried (Na_2SO_4), evaporated, and after reversed-phase PTLC (eluting with 95% aq. MeOH), yielded 4.1 mg (59%) of pure **3.23**. MP= 152-153°(Lit.[88] 149-151°) $[\alpha]_D$ + 19°(0.16,$CHCl_3$) ;^1H-NMR ($CDCl_3$) δ 5.29 (1H, br s), 4.71 (1H, s), 4.65 (1H, s), 3.84 (1H, m), 3.55 (1H, m), 1.05 (3H, s), 1.03 (3H, d, J=6.8), 1.01 (3H, d,J =6.8), 0.95 (3H, d,J =6.4), 0.70 (3H, s); ^{13}C-NMR ($CDCl_3$) d 156.8, 143.5, 125.5, 106.1, 73.3, 71.4, 55.9, 55.3, 48.2, 42.9, 41.7, 40.9, 39.5, 36.9, 36.4, 35.7, 34.7, 33.8, 31.6, 31.0, 28.5, 26.3, 22.0, 21.8, 21.1, 19.1, 18.7, 11.8; EIMS m/z (rel int.) $[M]^+$ 414.3489 ($C_{28}H_{46}O_2$ requires 414.3498), $[M-H_2O]^+$ 396.3392 ($C_{28}H_{44}O$ requires 396.3392), 378(9), 312(12), 143(8), 107(11).

3β,7β-DIHYDROXY-24,28-EPOXYERGOST-5-ENE **3.24**. Under argon, 3mg (.12 mmol) NaH in 2.5 ml DMSO was heated to 70° for 45 min., then cooled to room temperature and 2.5 ml THF was added. 1.0 ml of this solution was removed by syringe, and added to an argon filled 2-neck flask. This was cooled to 0° and 5

mg (.024 mmol) $(CH_3)_3SI$ in minimal DMSO was added slowly. The resulting mixture was stirred at 0° for 1 min., and 5 mg (0.012 mmol) of **3.22** in THF was added. This was stirred at 0° for ca. 1 min., then allowed to warm to room temperature. After 1 hr., reaction was worked up with H_2O/ether. The ether layer was dried (Na_2SO_4), evaporated, and after reversed-phase PTLC (eluting with 95% aq. MeOH), yielded 3.2 mg (63%) of pure **3.24**. Mp= 157-158° $[\alpha]_D$= + 14°(0.13,$CHCl_3$); ^1H NMR ($CDCl_3$) δ 5.29(1H, br s), 3.84(1H, m), 3.55(1H, m), 2.57(2H, m), 1.05(3H, s), 0.80-0.98(6H, four overlapping doublets), 0.69(3H, s); ^{13}C NMR ($CDCl_3$) δ 143.5, 125.4, 73.3, 71.4, 62.7, 55.9, 55.2, 55.0, 50.5, 48.2, 42.9, 41.7, 40.9, 39.5, 36.9, 36.4, 35.8, 35.7, 32.0, 31.7, 31.6, 30.3, 30.2, 28.5, 28.4, 27.7, 26.3, 21.0, 19.1, 18.7, 18.6, 18.4, 18.2, 17.9, 17.7, 11.8; EIMS *m/z* (rel int.) $[M]^+$ 430.3462 ($C_{28}H_{46}O_3$ requires 430.3447), $[M-H_2O]^+$ 412.3337 ($C_{28}H_{44}O_2$ requires 412.3341), $[M-2H_2O]^+$ 394.3236 ($C_{28}H_{42}O$ requires 394.3236), 353(5), 312(7), 269(10), 211(12), 143(35), 95(38), 55(51).

3β-HYDROXYCHOLEST-5-ENE,24-ONE [**3.25**]. 110 mg (0.25mmol) of acetate **3.13** in 5% methanolic KOH was stirred at room temperature for 2 hr. The solvent was removed under vacuum, and the resultant mixture was taken up in CH_2Cl_2, washed repeatedly with water, then dried over Na_2SO_4. After reversed-phase PTLC using 90% aq. MeOH as eluant, 98 mg (98%) of pure **3.25** was obtained. MP=136-138°(Lit[89] 137°);^1H NMR ($CDCl_3$) δ 5.34(1H, m), 3.53(1H, m), 2.60(1H, sept., *J* = 6.9), 1.08(6H, two overlapping doublets,*J* = 6.9), 0.99(3H, s), 0.90(3H, d, *J* = 6.8), 0.67(3H, s);^{13}C NMR ($CDCl_3$) δ 215.5, 140.7, 135.6, 127.6, 121.7, 71.8, 56.7, 55.9, 50.0, 42.3, 42.2, 40.8, 39.7, 37.2, 36.5, 35.4, 31.9, 31.6, 29.8, 28.1, 24.2, 21.1, 19.4, 18.5, 18.4, 18.3, 11.9; EIMS m/z (rel int.) $[M]^+$ 400.3341 ($C_{27}H_{44}O_2$

requires 400.3341), $[M- H_2O]^+$ 382.3242 ($C_{27}H_{42}O$ requires 382.3236), 367(41), 314(60), 312(53), 234(50), 213(80), 71 (95).

3β-HYDROXYERGOST-5,24(28)-DIENE [**3.26**]. Under argon, 1.1 mg (0.046 mmol) NaH in 0.5 ml dry DMSO was stirred at 70° for 45 min., then cooled to room temperature. To this solution 16 mg (0.046 mmol) of Ph_3PCH_3Br in 0.5 ml of DMSO was added and stirred for 10 min. Then 10 mg (0.023 mmol) of **3.25** was added in 0.2 ml DMSO and the mixture was stirred at 60% for ca. 2 hr. The mixture was cooled, diluted with ether and washed repeatedly with water. The ether layer was dried (Na_2SO_4), evaporated, and after PTLC (eluting with 30% EtOAc in hexane), yielded 7 mg (71%) of pure **3.26**. MP= 141-142°(Lit[90] 141°);^{1}H NMR ($CDCl_3$) δ 5.34 (1H, m), 4.71 (1H,s), 4.65 (1H, s), 3.53 (1H,m), 2.57 (2H, m),1.05 (3H, s), 1.03(3H, d, *J* = 6.8), 1.01 (3H, d, *J* = 6.8), 0.95 (3H, d, *J* = 6.5), 0.68 (3H, s); MS *m/z* (rel. int.) 398(5), 383(5), 314(55), 299(20), 271(25), 229(20), 213(25), 145(35), 133(20), 105(30), 91(40), 81(35), 69(100).

3β-HYDROXY-24,28-EPOXY-ERGOST-5-ENE [**3.27**]. Under argon, 11 mg of NaH in 2.5 ml dry DMSO was heated to 70° for 45 minutes, then cooled to room temperature and diluted with 2.5 ml of dry THF. 0.5 ml of this solution was removed and added to an argon filled 2 neck flask and cooled to 0°. $(CH_3)_3SI$ (9.4 mg) in 0.5 ml DMSO was added and stirred for 10 min. Sterol **3.25** (10 mg,0.023 mmol) was added in minimal THF and stirred at 0° for 1 min. then allowed to warm to room temperature and continued to stir for 2 hr. The reaction was worked up with ether and water. After drying the ether layer over Na_2SO_4, the crude product was subjected to PTLC, eluting with 30% EtOAc in hexane and

this yielded 3.6 mg(36%) of pure **3.27**. MP= 150-152°(Lit[90] 153°); IR ν max (KBr) cm^{-1} 3370, 2920, 2850, 1740, 1440, 1270, 1170, 1120, 1015, 690; ^{1}H NMR ($CDCl_3$) δ 5.34(1H, m), 3.53(1H, m), 2.57(2H, m), 1.05(3H, s), 0.80-0.98(6H, four overlapping doublets), 0.69(3H, s); ^{13}C NMR ($CDCl_3$) δ 140.7, 121.6, 71.8, 62.7, 55.9, 55.8, 50.4, 50.1, 42.33, 42.30, 39.8, 37.2, 36.5, 35.8, 35.7, 32.0, 31.90, 31.87, 31.70, 31.66, 30.3, 30.2, 29.7, 28.2, 28.1, 27.8, 24.2, 21.1, 19.4, 18.64, 18.61, 18.35, 18.17, 17.9, 17.7, 11.8; EIMS *m/z* (rel. int.) 414, (40), 396(35), 381(20), 329(15), 314(25), 303(15), 271(35), 213(30), 161(40), 145(50), 133(40), 119(35), 105(50), 95(55), 81(70), 69(60).

IV. CONCLUSIONS

Two approaches to the search for novel anticancer agents have been described in the preceding chapters. One approach, the isolation of compounds from plant material, was discussed in chapter 2, and new and interesting compounds were shown which possess selective DNA damaging activity.

Another approach, the synthesis of known bioactive natural products, was covered in chapter 3. The completion of this synthetic work allowed for structure proof of some previously isolated sterols and brought to light very interesting data concerning the structure-activity relationships of these compounds.

Although this work stands on its own, it also opens the door to further studies. For example, the naphthoquinones may be derivatized in order to enhance their activity, or alternatively they can serve as model structures for work directed at the synthesis of improved analogs. The sterols are not currently envisaged as anticancer drugs because of their relatively low activity, but they could serve as substrates for studies into the mechanism of cytotoxicity or DNA damage.

