PHYTOCHEMICAL INVESTIGATION OF CROTON CALIFORNICUS
FAMILY EUPHORBIACEAE

by
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ABSTRACT

The ethanol extract of the roots, stems, leaves, flowers, and fruits of *Croton californicus* family Euphorbiaceae demonstrated antileukemic activity against the P-388 lymphocytic leukemia test system in mice.

The crude ethanol extract was fractionated by a series of solvent partitions and extraction. The use of column chromatography and crystallization techniques led to the isolation of the following three compounds: (-)-hardwickic acid, 1-triacontanol, and (-)-methyl barbascoate.

The compounds were identified by a combination of physical methods, including infrared spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectroscopy. In addition, melting and mixed-melting point determinations as well as carbon, hydrogen analyses provided further evidence for the proposed structures. The structure of hardwickic acid was confirmed by the preparation of a known derivative. The observed optical rotation established hardwickic acid as the levorotatory isomer.
CHAPTER 1

INTRODUCTION

In recent years the genus Croton (Euphorbiaceae) has been of considerable pharmacological interest. Berenblum observed that the seed oil of Croton tiglium L. had a cocarcinogenic effect in tumorigenesis of mouse skin induced by carcinogenic aromatic hydrocarbons (Hecker and Schmidt 1974, p. 379). However, other Croton species were found to contain constituents that demonstrate antitumor activity (Farnsworth et al. 1969, p. 18). These observations developed as a result of the massive screening of plants for potential antitumor compounds. Preliminary investigations showed that an ethanol extract of Croton californicus Muell. Arg. demonstrated antileukemic activity against the P-388 lymphocytic leukemia test system in mice (Abbott et al. 1972, p. 9). Therefore, it was considered worthwhile to conduct a further investigation of Croton californicus.

Occurrence of the Plant

Croton californicus is an herbaceous shrub indigenous to the Sonoran Desert. The plant has been found to grow on dry sandy hillsides, mesas, and arroyos at elevations below four thousand feet (Munz 1935, p. 283).
region includes the Upper and Lower Sonoran Zones, north into Contra Costa County, California, south to southern Baja California, and east into Arizona and Sonora (Shreve and Wiggins 1964, p. 779). The plant investigated was collected near Yuma, Arizona, during September, 1975.

**Description of the Plant**

*Croton californicus* can best be described as a suffrutescent perennial 2-12 dm tall with stems branching, erect or spreading with stellate canescence throughout. The leaf blades are oblong to elliptic (1.5 to 5 cm long), silvery or hoary beneath and less densely pubescent and yellowish green above. The staminate flowers are racemose (10 to 15 mm long) and deciduous soon after anthesis. A few pistillate flowers are present in each inflorescence. The flowering season lasts from February to October (Jepson 1975, p. 596; Shreve and Wiggins 1964, p. 779).

**Extraction of the Plant**

The dried roots, stems, leaves, flowers, and fruits were ground to a coarse powder in a Wiley mill. A 14 kg sample of the powder was extracted exhaustively with 95% ethanol in a Lloyd-type extractor to yield 1.3 kg of air dried extract which was stored at -10°C.
CHAPTER 2

ISOLATION OF PLANT CONSTITUENTS

An extensive literature search revealed that the genus Croton contains several novel chemical entities. These include numerous esters of the phorbol nucleus and the only naturally occurring highly oxygenated cyclohexane derivative possessing a diepoxide functionality. Hecker isolated and identified the cocarcinogenic principles of croton oil (C. tiglium) as esters of the tetracyclic diterpene phorbol (Hecker and Schmidt 1974). In the continuing search for more effective antineoplastic agents, a phorbol ester demonstrating antileukemic activity was isolated from the seed oil of C. tiglium and identified as phorbol 12-tiglate 13-decanoate (Kupchan et al. 1976). Previously, Kupchan and coworkers had isolated and identified crotepoxide, a novel cyclohexane diepoxide tumor inhibitor, from the fruits of Croton macrostachys (Kupchan, Hemingway, and Smith 1969). More recently, the diterpene methyl barbascoate was isolated from Croton californicus (Wilson, Neubert, and Huffman 1976). Since no further chemical work had been done on this plant (Wilson 1978), a phytochemical investigation was undertaken. The appendix lists the structures of the above compounds.
Solvent Partition and Extraction

The ethanol extract of *Croton californicus* was fractionated by solvent partitions and extraction (Figure 1).

