

Novel nitrogenous heterocycles from the tunicate Clavelina picta
by Michael Franklin Raub

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University
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Abstract:
The goal of natural products chemistry is the isolation and identification of novel secondary metabolites from terrestrial or marine organisms. In some cases, nontraditional separation techniques must be developed in the course of the investigation. An equally important and hoped for outcome is that those compounds discovered will express potentially useful biological activity that can be identified and quantified. It was the purpose of this research to investigate the secondary metabolites of the Bermudian tunicate Clavelina picta towards those ends.

In the course of the investigation, thirteen novel compounds were isolated . Two additional compounds were characterized as a mixture. Of the fifteen, twelve were identified as quinolizidines, and three were identified as isomeric mixtures of indolizidines. The isolation of the compounds was achieved via high speed counter current chromatography. Four quinolizidines and the three indolizidine mixtures were found to possess both cytotoxic and antimicrobial activity.

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by

Michael Franklin Raub

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Chemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

August [illegible]90

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APPROVAL

of a thesis submitted by

Michael Franklin Raub

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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This thesis is dedicated to my parents, who supported me to the fullest no matter what I did.

"It happened in a season of dreams."
Eudora Welty

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ABSTRACT

The goal of natural products chemistry is the isolation and identification of novel secondary metabolites from terrestrial or marine organisms. In some cases, nontraditional separation techniques must be developed in the course of the investigation. An equally important and hoped for outcome is that those compounds discovered will express potentially useful biological activity that can be identified and quantified. It was the purpose of this research to investigate the secondary metabolites of the Bermudian tunicate *Clavelina picta* towards those ends.

In the course of the investigation, thirteen novel compounds were isolated . Two additional compounds were characterized as a mixture. Of the fifteen, twelve were identified as quinolizidines, and three were identified as isomeric mixtures of indolizidines. The isolation of the compounds was achieved via high speed counter current chromatography. Four quinolizidines and the three indolizidine mixtures were found to possess both cytotoxic and antimicrobial activity.

INTRODUCTION

The investigation of natural products is an ancient phenomenon. Medicinal lore from prehistoric times was surely based on a literal trial and error method. Eating or chewing on a plant was an albeit dangerous, but immediate, method of determining its nutritional, healing or toxic properties. Indeed, Socrates himself was aware of the powerful effect of a constituent of hemlock. The Phoenicians, or one of their many trading partners, developed the use of an exudate from a mollusk as a dye, royal purple. A Polynesian fisherman was probably the first person to observe the killing of fish in a lagoon in the presence of a particular coelenterate, one which harbored palytoxin[1]. Perhaps by dipping his spear in a colony of the organisms, he would insure himself an easier catch!

Based on these observations or simple investigations, a practical application of the natural product has come about. These applications have proven to be, for the most part, of a beneficial nature to mankind. In earlier times, it was enough to use the whole plant or animal responsible for the activity of interest. With the sophisticated methods of modern science, we can no longer satisfy ourselves with merely noting that by drinking tea

made from bitterroot, we are making use of an effective heart tonic[2]. We are finally capable in the twentieth century of isolating and characterizing even very minor metabolites which are responsible for bioactivity, as it now has been named. These methods also allow for the identification of compounds that may not display biological activity but are important nevertheless for their novelty or commercial utility.

In the realm of modern marine natural products, a multitude of novel and biologically active compounds have come to light in the last three decades. J.T. Baker and V. Murphy cite that the upswing of research in marine natural products took place in the mid 1960's[3]. The trend in the 1970's was that of discovering compounds both novel and biologically active. This interest led, for example, to Hoffmann LaRoche establishing a facility in Australia with the expressed purpose of finding marine natural products with potential as drugs[4]. The National Institutes of Health have expressed increased interest in recent years in natural products for cancer treatment. A vast program involving the collection of organisms and testing of abstracts with a wide array of cell lines is underway at present. It was felt that, given the few cell lines used earlier for testing, some biologically active compounds could have been overlooked. The testing

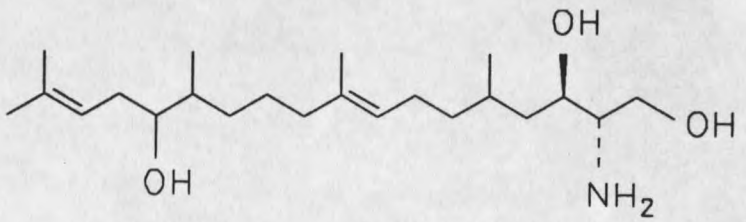
program now includes bioassays with the HIV-1 virus as well.

Not all types of marine organisms seem to have received the same amount of interest from natural products chemists. Very little work was done on tunicates prior to 1979, although there does not seem to be any specific reason for neglecting these invertebrates. It was perhaps the isolation and identification of the biologically active cyclic peptides by Rinehart[5] that gave impetus to an increase in interest in tunicates. Tunicates have been an object of study at the natural products laboratory at Montana State University since the early 1980's. This report will discuss the research into the biologically active metabolites of the Bermudian tunicate *Clavelina picta*.

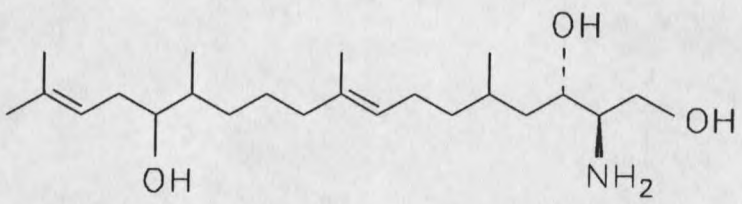
CHEMISTRY OF THE TUNICATES

Whereas most research interests were devoted traditionally to other marine invertebrates over the course of many decades, no marine natural products chemist can deny that tunicate metabolites deserve attention. The most important reason for this newly achieved notoriety is the substantial numbers of compounds that display a variety of biological activities. The basic structural characteristics are also not limited to one or two types; the only commonality among the compounds is that they are nitrogenous.

From an *Aplidium* species were isolated two compounds, the 2,3-*erythro*-isomer, **1**, and the 2,3-*threo*-isomer, **2**, of aplidiasphingosine. In the original paper by Carter and Rinehart, the compounds were cited as having antimicrobial properties and were also cytotoxic toward KB and L1210 tumor cells in tissue culture[6]. The relative configuration was determined in a later synthesis[7].

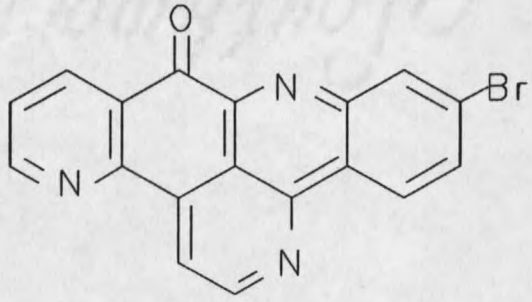


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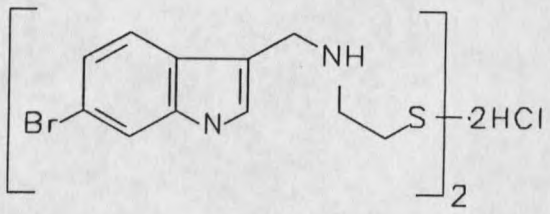
A highly aromatized pentacyclic compound, 2-bromoleptoclinidinone, **3**, was isolated from a *Leptoclinides* species found in the Truk Lagoon in Micronesia. The characterization of the new fused ring skeleton required extensive long range carbon correlation NMR experiments[8]. It was reported to display mild cytotoxicity, ED_{50} (PS=4µg/ml)[9].



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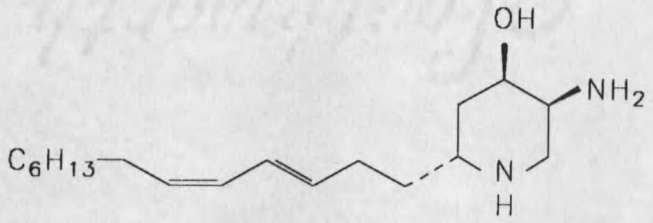
From the tunicate *Polycitorella mariae* was isolated citorellamine, **4**, which possesses insecticidal, cytotoxic and potent antimicrobial properties. The structure

originally proposed by Roll and Ireland[10] was revised and confirmed by total synthesis[11].

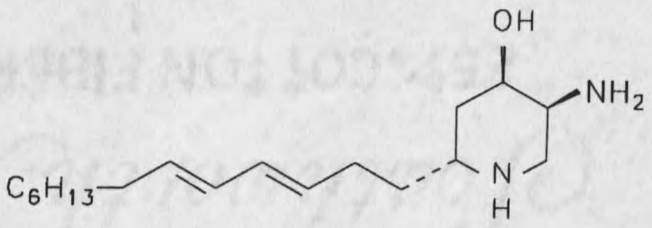


4

A Japanese group reported the isolation and characterization of two isomeric compounds from the Okinawan tunicate, *Pseudistoma kanoko*, pseudodistomin A, **5**, and B, **6**. The structure elucidation was done with the triacetate of the molecule, due to the natural product's proclivity for decomposition. The pseudodistomins represent the first piperidines isolated from the marine environment. They exhibit potent antagonistic activity toward calmodulin, a mediator regulating cellular function and a variety of cellular enzyme systems[12].