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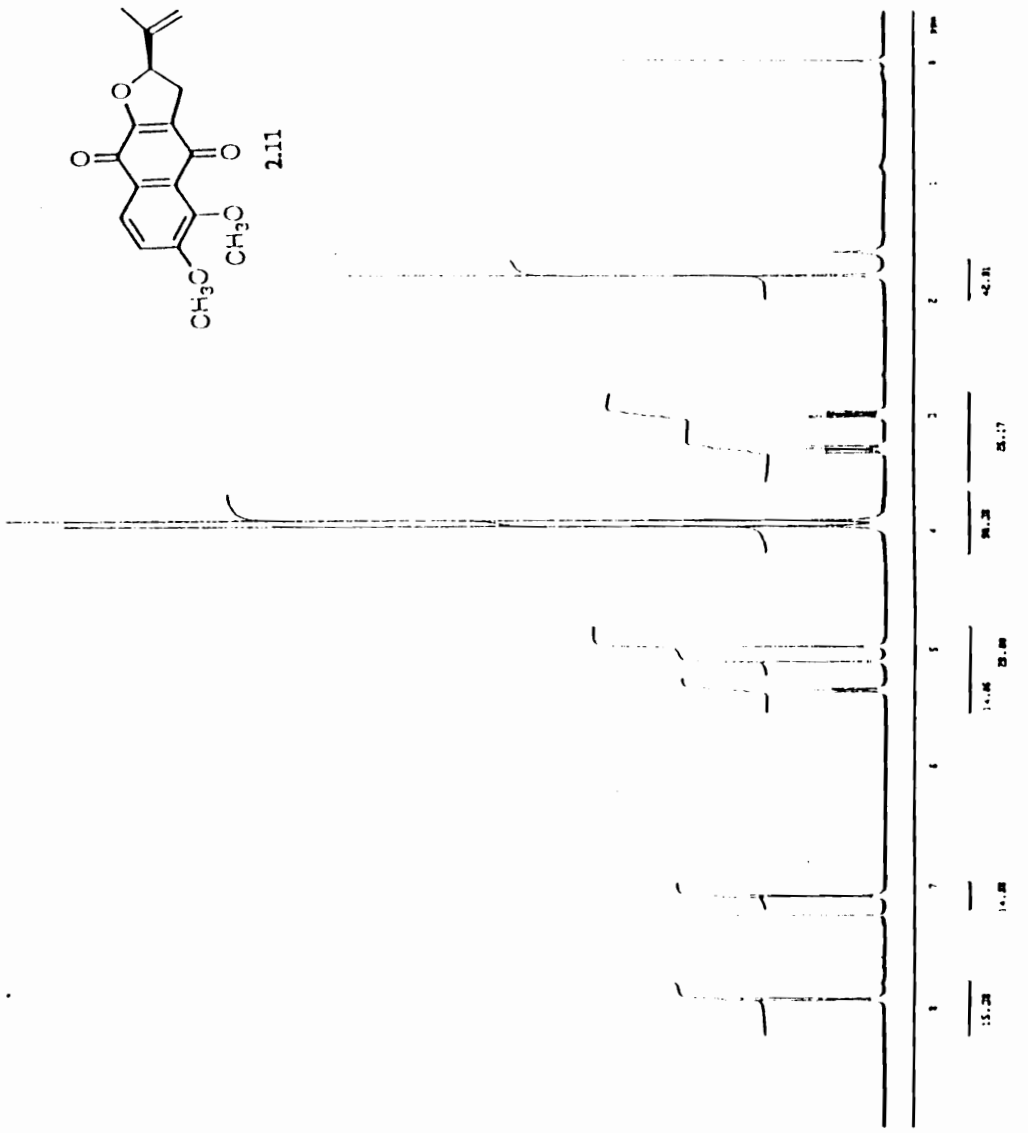
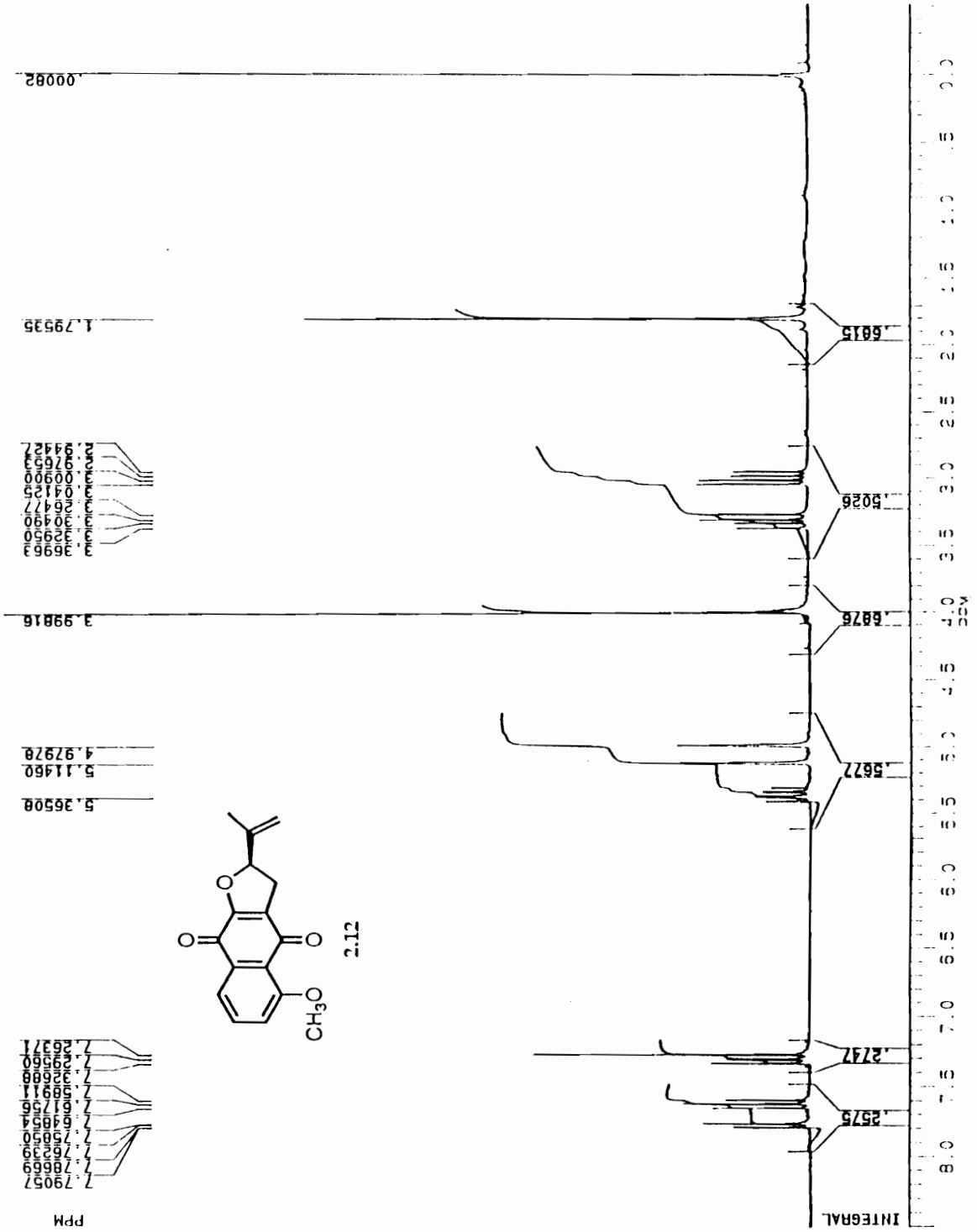
VI. APPENDIX

^1H NMR Spectra of Selected Compounds

in $CDCl_3$ (unless otherwise noted)



2.11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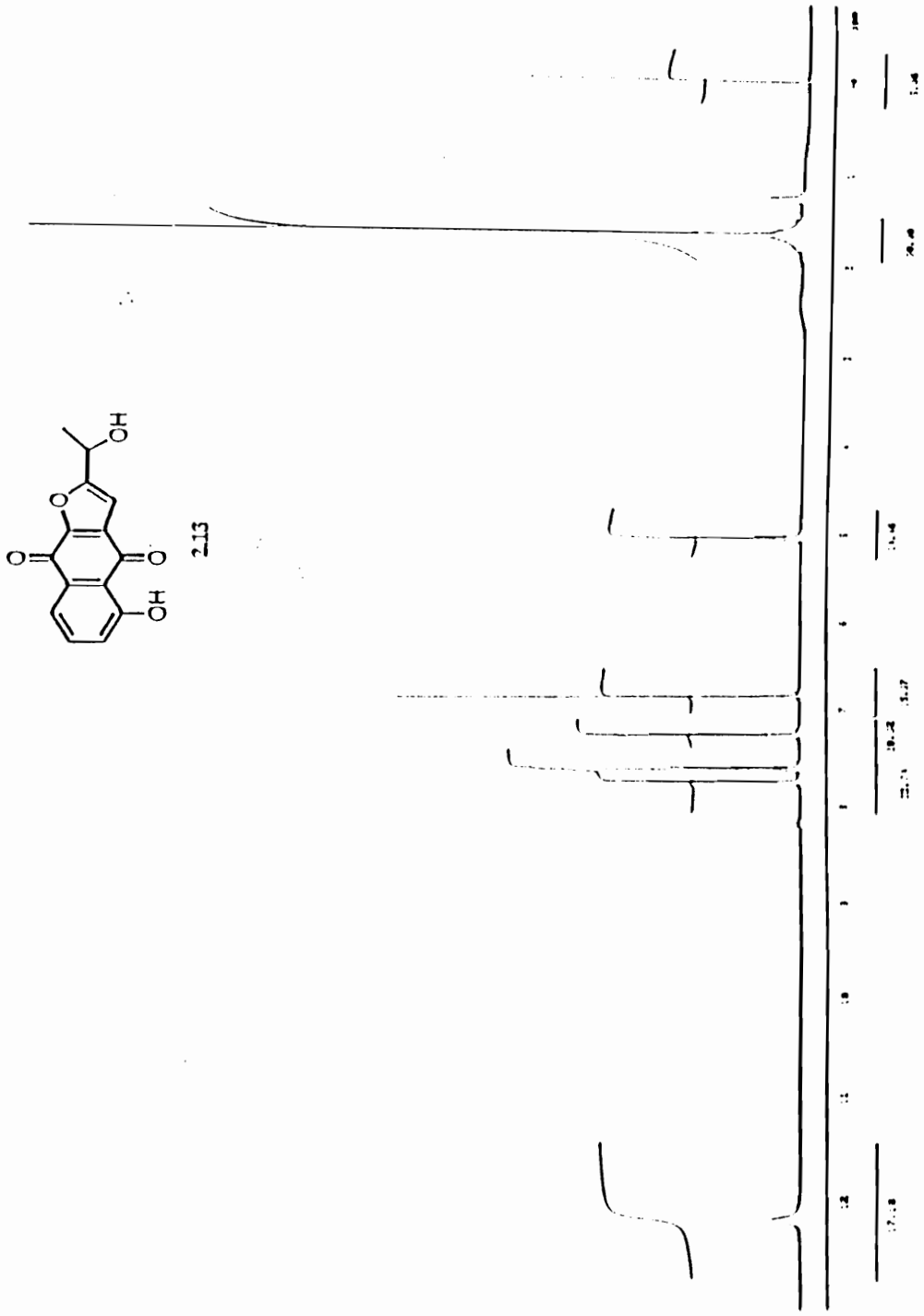
PPM
2.12
CH_3O