Silica Gel 60 Column Chromatography

Further fractionation of the petroleum ether fraction was accomplished by silica gel-60 column chromatography utilizing a modified gradient elution (*hexane:ether* 4:1 to 1:1) until the column was exhausted. Fractions (250 ml) were collected and combined on the basis of their thin-layer chromatogram pictures. The major component (compound I) of the *hexane:ether* (4:1) eluent (fractions 5-52 designated as fraction B) appeared on the chromatogram after 20 seconds of heating as a black spot with extensive tailing. Co-occurring with compound I was a minor component (compound II) observed at *R_f* 0.2 (*hexane:ether* 4:1). When no further trace of compound I was observable on the chromatograms, the concentration of ether was increased to 40% and all subsequent fractions were collected on an automatic fraction collector (see p. 31). After approximately 15 liters of eluent were collected, the concentration of ether was increased to 50%.

Isolation of Hardwickiic Acid

Fraction B was subjected to silica gel-60 column chromatography with the solvent system *hexane:ether:acetic acid* (40:5:0.5) serving as the eluent. Compound I
Figure 1. Solvent Partition and Extraction of *Croton californicus*
(hardwickiic acid) was concentrated in the first fraction (tubes 1-10). Subsequently, compound I was purified by crystallization from hexane to yield hardwickiic acid as colorless crystals.

Isolation of Triacontanol

The third fraction (tubes 40-80) obtained from the previous silica gel-60 column contained compound II (triacontanol) as the primary component. Compound II was isolated from the third fraction by eluting a silica gel-60 column with the solvent system chloroform:ether (45:5). Fractions (12 ml per tube) were collected with an automatic fraction collector (see p. 31). Compound II was concentrated in tubes 18 to 26 and purified by crystallization from methanol. Two recrystallizations from acetone resulted in the formation of white flakes.

Isolation of Méthyl Barbascoate

The ether-soluble extract of the butanol fraction was fractionated on a silica gel-60 column that was eluted with hexane:ether (3:2, 16 liters) followed by hexane:ether (1:1, 15 liters) and subsequently washed with hexane:ether (2:3, 8 liters). Fractions 263 to 317 were combined, the solvent removed under vacuum, and compound III was crystallized from ether. Recrystallization from ether resulted in the formation of methyl barbascoate as fine white needles.
CHAPTER 3

CHARACTERIZATION OF CONSTITUENTS

The structures of the three compounds isolated from the plant extract were determined by a combination of physical methods, preparation of a derivative, and comparison with previously reported data and/or data from authentic samples. The three compounds are the diterpene acid, (-)-hardwickiic acid (compound I); the long chain alcohol, 1-triacontanol (compound II); and the diterpene, (-)-methyl barbascoate (compound III).

(-)-Hardwickiic Acid

The colorless crystals of (-)-hardwickiic acid (compound I) obtained after crystallization from hexane had a melting point of 99-100°C and \([\alpha]_D^{25} -116.5^\circ\) (ETOH, \(c = 1.09\)); reported: 106-107°C and \([\alpha]_D -114.7^\circ\) (CHCl₃) (Misra, Pandey, and Dev 1964, p. 3752).

Infrared Spectrum

The infrared spectrum of compound I (Figure 2) was indicative of an \(\alpha,\beta\)-unsaturated carboxylic acid (Nakanishi 1966, p. 43; Silverstein, Bassler, and Morrill 1974, pp. 88, 99-101) with hydroxyl stretching 3300-2500 cm\(^{-1}\), carbonyl stretching at 1680 cm\(^{-1}\), and C=C stretching at 1632 cm\(^{-1}\).
Figure 2. Infrared Spectrum of Hardwickiic Acid
Absorption bands at 1500, 1055, 1015, and 868 cm\(^{-1}\) suggested the presence of a furan moiety (Nakanishi 1966, p. 52).

Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum of compound I (Figure 3) integrates for one carboxylic acid proton at \(\delta 10.75\) and one vinylic proton at \(\delta 6.8\) (triplet, \(J=4\) Hz). The three methyl groups can be accounted for as follows: the C-5 and C-9 methyl groups, singlets at \(\delta 1.26\) and 0.76, respectively; the C-8 methyl group, doublet at \(\delta 0.87\) (\(J=6\) Hz). In addition, the alpha furan protons at C-15 and C-16 appear at \(\delta 7.27\) (triplet, \(J=1.5\) Hz) and 7.17, while the beta furan proton at C-14 appears at 6.2.

Carbon, Hydrogen Analysis

The molecular formula \(\text{C}_{20}\text{H}_{28}\text{O}_3\) was supported by the carbon, hydrogen analysis (calculated: C, 75.92, H, 8.92; observed: C, 75.20, H, 8.61) and mass spectrum parent peak m/e 316.

Mass Spectrum

The mass spectrum of compound I (Figure 4) was identical to the previously reported mass spectrum of hardwickiic acid (George et al. 1974, p. 1248). Four of the major fragments can be accounted for by the following fragmentations:
Figure 3. NMR Spectrum of Hardwickiic Acid
Figure 4. Mass Spectrum of Hardwickiic Acid
The proposed fragments responsible for the peaks at m/e 81 (74%) and m/e 125 (base) have been reported (George et al. 1974, p. 1249) to be:

Methyl Ester Derivative of Hardwickiic Acid

After purification by preparative thick layer chromatography (p. 34), hardwickiic acid methyl ester was
obtained as an oil. The infrared spectrum (Figure 5) of the derivative lacked the broad O-H stretching absorption from 3300-2500 cm⁻¹. The shift in the carbonyl stretching frequency from 1680 cm⁻¹ to 1710 cm⁻¹ indicated the presence of an α,β-unsaturated ester (Silverstein et al. 1974, p. 1020).

The nuclear magnetic resonance spectrum (Figure 6) further verified the presence of the methyl ester with a peak at δ3.68 (Silverstein et al. 1974, p. 213) and the absence of the acidic proton (singlet, δ10.75).

1-Triacontanol

White flakes of compound II were obtained after two recrystallizations from acetone. No depression was observed with the mixed melting point at 86°C (Karrer 1958, p. 49) of an authentic sample of 1-triacontanol (Analabs, Inc., North Haven, Connecticut).

Carbon, Hydrogen Analysis

The carbon, hydrogen analysis (calculated: C, 82.11, H, 14.24; observed: C, 82.07, H, 14.38) was consistent with a C₃₀H₆₂O molecular formula.

Mass Spectrum

The mass spectrum of compound II (Figure 7) was indicative of a saturated long chain alcohol. The fragmentation pattern resembles that of the corresponding
Figure 5. Infrared Spectrum of Hardwickiic Acid Methyl Ester
Figure 6. NMR Spectrum of Hardwickeic Acid Methyl Ester
Figure 7. Mass Spectrum of Triacontanol
hydrocarbon with peak groups 14 mass units apart and de­
creasing in intensity with increasing fragment weight
(Budzikiewicz, Djerassi, and Williams 1967, p. 101). As
expected for alcohols, the molecular ion peak is absent
(Silverstein et al. 1974, p. 21).

The first prominent peak (m/e 420) in the high mass
region of the spectrum resulted from dehydration (M-18) of
the alcohol. Loss of water in long chain primary alcohols
may occur by one of two processes: thermal decomposition
prior to electron impact resulting in a 1,2-elimination or
electron bombardment induced dehydration by a 1,4-elimina-
tion mechanism (Budzikiewicz et al. 1967, p. 98). The fol-
lowing mechanism has been suggested for elimination by
electron impact (Silverstein et al. 1974, p. 21):

A series of peaks at m/e 392 (M-46), m/e 364 (M-74),
m/e 336 (M-102), etc. can be accounted for by the elimina-
tion of water coupled with the successive expulsion of
ethylene groups. The resulting M-(olefin + H₂O) fragments
are formed by the following process (Silverstein et al. 1974, p. 22):

\[
\begin{align*}
\text{R-CH} & \text{CH}_2 \\
\text{CH}_2 & \text{O}^+ \\
\text{H} & \rightarrow \\
\text{H} & \rightarrow \\
\text{CH}_2=\text{CH}_2 & \rightarrow [\text{CH}_2=\text{CHR}]^+ \\
\text{H}_2\text{O} & \rightarrow
\end{align*}
\]

The above observations were consistent with the previously reported mass spectrum of triacontanol (Ries et al. 1977, p. 1340).