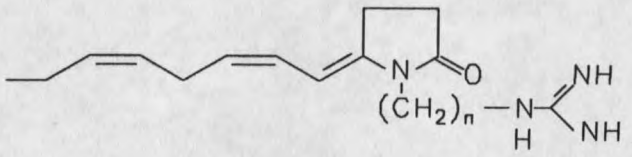


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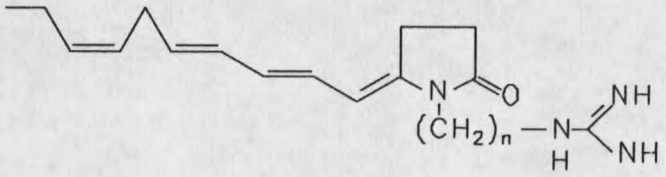


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Possessing both antimicrobial and cytotoxic activity are the polyandrocarpidines, A,B, **7,8** and C,D, **9,10** from the genus *Polyandrocarpa*[13,14].



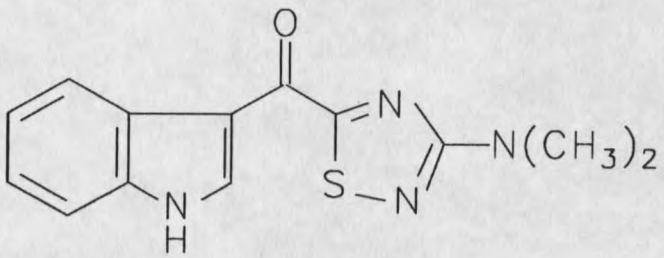
7, n=4
8, n=5



9, n=4
10, n=5

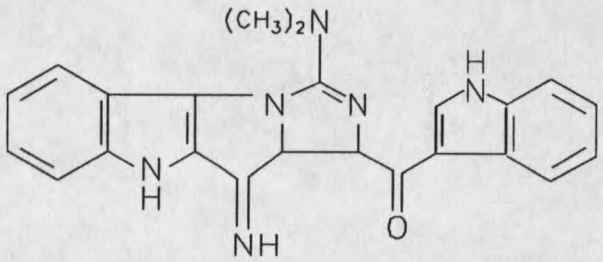
Unique to marine natural products is the 1,2,4-thiadiazole functionality of dendroine, **26**, whose

structure was established by X-ray analysis. Isolated from *Dendrodoa grossularia* from Brittany, it is slightly antimicrobial and displays L1210 inhibitory activity[15].



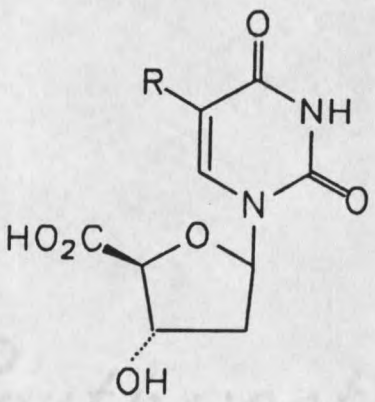
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Also isolated from the same tunicate was grossularine, **12**, whose structure was elucidated via NMR, UV, IR and MS[16].



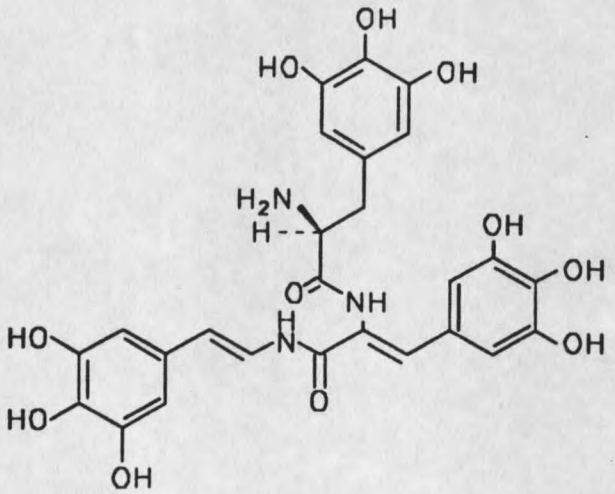
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Two unusual 2′-deoxy-nucleosides were discovered among the secondary metabolites of the Mediterranean tunicate, *Aplidium fuscum*, thymidine-5′-carboxylic acid, **13**, and 2′-deoxyuridine-5′-carboxylic acid, **14**[17].



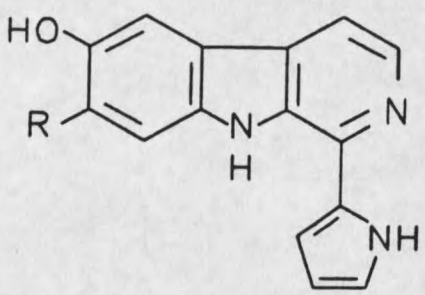
13 R=CH_3
14 R=H

Tunichrome B-1, **15**, isolated and characterized by Breuning, et. al., is a reducing blood pigment from *Ascidia nigra*. It is apparently constructed of three hydroxy-DOPA units. Due to the susceptibility of the compound to rapid air oxidation, 5,000 tunicates were sacrificed to obtain 500 µg of the pure compound[18,19].

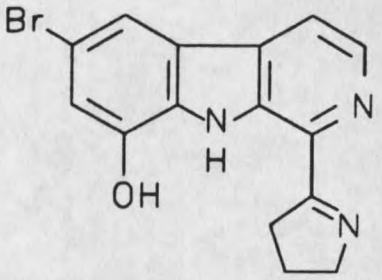


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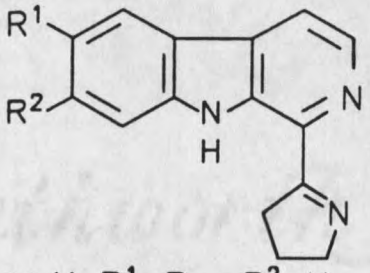
A large number of compounds are derived from tryptophan. Eudistomins A, **16** and M, **17**, from *Eudistoma olivaceum*, are pyrrolocarbolines[20,21]. A compound isolated from an Okinawan tunicate, *Eudistoma glaucus*, containing a dihydro pyrrole moiety, was eudistomidin A, **18**. The compound was characterized via spectral data, and it was found to be a calmodulin antagonist[22]. Five corresponding dihydro derivatives from *E. olivaceum* are G,H,I,P and Q, **19-23**. Completely lacking the pyrrole moiety are D,J,N and O, 24-27. The aforementioned eudistomins from *E. olivaceum* were tested from bioactivity against HSV-1, *Saccharomyces cerevisiae* and *Bacillus subtilis*. D,G,H,I,N and O displayed activity against the *Herpes* virus, whereas H,N,O and P were active against the yeast. Expressing activity against the Gram positive bacteria were D,I,N,O,P and Q[20]. Another group of eudistomins, C,E,F,K and L, **28-32**, possess the extraordinary oxathiazepine ring[19,23]. Preliminary assays performed with C,E,K and L showed activity against HSV-1 at 50 ng/12.5 mm disk. Investigation of the Bermudian *E. olivaceum* yielded three new β-carbolines, R,S, and T, **33-35**. The structures of R, S and T were determined via spectral methods and comparison with other eudistomins[24]. The structure of eudistomin T was later confirmed by synthesis[25].



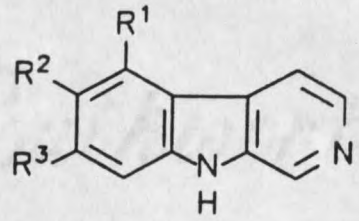
16 A R=Br
17 M R=H



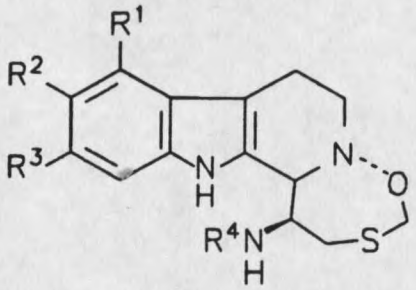
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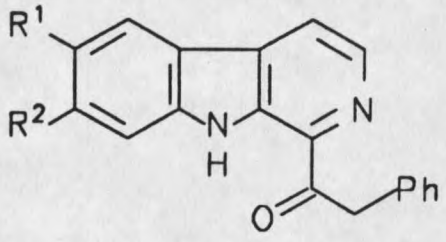
19 H R^1=Br, R^2=H
20 I R^1=R^2=H
21 Q R^1=OH, R^2=H
22 G R^1=H, R^2=Br
23 P R^1=OH, R^2=OH