2.13

OH
O
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63.62
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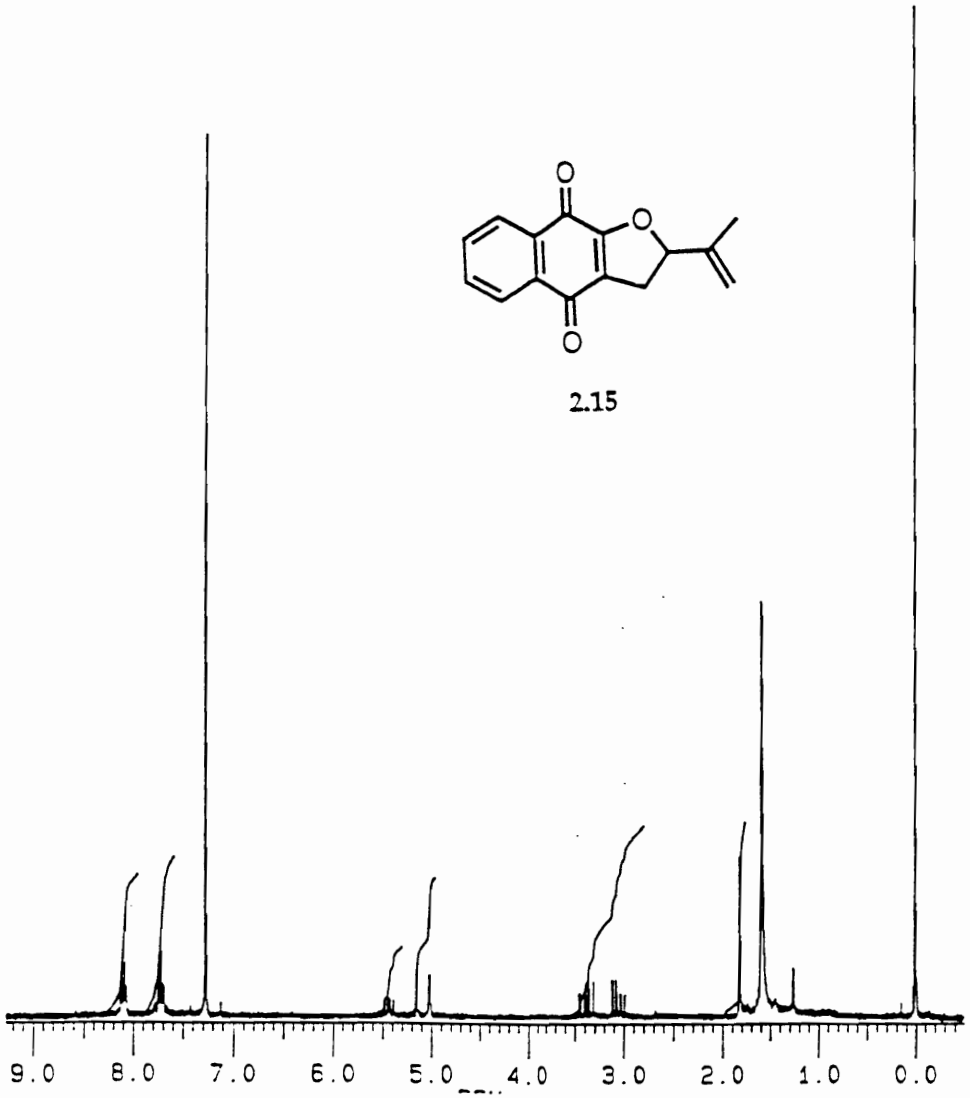
11.05
9.50
INTEGRAL
PPM

PROTON AT 200 MHZ

2.15



BRUKER

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BEH.002
DATE 26-9-93

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SI    16384
TD    16384
SW     2000.000
HZ/PT     .244

PW        6.0
RD         .500
AQ        4.096
RG      100
NS       71
TE      297

FW     2500
O2        0.0
DP   63L PO

LB        0.0
GB        0.0
CX       15.00
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PPM/CM    .666
SR     2340.34
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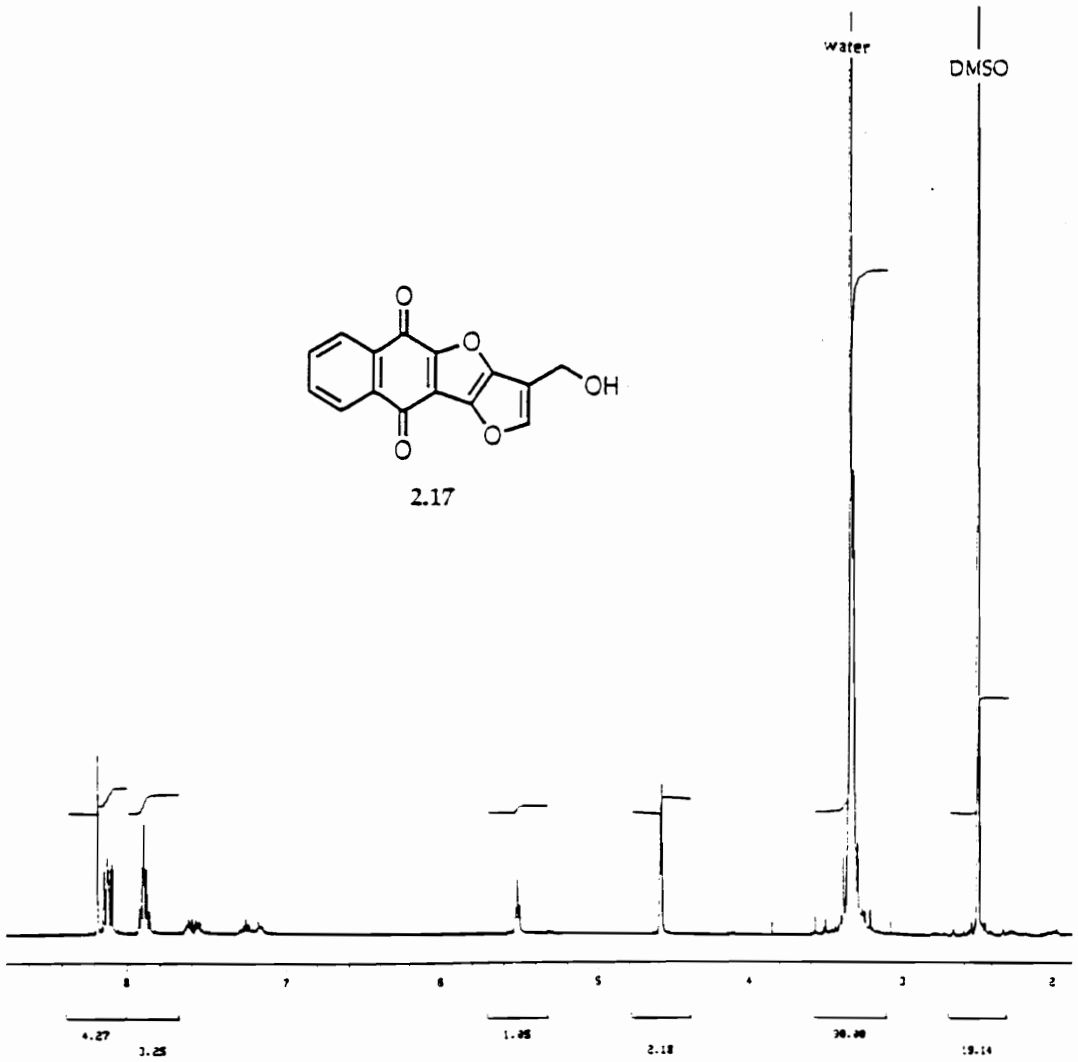
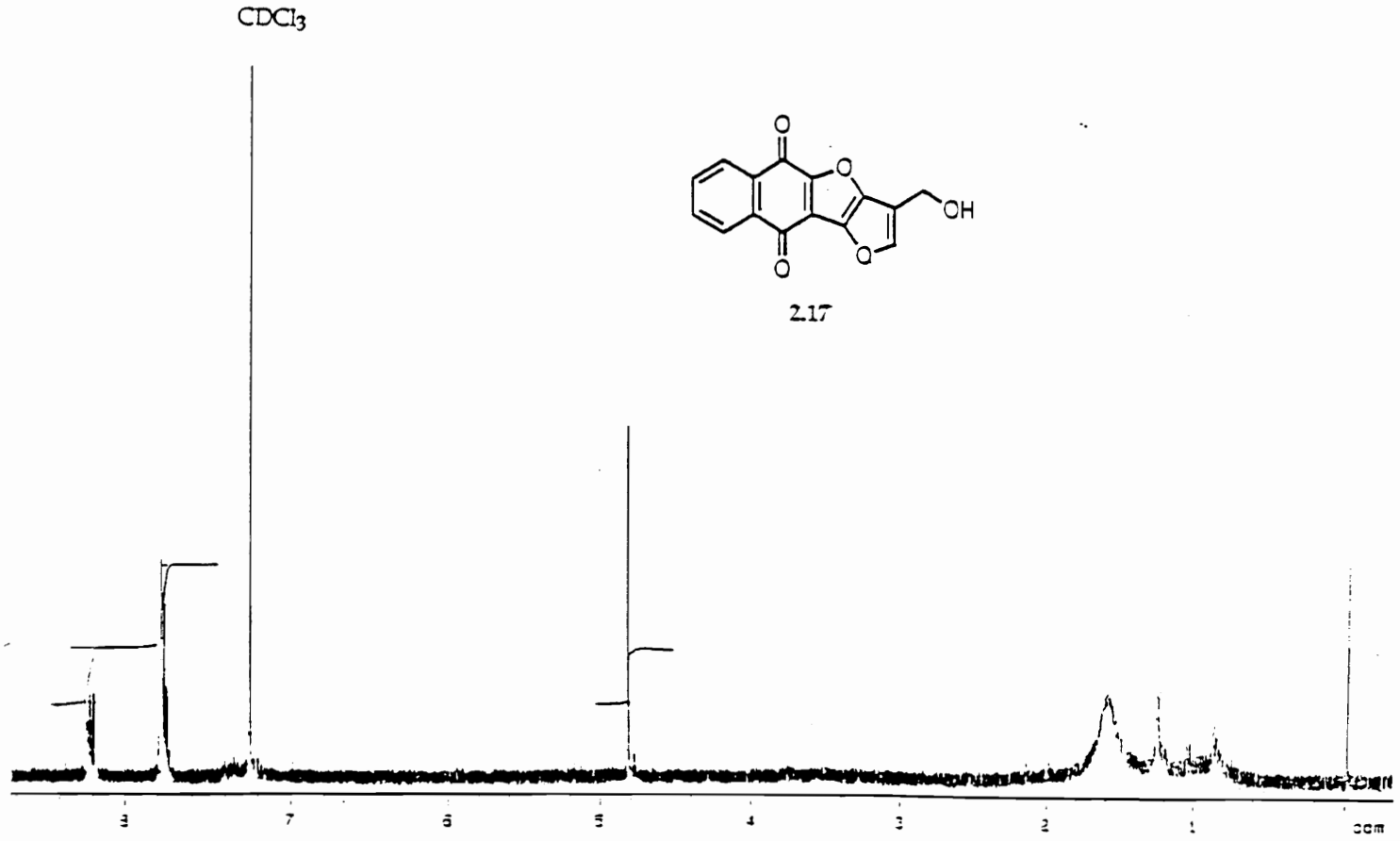
2.17

