CHEMICAL INVESTIGATION
OF
DICRANUM FULVUM
FOR
ANTICANCER ACTIVITY

by

Veronica R. Cadorette

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APPROVED:

D.G.I. Kingston, Chairman

L. T. Taylor

H. M. Bell

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Committee Chairman: David Kingston
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(ABSTRACT)

Biological screening of extracts of various bryophytes showed that the species *Dicranum fulvum* gave extracts with activity in both *in vitro* and *in vivo* bioassays. This plant was thus selected for extraction and fractionation, monitored by *in vitro* bioassays.

Isolation was guided by a combination of bioassay and chemical methods, and led to the isolation of three compounds, betulin, 9,19-cyclolanost-23-ene-3,25-diol, and β-sitosterol. Purification was achieved by open column, flash column, gel filtration, thin layer chromatography, the chromatotron and crystallization.

The isolated compounds were identified by comparisons of spectroscopic data with those of authentic samples and the matching of experimental and literature melting points and optical rotations.

ACKNOWLEDGEMENTS

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INTRODUCTION

It has been estimated by the American Cancer Society that approximately 4.5 million people will have died from cancer in the 1980's. In 1988, 494,000 people died of cancer in America alone, and it is estimated that 502,000 will die of cancer in 1989.[1] It is obvious that there is a great need for improved treatments. The best treatment of course would be prevention, and that idea has developed into the education of the general public to change life styles and diets. Some of these changes include avoiding tobacco products, high fat diets, overexposure to sun, large amounts of alcohol, and large doses of X-rays.[2] There is also a need for improvement of present treatments which are surgery, radiation therapy, chemotherapy, hormone therapy, and immuno therapy.[2] The best way to improve chemotherapy is to improve the drugs involved. The two ways of improving the drugs are either to synthesize more effective drugs that have fewer detrimental side effects or to find new natural products that can be used as found or modified to be more effective.

Plants have long been reputed to be effective against cancer both in folklore and literature.[3] Examples of compounds isolated from plant sources and having useful anticancer activity are vincristine and vinblastine extracted from *Catharanthus roseus* [4] and taxol extracted from *Taxus brevifolia*[5].

The most direct approach to finding the anti-cancer active constituent of a plant is to extract the plant and carry out a fractionation directed by an appropriate bioassay.[6] The three bioassays considered for use in this investigation were the P-388, the KB and the

brine shrimp[7] assays. The P-388 assay is an in vivo assay which uses the percent increase in the lifespan of mice with cancer over control mice; the higher the value the more active a compound is considered. The P-388 assay has a good correlation to effectiveness in humans, but it is very expensive, time consuming, and demands a large sample.. The next choice of assay is the KB assay which measures cytotoxicity against a human cancer cell line. The assay is an in-vitro assay where cancerous cells are grown in culture and the concentration of compound required to retard growth by 50% is determined and reported as the compound's ED_{50} value. The lower the effective concentration, and thus the lower the ED_{50} value, the more effective the compound is considered to be. The third choice was an in-house assay called the brine shrimp assay. The premise of this assay is that the fast growing body cells of the brine shrimp are similarly affected by drugs as are rapidly dividing cancer cells. The concentration at which half the brine shrimp die would be considered the ED_{50} of that compound. The shrimp are hatched in a salt water medium and allowed to grow for 24 hours. A known number of them are then placed in vials with varying concentrations of the compound being tested. Twenty-four hours later the shrimp are counted and an ED_{50} curve is constructed. This assay is inexpensive and since it can be done in-house it is also less time consuming than external assays like the KB assay and the P388 assay.

RESULTS AND DISCUSSION

Selection of Plant Material

Bryophytes as a class were selected for this investigation due to both the availability of the plant in large quantities and the lack of chemical investigations present in the literature. In work carried out before the beginning of this research, Dr. Kingston had arranged for the collection and bioassay of about 40 different species of bryophyte. These plants were then extracted with ethanol, and KB and P-388 bioassays were performed on the ethanol extracts of a wide variety of species of bryophytes. The KB assay results of a few of these can be seen in Table I.

There was a wide range of ED_{50} values for the bryophytes tested, ranging from >100 μg/ml for *Pseudo-cyphellaria anomala* to the lowest value of 0.3 μg/ml for *Dicranum fulvum* and *Diphysicum foliosum*. Due to the high activity, low collection cost and local availability, *Dicranum fulvum* was chosen as the plant for further investigation. There has been very little chemical investigation done on the *Dicranum* species in general. Most of the work on this species has been involved with the biological aspects of the mosses. Previous partial chemical investigations were on *Dicranum elongatum* and *Dicranum scoparium*. The chemical investigations of *Dicranum elongatum* produced some acyl lipids[8] and some steryl and wax esters[9,10,11] while the investigations of *Dicranum scoparium* uncovered a new biflavone[12], a new branched flavanoid triglycoside[13] and a new branched apigenin-7-triglycoside.[14] Up to now no work has been reported on *Dicranum fulvum*.

Table I: KB Assay Results

PLANTS	ED50
Hylocomium splendens	1.9
Dicranum fulvum	0.3
Diphysium foliosum	0.3
Bazzania trilobata	2.6
Calopaca trachphylla	7.0
Lecanora novomexicana	2.5
Peltigera polydactyla	1.1
Usnea arizonica	27
Hypnum imponens	24
Pseudocyphellaria anomala	>100

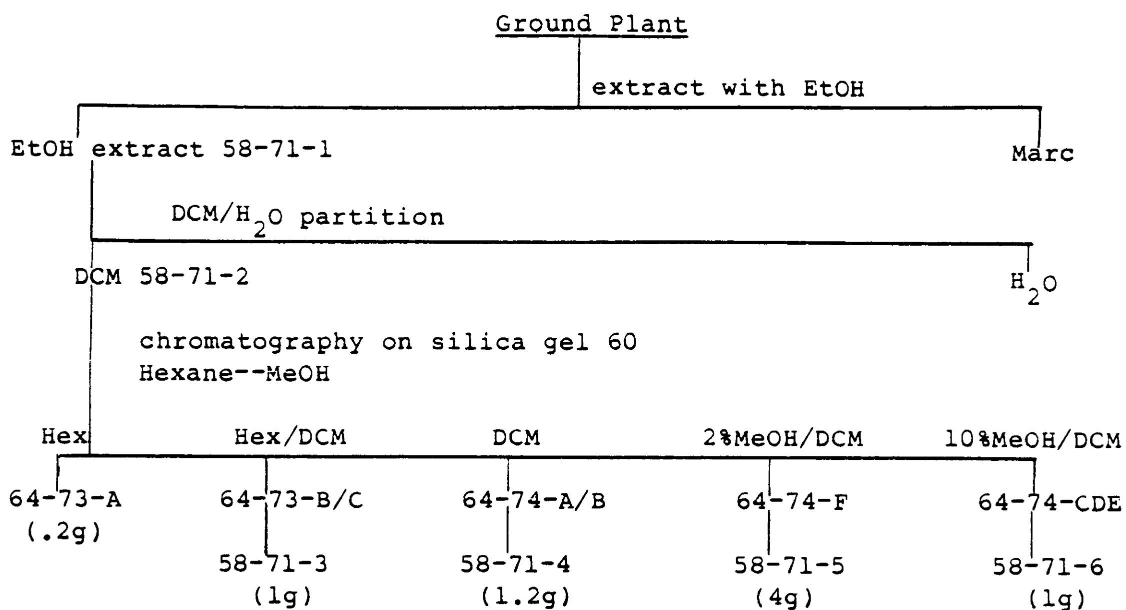
Selection of Bioassay

As noted above, *Dicranum fulvum* was selected for investigation based on its activity in the KB bioassay. This bioassay is still relatively expensive (about $30 a sample) and is also time consuming (approximately 6-8 weeks to receipt of data), and a faster and cheaper alternative was thus sought.

The brine shrimp bioassay was established according to the published protocols[7], and extracts of *Dicranum fulvum* and also of other bryophytes were tested. Regrettably, all the bryophyte extracts, including those showing cytotoxicity to KB cells, proved to be inactive in the brine shrimp bioassay. It thus proved necessary to use the KB bioassay. Because funds for this bioassay were used up during the course of this work, the latter part used the strategy of matching fractions by TLC comparison with earlier fractions which were known to be active from the initial studies. Once the fractions from the large scale reisolation were matched up to the active fractions from the initial small scale work, the objective then became the isolation of pure compounds from these fractions.

Fractionation Directed by Bioassay

Originally a small scale extraction of the plant was done by the method shown in Chart I. Separation of the methylene chloride (DCM) layer by chromatography on silica gel produced an active fraction 64-74-F which was relabelled 58-71-5 to simplify the confusing system used by a technician who had done the initial work. It was decided to repeat this work adding in an additional methanol(MeOH):water(H_2O)/hexane partition step to provide better separation of lipophilic materials. A

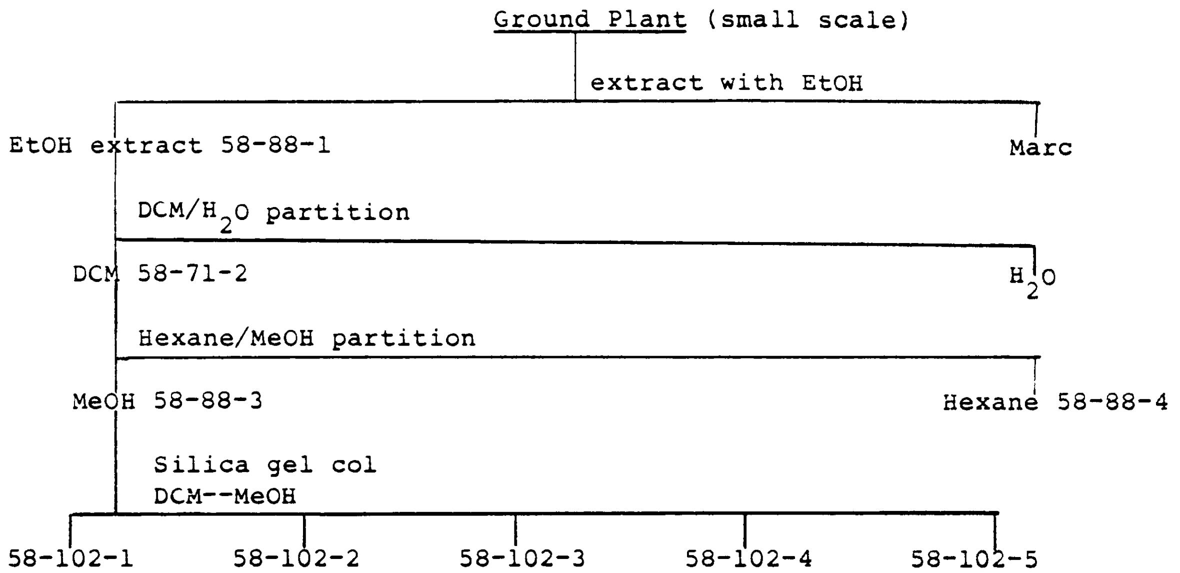


Samples sent to University of Miami

Sample	Ed_{50}
58-71-1	1.8
58-71-2	0.7
58-71-3	8.0
58-71-4	29.0
58-71-5	0.3
58-71-6	31.0

Chart I: Small scale extraction of plant without methanol:water/hexane partition

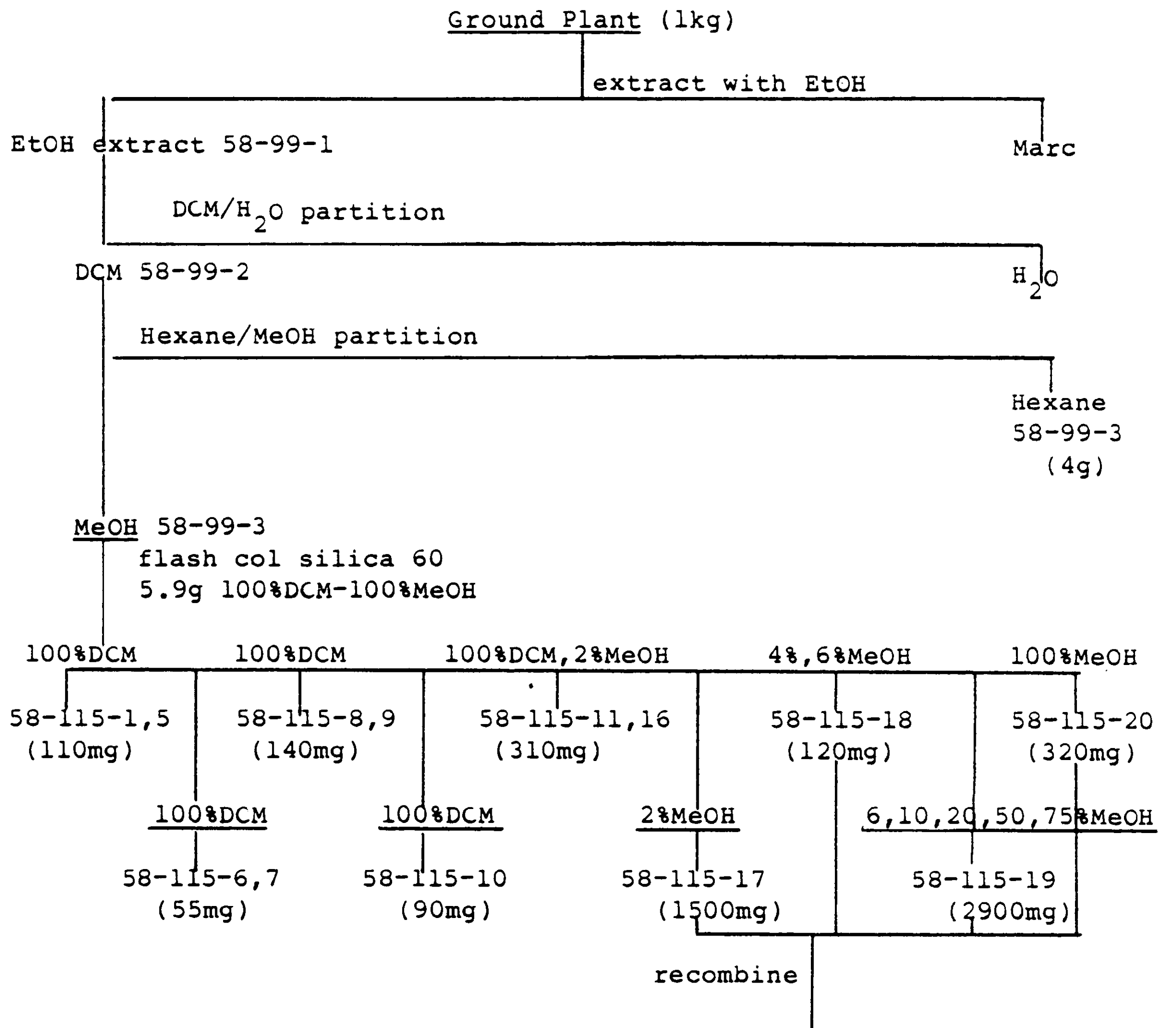
column was run on the methanol fraction 58-88-3 and samples were sent off for assay. Unfortunately the assay samples (58-102-1 to 58-102-5) were temporarily lost in the mail and when they were finally found and tested, they proved to be inactive (see Chart II). It was not known at this time if they had lost activity due to the long period between collection and testing or if the active component(s) were unstable enough to decompose on the column. Since the column that produced the initial series (Chart I) had not decomposed the active component(s), the second possibility was determined to be unlikely. A large scale extraction was thus performed on a kilogram of plant and a silica gel column was run on the methanol fraction 58-99-3 (see Chart III). This column produced the 58-115 series of fractions. Fractions 58-115-17 through 20 were recombined and another column was run producing the 58-118 series of fractions (see Chart IV). Fractions 58-118-B1 and B2 were very similar to the previously determined active fraction 58-71-5. These fractions plus an earlier fraction 58-115-11,16 were sent off for assay along with 58-99-3 and 58-71-5 to determine if the activity was still present and had not been lost over time (see Charts III and IV). While these fractions were being assayed, fraction 58-115-10 (see Chart V) was separated into its components, one of which was determined to β-sitosterol. The confirmation will be discussed later. When the bioassay data returned it showed that fraction 58-115-11,16 was not active, but fractions 58-118-B1 and B2 were active. In an attempt to remove a chlorophyll type compound that was making the TLCs difficult to run, it was decided to run 118-B1 and B2 through a column of Sephadex LH-20 which is a chemically modified Sephadex that allows the use of