Infrared Spectrum

The infrared spectrum of compound II (Figure 8) was indicative of a straight chain saturated primary alcohol. The hydroxyl stretching absorption is centered at 3350 cm\(^{-1}\) and the C-O stretching appears at 1055 cm\(^{-1}\). The remaining absorptions can be attributed to the various C-H vibrational modes as follows: symmetrical and asymmetrical methylene stretching at 2855 cm\(^{-1}\) and 2925 cm\(^{-1}\) respectively; symmetrical and asymmetrical methyl C-H bending at 1370 cm\(^{-1}\) and 1460 cm\(^{-1}\) respectively; methylene scissoring at 1470 cm\(^{-1}\); and methylene rocking at 720 cm\(^{-1}\) and 710 cm\(^{-1}\) (Nakanishi 1966, pp. 20, 30-31).

Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum of compound II (Figure 9) is typical of long chain saturated primary alcohols (Bhacca, Johnson, and Shoolery, 1962, spectrum number 282). The hydroxylic proton appears at \(\delta 1.88\), the C-1 methylene protons at \(\delta 3.66\) (\(J=6\) Hz), and methyl protons
Figure 8. Infrared Spectrum of Triacontanol
Figure 9. NMR Spectrum of Triacontanol
at δ0.96. The methylene protons adjacent to the primary alcohol appear at δ1.56, and the remaining methylene protons appear as a sharp singlet at δ1.26.

**(-)-Methyl Barbascoate**

The white crystalline needles of (-)-methyl barbascoate (compound III) obtained from ether had an identical melting point range (152-153°C) as previously reported (Wilson et al. 1976, p. 3670), and failed to depress the melting point of authentic methyl barbascoate (Wilson 1978).

**Infrared Spectrum**

The infrared spectrum of compound III (Figure 10) showed carbonyl stretching at 1710 cm⁻¹ and 1740 cm⁻¹ due to the α,β-unsaturated ester and δ-lactone (Nakanishi 1966, p. 44; Silverstein et al. 1974, pp. 102-103). The band at 1240 cm⁻¹ with shouldering resulted from the C-O stretching vibrations of the α,β-unsaturated ester and δ-lactone. The presence of a β-substituted furan was indicated by absorptions at 1500, 1070, 1015, and 865 cm⁻¹. The spectrum was superimposable upon that of the spectrum sent by Dr. Wilson.

**Nuclear Magnetic Resonance Spectrum**

Portions of the nuclear magnetic resonance spectrum of compound III (Figure 11) closely resemble those of hardwickiic acid methyl ester (Figure 6). The spectrum had
Figure 10. Infrared Spectrum of Methyl Barbascoate
Figure 11. NMR Spectrum of Methyl Barbascoate
peaks at $\delta3.68$ for methyl ester, $\delta6.61$ ($J=4$ Hz) for vinyl proton at C-3, and $\delta1.27$ and 1.01 for angular methyl groups at C-5 and C-9. In addition, peaks at $\delta6.43$ and 7.40 indicated a $\beta$-substituted furan. The C-12 lactonic proton appeared as a triplet at $\delta5.34$ ($J=9$ Hz). The spectrum of compound III was identical with that of the spectrum sent by Dr. Wilson.

Carbon, Hydrogen Analysis

The molecular formula $C_{21}H_{26}O_5$ was substantiated by the carbon, hydrogen analysis (calculated: C, 70.37, H, 7.31; observed: C, 70.49, H, 7.53) and mass spectrum parent peak m/e 358.