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O
O
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5
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3
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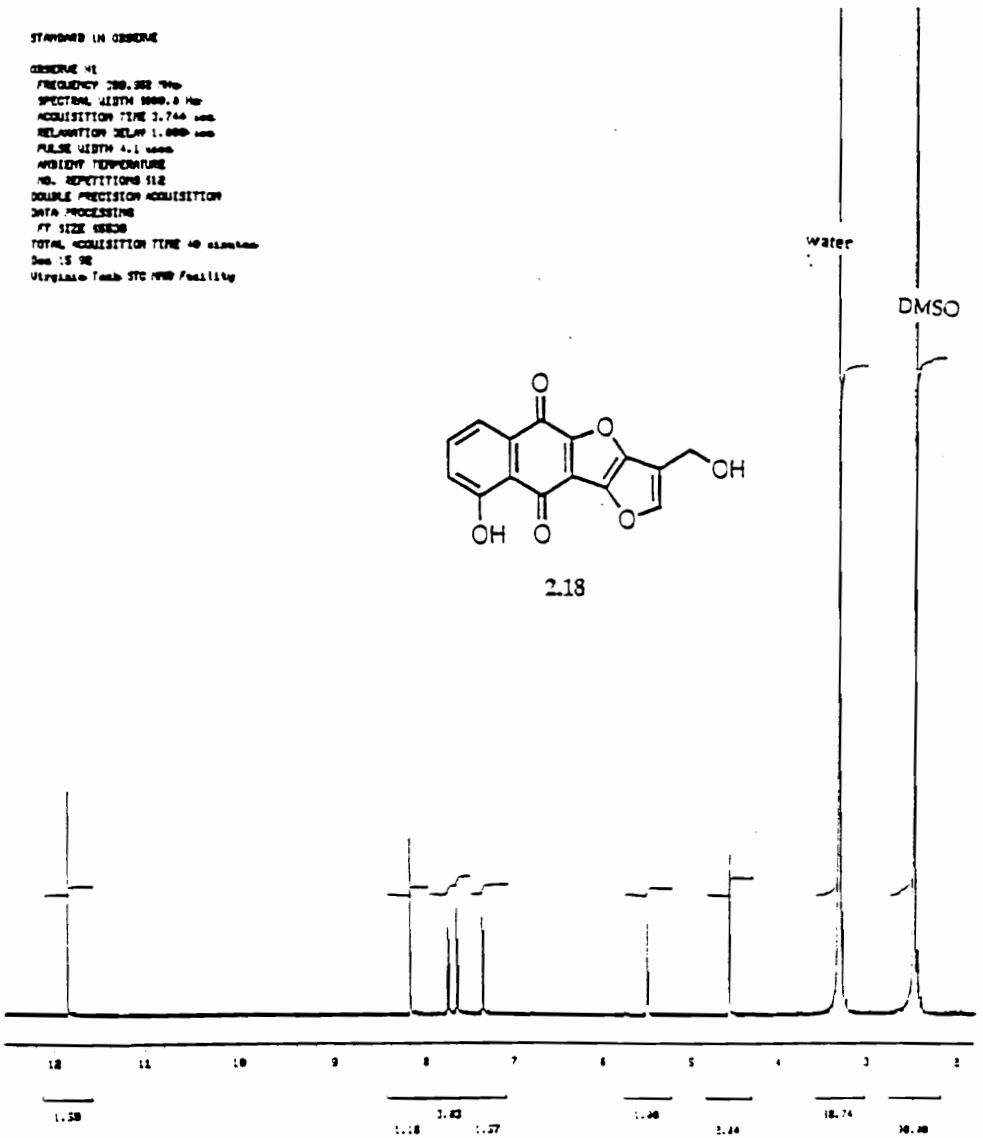
ppm