Samples sent to University of Miami

Sample	ED_{50}	Sample	ED_{50}
58-88-1	1.5	58-102-1	>100
58-88-2	2.1	58-102-2	>100
58-88-3	0.3	58-102-3	28
58-88-4	42	58-102-4	100
		58-102-5	20

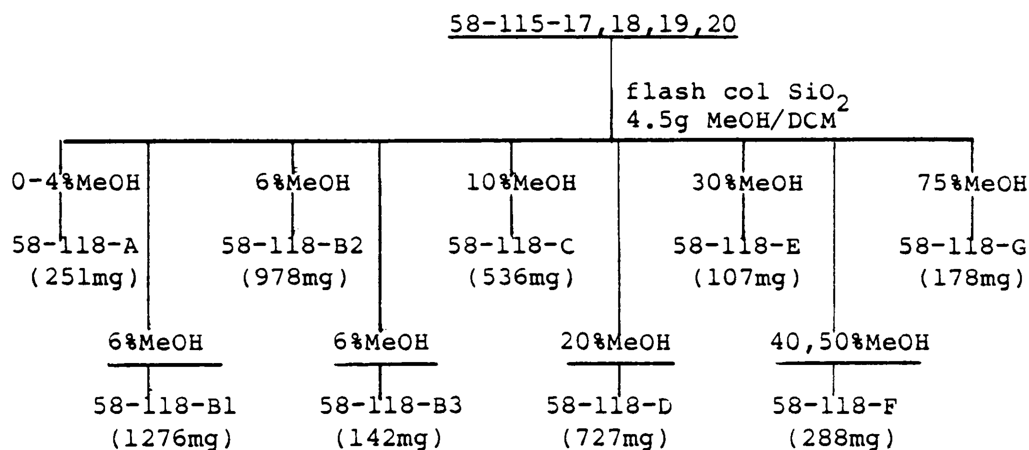
Chart II: Small scale extraction of plant with methanol:water/hexane partition



Samples sent to University of Miami

Sample	ED_{50}
64-74-F	.10
58-99-3	.30

Chart III: Formation and Fractionation of 58-99-3



Samples sent to University of Miami

Sample	ED_{50}
58-118-B1	.30
58-118-B2	.35
58-115-11,16	31.0

Chart IV: Refractionation of 58-115-17,18,19,20

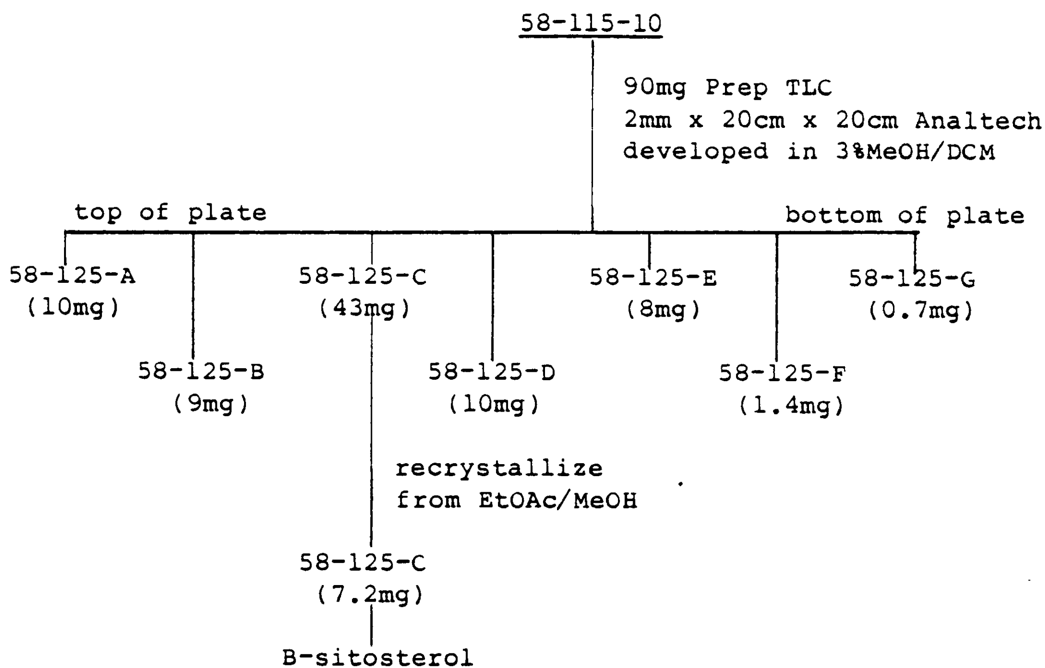
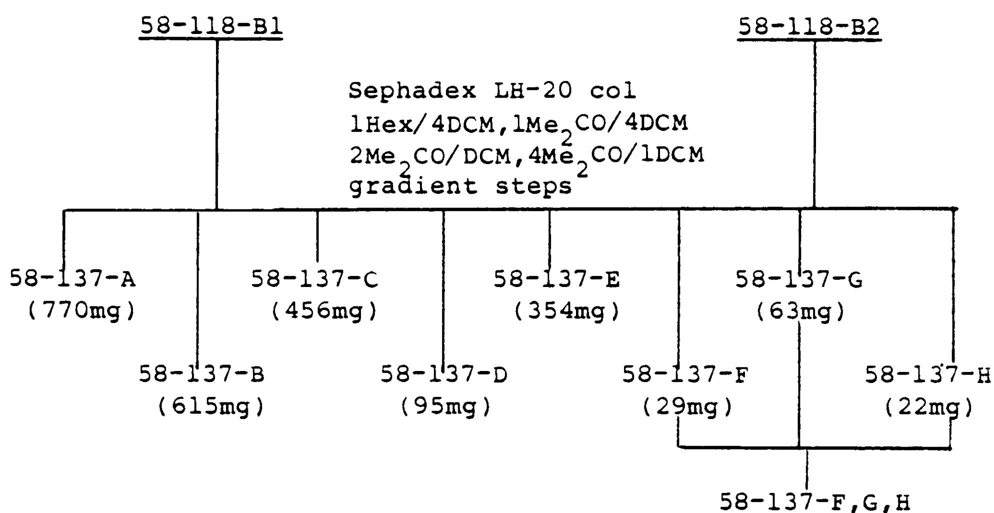


Chart V: Fractionation of 58-115-10

organic solvents instead of being restricted to aqueous solvent systems. The mechanism of separation with Sephadex is the mechanism involved in gel permeation instead of the mechanism of adsorption when using silica gel. The hope was that a molecule like chlorophyll would move off the column quickly leaving the other components of interest to be washed off the column later. A method was found in the literature for gradient elution using Sephadex;[15] this method allowed for the possibility of not only ridding the fractions of chlorophyll-type compounds but also getting another separation step done all in one column. The first running of the Sephadex column produced some separation, but the bands moved very quickly when the second step of the solvent system was introduced. In order to modify this occurrence an extra solvent system step was added, but this did not affect the rate of separation so it was not used again. Multiple Sephadex columns were run and finally produced fractions 58-137-A through H (Chart VI). Fractions F, G and H were recombined for assay since they were small compared to the rest of the fractions. While these fractions were sent off for assay, it was decided to work on fraction 58-137-E since it had a single deep purple spot that was UV active and appeared as if it could easily be isolated. Fractions 58-138-1 through 6 were produced by preparative HPLC separation of 58-137-E (Chart VII). Fraction 58-138-5 contained the purple spot which was pure according to both HPLC and TLC. Attempts to recrystallize the solids this fraction formed upon drying were unsuccessful, and a mass spectrum did not give repeatable results with any of the currently available excitation methods. Since the assay results had returned by this time and the results showed 58-137-E not to



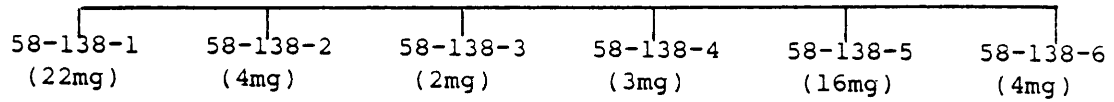
Samples sent to University of Miami

Samples	ED_{50}
58-137-A	0.2
58-137-B	0.2
58-137-C	0.4
58-137-D	>10
58-137-E	2.2
58-137-F,G,H	>10

Chart VI: Fractionation of 58-118-B1 and B2

58-137-E (103mg)

HPLC RP-18
85/15 MeOH/H_2O
3ml/min



Samples sent to University of Miami

Samples	ED_{50}
58-138-5	2.9

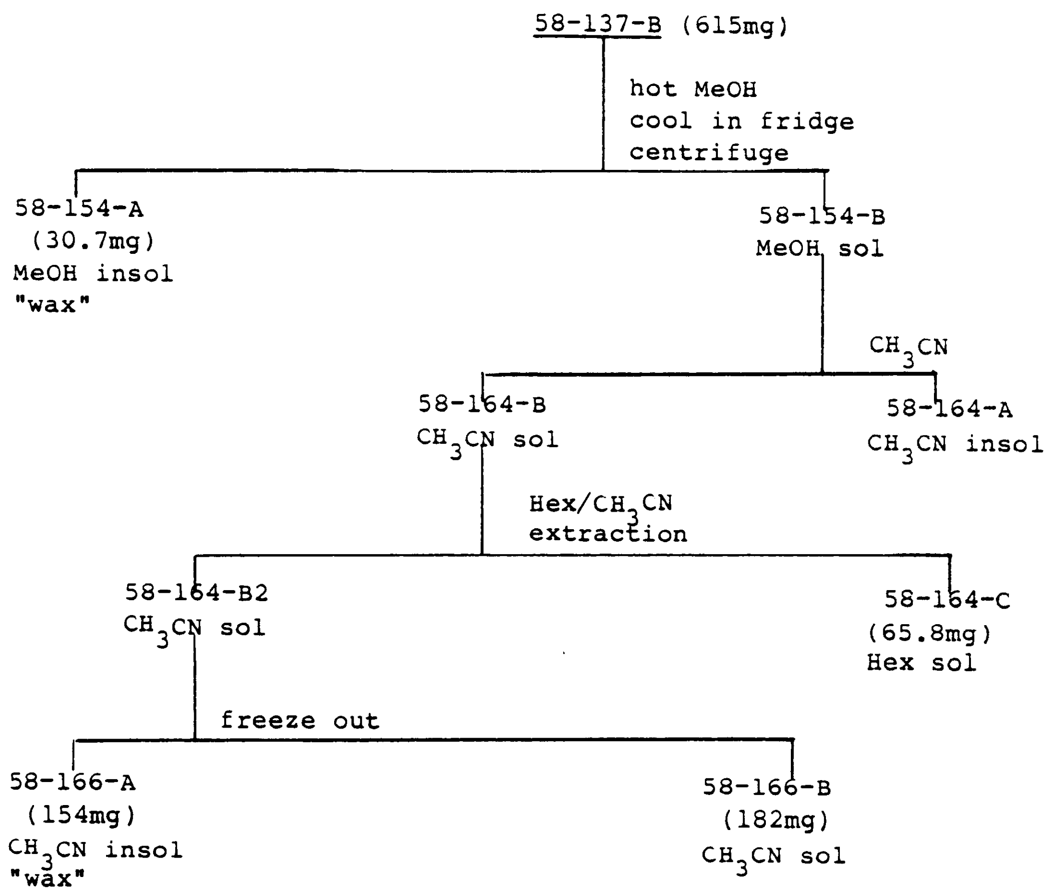
Chart VII: Fractionation of 58-137-E

be the most active fraction, it was decided to leave fraction 58-138-5 alone except to assay it; there was no improvement in the activity, and this fraction was thus dropped from active investigation.

The assay results showed that fractions 58-137-A and B were both very active, with each having an ED_{50} of 0.2 μg/ml. Since 58-137-B did not contain any of the chlorophyll-type compound and was active, it was decided to work with it. An initial study by HPLC was complicated by problems with poor solubility and wax build-up in the lines, and a hot-cold separation of 137-B was thus carried out (Chart VIII). The general methodology for the extraction was to dissolve the fraction in as little of the hot solvent as possible and then place it in the freezer for an hour. The conical test tube containing the sample was centrifuged and the liquid was decanted leaving the "wax" behind. Fraction 166-A still contained some of 166-B since the wax was a black color like 166-B, not the cream color of the other wax fractions. Fractions 58-154-A, 58-166-A and 58-166-B were sent off for assay; fractions 166-A and B came back as active fractions. A silica gel column was run on fraction 58-166-B to produce the 58-177 series of fractions (see Chart IX). The active fraction was 58-177-3; the result on fraction 58-177-2 was not reported back to us.

Fractionation Directed by TLC Match-up of Bioassayed Fractions

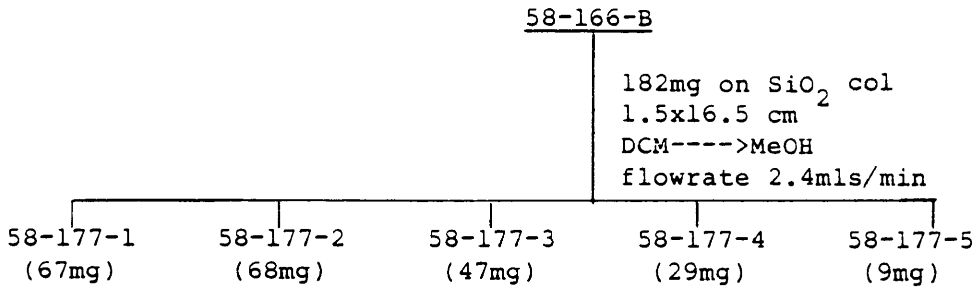
At this point in the study we were down to small amounts of each fraction and still had a number of separations to go, so we decided to do an even larger scale extraction of the plant. Since funding for the bioassays ended at this point, we planned to determine which fractions to work on by TLC matches with previously assayed fractions with the



Samples sent to University of Miami

Sample	ED_{50}
58-154-A	2.8
58-166-A	0.23
58-166-B	0.23

Chart VIII: Hot-cold partition of 58-137-B



Samples sent to University of Miami

Sample	ED_{50}
58-177-1	14.0
58-177-2	----
58-177-3	0.02
58-177-4	20.0
58-177-5	8.5

Chart IX: Fractionation of 58-166-B

ultimate aim of obtaining a larger quantity of a fraction comparable to 58-177-3.

The largest scale extraction was of five kilograms of plant (Chart X). Separation of the methanol extract by chromatography yielded fractions 58-190-4 and 5 which matched closely by TLC to the active fractions 58-118-B1 and B2. A second silica gel column was run on 58-190-5 since 190-4 contained the chlorophyll type compound and therefore needed to be cleaned up with a Sephadex column. The silica gel column produced the 58-194 series of fractions (Chart XI). Fractions 58-194-1 and 58-194-2 were too small to be worked on conveniently. Fraction 58-194-3 was closely matched by TLC to the moderately active 58-137-E. Fractions 58-194-5,6,7 had TLCs that matched up with components in the previous inactive fractions 58-137-E,F,G and H. Fraction 58-190-4 was the best match to the previous active fraction 58-137-B, except for some spots with higher R_f values and the presence of the large amount of the chlorophyll type compound.