Mass Spectrum

The mass spectrum of compound III (Figure 12) was identical with the mass spectrum of methyl barbascoate (Wilson et al. 1976, p. 3670). The base peak at m/e 94 results from the following fragmentations:

![Diagram of mass spectrum fragmentations]
Figure 12. Mass Spectrum of Methyl Barbascoate
The m/e 264 fragment undergoes successive loss of carbon dioxide and methyl radical to account for the intense peak at m/e 205 (55%).
CHAPTER 4

EXPERIMENTAL

This chapter deals with the procedures and equipment used for grinding and extracting the plant, along with a description of methods and chromatographic techniques utilized for the separation and isolation of plant constituents. The remainder of the chapter deals with the methods and types of equipment used to obtain the necessary physical data, and the chemical procedure used in the preparation of the derivative.

Grinding of the Plant

A Wiley Mill (Arther H. Thomas Co., Philadelphia, Pennsylvania) equipped with a 3 mm sieve was utilized in grinding the dried roots, stems, leaves, flowers, and fruits of the plant to a coarse powder.

Extraction of Plant

A 14 kg sample of the dried powder was extracted exhaustively with 95% ethanol in a Lloyd-type extractor (Brighton Copper Works, Cincinnati, Ohio), yielding 1.3 kg of air dried extract.
Solvent Partitioning and Extraction

The scheme outlined in Figure 1 summarizes the results of solvent partitioning and extraction of the crude ethanol extract. An extract portion (260 g) was dissolved in an equal volume (1.5 liters) of 20% aqueous methanol and petroleum ether, transferred into a four-liter separatory funnel, and shaken vigorously. Subsequently, the petroleum ether phase was removed and the 20% aqueous methanol layer was partitioned with an additional 1.5 liters of petroleum ether. Interphases containing an insoluble powder were combined, filtered, and the filtrate partitioned twice with equal volumes of petroleum ether (1 liter).

Solvent partitioning of the 20% aqueous methanol fraction between n-butanol and water was accomplished by utilizing the same technique as described above. The 200 g portions were partitioned between n-butanol (saturated with water) and water (saturated with n-butanol), with the n-butanol layers being partitioned twice with water.

The solvent extraction was accomplished by suspending the butanol fraction (116 g) in ether (600 ml) with the aid of an automatic stirrer (Scientific Products, McGraw Park, Illinois). After a few hours the mixture was filtered and the procedure repeated until the filtrate was nearly colorless. All fractions obtained through solvent partitioning and extraction were air dried and stored at -10°C.
Chromatography

The silica gel-G, silica gel-PF$_{254}$, and silica gel-60 (Merck) used for thin-layer, preparative thick-layer, and column chromatography was distributed by Brinkman Instruments, Inc., Westbury, New York.

Thin Layer

The plates were prepared with an automatic plate maker (Camag, Homburgerstrasse 24, Muttenz, Switzerland) distributed by Gelman Instrument Co., Ann Arbor, Michigan. The plates were covered with a uniform layer of silica gel-G 0.3 mm thick. Prior to use the plates were activated by heating for one hour or more in an oven at 110°C. After developing in the various solvent systems (see Chapter 2), the plates were air dried, sprayed with ceric sulfate solution (20 gm of ceric sulfate mixed with 56 ml of concentrated sulfuric acid and one liter of water), and warmed gradually with a heat gun (The Lab Apparatus Co., Cleveland, Ohio) until the spots appeared and became charred.

Preparative Thick Layer

The same method was employed in making the preparative plates as the thin layer plates with the exception being a 1.5 mm thickness of silica gel-PF$_{254}$ instead of 0.3 mm of silica gel-G. The material was dissolved in chloroform and applied with a pipette at concentrations of 40-50 mg per plate. After development in the suitable solvent
system, the far left edge of the plate was sprayed with ceric sulfate solution and gradually heated until the spots became visible. The plates were then observed under ultraviolet light (254 nm) and the desired sections removed with a razor blade. Each recovered section was mixed with chloroform:methanol (1:1), allowed to soak for at least 30 minutes, filtered, and the solvent removed under vacuum resulting in the purified compound.