STANDARD IN OBSERVE
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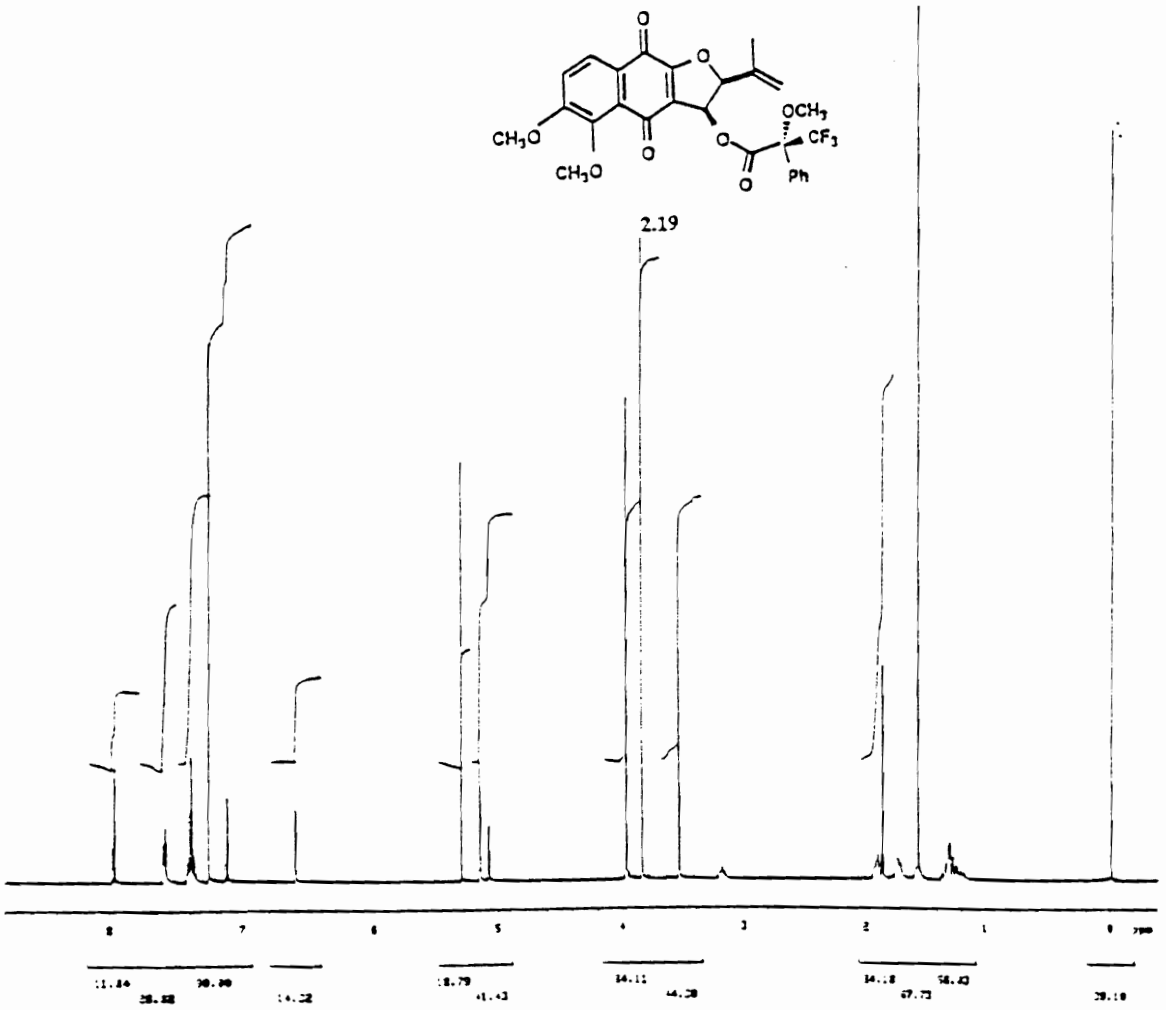
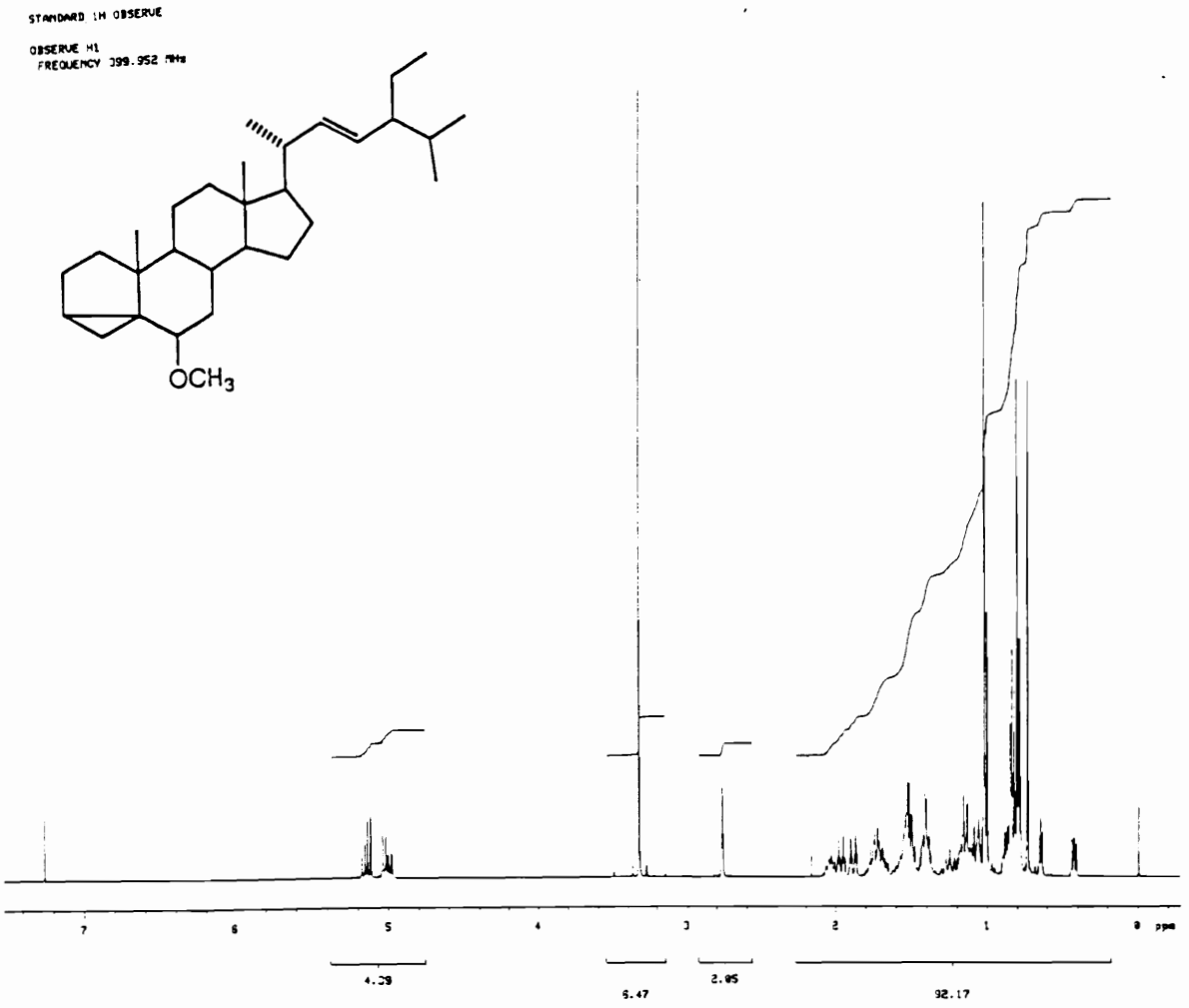
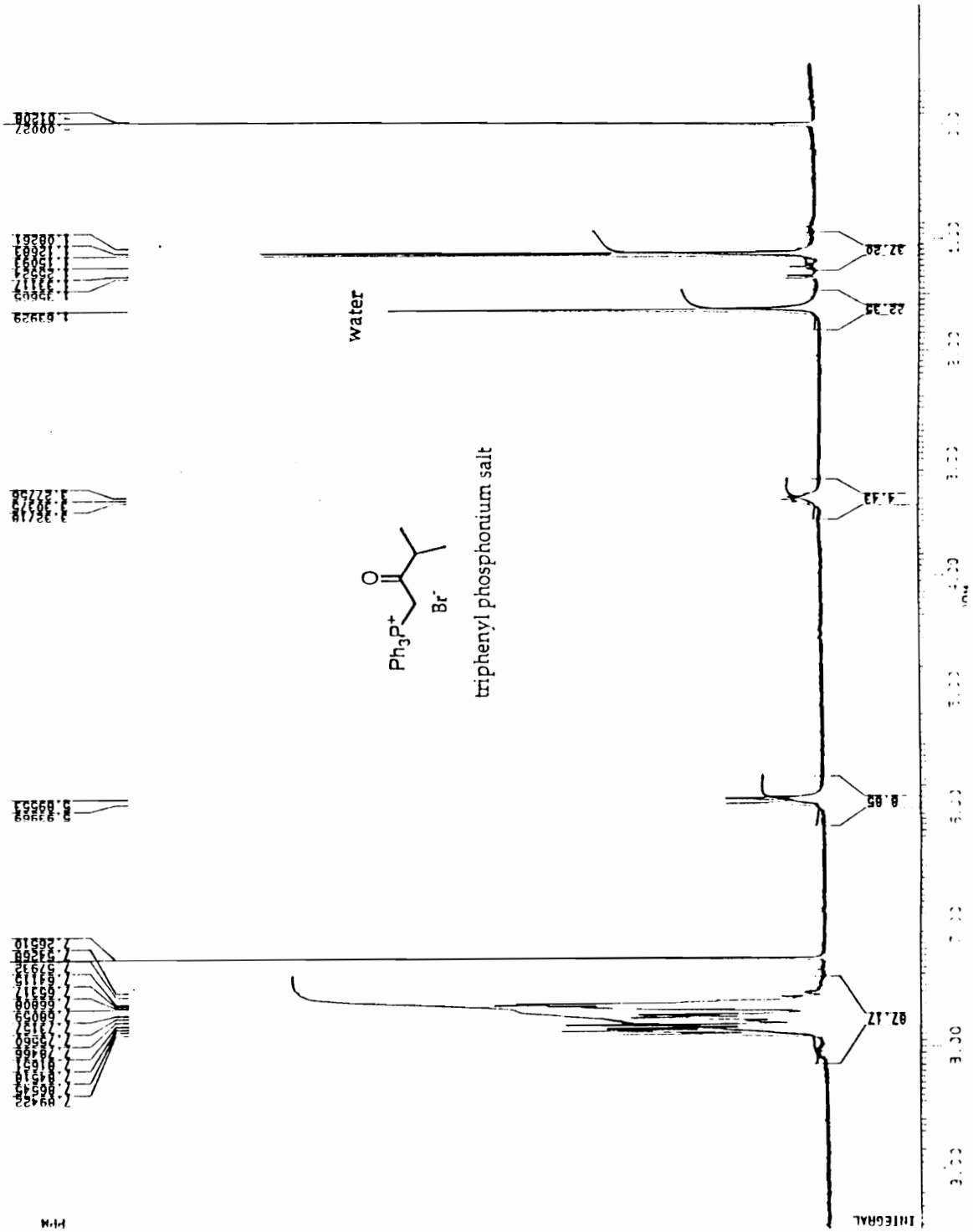
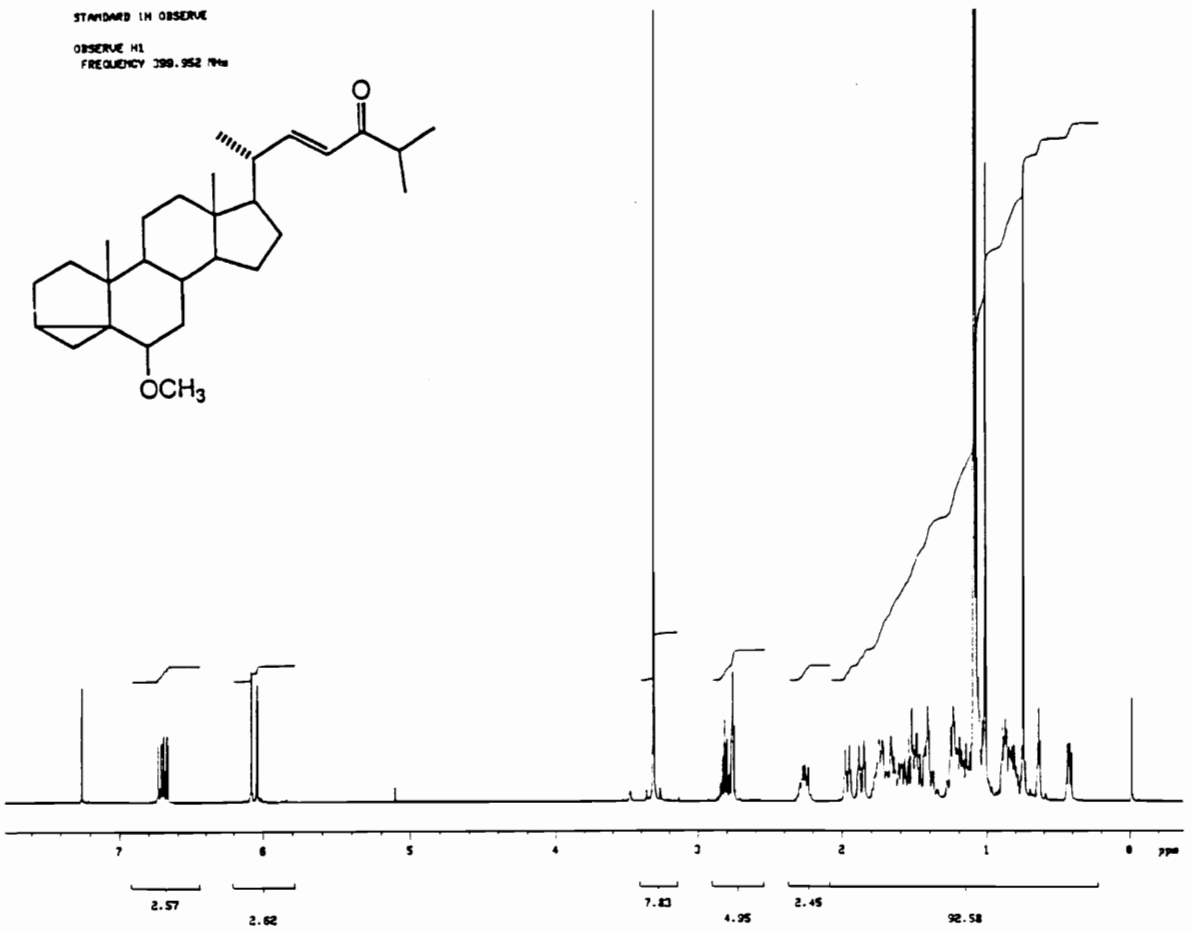
SPECTRAL WIDTH 5000.0 Hz
ACQUISITION TIME 3.744 sec
RELAXATION DELAY 1.000 sec
PULSE WIDTH 4.1 usec
AMBIENT TEMPERATURE
NO. REPETITIONS 512
DOUBLE PRECISION ACQUISITION
DATA PROCESSING
FT SIZE 65536
TOTAL ACQUISITION TIME 40 minutes
Done :S 98
Virginia Tech STC NMR Facility
Water
DMSO
OH
OH
O
O
O
O
2.18
12
11
10
9
8
7
6
5
4
3
2
1.58
3.83
1.18
1.27
1.48
2.44
18.74
30.00