Because of the presence of the chlorophyll-type compound, a separation over Sephadex LH-20 was next carried out. The initial attempt using a short fat column was not satisfactory, but did yield a fraction 58-198-2, eluted as a late band with acetone, which resembled the inactive or weakly active fractions 58-137-D,E, and F. Rechromatography of the early fractions (58-198-1) over Sephadex LH-20 using a longer and narrower column gave a useful separation into fractions 58-203-1 to 58-203-4 (Chart XII). Fraction 58-203-1 looked like a pre-137-A fraction having some R_f values higher than those of fraction 58-137-A. Fraction 58-203-2 matched closely by TLC to 58-137-

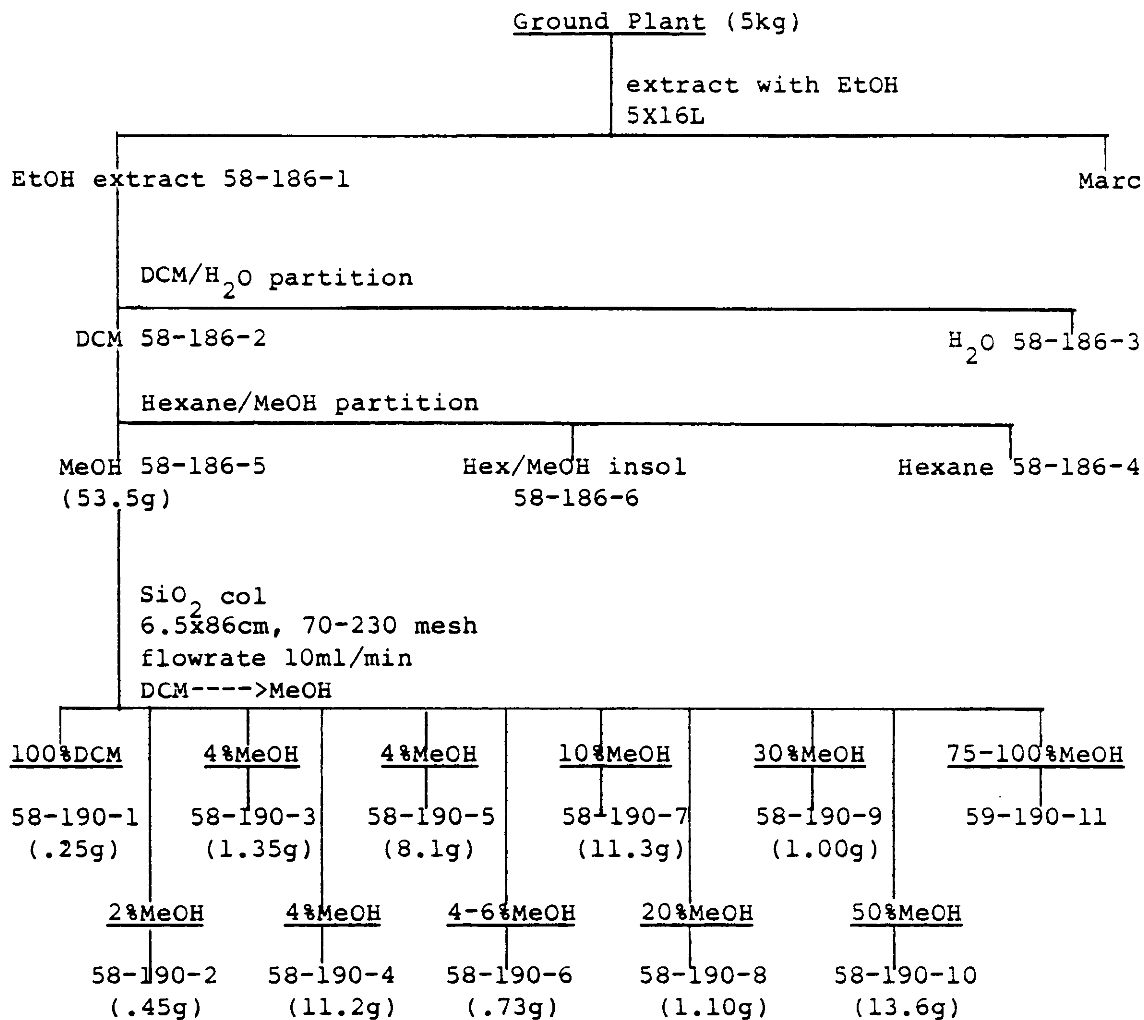


Chart X: Large scale extraction of plant and fractionation of methanol extract 58-186-5

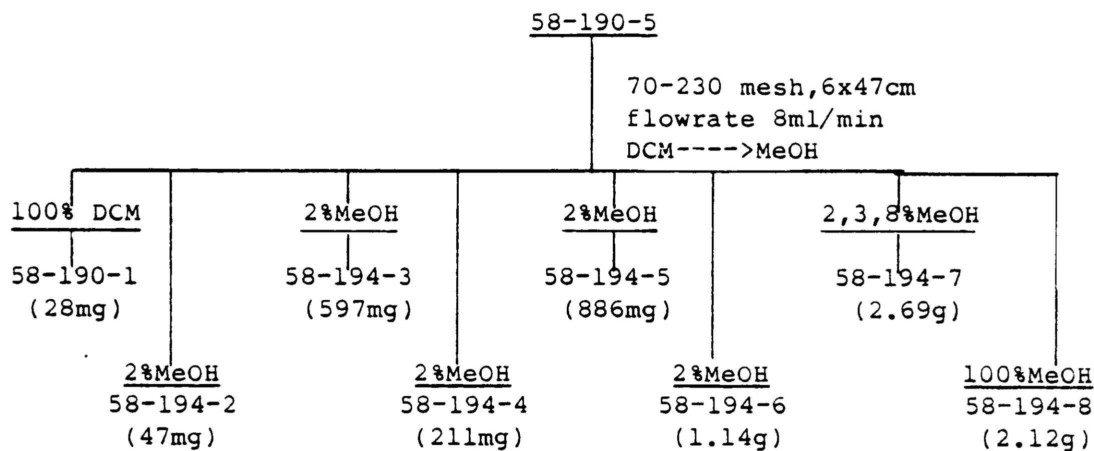


Chart XI: Fractionation of 58-190-5

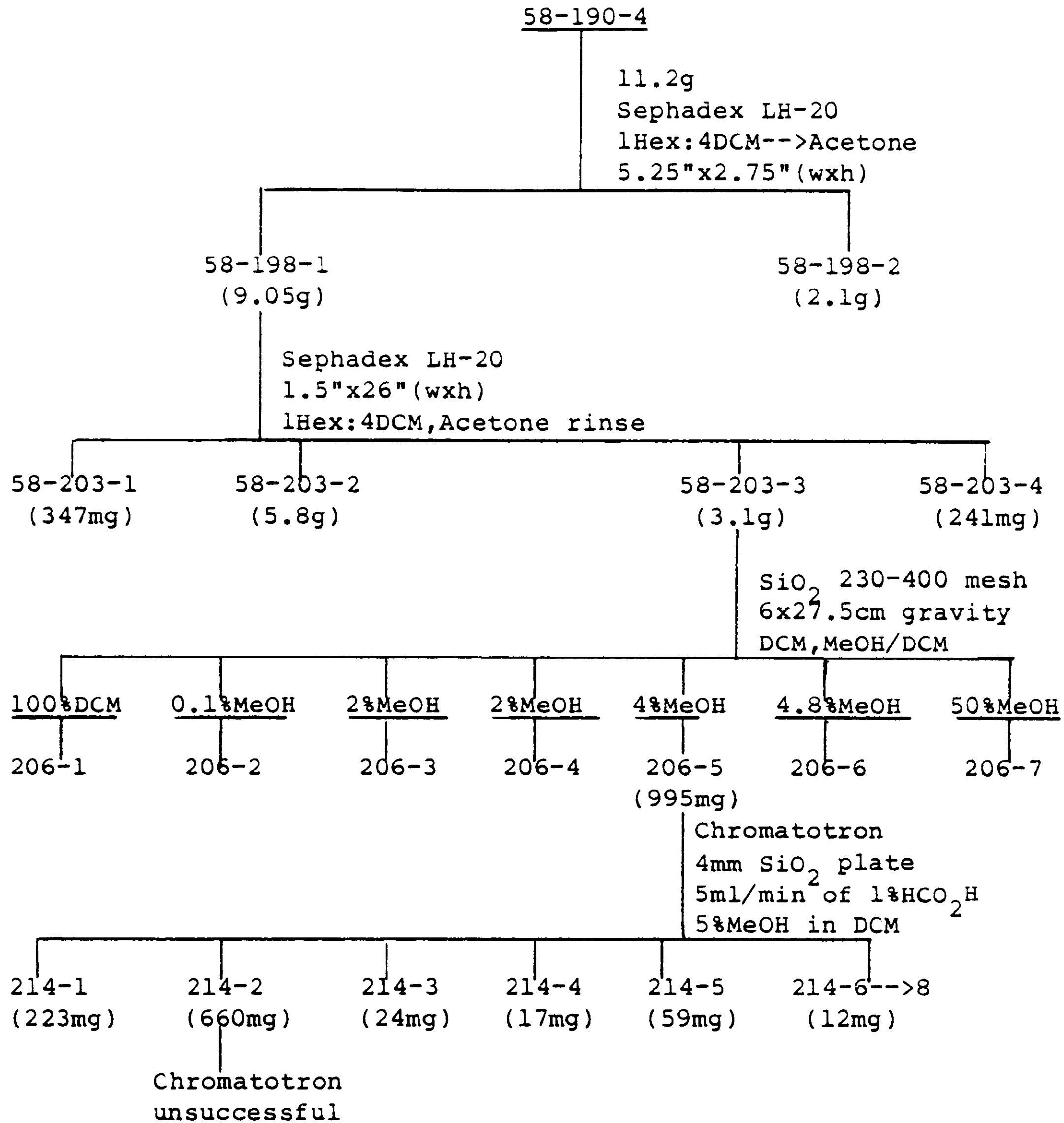


Chart XII: Fractionation of 58-190-4, 58-198-1, 58-203-3 and 58-206-5

A; fraction 58-203-3 matched closely by TLC to a combination of 58-137-B and 137-C. Fraction 58-203-4 matched well with 58-137-D. Since fraction 58-203-3 had a partially matching TLC with the active fraction 58-137-B, it was chosen to be worked on next. A silica gel column was thus run on fraction 58-203-3. This column produced the 58-206 series of fractions (see Chart XII) of which 58-206-5 seemed to contain a lot of the components in 58-177-3. Unfortunately fraction 58-177-3 type components were also present in 58-206-4 and 58-206-6, so that there was no clear cut choice as to which fraction contained the active component(s).

At this point there was thus no longer any clear cut fraction that was known to contain activity, so the objective then became the isolation of compounds from fractions which showed some overlap with 58-177-3 in order to identify them. It was hoped by this approach to obtain active compounds from these fractions.

The TLC results described above were obtained with a dichloromethane-methanol solvent mixture on silica gel plates. Although this system gave adequate resolution for comparison of fractions, it did give significant streaking which reduced its value for preparative purposes. After investigation of various other solvent systems and plates, it was found that the addition of 1% formic acid to 5% methanol in dichloromethane gave the best separation of the desired fractions.

The Harrison Chromatotron was selected as the chromatographic method. This device for centrifugally accelerated radial chromatography offers all of the advantages of preparative TLC without the disadvantages of scraping and eluting the plate with its resulting

contamination; it can also handle larger samples in a single run than preparative TLC, and the progress of elution can be monitored by UV light.

Fraction 58-206-5 was the first fraction subjected to the Chromatotron, and it yielded fractions 58-214-1 to 58-214-8 (Chart XII). Fraction 58-214-2 was the only fraction of significant weight, but attempts to separate it into its components have been so far unsuccessful.

The next fraction worked with was 58-206-1, which when dried down contained solids in a yellow-orange oil. The solids were washed with hexane to remove the oil. The hexane solubles, fraction 58-224-1, were then run on the Chromatotron and produced the 58-229 series of fractions (see Chart XIII). Fraction 58-229-6 was fairly pure and was further purified by running the Chromatotron on it again producing the 58-233 series of which 58-233-3 was a white solid which was later recrystallized in methanol/dichloromethane and identified as 9,19-cyclolanost-23-ene-3,25-diol. Fraction 58-224-2, the hexane insolubles of 58-206-1, was the next fraction to be run on the Chromatotron and produced the 58-245 series. Fraction 58-245-2 contained yellowish crystals and was slightly UV active. A new solvent system was needed to remove the impurities from this fraction; this new solvent systems was found to be methanol:ethyl acetate:hexane,2:25:73. When 58-245-2 was taken up in this solvent white solids remained in the flask and would not dissolve unless a large amount of solvent was used. Fraction 58-245-2 was redried down and a very small amount of the solvent was used to wash out the UV active impurities which were labelled 58-245-2B.

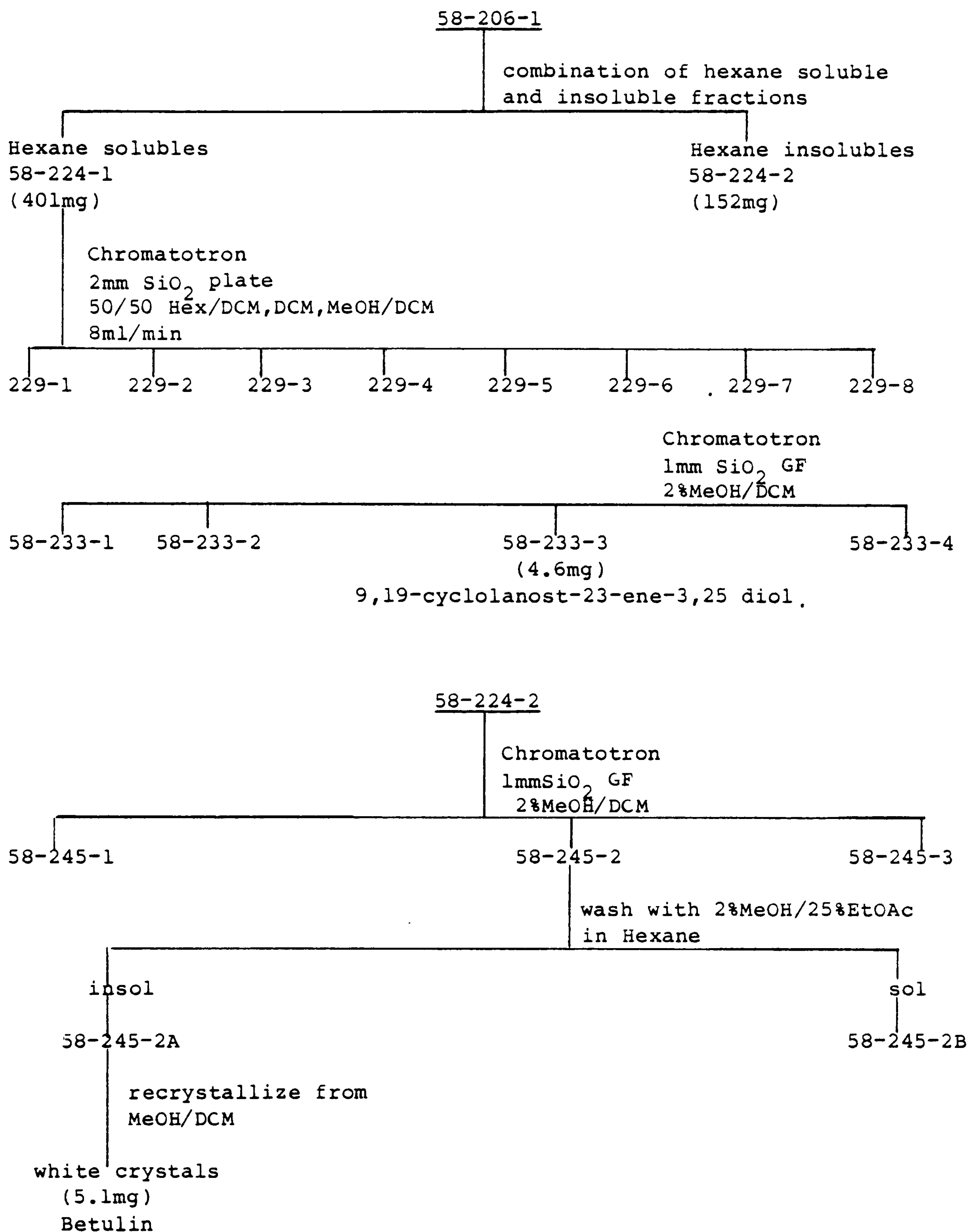


Chart XIII: Fractionation of 58-206-1, 58-224-1, 58-229-6, 58-224-2 and 58-245-2

The insolubles, fraction 58-245-2A, were recrystallized from methanol/dichloromethane and later identified as betulin.