Silica Gel-60 Columns

The petroleum ether extract was fractionated on three silica gel-60 columns. The following technique was used in packing the glass column with silica gel-60: a cotton plug was inserted into the stem of the column directly above the stopcock, followed by the introduction of another cotton plug at the bottom of the column. After alignment of the column in a stationary position, a small quantity of dry silica gel-60 was added to form a thin layer above the cotton. With the stopcock open, the column was filled and maintained at three-fourths volume capacity with the solvent system hexane:ether (4:1) and the drop rate was adjusted to a steady stream; 1.3 kg dry silica gel-60 was slowly added over a period of a few hours to allow for adequate displacement of air; after the addition was completed, the column was filled with the solvent system and the drop rate adjusted to one every few seconds. The column
was left standing for a period of several hours to allow for further settling of the adsorbant. The dried extract (50 gm), dissolved in chloroform and adsorbed on 100 gm silica gel-60 the previous day, was introduced onto the column (7 cm x 65.5 cm) after the solvent level had been lowered to just a few cm above the silica gel-60 layer. Subsequently, the adsorbed material was covered with 3-4 cm of dry silica gel-60. After the column was refilled with the solvent system, the drop rate was set to one drop per second. Separation of the fractions (25 ml per tube) was accomplished with the aid of an automatic fraction collector (Buchler Instruments, Inc., Fort Lee, New Jersey).

The subsequent silica gel-60 columns utilized in the fractionation process of the petroleum ether extract were packed in the same manner as before. However, the column (4 cm x 38 cm) used to isolate hardwickiic acid was packed with silica gel-60 (190 gm) and the extract (3.8 gm) was dissolved in dichloromethane and methanol. The solvent system was hexane:ether:acetic acid (40:5:0.5).

Triacontanol was isolated from a 1.5 cm by 40 cm dimension column which had been packed with 40 gm silica gel-60 for a 1.1 gm sample (dissolved in hexane/dichloromethane). Elution was carried out with chloroform:ether (45:5).

A packing method slightly different from the previous one was used for the silica gel-60 column of the
ether-soluble fraction, the difference being in the introduction of 1.5 kg silica gel-60 as a slurry with the solvent system (hexane:ether 3:2) instead of the addition as dry silica gel-60 into the solvent-filled column. The 7 cm diameter column was packed to a height of 108.5 cm, with a 49 gm sample (dissolved in chloroform/methanol).

**Physical Data**

Unless otherwise indicated, the physical data were collected at the Arizona Health Science Center, the Department of Chemistry, and the College of Pharmacy, The University of Arizona, Tucson, Arizona.

**Melting Point Determination**

The uncorrected melting points were determined on a Kofler hot-stage apparatus.

**Carbon, Hydrogen Analysis**

The carbon and hydrogen analyses were performed by Chemalytics, Inc., 2330 South Industrial Park Drive, Tempe, Arizona. Prior to analysis, samples were dried under vacuum at 56°C for at least 15 hours and weighed 10-15 mg.

**Infrared Spectroscopy**

Most of the infrared spectra were run in spectra grade chloroform (0.5 mm KBr cells) on a Beckman IR-33 (Beckman Instruments, Inc., Fullerton, California). The
spectrum of compound II was obtained from a potassium bromide pellet.

Nuclear Magnetic Spectroscopy

The nuclear magnetic spectrum of methyl barbascoate was obtained on a Varian T-60, while a Varian EM-360L (Varian Associates, 611 Hansen Way, Palo Alto, California) was utilized for all remaining spectra. The spectra of all compounds and the derivative were run in deuterated chloroform in 20 per cent concentration using tetramethylsilane as an internal standard.

Mass Spectrometry

All mass spectra were obtained on a Finnegan 300-6110 Quadrupole gas chromatograph-mass spectrometer (Finnegan Corporation, 845 W. Maude Avenue, Sunnyvale, California).

Optical Rotation

The optical rotation of compound I was taken on a Perkin-Elmer 241 MC Polarimeter (Perkin-Elmer, Uberlingen, West Germany).

Esterification Procedure

Preparation of Diazomethane

A solution of 4 ml of 40% aqueous potassium hydroxide and 20 ml of diethyl ether was cooled to 4°C in
an ice-salt bath. To this solution, 1.5 g of N-methyl-N'-nitro-N-nitrosoguanidine was added portionwise with swirling during a 15 minute period. The reaction mixture was transferred to a 60 ml precooled separatory funnel, the aqueous layer removed, and the ethereal diazomethane transferred to a 50 ml dry precooled Erlenmeyer flask. A few potassium hydroxide pellets were added to the flask before it was tightly stoppered and refrigerated for at least two hours.