24 R^1=Br, R^2=OH, R^3=H
25 J R^1=H, R^2=OH, R^3=Br
26 N R^1=R^3=H, R^2=Br
27 O R^1=R^2=H, R^3=Br

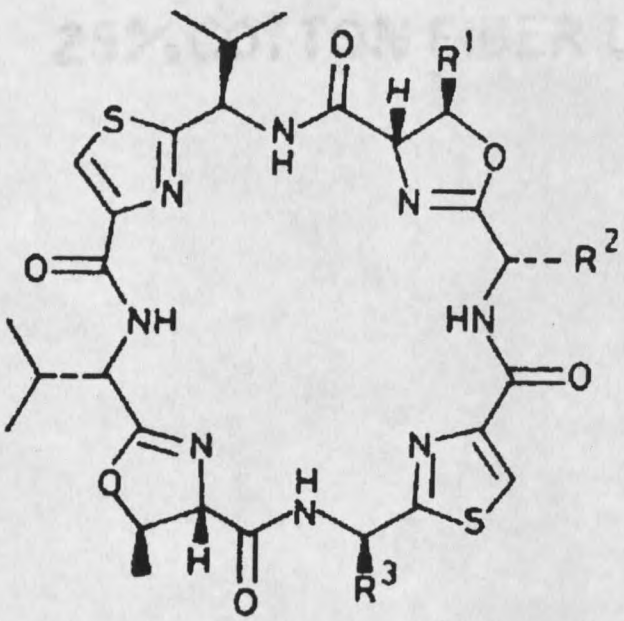


28 C R^1=R^4=H, R^2=OH, R^3=Br
29 E R^1=Br, R^2=OH, R^3=R^4=H
30 F R^1=H, R^2=OH, R^3=Br, R^4=$C_2H_3O_2$
31 K R^1=R^2=R^4=H, R^3=Br
32 L R^1=R^3=R^4=H, R^2=Br



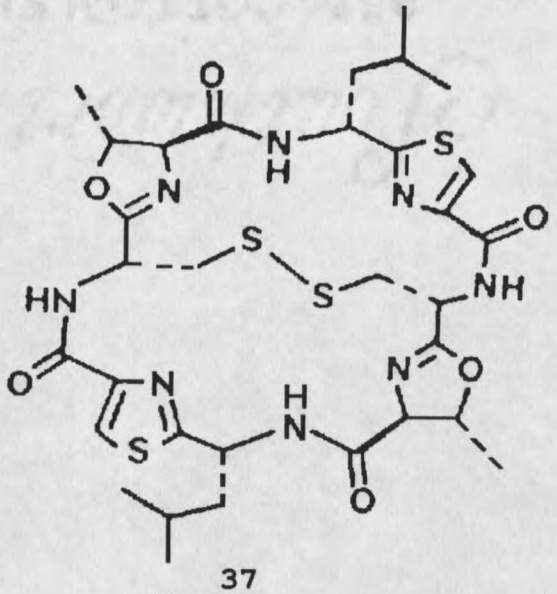
33 R R^1=H, R^2=Br
34 S R^1=Br, R^2=H
35 T R^1=R^2=H

Another remarkable set of compounds are the thiazole ring-containing cyclic peptides. Ascidiacyclamide, **36**, is a cytotoxic metabolite from an unidentified ascidian collected in the Barrier Reef. The structure, containing both thiazole and oxazole rings, was determined by X-ray crystallographic analysis[26].

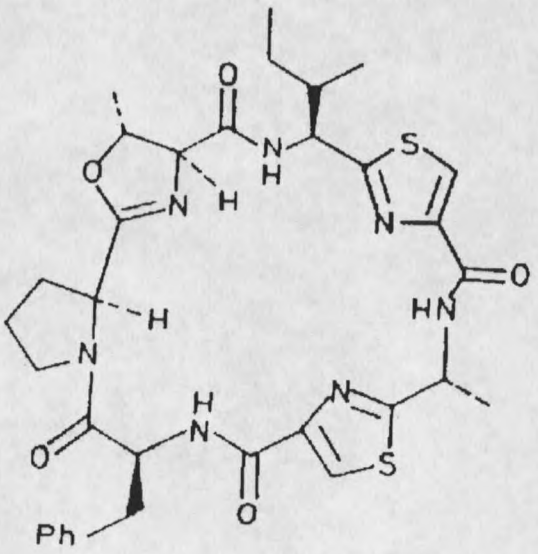


36

Ulithiacyclamide, **37**, with a disulfide bridge, and ulicyclamide, **38**, were the first two cyclic peptides reported from tunicates, isolated from *Lissoclinum patella*. Ireland and Scheuer based their structure elucidation on UV, IR, MS and NMR data[27].



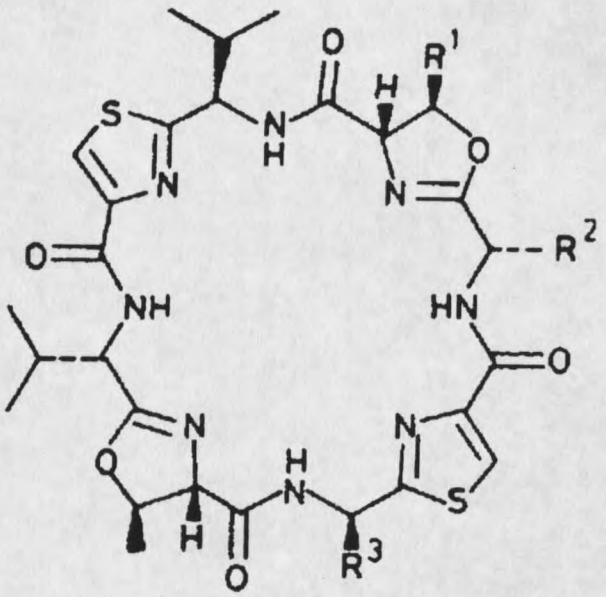
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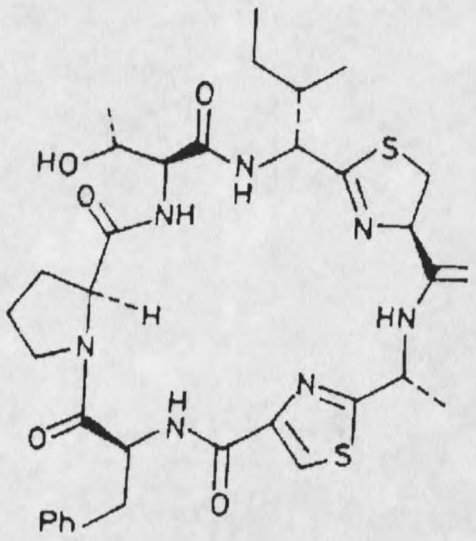
The patellamides A, B and C, **39,40,41**, characterized on the basis of NMR and degradation studies, express

activity against L1210 murine leukemia cells. Also, ulithiacyclamide proved to be six times more potent than the most active patellamide[28].

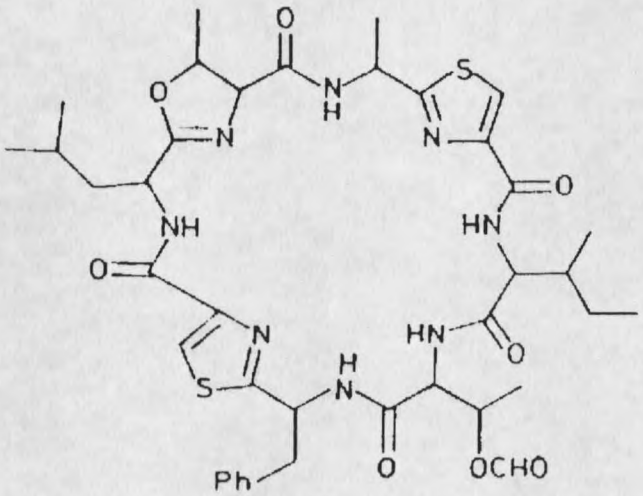


	R1	R2	R3	R4
39	A			H
40	B	CH_3	Ph	CH_3
41	C	CH_3	Ph	CH_3

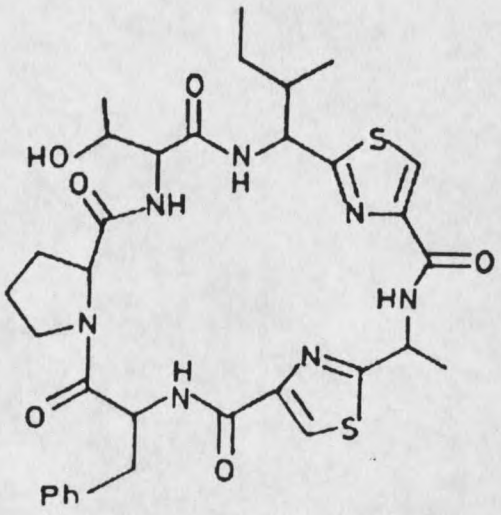
Spectral data for A, B and C were re-assigned, leading to the elucidation of prelissoclinamide-2, **42**, prepatellamide-B formate, **43**, and preulicyclamide, **44**. Amino acid sequences were determined by NIFAB and PIFAB[28].



42

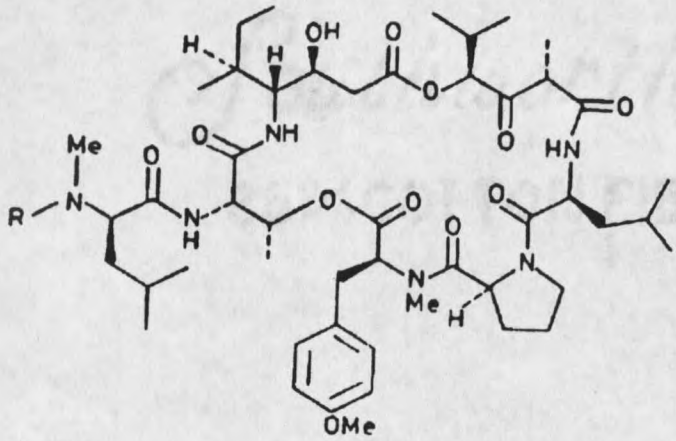


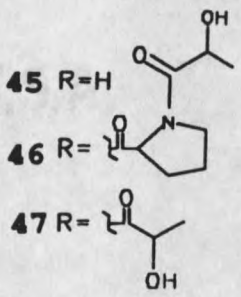
43



44

Cyclic depsipeptides are metabolites of the Caribbean didemnid tunicate, **45,46,47**, possessing potent antitumor and antiviral activity. Among the didemnids, didemnin B, **46**, entered Phase 2 clinical trials in 1987[29]. The original structure was based on FDMS and degradation studies[30] and later revised by synthesis[31].