There were some other fractions that were worked with but they did not yield any results and therefore were not discussed though the work done can be found in the experimental.

Identification of Pure Compounds

β-sitosterol

A library match of the mass spectrum tentatively identified fraction 58-125-C as β-sitosterol (see Figure 1). A comparison of the 270 MHz ^{1}H NMR of 58-125-C (Figure 2) to a 60 MHz ^{1}H NMR of β-sitosterol from Sadtler showed the similarities between the two (see Figure 3). The only difference between the spectra was the resolution. The doublet at δ5.35 and the multiplet at δ3.2-3.7 are present in both spectra and the aliphatic region have similar patterns. Comparison of the FT-IR of 58-125-C (Figure 4) and the Sadtler IR of β-sitosterol shows that they are very similar (Figure 5). Fraction 58-125-C has the O-H stretch between 3200 and 3600 cm^{-1} and the aliphatic C-H stretch as well as the C=C stretch at 1600-1750 cm^{-1} as does β-sitosterol. All the above evidence as well as an uncorrected melting point of 138-141°C (lit. 139-142°)[16] confirmed that fraction 58-125-C was β-sitosterol.

9,19-cyclolanost-23-ene-3,25-diol

Fraction 58-233-3's identification was also first indicated by a library match of the mass spectrum (Figure 6). Identification of 58-233-3 through spectra was a little more difficult since no NMR nor IR spectrum was found in the literature. A 270 MHz ^{1}H NMR spectrum of 58-233-3 (Figure 7) was taken; the regions of primary interest were the

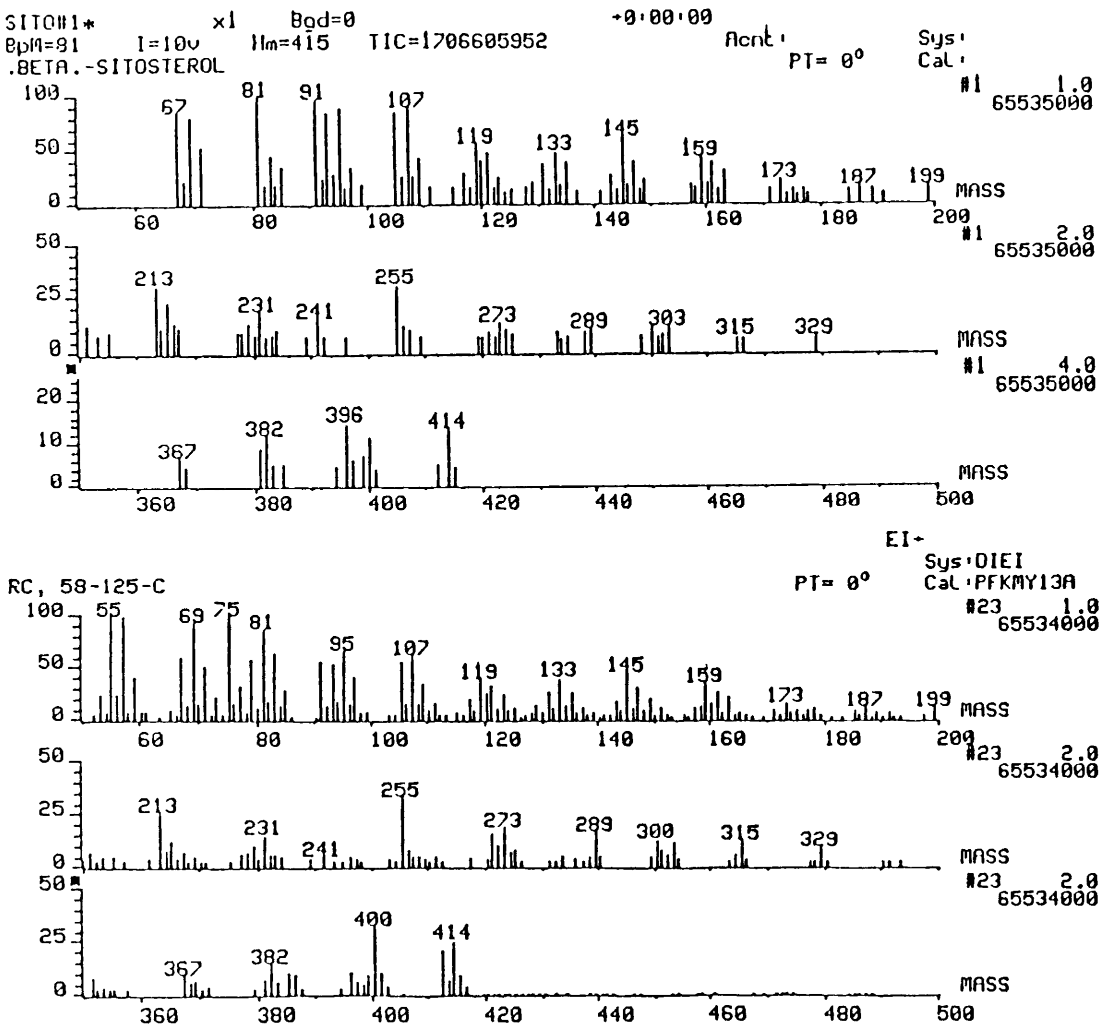


Figure 1: Mass Spectrum library match of B-sitosterol and 58-125-C

β-SITOSTEROL

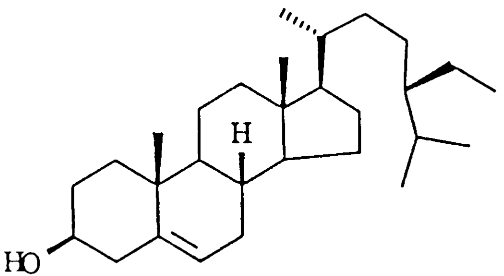


Figure 2: 270 MHz ^{1}H NMR of 58-125-C

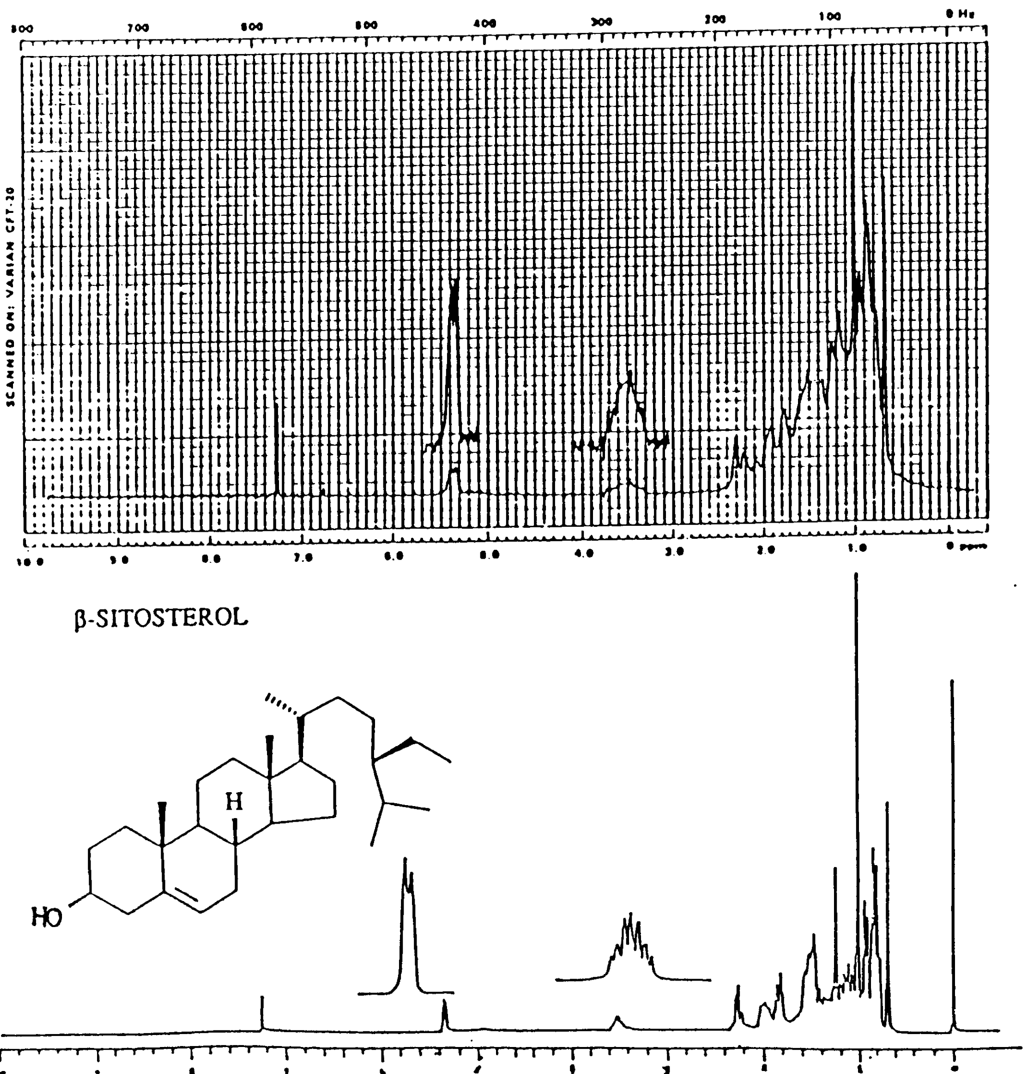


Figure 3: Comparison of 270 MHz ^{1}H NMR of 58-125-C and 60 MHz ^{1}H NMR of B-sitosterol