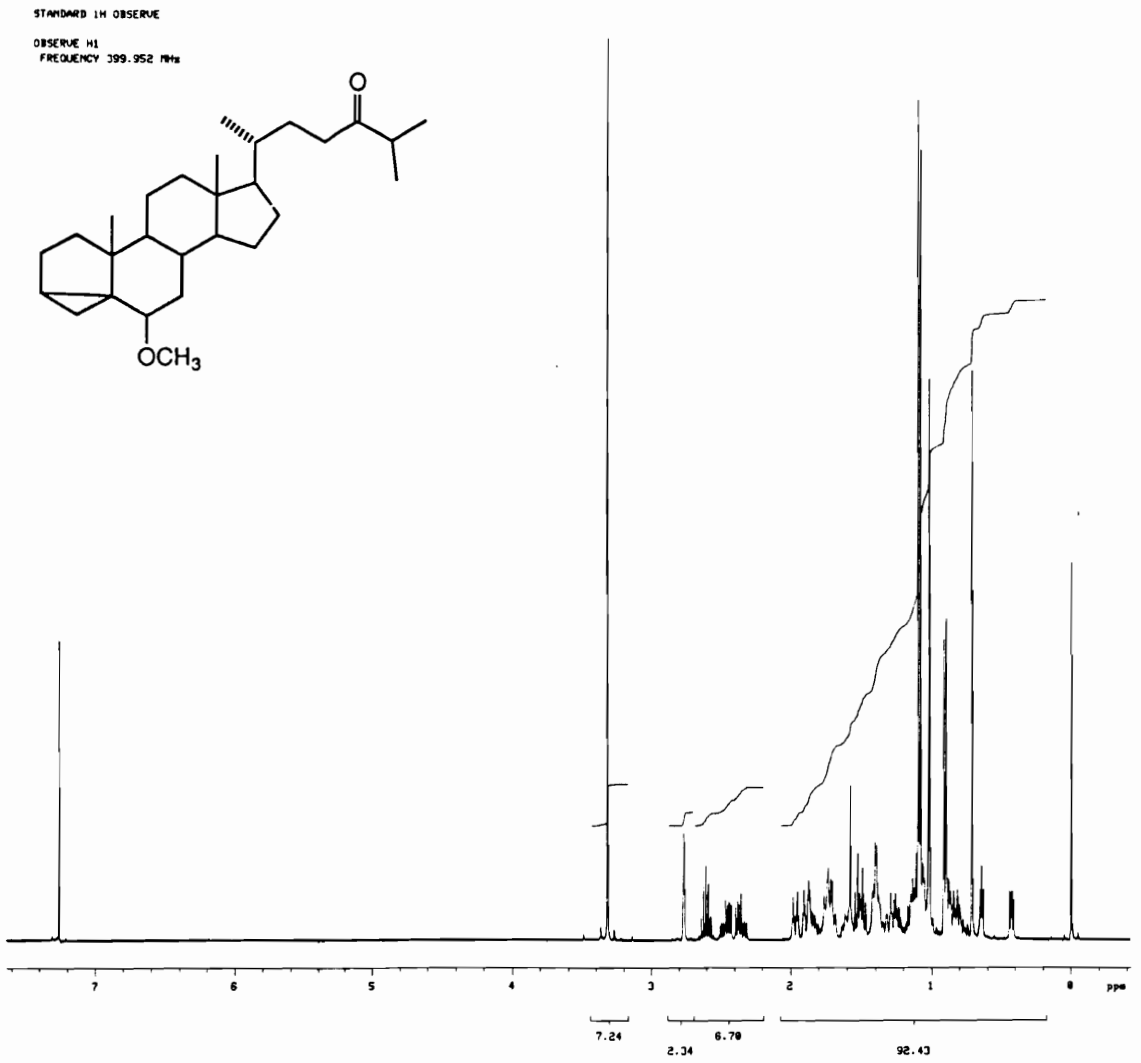

O
O
O
CH3O
CH3O
O
O
OCH3
CF3
Ph
2.19


STANDARD 1H OBSERVE
OBSERVE H1
FREQUENCY 399.952 MHz
OCH3
7
6
5
4
3
2
1
0 ppm
4.29
6.47
2.05
92.17


water
triphenyl phosphonium salt
Ph3P+
Br-
O
INTEGRAL
32.20
22.33
4.43
8.85
82.12