Since research efforts in our own group had been successful in bringing novel compounds from *Eudistoma* to light[24], it was hoped that other Bermudian tunicates would also yield unique metabolites. *Clavelina picta*, a light purple tunicate indigenous to Bermuda, is readily found on bridge pilings and in shallow inshore waters. Although it shares its immediate surroundings with various tunicates, other invertebrates and algae, it competes effectively for space. Nor does it appear to be a victim of predation. These general underwater observations indicated the possible existence of chemically induced self-preservation.

A KB assay performed under the auspices of the National Cancer Institute revealed that the organic crude of *C. picta* was active at approximately 10μg/ml. In-house testing also determined that the organic crude displayed appreciable antimicrobial and slight insect-antifeedant activity. A computer-aided literature search on *Clavelina* species indicated that no investigations into the secondary metabolites had been reported. In light of the range of activities displayed and the lack of research undertaken on that species, it presented itself as an ideal target for analysis.

STATEMENT OF THE PROBLEM

The main goal of natural products chemistry is, in general, the isolation and identification of novel secondary metabolites. In some cases, non-traditional separation techniques must be developed in the course of the investigation. An equally important and hoped for outcome is that those compounds discovered will express potentially useful biological activity that can be identified and quantified. It was the purpose of this thesis to investigate the secondary metabolites of *Clavelina picta* towards those ends.

INITIAL ISOLATION ATTEMPTS

The use of a partitioning scheme with the organic crude extract resulted in the separation of its constituents by polarity into three portions, hexane, chloroform and methanol/water solubles. Both antimicrobial and brine shrimp tests indicated that activity was fairly evenly dispersed between the latter two polar fractions. Bioassay guided fractionation was used to facilitate the isolation of the active material.

Preliminary chromatographic separations were performed on the chloroform solubles via size exclusion gels. First, one to two gram aliquots were applied to Bio-Beads S-X4 gel; elution with hexane-methylene chloride-ethyl acetate (4:3:1) yielded eight fractions. Those fractions from the S-X4 chromatography which displayed activity were then applied separately to Sephadex LH-20 gel in methanol-methylene chloride (1:1), giving eight fractions. The following chromatographic separation, utilizing Bio-Beads S-X8 gel and methylene chloride-cyclohexane (3:2), was primarily for stripping the appropriate fractions of excess fatty material. Subsequent application of the eluted fractions to Sephadex LH-20 gel in methylene chloride-methyl t-butyl

ether-isopropanol (1:1:1) proved of little value, and this step was later excluded.

Being of a highly polar nature, the fractions containing the target molecules were then subjected to reverse phase flash chromatography. A step gradient solvent system was used with a short column containing a C_{18} gel: step 1) 70% methanol in water; step 2) 90% methanol; step 3) 100% methanol. By weight, most of the material applied eluted with 70% methanol. ^1H NMR spectra revealed that each fraction had a very similar profile. The spectra implied the presence of structurally similar compounds with differing polarity.

The optimum separation achieved with this system was a fraction containing two major components, with one compound comprising approximately 80% of the mixture by weight, (Figure 1). EIMS revealed the molecular weight of the major compound to be m/z 347, while the other compound appeared to have a molecular weight of m/z 319 (see Figures 2 and 3). The low resolution determinations were substantiated by HRMS, which suggested a molecular formula of $C_{22}H_{37}NO_2$ for the former and $C_{20}H_{33}NO_2$ for the latter compound. Characteristic of the two compounds was the apparent loss of an acetate, m/z 287 and 259 (M^+-HOAc). Infrared analysis of the fraction indicated the presence of an isolated acetate, absorbing at 1735 cm^{-1}

and a protonated tertiary amine, with a broad, moderately strong peak centered at 2500 cm^{-1}.

In order to separate the compounds from the mixture, a series of solvent systems were tested via thin layer chromatography to check the viability of either a normal phase or a reverse phase chromatographic separation by high performance liquid chromatography. The compounds did not move on silica gel, thus obviating the use of a silica column. Reverse phase plates (C_{18}) indicated that a successful separation should be realized with 100% methanol.

A number of separations were attempted with both semi-prep and/or analytical columns, including octadecylsilyl, cyano, diphenyl, amino, β-cyclodextrin and polystyrene-divinylbenzene columns. The first five columns utilize silica as a solid support, whereas the last column is composed only of the aforementioned packing. Normal phase chromatography was also attempted with those columns which could be run in both normal and reverse phase, namely, the cyano, amino and diphenyl columns.

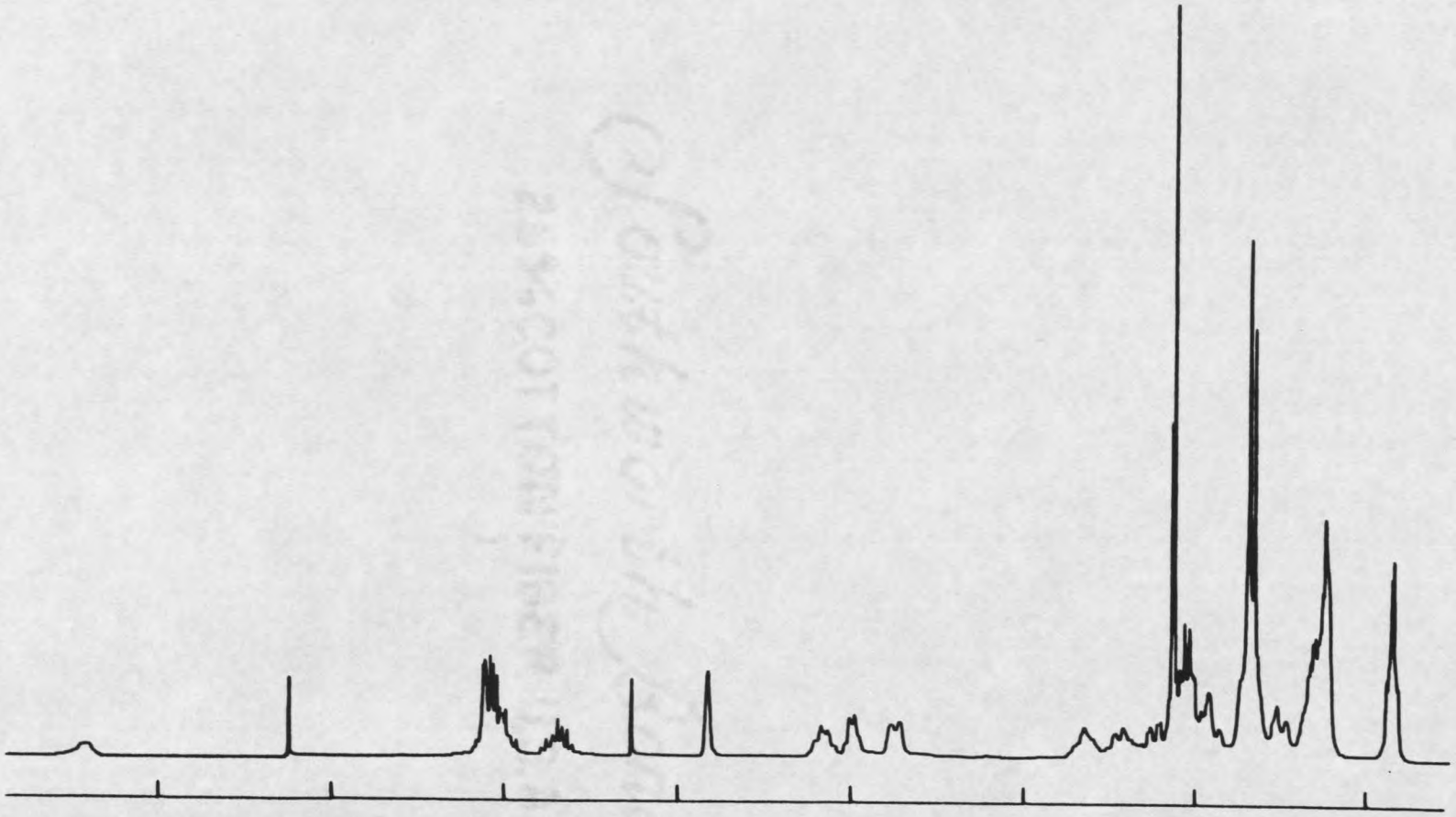


Figure 1. 250 MHz ^{1}H NMR Spectrum of **51** and **65** in $CDCl_3$.