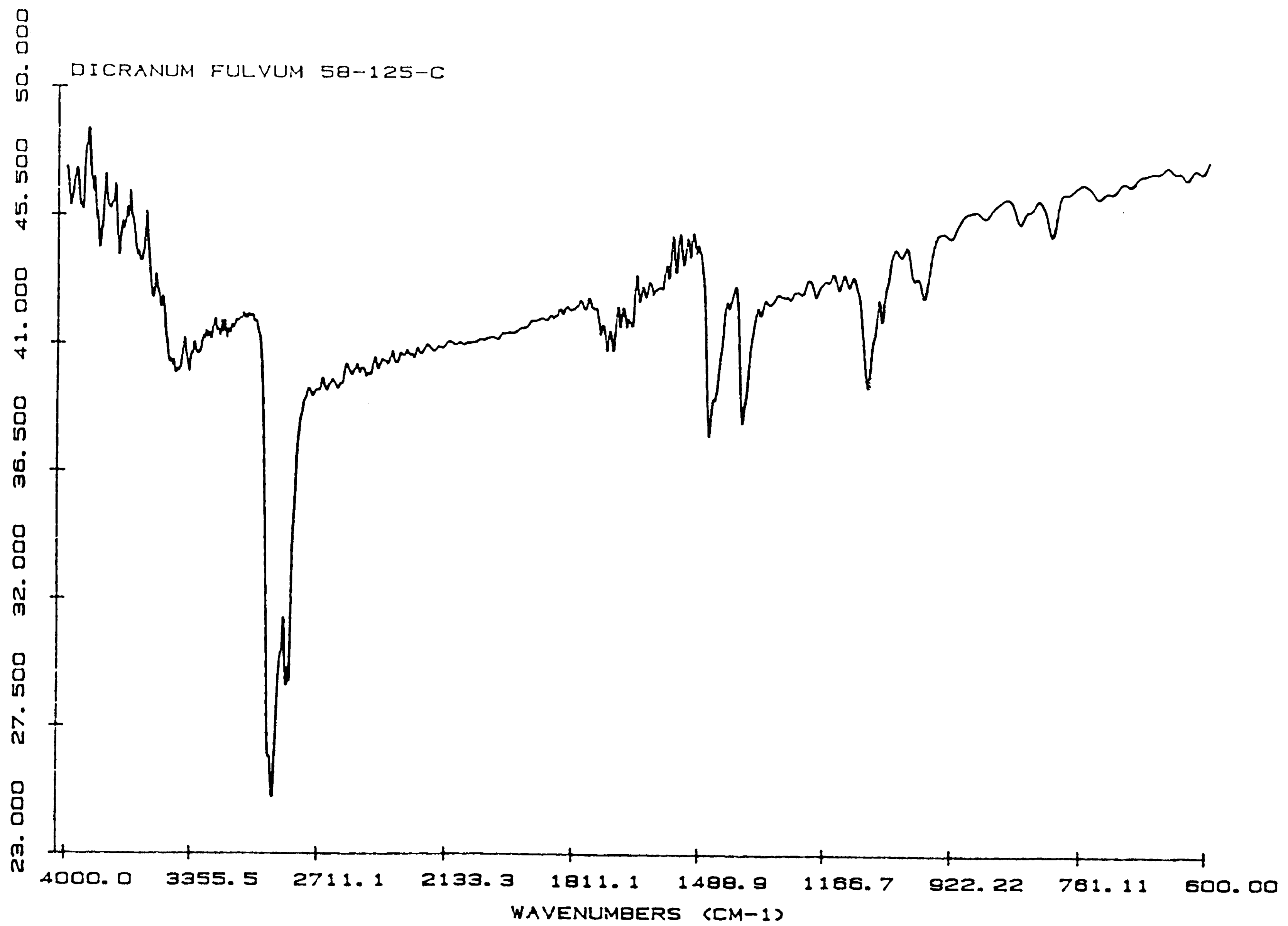


Figure 4: FTIR of 58-125-C

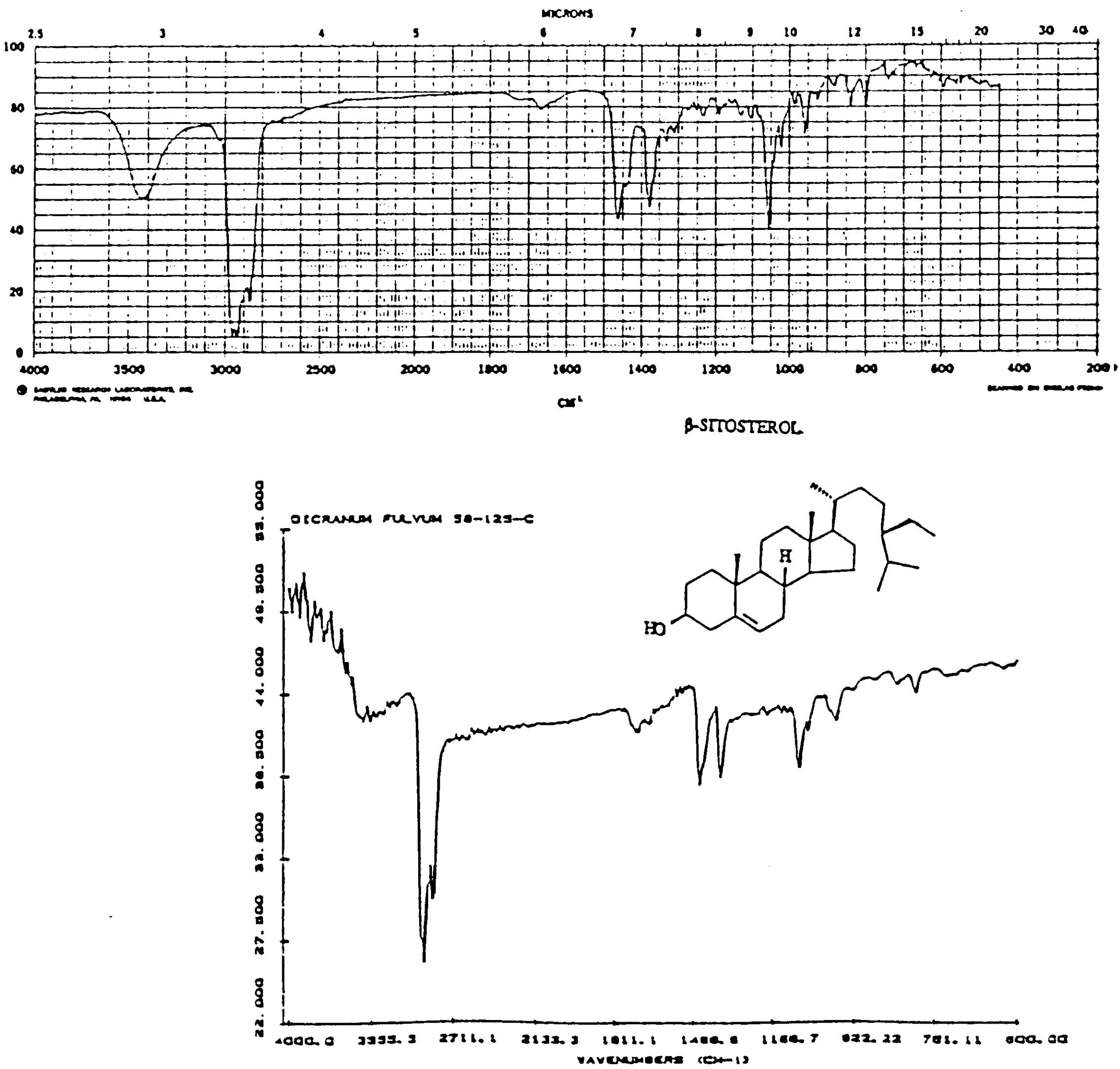
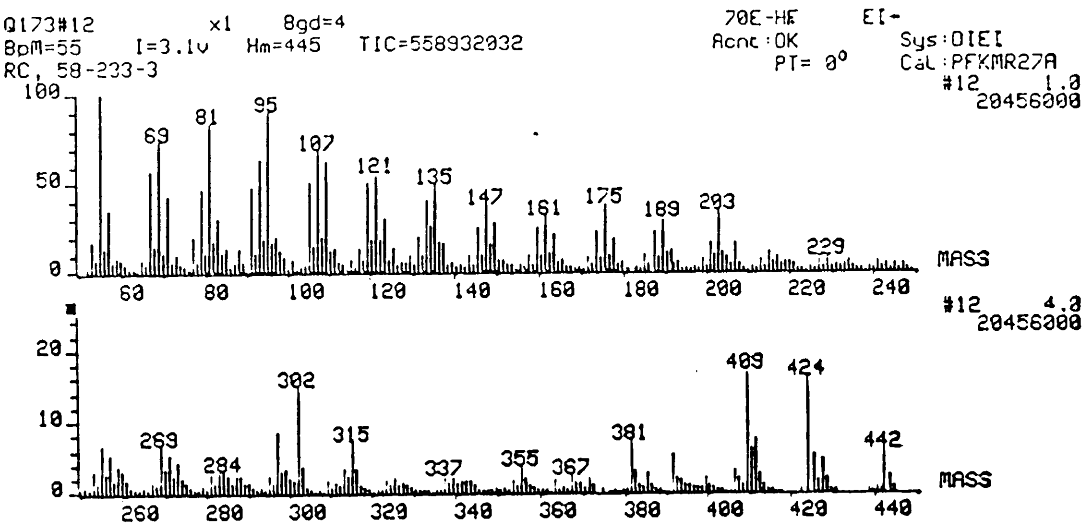


Figure 5: Comparison of FTIR of 58-125-C and grating IR of B-sitosterol



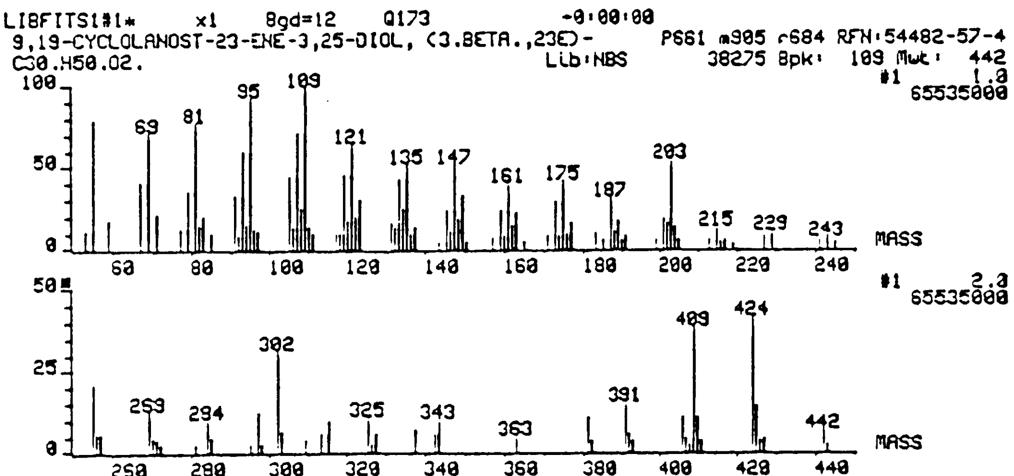


Figure 6: Mass spectrum library match of 9,19-cyclolanost-23-ene-3,25-diol and 58-233-3

9,19-CYCLOLANOST-23-ENE-3,25-DIOL

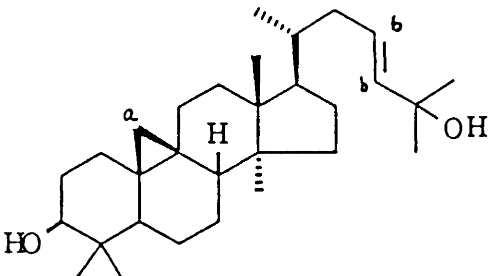


Figure 7: 270 MHz ^{1}H NMR of 58-233-3

<δ1.0 and δ4-5. The cyclopropyl protons were reported in literature at δ0.3 and 0.58[17], and signals at these positions were observed for 58-233-3. The vinyl protons were located between δ4.5 and δ5 but the literature made no mention of the pattern. The FT-IR spectrum of fraction 58-233-3 (Figure 8) showed the characteristic O-H stretch along with aliphatic C-H, C=C-H, C-C and C=C stretches. The literature[17] reported the O-H stretch of 3595, 3430 cm^{-1} and the gem dimethyls of 1375, 1365 cm^{-1} which match up well with the FTIR spectrum. 9,19-Cyclolanost-23-ene-3,25-diol was usually identified in the literature by a combination of melting point and optical rotation. The uncorrected melting point of 199-203°C matched well with the literature[18] value of 200-204°C and the $[\alpha]_D^{27}$ = +38° in chloroform of 58-233-3 was identical to the literature value. This evidence, together with the mass spectral identity discussed earlier and the partial NMR evidence, demonstrates that 58-233-3 has the structure 9,19-cyclolanost-23-ene-3,25-diol.

Betulin

Fraction 58-245-2A was easily identified through spectral comparisons and melting point as betulin. Once again the initial information came from a library match of 58-245-2A's mass spectrum (Figure 9) with that of betulin. A 270 MHz ^1H NMR spectrum of 58-245-2A (Figure 10) was taken and compared to a Sadtler 60 MHz ^1H NMR spectrum of betulin (Figure 11) and aside from resolution they matched very well. An FTIR spectrum was also run on 58-245-2A (Figure 12) and compared to a Sadtler IR spectrum from the literature (Figure 13). All the bands present in the spectrum of 58-245-2A are present in the literature spectrum. An uncorrected melting point of 58-245-2A was taken and found