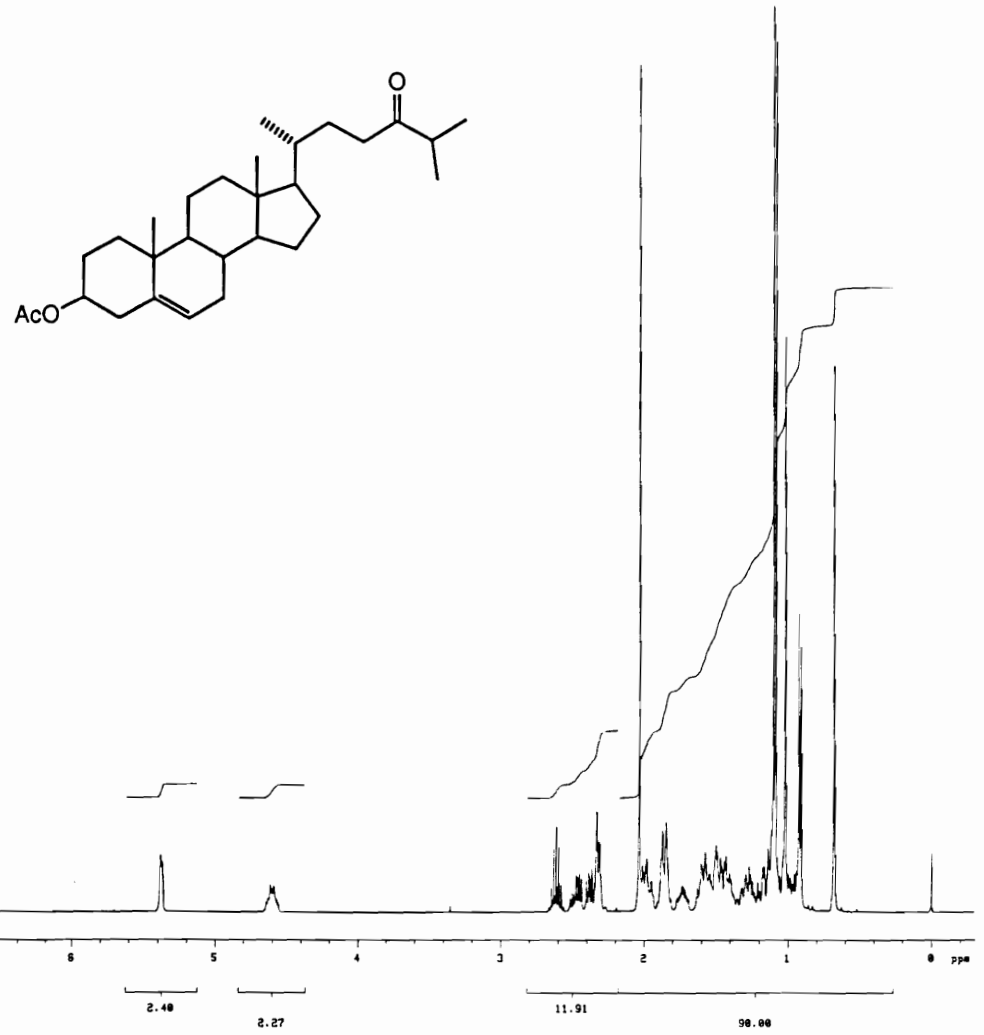
water
3.10
Ph3P
O
INTEGRAL
PPM

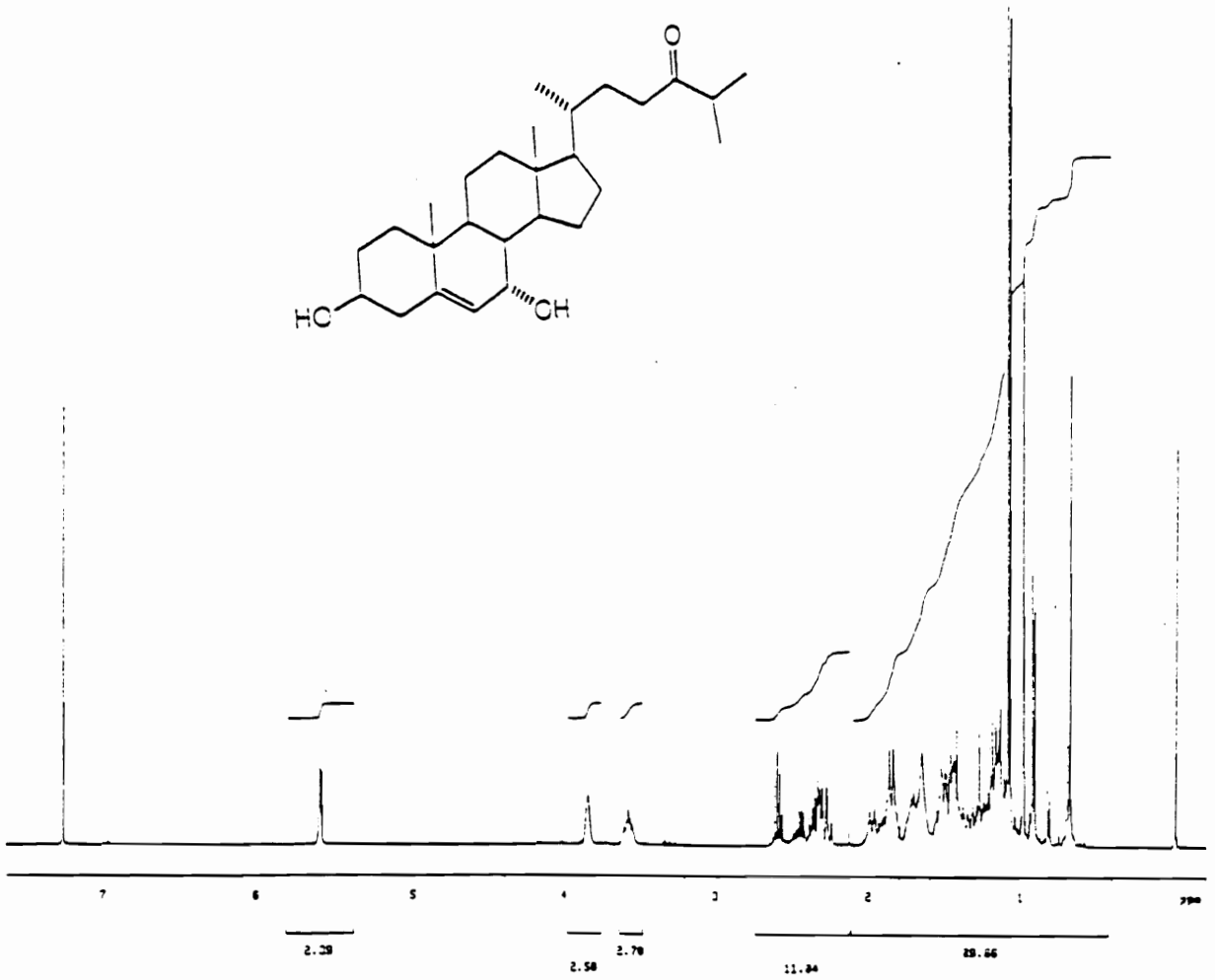

STANDARD 1H OBSERVE
OBSERVE H1
FREQUENCY 399.952 MHz
O
OCH3
7
6
5
4
3
2
1
0
ppm
2.57
2.62
7.83
4.95
2.45
92.58


STANDARD 1H OBSERVE
OBSERVE H1
FREQUENCY 399.952 MHz
O
OCH3
7
6
5
4
3
2
1
0
ppm
7.24
2.34
6.70
92.43

STANDARD 1H OBSERVE

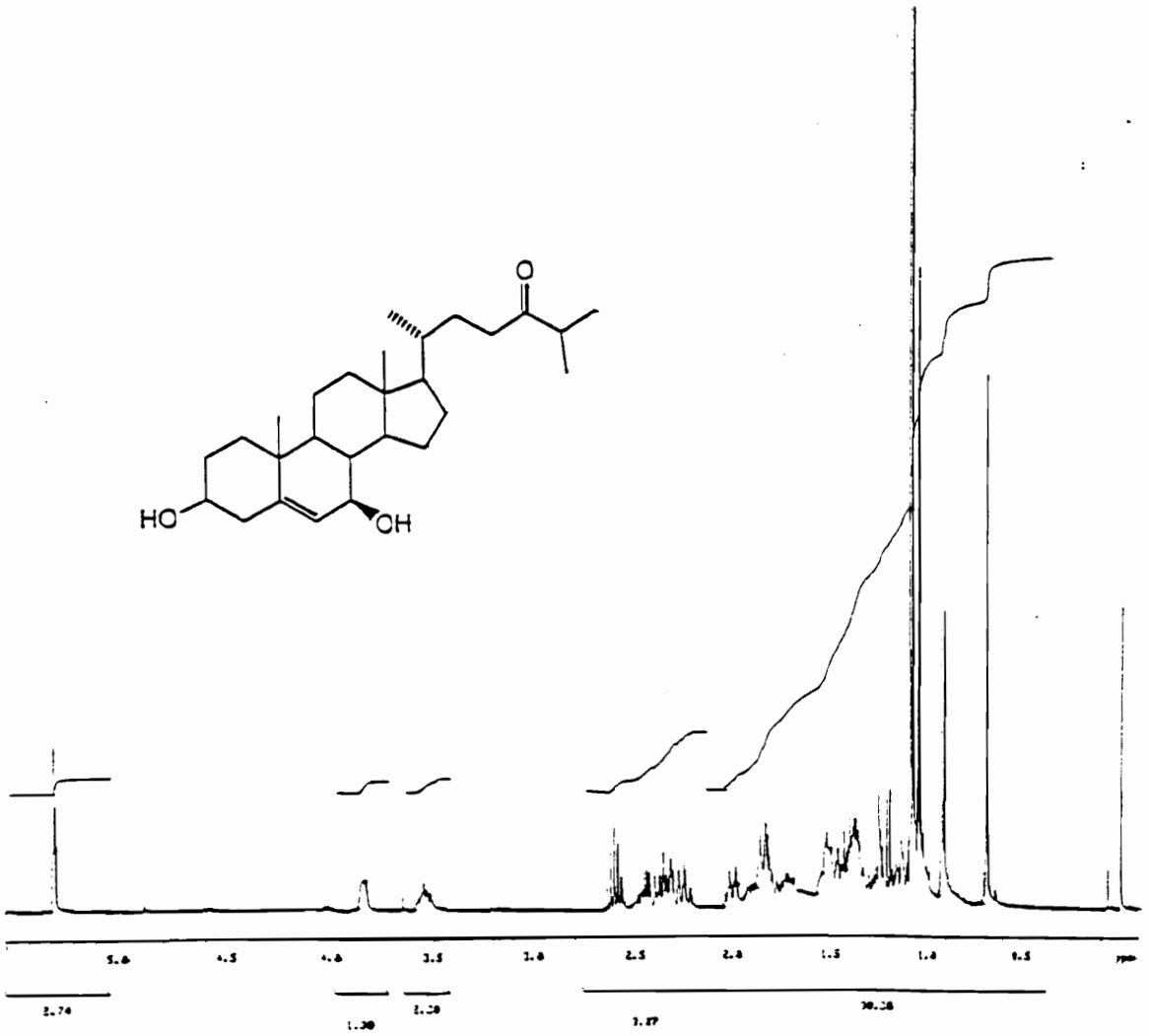
OBSERVE H1
FREQUENCY 399.952 MHz
SPECTRAL WIDTH 5000.0 Hz
ACQUISITION TIME 3.744 sec
RELAXATION DELAY 1.000 sec
PULSE WIDTH 4.1 usec
AMBIENT TEMPERATURE
NO. REPETITIONS 16
DOUBLE PRECISION ACQUISITION
DATA PROCESSING
FT SIZE 65536
TOTAL ACQUISITION TIME 1 minutes
Apr 7 92
Virginia Tech STC NMR Facility