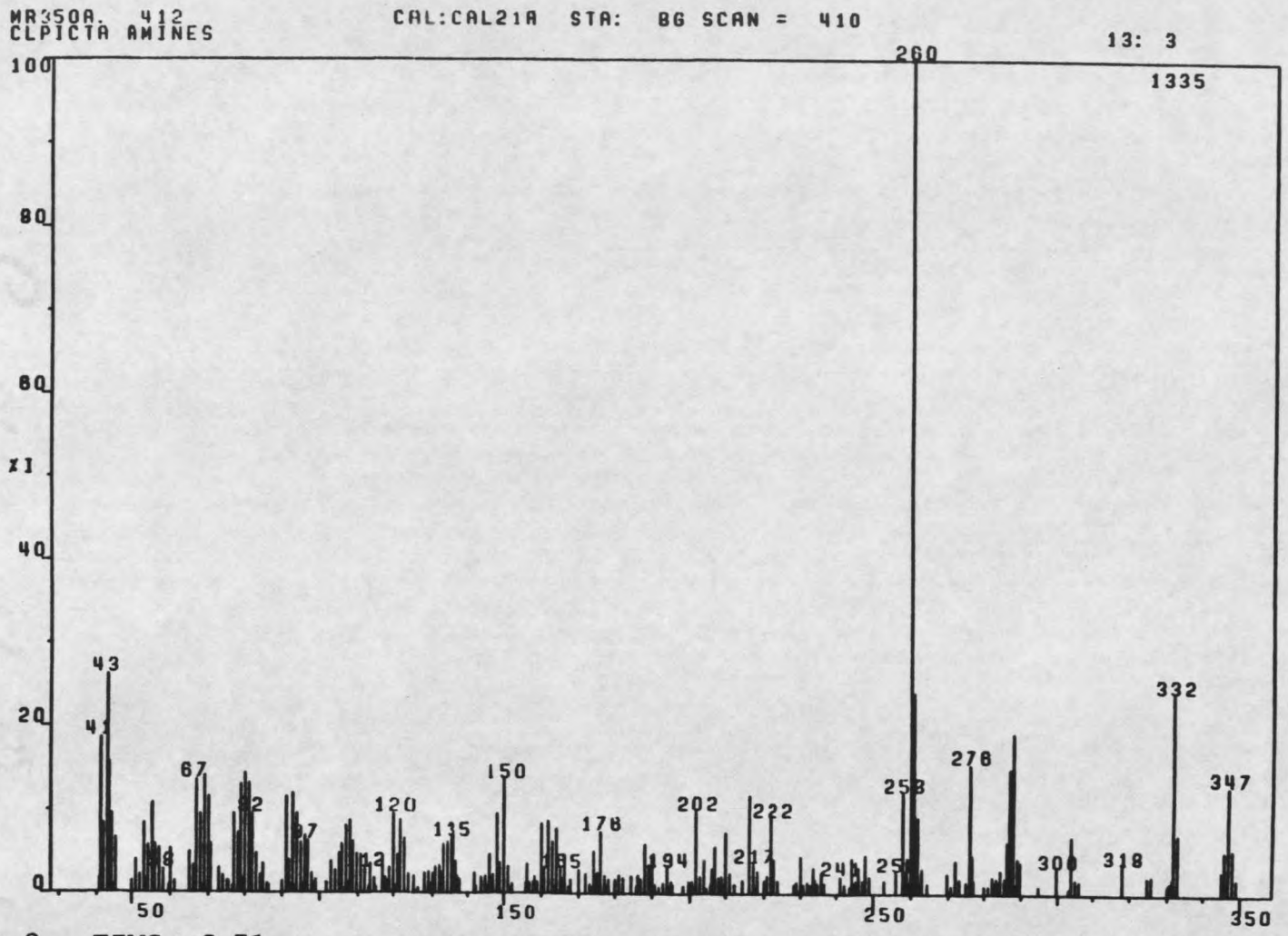


Figure 2. EIMS of **51**.

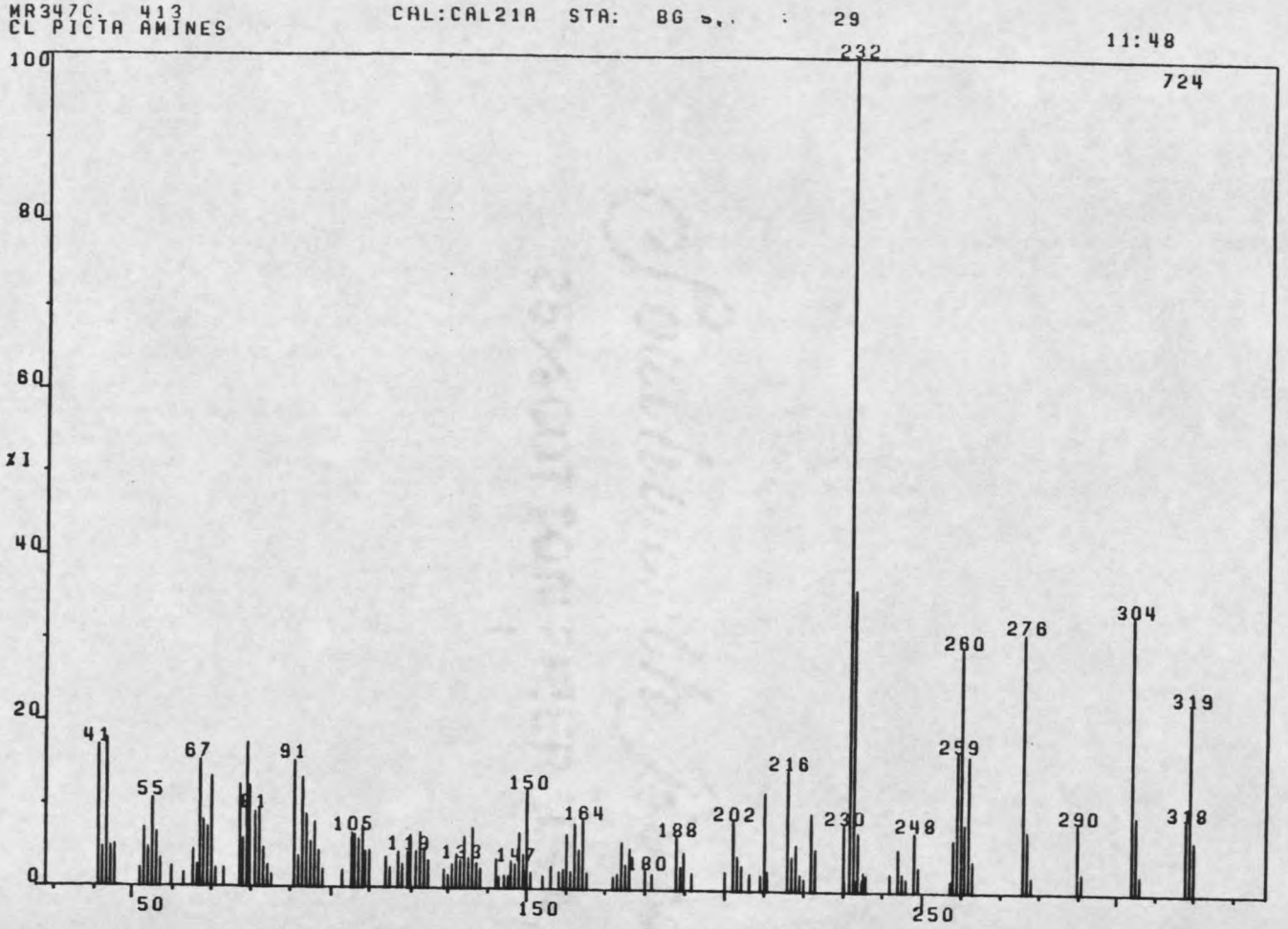


Figure 3. EIMS of **65**.

As hoped, the protonated species of the compounds were readily eluted from the columns regardless of the mode, reverse or normal phase, or the solvent system employed. Sharp Gaussian peaks were observed with the elution of the polar species, but resolution was very poor. Subsequent to the elution of the protonated species, the elution of other compounds was characterized by low, broad peaks with extensive tailing and unacceptable, extended retention times. Examination of the nonpolar eluants from these trials indicated that the fractions contained the deprotonated species. The fractions applied to the HPLC columns had not been shown to contain both protonated and deprotonated forms of the compounds, and this elution phenomenon was unexpected. It is possible that an equilibrium mixture of protonated and deprotonated species was formed, but no attempt was made to establish this hypothesis.

Tertiary amines have been cited often in the literature as having this notorious property, especially in the case of silica or silica-based columns. As has been described by Stadalius, Berus and Snyder[32], unreacted or uncapped silanol groups on the silica surface are capable of interacting with basic samples. One of the most common ways of dealing with this problem is by solvent modification, that is, typically by

addition of 0.1% triethylamine or trifluoroacetic acid. Unfortunately, the addition of such highly basic or acidic modifiers can lead to rapid decomposition of the solid HPLC packing. Nevertheless, 0.1% TFA was used as a modifier in a methanol-water system using an analytical octadecylsilyl (ODS) column; the observed UV trace revealed that the modifier had little effect on tailing. This could have been due to too large a sample or the fact that the pH was 3.5 and not 2, a typical acid pH for this type of modified system. A system employing a lower pH was not tested.

In two other cases, 0.1% triethylamine was employed as a modifier. In the case of the ODS column, it had some effect, but improvement was not considerable. A further attempt was made by applying the fractions to a column with a non-silica based polystyrene-divinylbenzene packing. The UV trace yielded more Gaussian peaks and curtailed tailing, but the run duration of a 1 mg injection was still protracted; it was sixty minutes long.