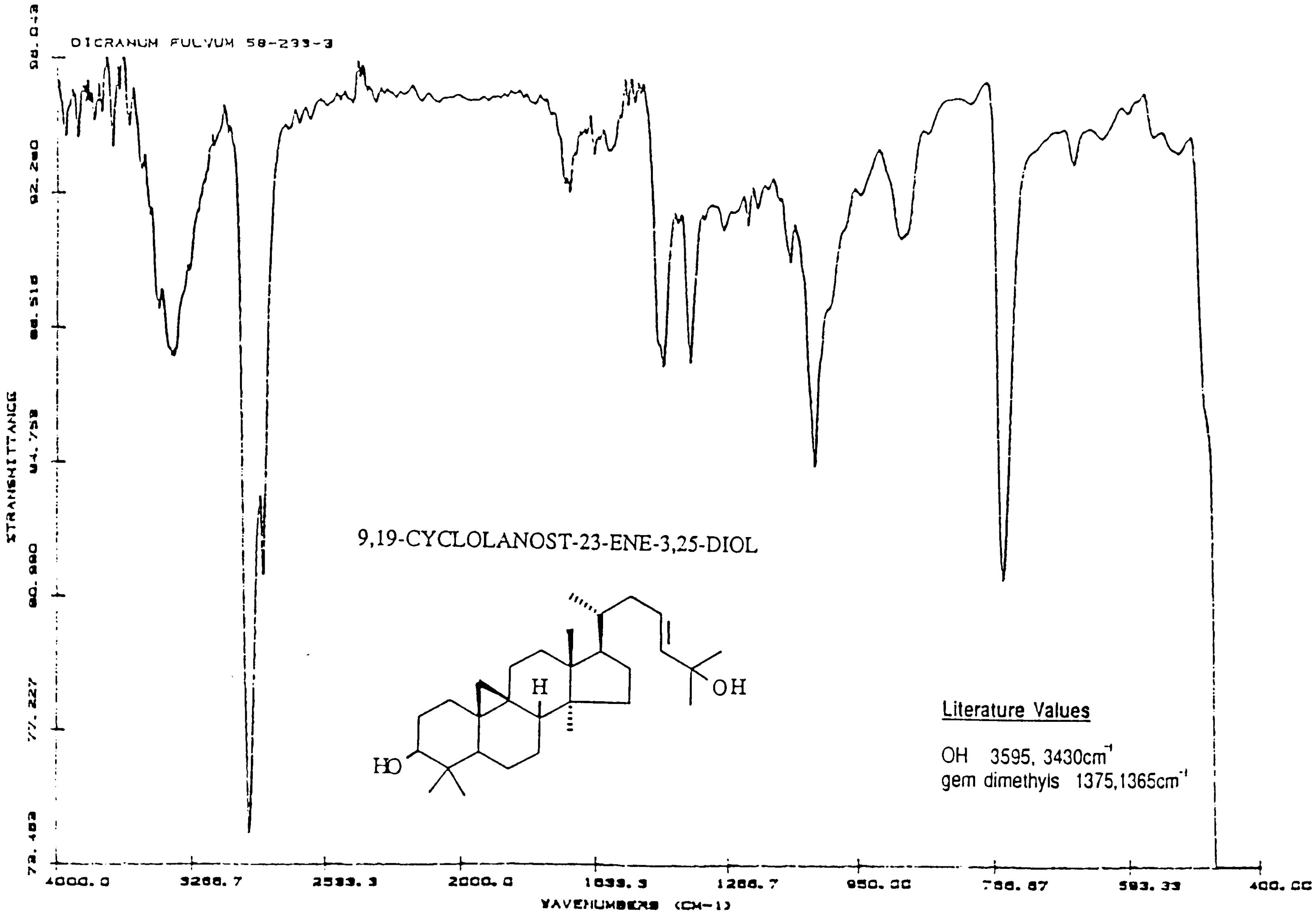


Figure 8: FTIR of 58-233-3

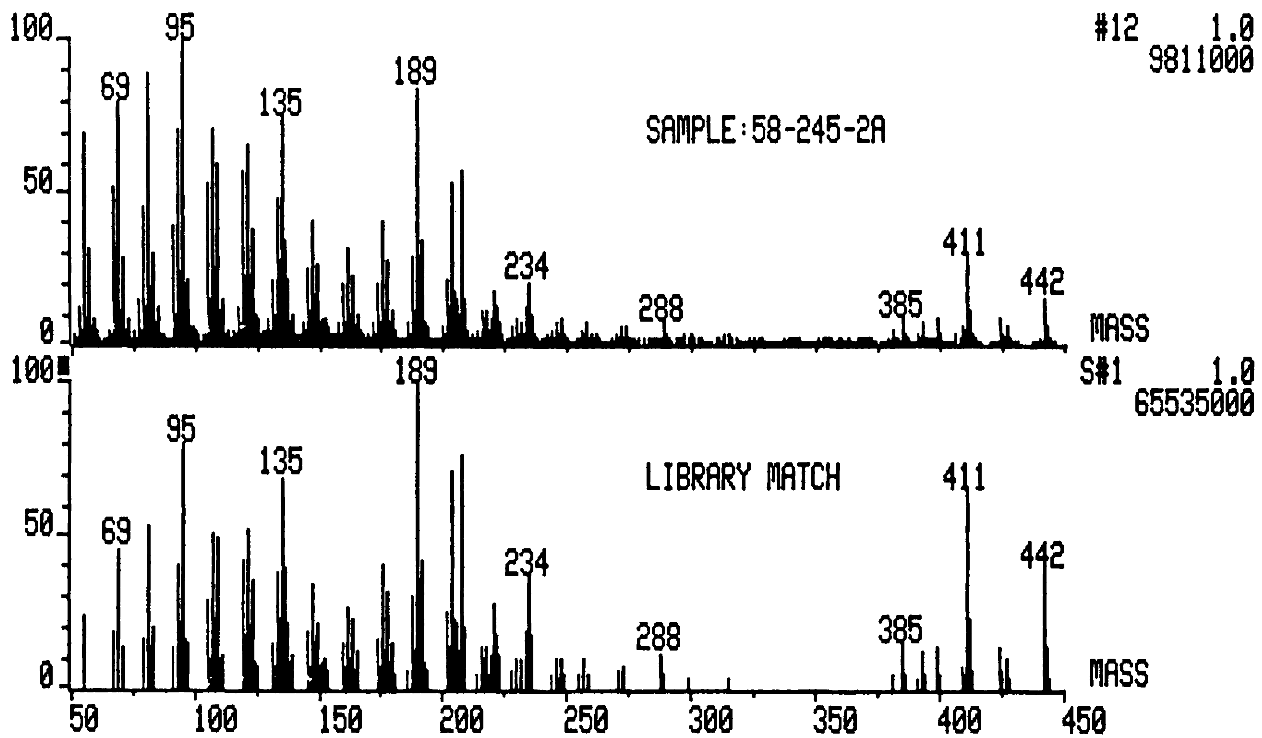


Figure 9: Mass spectrum library match of betulin and 58-245-2A

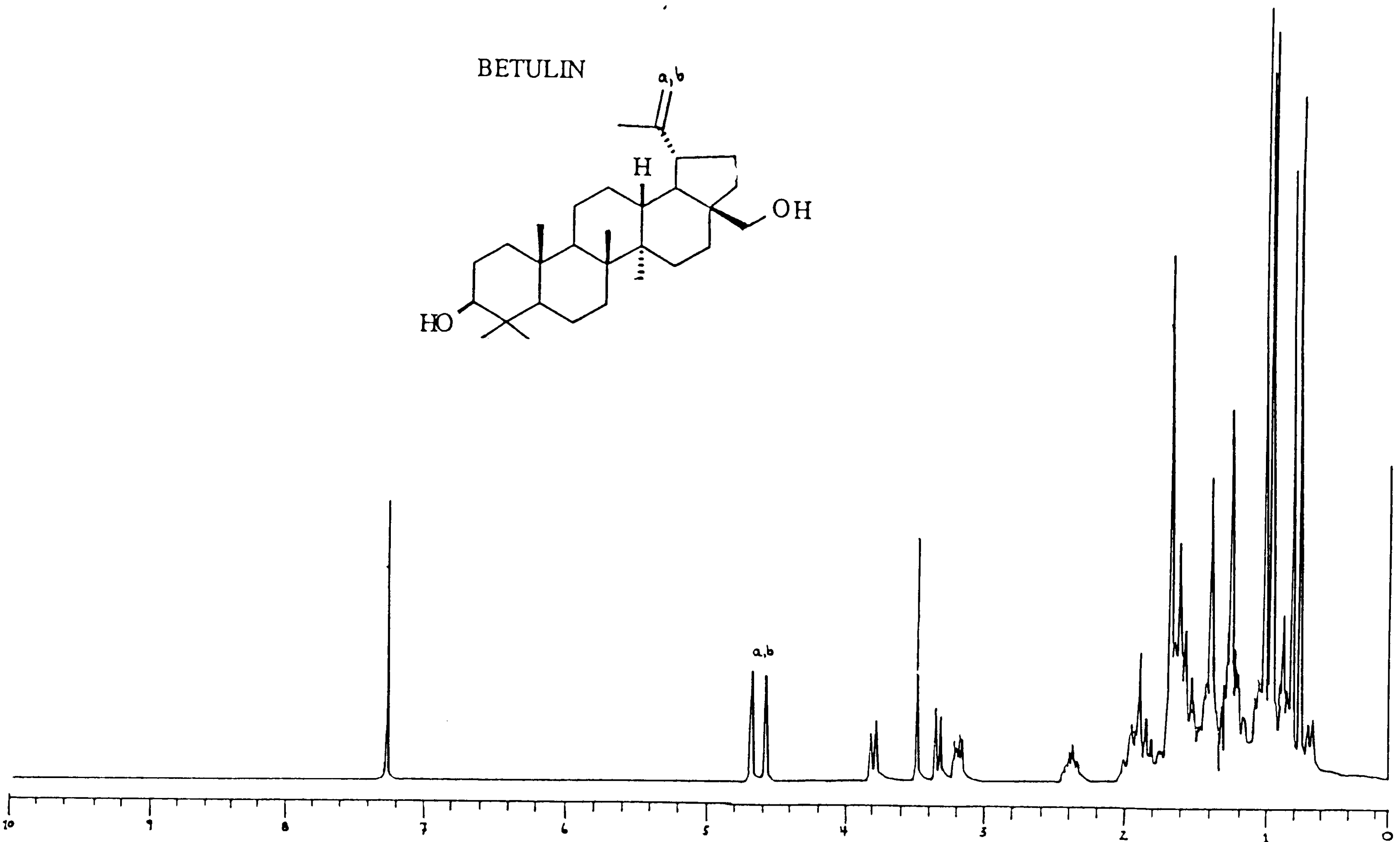


Figure 10: 270 MHz ^1H NMR of 58-245-2A

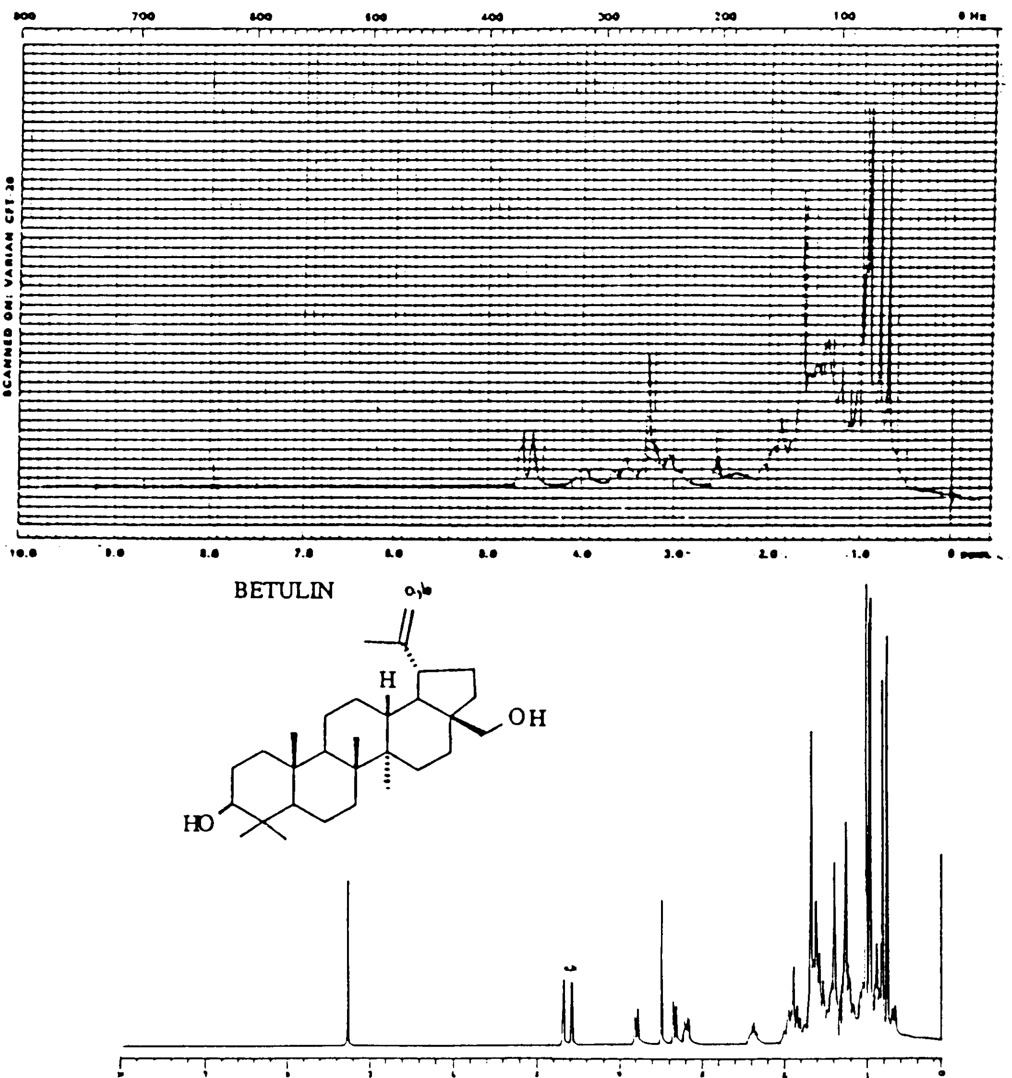


Figure 11: Comparison of 270 MHz ^{1}H NMR of 58-245-2A and 60 MHz ^{1}H NMR of betulin

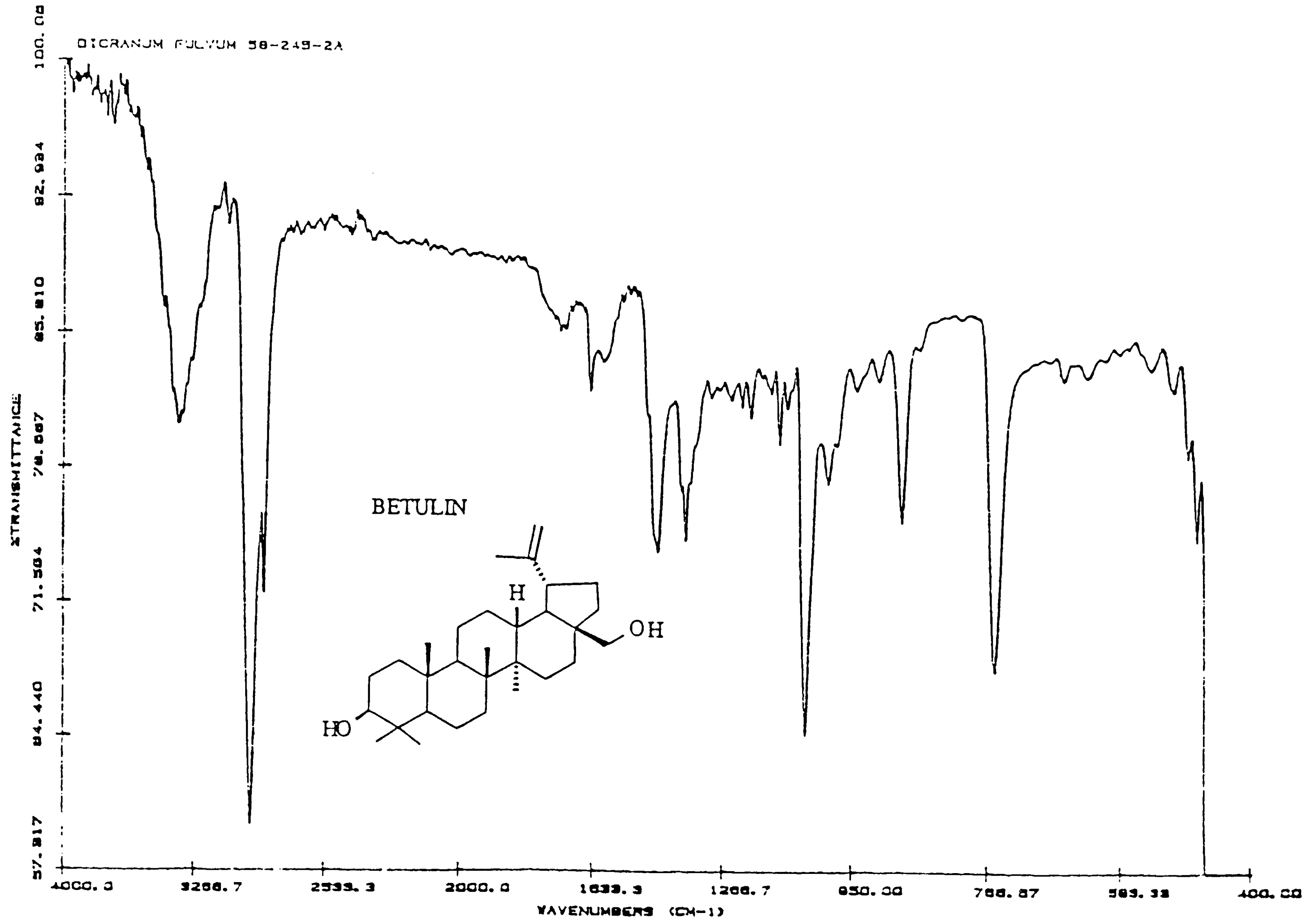


Figure 12: FTIR of 58-245-2A

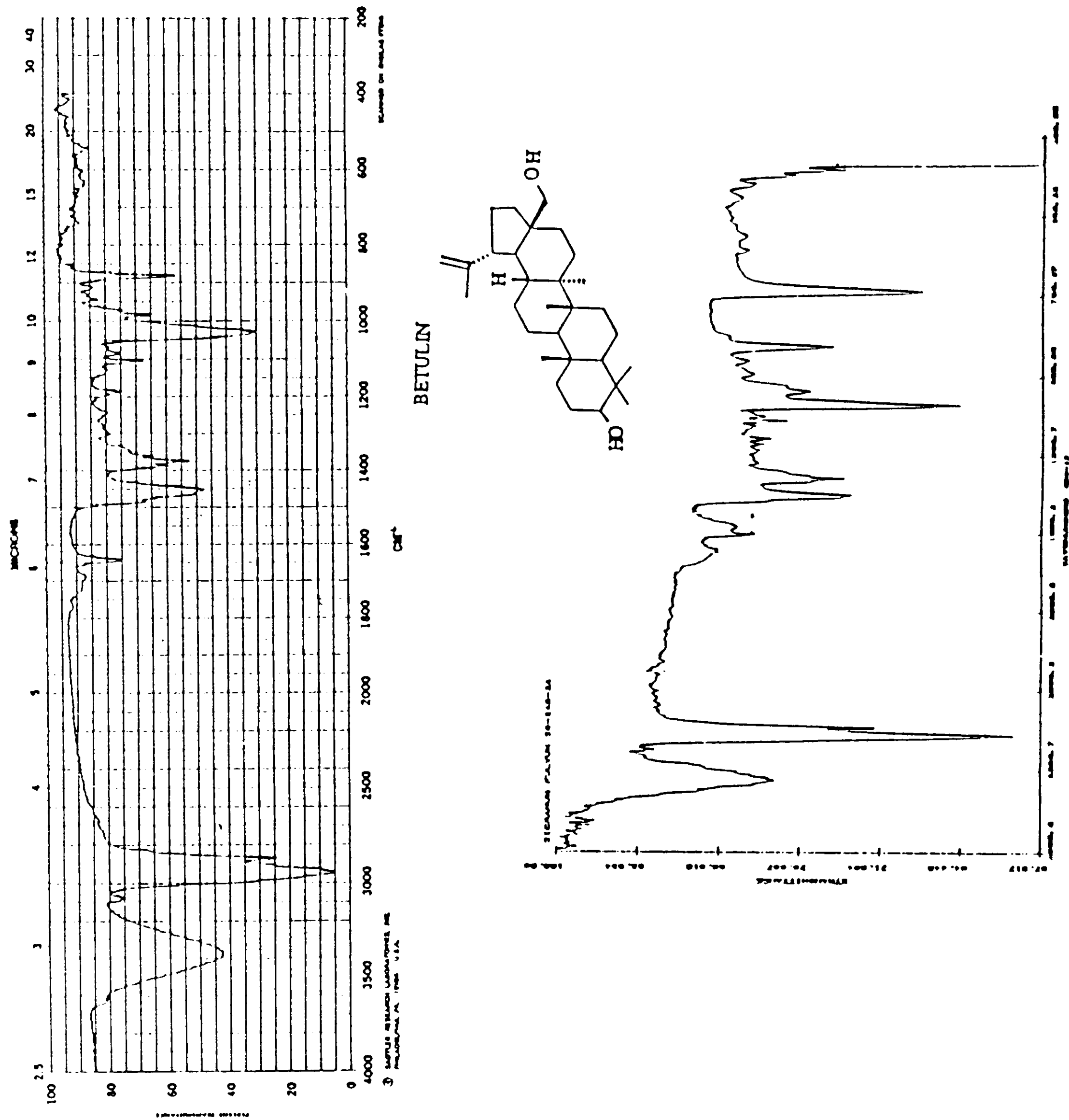


Figure 13: Comparison of FTIR of 58-245-2A and grating IR of betulin

to be 249-250°C. The literature melting point[19] of betulin is 248-251°C. According to the spectral and melting point matches fraction 58-245-2A was betulin.

Conclusions

Three compounds were isolated and identified for the first time from the plant Dicranum fulvum. These three compounds were β-sitosterol, 9,19-cyclolanost-23-ene-3,25-diol and betulin.

Experimental

General Information

Melting points, uncorrected, were obtained on a Gallenkamp Melting Point Apparatus.

Solvents employed were reagent grade and were redistilled in glass except for the large scale extractions and fractionations where large amounts of solvent were needed, in which case the solvents were used as is. HPLC grade solvents were for HPLC.

The infrared spectra of the pure compounds were obtained on a Nicolet 5-DX FTIR. Samples were dissolved in chloroform and allowed to air dry onto NaCl plates.

The nuclear magnetic resonance spectra of the extracted compounds were obtained on a Bruker WP-270 in deuterochloroform with tetramethyl silane as the internal standard.

The mass spectra of the purified compounds and the library matches were obtained on a VG 7070-EHF mass spectrometer.

The thin-layer chromatography plates produced by Merck were of the aluminum back variety. The adsorbant was 0.2mm of silica gel 60 F_{254}, 20mm x 20mm, cut to the size needed. The plates were visualized first by a UV lamp model UVG-11 short wave UV-254 nm manufactured by UVP, Inc. The plates were then sprayed for visualization using 10% phosphomolybdic acid in ethanol. The preparative TLC plates were 2mm Analtech plates.

The optical rotations were determined using a Perkin-Elmer Model 241 Polarimeter. Sample was dissolved in chloroform and the temperature of the chamber was measured with an uncorrected thermometer.