Methyl Ester of Hardwickiic Acid

An 80 mg sample of compound I was dissolved in 40 ml diethyl ether and cooled to 4°C in an ice-salt bath. The cold ethereal diazomethane was added to the solution over a period of five minutes. The reaction mixture was allowed to stand at room temperature for two hours, followed by evaporation of the solvent in a hood. The resulting residue was purified on preparative plates with hexane:ether:acetic acid (40:5:0.5) as the developing solvent system. The purified compound was an oil. This procedure was a modification of a known procedure (Lane 1969).
CHAPTER 5

SUMMARY AND CONCLUSIONS

Two compounds were isolated from the petroleum ether fraction and one compound was isolated from the ether soluble fraction of Croton californicus, family Euphorbiaceae. One of the three compounds was identified as 1-triacontanol, a long chain alcohol. The remaining two compounds were diterpenes characterized as (-)-hardwickiic acid and (-)-methyl barbascoate.

Each of the three compounds isolated from the ethanol extract of Croton californicus will undergo in vivo testing against the P-388 lymphocytic leukemia test system in mice to determine if they are responsible for the antitumor activity demonstrated by the crude extract. Based on the types of compounds which have previously demonstrated antitumor activity (Hartwell 1976), it would seem unlikely that a relatively unreactive compound such as triacontanol would possess antineoplastic activity. However, antitumor activity has been demonstrated by several diterpenoids isolated from various plant families, including the Euphorbiaceae (Hartwell 1976, pp. 1036-1037; Torrance et al. 1976). Additional material is being collected for further evaluation.
APPENDIX A

MOLECULAR STRUCTURES OF COMPOUNDS ISOLATED FROM OTHER CROTON SPECIES

![Phorbol structure](image)

**Phorbol**  \( R_1 = R_2 = H \)

<table>
<thead>
<tr>
<th>Croton Oil Factor</th>
<th>( R_1 )</th>
<th>( R_2 )</th>
</tr>
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<tr>
<td>A1</td>
<td>(-\text{CO(CH}<em>2\text{)}</em>{12}\text{CH}_3)</td>
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<tr>
<td>A2</td>
<td>(-\text{CO(CH}<em>2\text{)}</em>{8}\text{CH}_3)</td>
<td>(-\text{COCH}_3)</td>
</tr>
<tr>
<td>A3</td>
<td>(-\text{CO(CH}<em>2\text{)}</em>{10}\text{CH}_3)</td>
<td>(-\text{COCH}_3)</td>
</tr>
<tr>
<td>A4</td>
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<td>(-\text{COCH}_3)</td>
</tr>
<tr>
<td>B1</td>
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<td>(-\text{CO(CH}<em>2\text{)}</em>{10}\text{CH}_3)</td>
</tr>
<tr>
<td>B2</td>
<td>((+)-S\ \text{COCH(CH}_3\text{)}\text{CH}_2\text{CH}_3)</td>
<td>(-\text{CO(CH}<em>2\text{)}</em>{8}\text{CH}_3)</td>
</tr>
<tr>
<td>B3</td>
<td>(-\text{CO(CH}_3\text{)}\text{C=CHCH}_3)</td>
<td>(-\text{CO(CH}<em>2\text{)}</em>{8}\text{CH}_3)</td>
</tr>
<tr>
<td>B4</td>
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<td>(-\text{CO(CH}<em>2\text{)}</em>{10}\text{CH}_3)</td>
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<tr>
<td>B7</td>
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<td>(-\text{CO(CH}<em>2\text{)}</em>{8}\text{CH}_3)</td>
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</table>

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CROTEPOXIDE
REFERENCES CITED


Lane, Clinton F. Research Chemist, Aldrich Chemical Co., Milwaukee, private communication, 1969.


Wilson, Stephen R. Assistant Professor, Department of Chemistry, Indiana University, Bloomington, private communication, 1978.