The only column which caused no tailing and no extended retention times was an amino column, which, curiously enough, was silica-based. Less endearing was the fact that the eluants had no recognizable ^{1}H NMR spectra. It appeared that the compounds were either

rearranging or decomposing on the column. GC/EIMS did indicate the presence of compounds having the molecular weight of the target molecules, but no other GC peaks were observed. However, if polymerization were taking place, those compounds would not elute from the GC column. No further attempts to separate the compounds were made with the column.

SEPARATION ATTEMPTS WITH COUNTER CURRENT CHROMATOGRAPHY

It was obvious that a more traditional methodology for isolation of the tertiary amines was not effective. A relatively new alternative for chromatographic separation is high speed, centrifugal counter current chromatography, CCC, developed by Ito as a faster alternative to droplet counter current chromatography, DCCC[33]. In recent years a number of publications has attested to the success of CCC for isolations[34]. Over the past six years, the natural products laboratory at this university has been involved in developing CCC specifically for the isolation of natural products on semi-preparative and analytical scales. An expansion on Ito's earlier separations of nucleosides, purines and pyrimidines has been developed by this author[35]. It has been demonstrated as a means of isolating these molecules from the aqueous extracts of sponges[35]. Coumarins differing by only one sugar unit have also been successfully separated[36]. Baseline separation of diastereomeric diketopiparazines has been achieved as well[37].

CCC has a great advantage over HPLC in that all that is required is a two-phase solvent system; it can be run in normal or in reverse phase. Modifiers resulting in

high or low pH have no deleterious effects on the column. While the number of theoretical plates is low, efficiency and resolving power are enhanced by a highly dynamic partitioning process. A further and perhaps the most important consideration for the natural products chemist is that no material is lost. Thus, samples in the 100 microgram range can be chromatographed with the assurance of recovering all the material.

A series of solvent systems (Table 1) was tested to see whether the separation of the compounds in the polar fractions from flash chromatography could be achieved.

Table 1. Solvent Systems Used for CCC.

Protonated Amines	
Solvent System	Solvent Ratio
$CHCl_3$-MeOH-H_2O	24:35:20
C_6H_{14}-$CHCl_3$-MeOH	1:1:1
C_6H_{14}-C_6H_{12}-MeOH	1:0.5:1
C_6H_{14}-CH_2Cl_2-CH_3CN	10:15:35
CH_2Cl_2-MeOH-H_2O	5:5:2
Deprotonated Amines	
Solvent System	Solvent Ratio
C_6H_{14}-CH_2Cl_2-CH_3CN	50:15:35
C_6H_{14}-EtOAc-CH_3CN	50:15:35
C_6H_{12}-C_6H_{14}-EtOAc-CH_2Cl_2-CH_3CN	10:10:1:2:10
C_6H_{14}-CH_2Cl_2-CH_3CN-C_6H_{12}-MeOH	4:3:6:15:6

Without exception, all of the material eluted with the solvent front of the mobile phase; one large peak was observed on the UV trace. This suggested that the overriding determinant for separation was the polarity of the compounds. They apparently had such similar partition coefficients that a successful separation was not to be realized. What was required, then, were compounds that did not have such similar coefficients.

As has been stated previously, essentially all of the fractions eluted from the reverse phase flash column contained what appeared to be structurally similar compounds. The only major differences between the compounds eluted at 70% methanol and the compounds eluted at 100% methanol were upfield shifts of the one-proton signals and the methyl doublet in the ^{1}H NMR spectrum. The ^{1}H NMR spectra of those HPLC fractions eluted with protracted tailing were identical to the fractions eluted at 100% methanol, suggesting that they, too, were the deprotonated forms of the desired compounds. A signal indicating a protonated tertiary amine was lacking in the IR of these fractions. To further substantiate this hypothesis, a 5 mg aliquot of a fraction eluted at 100% methanol was partitioned between methylene chloride and dilute acid, HCl, pH2. A ^{1}H NMR spectrum of the methylene chloride phase showed a broad peak at δ11.5,

the resonance for the N-H proton. IR showed a broad peak centered at 2500 cm^{-1}, identical to that of the naturally occurring protonated compound. The spectral data were supportive evidence that the naturally occurring protonated compounds were the amine hydrochloride salts.

It was hypothesized that the nonprotonated compounds would be more amenable to separation, if the partition coefficients of the non-protonated species were different enough to allow separation. Previous ^{13}C NMR spectra had indicated the presence of the nonprotonated compounds in the hexane solubles of the organic crude extract of the tunicate. One gram aliquots of the hexane solubles of the organic crude extract were chromatographed on Bio-Beads S-X4 gel in hexane-methylene chloride-ethyl acetate (4:3:1), yielding eight fractions with each chromatography. After the fractions were analyzed via NMR, the fractions which contained the target compounds were pooled and subjected to CCC, in reverse phase mode with n-hexane-methylene chloride-acetonitrile (10:3:7). The chromatography was run for three hours and thirty minutes. This time a remarkably different UV trace was obtained (see Figure 4).

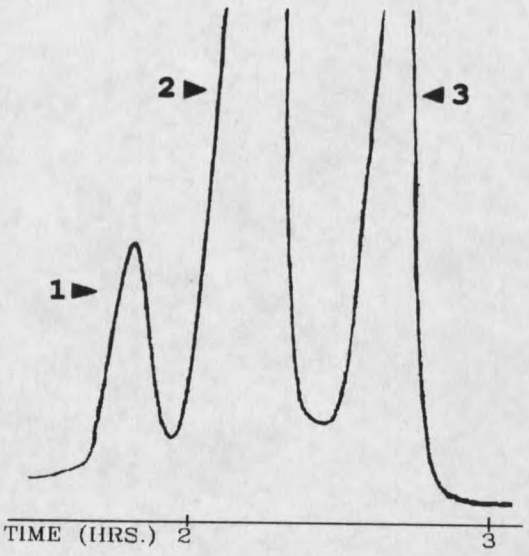


Figure 4. CCC Chromatogram of Quinolizidine Mixture.

Fraction 1 was composed of one compound, according to EIMS, m/z 317. Fraction 2 comprised two compounds, m/z 345 and 319, while fraction 3 was pure, m/z 347. This represented the first isolation of **51** and **70**.

ISOLATION OF OTHER QUINOLIZIDINE HOMOLOGUES AND ANALOGUES

Quinolizidine Dienes and Trienes **69, 65, 70, 67, 66**

Since the reverse phase flash column had demonstrated the ability to concentrate the protonated compounds using 70% methanol, it was considered germane to see if a slight modification of the solvent system could resolve the mixtures further. The modification employed was simply to begin the chromatography with a 1:1 mixture of methanol-water. A 100 mg aliquot of the fraction eluting at 70% methanol was reapplied to the column with the utilization of the aforementioned solvent system. Although this protracted the elution of the compounds, it did result in the elution of five fractions. The major fraction by weight, fraction 5, was eluted with 70% methanol. A thorough analysis of fractions 1, 2, 3 and 4 revealed that fractions 3 and 4 no longer appeared to contain as many quinolizidine analogues or homologues, as was common to earlier flash chromatographies.

Mass spectral analysis of fraction 3 indicated the presence of the C_{22} acetate diene, **51**, and a compound with m/z 345, **69**. Minor traces of two other compounds were observed, m/z 291, **67**, and m/z 317, **70**. Fraction 4 appeared to contain one major compound, m/z 319, **65**, and other very minor, unidentified constituents. Finally,

fraction 5, again eluted at 70% methanol, seemed to be comprised of **51**, **69**, a mixture of compounds with m/z of 263, 261 and 259, the homologous mixtures of **86**, **88** and **89**. Also present was the C_{20} alcohol, **74**.

The isolation of compound **1**, in its deprotonated form, had already been demonstrated with CCC. It was also suspected, that, given fractions containing less complicated mixtures of the quinolizidines in their deprotonated form, the reverse phase flash gel might also provide an adequate means for isolating individual quinolizidine types. In order to test this hypothesis, the constituents of fraction 5 were first deprotonated by spiking an aliquot of the fraction with 1 µl of triethylamine per 10 mg of compound. The aliquot was applied to the flash column and run with 90% methanol. It was thought that beginning with 90% methanol rather than with 100% methanol would enhance the separation. EIMS revealed that the major fraction eluted from the column contained only the diene and triene, **51** and **69**. This two compound mixture was then applied to CCC with the same solvent systems used for the isolation of the C_{22} diene, now resulting not only in the recovery of the C_{22} diene, but in the isolation of **69**, the C_{22} triene.

Another gratifying result was obtained when fraction 4 was deprotonated and chromatographed once again with

reverse phase flash chromatography. The fraction eluted contained the C_{20} diene analogue, **65**, and some unidentified coeluents in very minor amounts. Application of this fraction to CCC resulted in the removal of the unidentified contaminants, yielding clean **65**.

In retrospect, when the very first successful isolation of **41** was accomplished via CCC, an investigation of the eluents immediately prior to the elution of **51** revealed that **69** and **65** had coeluted. Utilization of the modified reverse phase flash scheme eliminated this problem.

When fraction 3 was deprotonated and applied to the reverse phase flash column with 90% methanol, two fractions were recovered. The first contained a mixture of the C_{22} acetate diene, **51**, and the C_{20} acetate triene, **70**, according to mass spectral analysis. These two compounds could be baseline separated with CCC.