The Chromatotron used was the Model 7924 from Harrison Research

with a quartz lid for UV visualization purposes. Solvent was pumped into the system via a FMI lab pump manufactured by Superior Electric.

High pressure liquid chromatographic separation was performed using a Perkin-Elmer series 100 pump, a Waters C-18, 5μ, Radial Pak LC Cartridge, 8mm i.d., 8NVC18 analytical column, a Perkin-Elmer Tri-det detector in the UV mode and a Perkin-Elmer R50 recorder. For preparative scale work a stainless steel Dynamax™ Macro HPLC C-18 column Serial #10054 25 cm long was used in place of the Waters Radial-Pak column.

General Extraction Method

The plant was placed in a Waring blender and was ground with enough ethanol to make a viscous slurry. The plant and ethanol was blended for three minutes at high speed. The slurry was then poured into a straight-sided container, either a four liter beaker or a thirty-two liter capacity stainless steel pot. The slurry was stirred for three hours with a mechanical stirrer with a stainless steel paddle set at a speed sufficient to keep all the solid matter in motion and therefore in contact with the extracting solvent. After three hours the stirrer was removed and the solid matter was allowed to settle and the ethanol was decanted and filtered through fluted filter paper into four litre flasks. The solid matter was extracted repeatedly in the same manner until a fifty milliliter aliquot in a one hundred milliliter beaker was light green in color. The number of extractions and volumes of ethanol were varied accordingly to achieve the above result. The marc was discarded and the ethanol extraction was rotary evaporated to almost total dryness; an aliquot was rotary evaporated to dryness

to determine the weight of the total extract. This extract was taken up as a whole or in parts into methylene chloride. The methylene chloride was washed repeatedly with equal volumes of distilled water. Both the methylene chloride and the water layers were concentrated, and aliquots were rotary evaporated to dryness to determine total fraction weight. The methylene chloride layer was taken up in equal volumes of hexane and a 90:10 mixture of methanol:water. Each layer was extracted by the counter solvent three times which usually produced a counter solvent that was only slightly colored. All the hexane and all the methanol/water extracts were combined with themselves and dried down using the rotary evaporator. The water bath of the rotary evaporator was never allowed to rise above 37°C and the vacuum was formed by a water aspirator.

General Fractionation Method

Gravity Column Chromatography

A thirty to one weight ratio of silica gel to sample was weighed out and a slurry was made with the eluant of choice. The silica gel used in the open columns was silica gel 60, 70-230 mesh from Merck. A plug of cotton was placed in the tip of a large open mouth glass column with a teflon stopcock to control flow when necessary. A layer of sea sand was placed on top of the cotton plug and the eluant was poured on top of this so the addition of the slurry would not disturb the sand bed. The stopcock was opened to allow the eluant to flow through and the slurry of silica gel was poured in the top of the column. The column was allowed to settle and then a layer of sand was placed on top of the column. The sample was then loaded onto the column in as small

volume of the eluant as possible. It was pulled onto the column by opening the stopcock until the eluant level was just below the top of the sand layer, then eluant was added on top again. This was repeated until there was no sample in the eluant head above the sand. After the samples had been pulled onto the column the reservoir was filled, glass wool was placed in the solvent reservoir and the column run at the specified flow rates. Fractions were collected in flasks of appropriate size to hold the fraction sizes collected. Collection of fraction was initially determined by colored bond collection until there were no distinct bonds forming. Then fractionation was determined by collection of the different solvents systems used in the gradient elution; final fraction determination was by TLC. Some fractions were recombined on the basis of similar TLC patterns.

Flash Column Chromatography

The same packing method was used for packing flash columns as for packing open columns except that the silica gel was 230-400 mesh and instead of free flowing eluant, the column was packed and run under pressure.[18] Piped air was used as the non-purified pressurizing gas. The pressure was adjusted through a flow controller so that the rate the eluent head fell in the column was two inches per minute.[18] Determination of fractions was the same as for the gravity columns except more care was taken to keep purer fractions separated.

Sephadex Column Chromatography

A weighed amount of Sephadex LH-20 was allowed to swell overnight in hexane:dichloromethane,1:4. The solvents used in the Sephadex LH-20 fractionation were all spectral grade. Old casings of pre-packed silica

gel columns were used to hold the column. A cotton plug was placed on top of a filter in the bottom of the column before the swelled Sephadex LH-20 was poured into the column. The tubing that was the means by which the sample eluted was sealed off until the sample was placed onto the top of the column. The sample was drawn onto the column by opening the plug in the tubing and the eluant dripped out via gravity. The column was attached to an autofractionator with a solvent reservoir of approximately one litre under initialized suction for a constant flow of solvent. Collections were put into test tubes at time intervals between one and three minutes. The flow rates varied due to the shrinkage of the Sephadex LH-20 as the eluting solvent was changed. The solvent gradient was a stepwise process. When the bands that had been formed by the solvent system did not seem to move down the column the solvent was changed to the next step of the solvent gradient.[15]

The Chromatotron

The Chromatotron chamber containing a silica gel GF plate of either one, two or four millimeters thickness was placed under a nitrogen atmosphere. Solvent was pumped onto the plate that was spinning at a rate of 750 rpm. The solvent system was determined as the system which separated the components with at least a ΔR_f of 0.1-0.2 and had an R_f of 0.5 or less.[21] The flow rate varied depending on which plate was used, which was determined by sample size.[22] Once the chamber was in equilibrium and the drop rate constant, the sample was loaded in the same solvent system via the pump. After the sample was on the spinning plate, the solvent was started again. As the plate was run, it was visualized by UV unless the sample was not UV active. When the sample

was UV active the separated components were collected in flasks. When the sample did not contain UV active components small fractions were collected in test tubes. Whichever way the fractions were collected they were then checked by TLC to determine the regrouping of test tubes or the purity of a UV active fraction.

Detailed Fractionation

Formation of 58-71-1 through 6

The plant (500g) was ground with 6L of ethanol and partitioned with methylene chloride and water. A gravity flash column was run on the methylene-chloride layer. Fractions were collected as shown in Chart I. The samples were submitted for assay; the results are in Chart I.

Formation of 58-102-1 through 5

The plant (100g) was ground with ethanol and produced 2.5g of ethanol (EtOH) extract. This was partitioned between dichloromethane (DCM) and water (H_2O) producing 1.2g of dichloromethane extract. This was partitioned between methanol:water,90:10, and hexane to produce .42g of methanol:water extract and .43g of hexane extract. A 2x17 cm flash column was used to fractionate the methanol:water extract using 100% dichloromethane taking 20 mL fractions for the first 36 fractions. The column was then washed off with methanol:dichloromethane,20:80, which yielded fractions 37 and 38 with a total volume of 90 mL. Fraction 39 was eluted with 40 mL of 100% ethyl acetate (EtOAc). Fraction 40 was eluted with 50 mL of ethanol:methanol,50:50 and 41 was eluted with 60 mL of 100% methanol. TLC's were run in 100% dichloromethane and fractions 1-4 became 58-102-1 weighing 116 mg, fractions 5-9 became 58-102-2 weighing 38 mg, fractions 10-26 became 58-102-3 weighing 44 mg,

fractions 27-36 became 58-102-4 weighing 31 mg and fractions 37-41 became 58-102-5 weighing 148 mg. All these fractions were sent off for assay and the results are reported on Chart II.

Formation of 58-99-3

The plant (1 kg) was extracted with 4L of ethanol three times producing 19.3g of the extract. The dichloromethane:water partition produced 10.6g of dichloromethane extract which was partitioned into 3.9g of hexane extract and 5.9g of methanol extract (58-99-3) which was sent off for assay (see Chart III).

Fractionation of 58-99-3

Silica gel 60 (300g) was slurry packed into a 6 cm wide glass column with 100% dichloromethane. The solvent system for the flash column ranged from 100% dichloromethane to 100% methanol. The collection of fractions and weights can be seen on Chart III. Fractions 58-115-17,18,19,20 were recombined and a smaller scale flash column separation was carried out using 225g silica gel with the solvent system of 100% dichloromethane to methanol:dichloromethane,75:25, (see Chart IV). The collection of fractions and weights can be seen on Charts III and IV. Fractions 58-99-3, 58-118-B1 and B2 and 58-115-11,16 were submitted for assay; the results are also in Charts III and IV.

Fractionation of 58-118-B1

A 2.5x18 cm Sephadex LH-20 column was poured and allowed to sit overnight. Only 394 mg of 58-118-B1 was placed on the column to make sure a separation would occur. The flow rate was 5 mL/min at the beginning of the run. Hexane:dichloromethane,1:4, was the first solvent system used and fractions 58-121-A through F (see Appendix Table I) were

collected from the bands formed. The next step in the solvent system was dichloromethane:acetone,3:2, which yielded fractions 58-121-G,H,I. The above column was reequilibrated by stepping back the solvent from dichloromethane:acetone,1:4, to dichloromethane:acetone,3:2, then to hexane:dichloromethane,1:4 using 500 ml of each solvent. Fraction 58-118-B1 (937 mg) was placed on the column in hexane:dichloromethane,1:4, in 3 mL of solvent. The column was run as before except an extra step of 100% dichloromethane was added in after the hexane:dichloromethane,1:4, in hope of getting a less drastic movement along the column. The flow rate of the column dropped by half so the acquisition time was doubled from one minute to two. There was no movement with 100% dichloromethane so another step of dichloromethane:acetone,4:1, was added to the gradient elution. The flow rate recovered back to the original 5 mL/min, and the bands were collected as before. The column was not totally clean after the dichloromethane:acetone,4:1, so the solvent was then stepped up to dichloromethane:acetone,3:2. The fractions tabulated in the Appendix Table I were combined according to their TLC's developed in methanol:dichloromethane,6:94. The two column fractions were combined as noted in Appendix Table II due to similar TLC patterns.

Fractionation of 58-118-B2

Fraction 58-118-B2 (0.997 g) was loaded onto a 2x24 cm column of Sephadex LH-20 packed in hexane:dichloromethane,1:4. The column had an initial flow rate of 6 mL/min. As with 118-B1 fractions were collected at one minute intervals and solvents were stepped up to the next solvent strength when band movement ceased or slowed dramatically. Solvents

used and weights obtained are tabulated in the Appendix Table III.

Formation of Fractions 58-137-A through H

Similar fractions from the Sephadex LH-20 columns of 58-118-B1 and 58-118-B2 were combined (Appendix Table IV) and sent off to assay; the assay results and weights can be seen on Chart VI.

Fractionation of 58-137-E

The HPLC system described earlier was used, initially using the analytical column to find the optimum parameters. Optimum analytical parameters were methanol:water,85:15, at a flow rate of 0.75 mL/min (see Appendix figure I). For preparative work the optimum flow rate was 3 mL/min. Samples of 50 μL were injected into a 63 μL loop; the sample's concentration was 57.6 mg/mL. The fractions taken are shown in the Appendix figure II which is the preparative chromatogram. The large single peak separated, 58-138-5, was sent off for assay; this result and weights are on Chart VII.

Formation of 58-166-B

Sample 58-137-B (615 mg) was dissolved in as small an amount of hot methanol as possible and then refrigerated for 30 min. The cold sample was centrifuged and the methanol-soluble fraction 58-154-B was pipetted away from the insoluble wax, 58-154-A. Fraction 58-154-B was then dissolved in hot acetonitrile, cooled, and the wax was removed again leaving the acetonitrile soluble fraction 58-164-B. Fraction 58-164-B was extracted with hexane and acetonitrile, and the acetonitrile fraction was refrigerated again to force out any more wax left in the sample. The methanol wax and the two final fractions were sent off for assay; the results and weights are in Chart VIII.

Fractionation of 58-166-B

A 1.5 x 16.5 cm (10g) column of silica gel was packed as an open column. Pressure was added so the flow rate was 2.4 mL/min. Fraction 58-166-B (182 mg) was placed on the column in 100% dichloromethane. Fractions were collected as bands developed. Fractions eluted with 100% dichloromethane and methanol:dichloromethane,1:99, were combined into 58-177-1 and weighed 67 mg. Fractions eluted with methanol:dichloromethane,2:98 and 4:96, were combined into 58-177-2 and weighed 68 mg, while those with methanol:dichloromethane,6:94,8:92, and 10:90 were combined into 58-177-3 and weighed 47 mg. The fractions eluted with methanol:dichloromethane,20:80, and 40:60, were combined into 58-177-4 and weighed 29 mg. Finally the methanol:dichloromethane,80:20 and 100:0 fractions were combined into 58-177-5 and weighed 9 mg. The assay results are in Chart IX.

Formation of 58-186-5

The plant (5 kg) was extracted with 16L of ethanol five times; partitioning of the ethanol extract between dichloromethane and water followed by partitioning of the dichloromethane extract between methanol:water and hexane produced 53g of methanol extract.

Fractionation of 58-186-5

A 6.5 x 86 cm column of silica gel was slurry packed with dichloromethane which had a flow rate of 10 mL/min. Fraction 58-186-5 (53g) was placed on the column; the solvent systems used and the weights of the fractions produced can be seen in Chart X. Fractions 58-190-4 and 58-190-5 matched 58-118-B1 and B2 very closely by TLC. They both

came off with methanol:dichloromethane,4:96, and weighed 11g and 8g respectively.

Fractionation of 58-190-5

A gravity column (6 x 47 cm) containing 400g of silica gel was slurry packed and had a flow rate of 8 ml/min. TLC's were checked as each band came off. The column started with 100% dichloromethane but this eluted bands much too slowly so solvent was changed to methanol:dichloromethane,2:98, after collection of an early band. It was stepped up to 3:97 after there seemed to be little movement off of the column. The whole column was black so the column was run by the change in darkness of eluent. TLC showed fractions were not matching what was being looked for, so the column was washed with methanol:dichloromethane,8:92, then followed by 100% methanol (see Chart XI).

Fractionation of 58-190-4

Sephadex LH-20 (600g) was swelled overnight in hexane:dichloromethane,1:4. The swelled Sephadex LH-20 was poured into a 2-L straight-sided fritted funnel. The column dimensions were 2.75" x 5.25". Two fractions could be separated with this crude column; they were 58-198-1 and 58-198-2 (see Chart XII).

Fractionation of 58-198-1

Fraction 58-198-1 (9g) was placed on a Sephadex LH-20 column with the dimensions 1.5" x 26". Samples were collected at 0.5 min because the sample was moving so fast down the column. Seventy-seven tubes were collected. Tubes 1-13 were combined to become 58-203-1; tubes 14-56 were combined to become 58-203-2; tubes 57-77 and flasks 1-4 each of

which contained 30 mL were combined and became 58-203-3 (see Chart XII). Flasks 1-4 all contained one band that spread over a great distance on the column. All the sample except for a small band left at the top of the column had come off with hexane:dichloromethane,1:4. The top band was washed off with a dichloromethane:acetone,3:2, wash. The TLC of the wash (58-203-4) showed it to be residual 58-198-2.

Fractionation of 58-203-3

A gravity column with dimensions of 6 x 27.5 cm was packed and 3 g of 203-3 was loaded onto the column with 100% dichloromethane. The fractions collected were combined on the basis of elution solvent and TLC pattern. Fractions were labelled 58-206-1 through 7 (see Chart XII).

Fractionation of 58-203-2

A column of silica gel of dimensions 6 x 30 cm was slurry packed with hexane. Fraction 58-203-2 (5.8g) was rotary evaporated with enough silica gel so a 6 x 6 cm plug of sample could be put at the head of the column. After 2L of hexane there was no movement and the column began to crack. Pressure had to be added to the column to close the cracks. The solvent system was moved up to hexane:dichloromethane,50:50, but still nothing moved, so it was stepped up to 100% dichloromethane. One band moved down the column and was collected as 58-210-1. The solvent was increased to methanol:dichloromethane,0.5:99.5, and fractions 58-210-2 and 58-210-3 were collected. When the solvent was moved up to methanol:dichloromethane,1:99, fraction 58-210-4 was collected; fraction 58-210-5 was methanol:dichloromethane,3:97. When the solvent polarity was increased to methanol:dichloromethane,4:96, seven 200 mL

fractions were collected, the first three becoming 58-210-6 and the last four became 58-210-7. Methanol:dichloromethane,6:94, was the solvent eluting fraction 58-210-8. Fraction 58-210-9, 58-210-10, and 58-210-11 were collected in solvents containing methanol:dichloromethane,10:90,20:80, and 50:50, respectively. Fraction 58-210-12 was a methanol wash of the column.