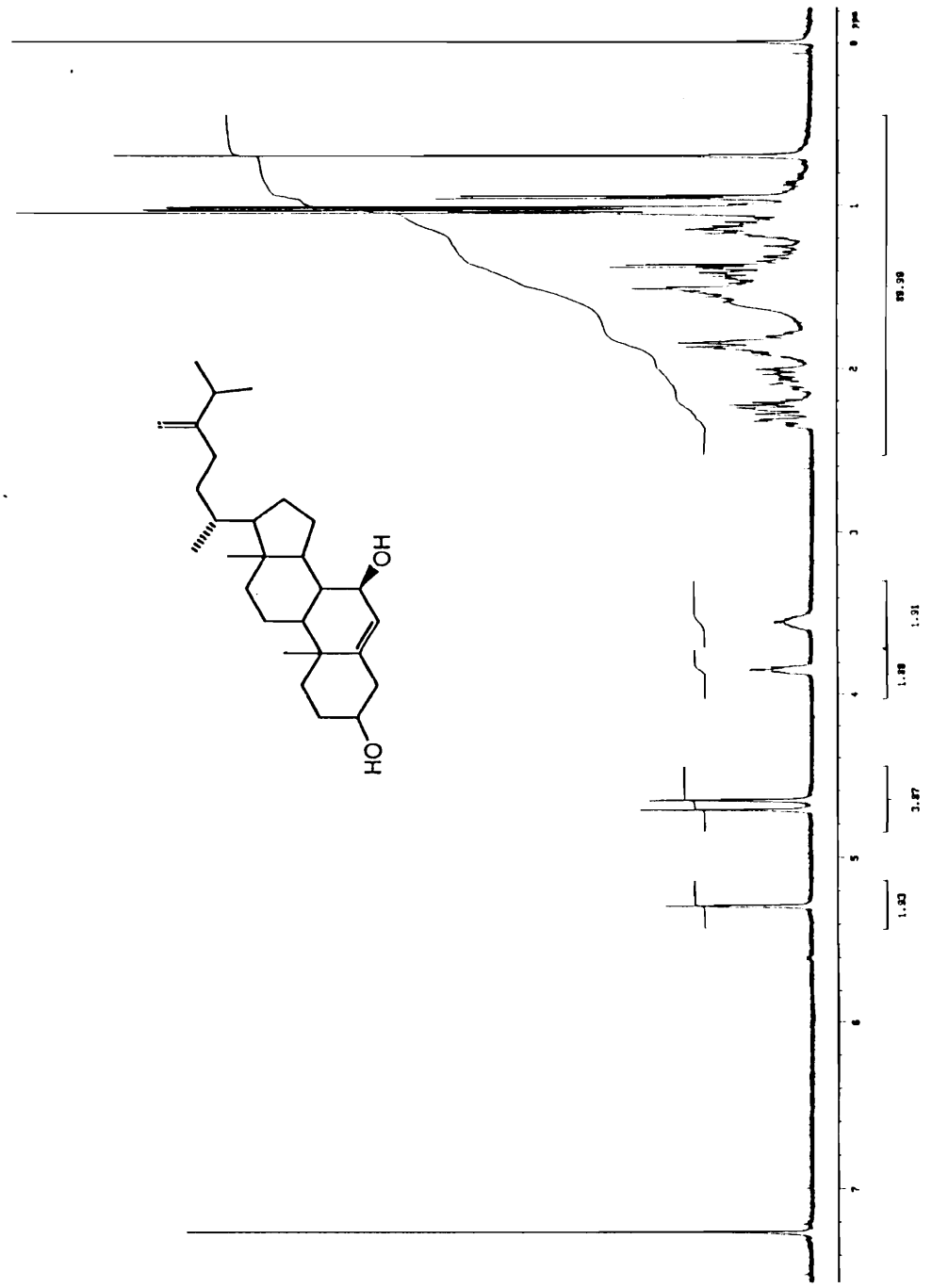


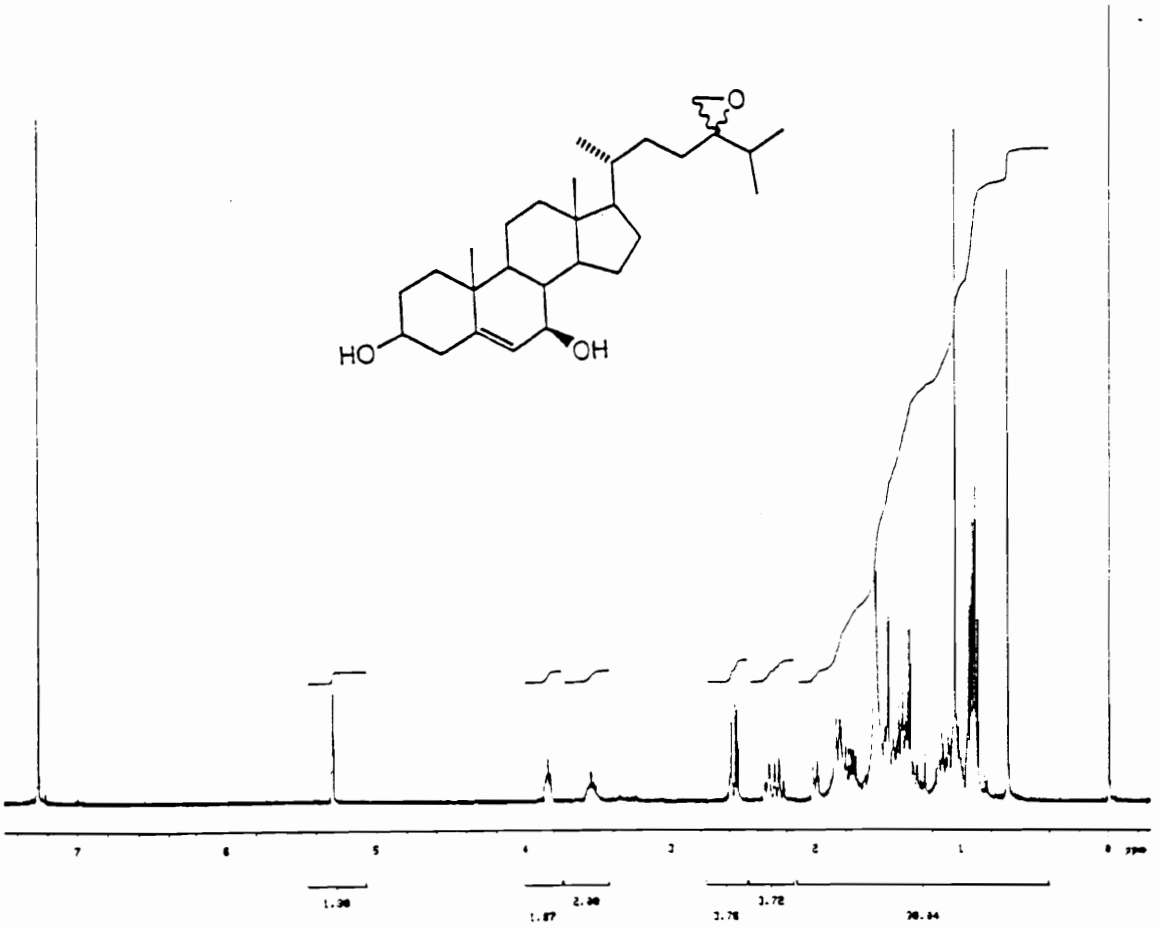

O
HO
OH
7
6
5
4
3
2
1
ppm
2.39
2.58
2.78
11.84
29.66

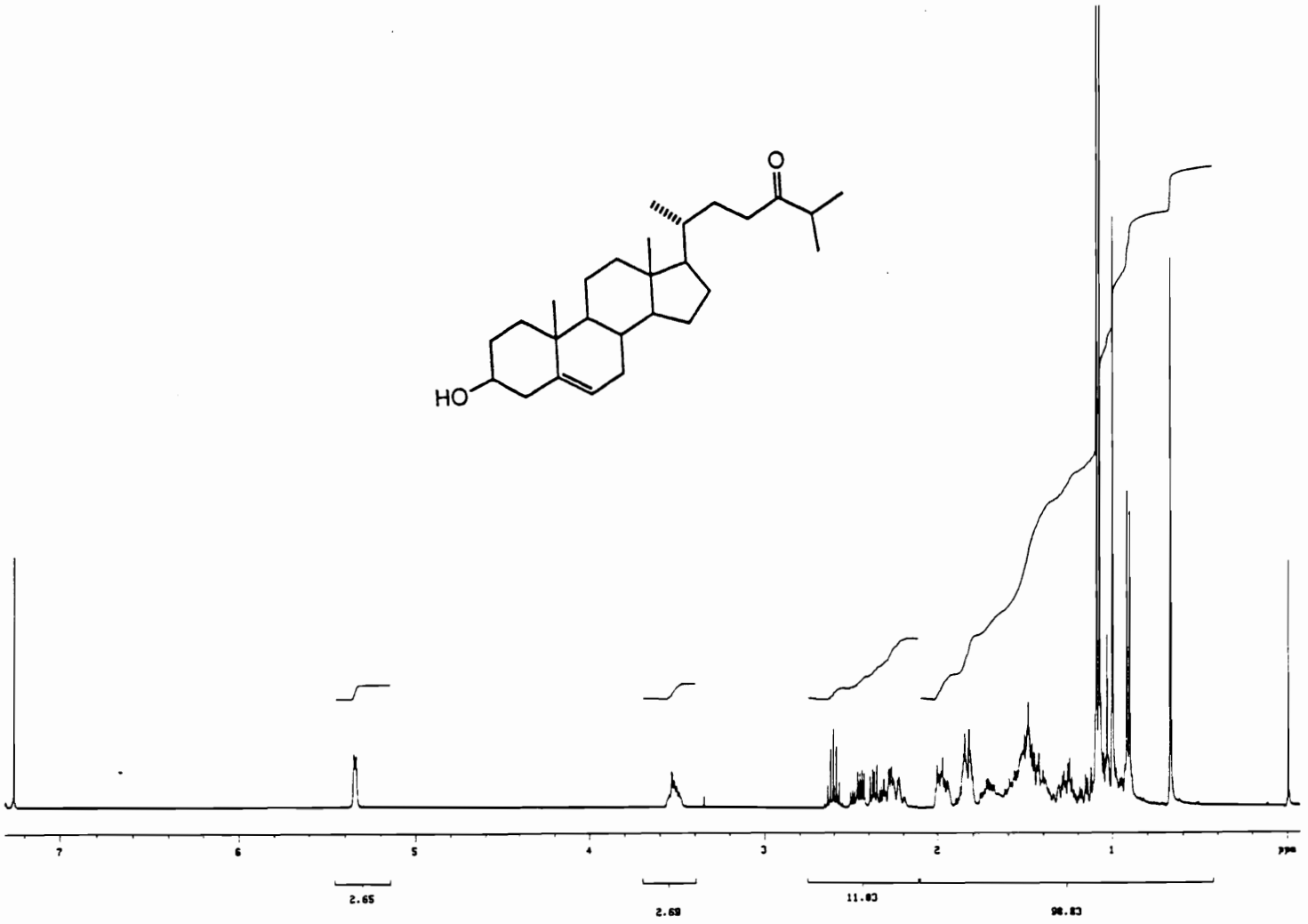
HO
OH

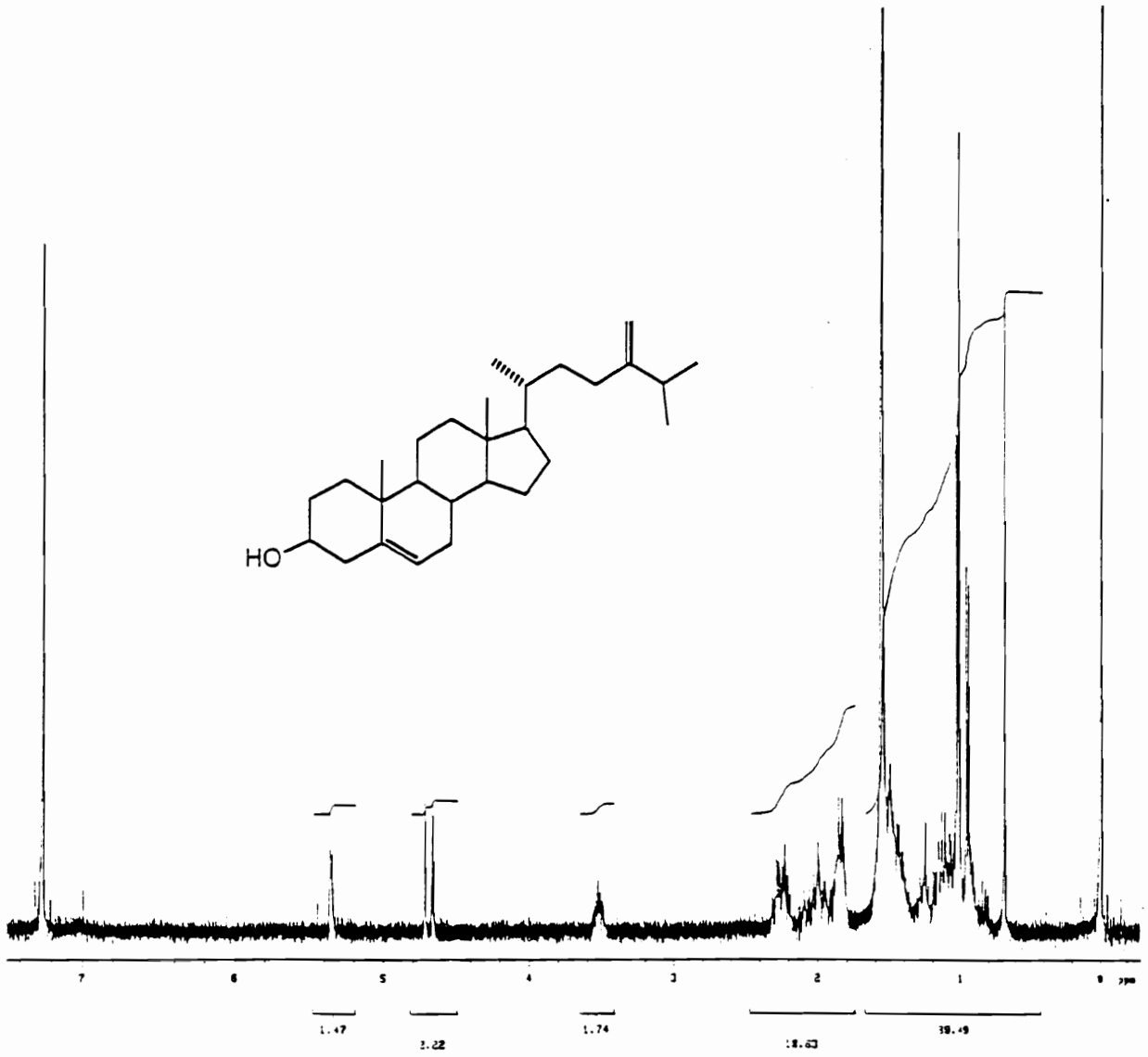
O
OH
HO


HO
OH
O
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
2.74
1.30
2.20
3.27
30.28


OH
HO


HO
OH
O
7
6
5
4
3
2
1
0 ppm
1.00
2.00
3.72


HO
O
7
6
5
4
3
2
1
ppm
2.65
2.69
11.83
98.83


HO
7
6
5
4
3
2
1
0 ppm
1.47
2.22
1.74
18.63
39.49

O
HO

VITA

Carl E. Heltzel was born on October 3, 1961 in Washington D.C. He married Amanda Hanson in 1983, and has since seen the birth of two daughters, Madison Lee and Savannah Lynn Heltzel. He completed his Bachelor of Science degree in chemistry at Radford University in 1989. In 1993 he was awarded a Ph.D. in organic chemistry from Virginia Polytechnic Institute and State University, where he studied under the direction of Professor David G.I. Kingston. He currently holds a research position at the University of Hawaii, department of chemistry.