Quinolizidine Alcohols **74**, **75**, **77**, **78**

The methanol/water portion of the organic crude extract appeared to harbor compounds similar to compounds **51** and **69**. For example, some methine signals similar in chemical shift to the acetate methines were present. Indeed, a ^1H NMR of the contents of the polar

constituents displayed four clearly delineated one-proton signals in the olefinic region, in contrast to the three-proton envelope encountered with the acetates in $CDCl_3$.

Aliquots of the polar solubles were first fractionated on Sephadex LH-20 gel in methanol-methylene chloride (1:1), to give eight fractions. The third fraction was subsequently applied to another Sephadex LH-20 gel in methylene chloride-methyl t-butyl ether-isopropanol (1:1:1), yielding six fractions. The second fraction was deprotonated by the addition of 10 µl of triethylamine immediately prior to the chromatography, CCC. The solvent system used for the isolation of the C_{22} acetate, **51**, was also used in these cases, again with the lower phase as the mobile phase, that is, in reverse phase mode.

At 1.3 hours and 1.7 hours into the run, respectively, two compounds eluted with baseline separation see Figure 5). EIMS indicated the first to be an alcohol, **75**, whereas the second compound was an alcohol of higher molecular weight, **74**.

Another CCC run was performed on a different fraction which had eluted from the second Sephadex chromatography. A peak on the UV trace which was a narrow shoulder of a peak containing **80** yielded a ^1H NMR

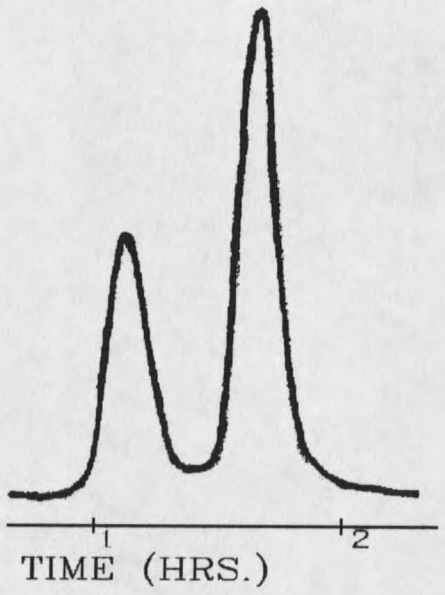


Figure 5. CCC Chromatogram for the Isolation of **74** and **75**.

spectrum that indicated the presence of three acetate-containing compounds (see Figure 6). DIP/EIMS confirmed the presence of **67** and **77**, but the third compound could not be identified. The fraction was chromatographed once again via CCC, using a slower chart speed than normal with the UV detector. This yielded a UV trace of a single peak with a slight shoulder eluting first. The ^{1}H NMR spectrum of the shoulder, 0.9 mg, contained only two acetate peaks, while the major peak still contained all three compounds. The shoulder contained **77** and an unidentified compound. As this was the cleaner of the two fractions, it was acetylated with acetic anhydride,

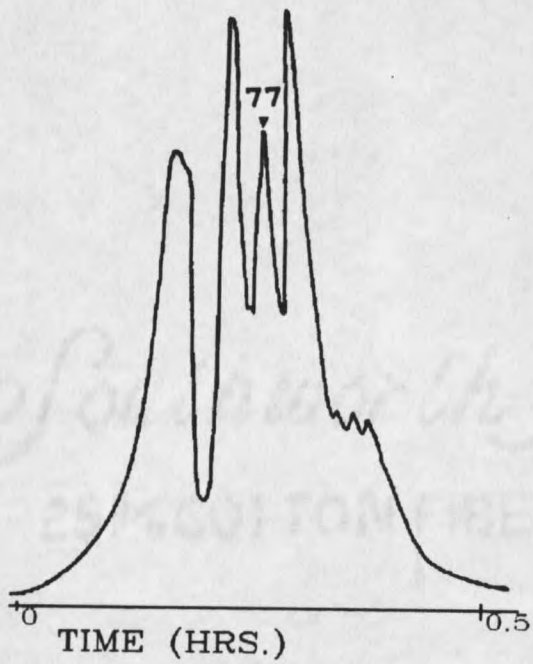


Figure 6. CCC Chromatogram for the Isolation of **77**.

forming a triacetate. The reaction mixture was subjected to CCC once again, yielding two fractions. The major fraction, 0.7 mg, contained the triacetate.

Finally, another alcohol, **78**, was isolated in approximately 90% purity, not via CCC, but rather with reverse phase flash chromatography. The compound was found to be a constituent of one of the compound mixtures that had been used in the experiments with deprotonated compounds on the reverse phase flash column. The fraction contained a mixture of **51**, **70** and **78**. When the deprotonated fraction was applied once again to the reverse phase flash gel at 90% methanol, **78** was readily separated from **51** and **70**.

Quinolizidine Aldehydes **80**, **81**

Although many of the proton spectra from very early gravity chromatographies indicated the presence of two compounds containing an aldehyde or a dialdehyde, it was not until fractions containing the aldehydes were treated with triethylamine and run with CCC that isolation was achieved. Utilizing the same solvent system and running in the same mode as that for the isolation of the C_{22} acetate, two aldehydes, **80** and **81**, eluted within the first thirty minutes of the injection. It was perhaps fortuitous that some aldehyde-containing fractions were comprised of **80** as the very major compound and other fractions had **81** as the very major constituent; both compounds coeluted upon CCC. No other solvent system proved capable of separating the two compounds. Derivatization of the aldehydes could not be considered as a viable alternative, since the quantities of compound were too small to sacrifice for such attempts.

ISOLATION OF THE INDOLIZIDINES

All of the quinolizidine separations via CCC had been accomplished using reverse phase mode. The chromatography was usually continued for approximately twenty-five minutes after the elution of the acetate **51**, but the elution of no other compounds was noted on the uv trace. The stationary phase of the CCC column was then removed by flushing the column with 300 ml methanol. After the stationary phase from the chromatography had been removed *in vacuo*, a ^{1}H NMR of the organic residue revealed the presence of a mixture of the compounds which displayed potent antimicrobial activity and brine shrimp toxicity, **86**, **88**, and **89**. Due to the fact that they were highly lipophilic in nature, it was thought that they would be more amenable to normal phase CCC as opposed to reverse phase chromatography. The same solvent system, n-hexane-methylene chloride-acetonitrile (5:3:7), was utilized, with the lower, more polar phase serving as the stationary phase in this case. The UV trace from the chromatography (see Figure 7) showed the elution of three fractions, A, B and C, within the first sixty minutes of the chromatography. Fraction A, by far the largest fraction by weight, contained a mixture of the C_{18} indolizidine monoene, **86**. The second fraction, Fraction B, contained the indolizidine diene, **88**, and one

monoene, while fraction C contained the isomeric triene mixture of the indolizidines, **89**.

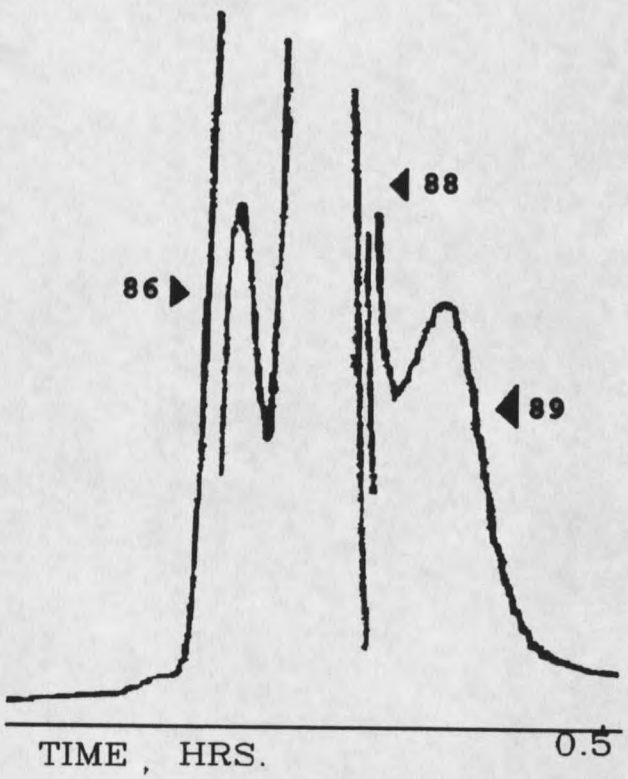


Figure 7. CCC Chromatogram of Indolizidine Mixture.

When more substantial amounts of the compounds were found in other fractions from previous gravity chromatographies, these fractions were pooled and partitioned between 5% aqueous triethylamine and methyl-t-butyl ether. Aliquots from the organic layer containing the deprotonated species were fractionated via

CCC in the same fashion as before, yielding larger quantities of the indolizidines.

STRUCTURE ELUCIDATION OF PROTONATED AND DEPROTONATED QUINOLIZIDINES

Protonated Quinolizidine Mixture **51, 65**

As has been stated, some spectral data had previously been obtained with the fraction eluted with reverse phase flash chromatography. A partial structure analysis was first carried out with the mixture of the two protonated compounds **51** and **65**, as they appeared to be homologous, differing only in the number of methylenes. Apart from the data already mentioned, a UV spectrum showed an absorption maximum at 230 nm, indicative of a heteroannular conjugated diene[38]. A Beilstein test was also positive for the presence of halogen, providing further evidence for an amine hydrochloride salt.