Fractionation of 58-206-5

Fraction 58-206-5 (0.995g) was pumped onto the Chromatotron with a 4 mm rotor which was equilibrated with formic acid:methanol:dichloromethane,1:5:94. The flow rate was 5 mL/min. As bands developed they were collected in flasks and this produced fractions 58-214-1 through 5. Fraction 58-214-6 through 8 were washes of the rotor with tetra hydrofuran and methanol (see Chart XII).

Fractionation of 58-214-2

Both the Chromatotron and preparative TLC were attempted to separate 214-2, but neither were successful.

Fractionation of 58-206-1

Fraction 58-206-1 (554 mg) was washed with 100% hexane five times. The oily fraction was dissolved in the hexane and was labelled 58-224-1 and the hexane insoluble solids became 58-224-2 (see Chart XIII).

Fractionation of 58-224-1

The 2 mm thick rotor of the Chromatotron was equilibrated with hexane:dichloromethane,50:50, with a flow rate set at 8.5 mL/min. After 401 mg of 58-224-1 was loaded onto the plate, two UV active bands separated from the center band and became fractions 58-229-1 and 58-229-2 (see Chart XIII). The eluting solvent was changed to 100%

dichloromethane, but nothing seemed to move; this washing was collected as 58-229-3. The solvent was stepped up to methanol:dichloromethane,1:99, and again there was little movement; this became fraction 58-229-4. Fraction 58-229-5 was the washing of the plate with methanol:dichloromethane,2:98, before the next UV band which was collected as fraction 58-229-6. Fraction 58-229-7 was a UV band that followed 58-229-6. Fraction 58-229-8 was a methanol wash of the rotor.

Fractionation of 58-229-1

Fraction 58-229-1 was loaded onto a 1 mm silica gel rotor of the Chromatotron that had been equilibrated with 100% hexane with a flow rate of 1.5 mL/min. The UV band formed was collected as fraction 58-231-1. Fraction 58-231-2 was a hexane:dichloromethane,50:50, wash of the rotor, and fraction 58-231-3 was a combination of a 100% dichloromethane wash followed by a methanol:dichloromethane,2:98, wash. A ^{1}H NMR spectrum of the 3 mg of 58-231-1 was obtained, but the sample decomposed and no further data on it was accumulated.

Fractionation of 58-229-6

Fraction 58-229-6 was loaded onto a 1 mm silica gel thick rotor of the Chromatotron which had been equilibrated with methanol:dichloromethane,2:98, having a flow rate of 2 mL/min. Since the two spots interested in were not UV active but only visible upon acid charring, the fractions were collected and the TLCs developed in methanol:dichloromethane,4:96, determined the combinations to be made. Fraction 58-233-1 was early contaminants; fraction 58-233-2 was the two spots unseparated, and 58-233-3 was some of the pure lower spot.

Fraction 58-233-4 was some base line contaminants (see Chart XIII).

Fractionation of 58-231-1

Fraction 58-231-1 was loaded onto the 1 mm silica gel rotor of the Chromatotron that had been equilibrated with 100% hexane with the flow rate of 4.2 mL/min. The UV active band that came off was of such a small amount that a ^{1}H NMR spectrum was only obtained with great difficulty. This UV band was labelled 58-243-1. Fraction 58-243-2 was a 100% dichloromethane and methanol:dichloromethane,2:98, wash of the rotor. Fraction 58-243-3 was residue left after the sample was loaded onto the rotor that is hexane insoluble.

Fractionation of 58-233-2

Fraction 233-2 was loaded onto a 1 mm silica gel rotor of the Chromatotron that had been equilibrated with methanol:ethyl acetate:hexane,2:25:73, with a flow rate of 4 mL/min. Fraction 58-233-2 separated after multiple runs into its two components. The top spot was labelled 58-233-2, and the purified 58-233-3 was added to the previously purified 58-233-3.

Fractionation of 58-224-2

Fraction 58-224-2 (152 mg) was loaded onto a 1 mm silica gel rotor of the Chromatotron which had been equilibrated with methanol:dichloromethane,2:98, with a flow rate of 4 mL/min. Samples of 0.5 mL were collected in 85 test tubes. The first 23 tubes were combined as fraction 58-245-1; tubes 24-34 had a white residue upon solvent evaporation and they were combined into fraction 58-245-2. Around test tube 40 the solvent was increased to methanol:dichloromethane,4:96, and moved up to

methanol:dichloromethane,10:90, at test tube 60 (see Chart XIII).

Fractionation of 58-206-2

Fraction 58-206-2 (144 mg) was loaded onto a 1 mm silica gel rotor of the Chromatotron equilibrated with hexane:dichloromethane,25:75, at a flow rate of 3.5 mL/min. A UV band developed and was collected as 58-225-1. The solvent was moved to 100% dichloromethane, methanol:dichloromethane,1:99, and methanol:dichloromethane,2:98. Nothing moved until the methanol:dichloromethane,2:98; a strong UV band moved out with a visible yellow band following. The UV band was collected as 58-225-2 and the visible yellow band was collected as 58-225-3.

Purification Methods

Fraction 58-115-10

A 2 mm thick 20 x 20 cm silica gel Analtech glass backed plate was streaked with 85 mg of 58-115-10 with a small interruption in the streak 5 mm from the end. The plate was developed twice in methanol:dichloromethane,3:97, with fifteen minutes between the developments to dry the plate. A channel was scraped into the plate to separate the 5 mm streak away from the majority of the plate. The plate was placed on a glass sheet that had been in the freezer for a half hour. Another piece of glass covered the major portion of the developed plate. The 5 mm wide strip was sprayed with phosphomolybdic acid solution and heated with a heat gun until charring occurred. The bands that had developed were scraped and collected as can be seen in Chart V. Fraction 58-125-C was a 43 mg pure compound. 58-125-C was recrystallized by dissolving the sample in hot ethyl acetate. Room

temperature hexane was added until the solution clouded and then the cloudy solution was refrigerated for 15 minutes. The mother liquor was separated and 7 mg of white crystals were left behind.

Fraction 58-233-3

Fraction 58-233-3 was recrystallized by dissolving it in hot methanol; dichloromethane was added until a cloudiness appeared. The same was then refrigerated for 2 hours. The mother liquor was separated away leaving 4.6 mg of a white powder like crystals.

Fraction 58-245-2

Fraction 58-245-2 was washed with methanol:ethyl acetate:hexane,2:25:73. After drying the insolubles, fraction 58-245-2A were recrystallized from methanol and dichloromethane. 58-245-2A was dissolved in hot dichloromethane and methanol was added until cloudiness appeared. The solution was cooled in the refrigerator for thirty minutes. The mother liquor was removed and 5.1 mg of white crystals were allowed to air dry.

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APPENDIX

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TABLE I

FRACTION COMBINATION OF 58-118-B1

Test tube	Fraction	Eluting Solvent	wt (mg)
First Column			
1-7	58-121-A	1 Hex: 4 DCM	203
8,9,10	58-121-B		44
11-45	C		113
46-56	D		7
57-160	E		15
161-241	F		8
242-246	G	3 DCM/2 Acetone	7
247-254	H		9
255-294	I		19
Second Column			
1-7	58-129-A	1 Hex:4 DCM	419
8,9,10	B		106
11-45	C		244
46-56	D		12
57-111	E		64
112-150		100% DCM	
151-174	F	1 Acetone:4 DCM	12
175-214	G		14
215-233	H		46
234-254	I	2 Acetone:3 DCM	18

TABLE II

COMBINATION OF TWO SEPHADEX COLUMNS ON 58-118-B1

Fractions	Combine Into
121-A + 129-A	58-131-A
121-B + 129-B	58-131-B
121-C + 129-C	58-131-C
121-D + 129-D	58-131-D
121-E + 129-E	58-131-E
129-F	58-131-F
121-F + 129-G	58-131-G
121-G + 121-H + 129-H	58-131-H
121-I + 129-I	58-131-I

TABLE III

FRACTIONATION OF 58-118-B2

Test Tube	Fraction	Eluting Solvent	wt (mg)
1-4	58-133-A	1 Hex/4 DCM	167
5-12	58-133-B		159
13-25	58-133-C		249
26-34		1 Acetone/4 DCM	
35-41	58-133-D		151
42-63	58-133-E		208
63-111	58-133-F	2 Acetone/3 DCM	61
111-115		4 Acetone/1 DCM	

TABLE IV

COMBINATION OF FRACTIONS FROM 58-118-B1 and B2

Fractions	Combine Into
131-A + 133-A	58-137-A
131-B,C + 133-B	58-137-B
133-C + 133-D	58-137-C
131-D + 131-E	58-137-D
131-H + 133-E	58-137-E
131-F + 131-G	58-137-F
133-F	58-137-G
131-I	58-137-H

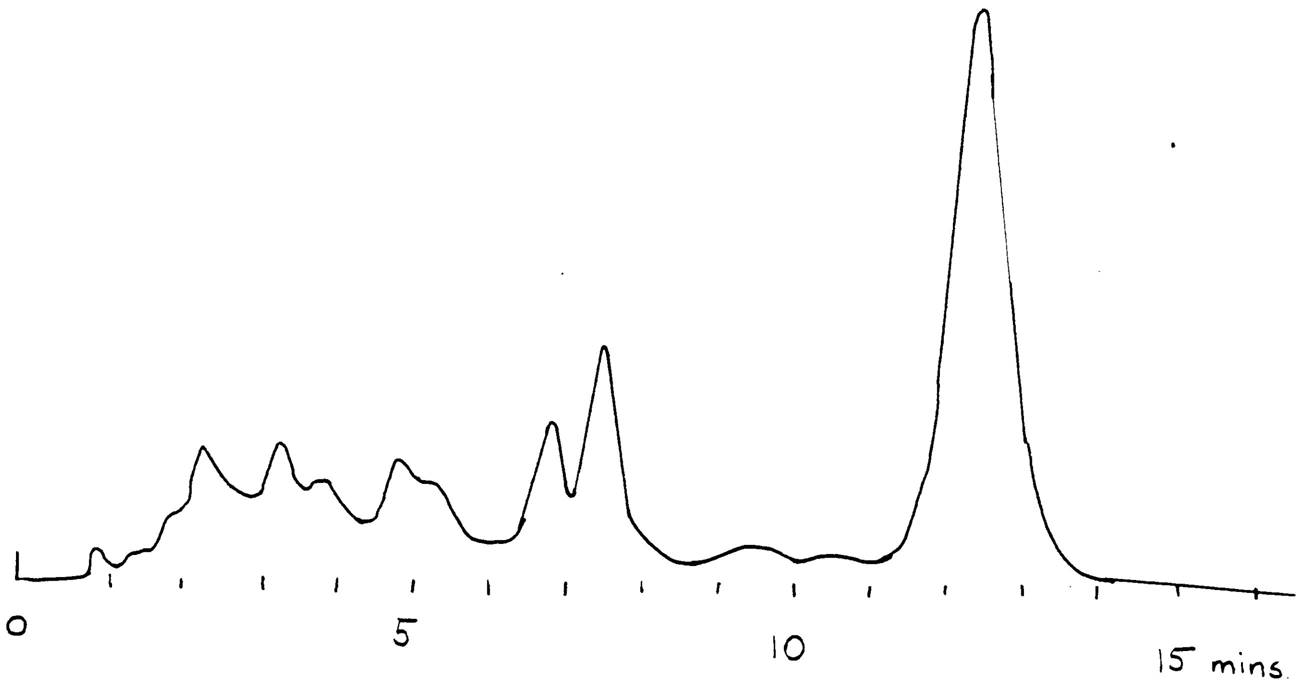

Figure I

Chromatogram from Analytical HPLC of 137-E

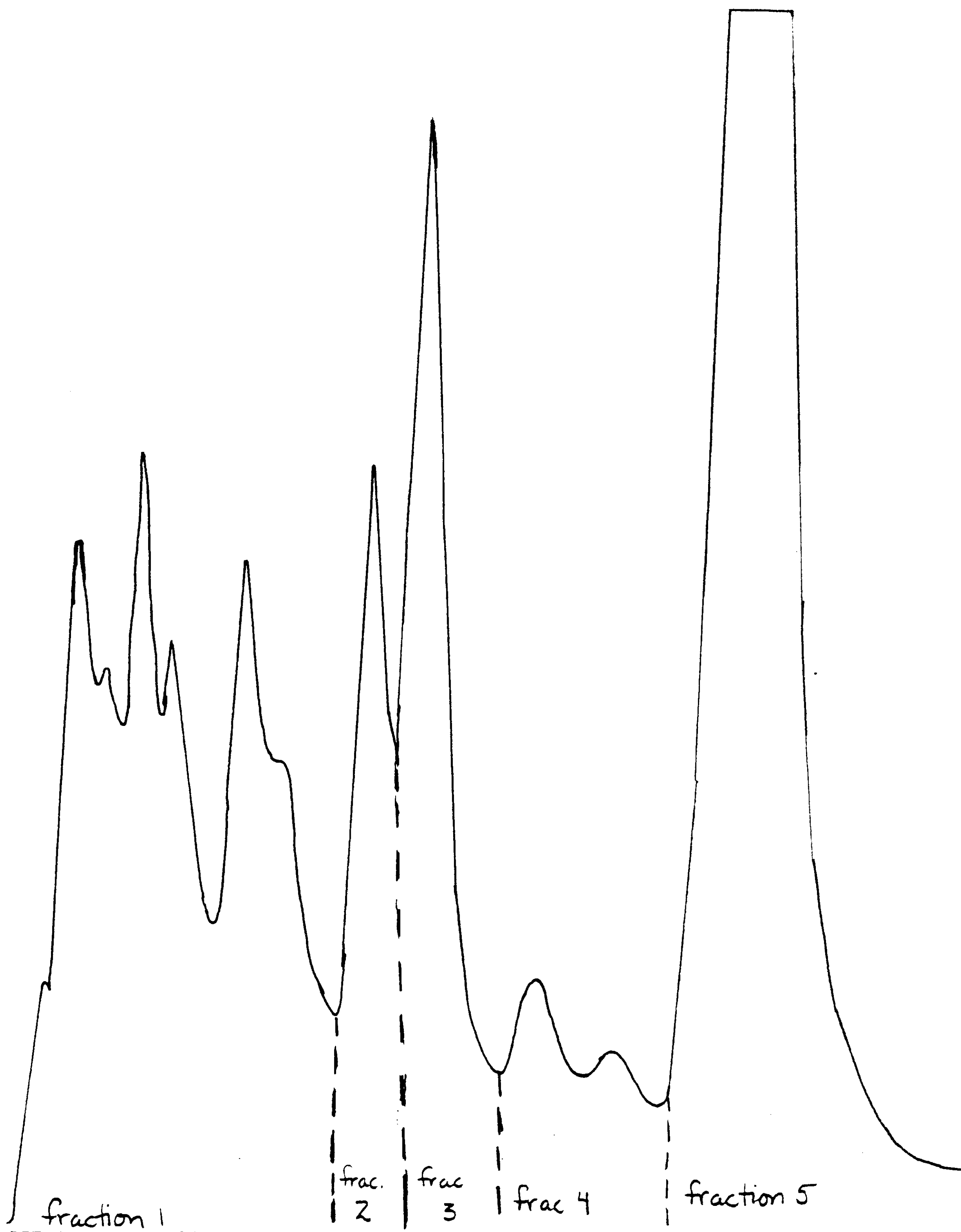

Figure II

Chromatogram from Preparative HPLC of 137-E

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