Analysis carried out via ^1H NMR ($CDCl_3$) supported the existence of an acetate with a sharp singlet at δ 2.1. Centered at δ 11.5 was a broad singlet, suggestive for the nitrogen-bonded proton of an NR_3H^+ moiety[39]. Other readily identifiable signals were a methyl doublet at δ 1.7, four olefinic protons from δ 6.1 to 5.5, and seven one-proton resonances at δ 4.8, 4.0, 3.8, 3.6, 2.6, 2.4 and 2.2, respectively. A multiple-proton signal was superimposed on the methyl doublet at δ 1.7. A large, broad methylene envelope was centered about δ 1.2, typical of methylenes in a long-chain unsaturated alkyl.

The broad methyl triplet δ 0.9 indicated that the alkyl chain contained a terminal methyl group. The chemical shift of the four protons at δ 4.8 to 3.6 could be explained only by invoking a system containing three methines attached to nitrogen and an acetate-bearing methine, since there were no other heteroatoms in the molecule to induce such chemical shifts.

Homodecoupling and difference decoupling experiments were undertaken to determine connectivities. In most cases $CDCl_3$ was used for the data obtained.

Irradiation of the methylene envelope centered about δ 1.2 collapsed the methyl signal at δ 0.9, lending credence to the idea of a terminal methyl group on an alkyl chain. Irradiation of the shoulder abutting the methylene envelope at δ 1.3 caused partial collapse of the quartet at δ 2.1. The chemical shift of the quartet is in agreement with that of an allylic methylene, and upon irradiation of this signal, the vinyl signal at δ 5.8, a multiplet, collapsed to a less well defined multiplet. It was clear from irradiation of this vinyl signal that it was coupled into the three-proton multiplet at δ 6.1, but the exact position of the neighboring vinyl proton could not be determined. However, in benzene-d_6 the three-proton multiplet at δ 6.1 was resolved into a two-proton signal at δ 5.9 and a

broad doublet of doublets at δ 6.4 (see Figure 8). Irradiation of the multiplet at δ 5.7 now collapsed the upfield portion of the two-proton signal at δ 5.9. The downfield portion of the two-proton multiplet collapsed when the signal at δ 6.4 was irradiated. Owing to the coincidental overlap of the two protons centered at δ 5.9, it was not possible to determine if they were adjacent to each other in the compound. However, the UV had provided strong evidence for a conjugated diene. Only one other connectivity was clearly assigned in benzene-d_6. The methine signal, δ 4.1 in $CDCl_3$ and δ 3.9 in benzene-d_6, collapsed to a poorly defined broad singlet when the doublet of doublets at δ 6.4 was irradiated. The calculated chemical shift an allylic methine attached to a carbon which is alpha to a protonated tertiary amine is in good agreement with the shift noted in the ^1H NMR spectrum[40]. All remaining assignments were determined in $CDCl_3$.

The bonding of a methyl group, the methyl doublet at δ 1.7, to a methine was demonstrated by the irradiation of the methine at δ 4.0, a doublet of quartets; the methyl doublet collapsed to a singlet. No other connectivities were apparent in homodecoupling, but

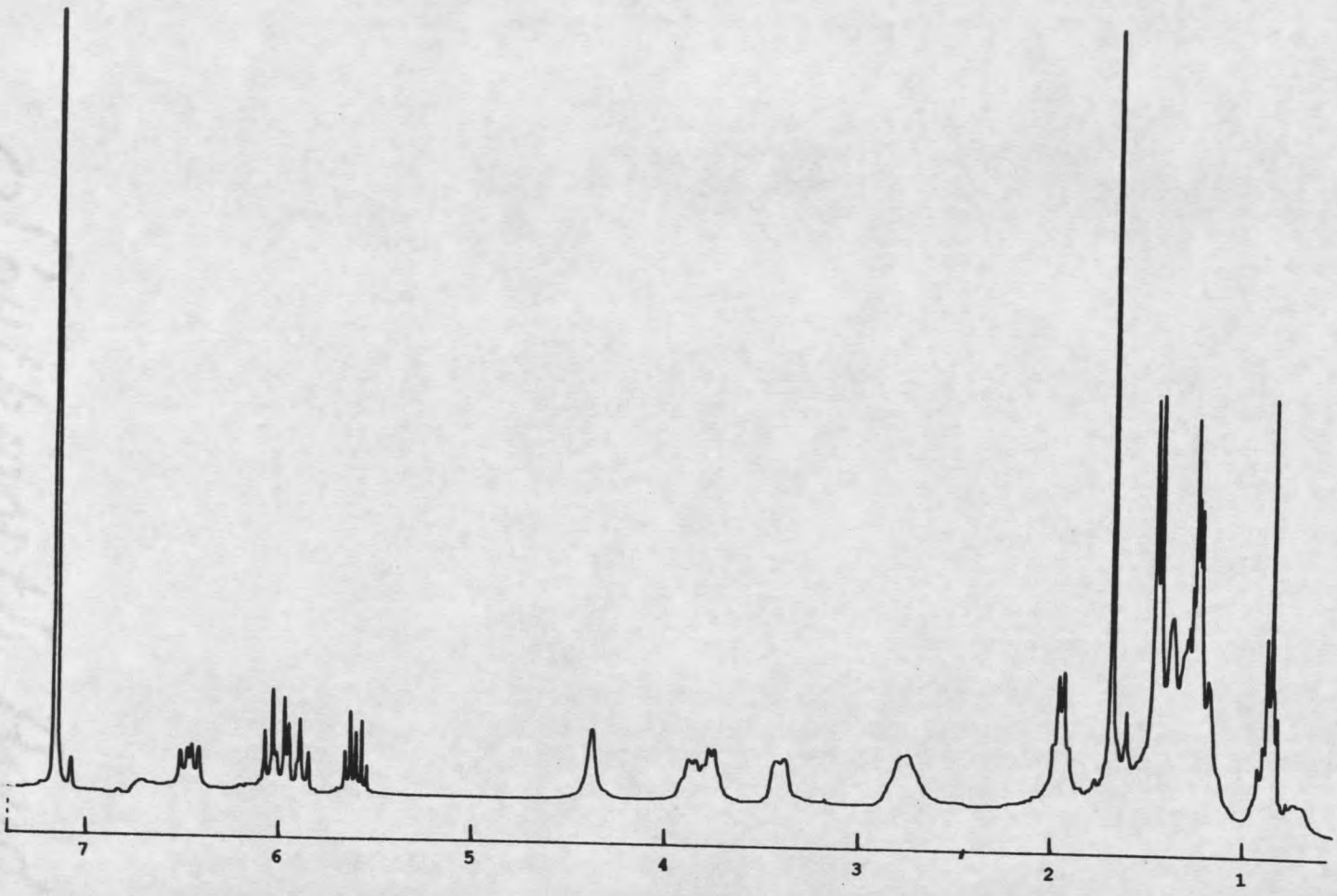
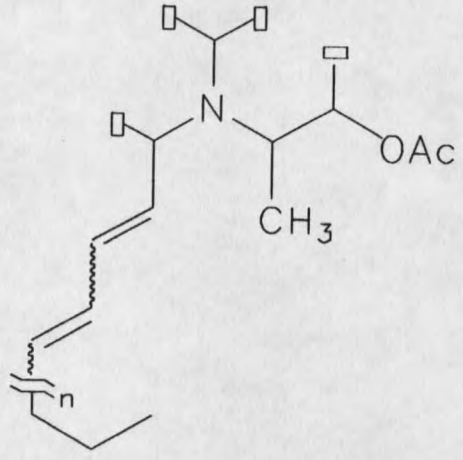


Figure 8. 250 MHz ^{1}H NMR Spectrum of a Mixture of **51** and **65** in Benzene-d_6.

difference decoupling indicated that the methine at δ 4.8 was adjacent to the methyl-bearing methine. A chemical shift to δ 4.8 was unlikely for a methine alpha to nitrogen, but the shift was in good accord with the shift of an acetate-bearing methine[40]. Again, difference decoupling experiments suggested coupling for the acetate-bearing methine to a signal centered at δ 2.0, but further delineation of that area was rendered impossible in light of the crowded nature of that region of the spectrum. Difference decoupling was once again utilized to elucidate other connectivities of the allylic methine and the third apparent alpha methine, the broad doublet at δ 3.8. Irradiation of the allylic methine revealed the connection of the methine to the one-proton resonance at δ 2.4. With irradiation of the broad methine doublet at δ 3.8, it was readily apparent that it was adjacent to each of the one-proton signals at δ 2.6 and 2.2, respectively. It was first thought that the two signals were geminal, but irradiation of each signal in separate experiments showed that this was not the case. Evidence for any connectivity between the two signals at δ 2.6 and 2.2 was not demonstrable. This implied either no adjacency or a Karplus angle of approximately 90^0 for adjacent protons[41].

In summary, a number of structural elements had been determined. The three methines alpha to nitrogen were located, and the positions of the three substituents, the methyl group, the acetate, and the alkyl chain bearing the diene were designated, **48**. This facilitated the elucidation of the remaining structural pieces when the pure deprotonated compound, **51**, was isolated.



48

Deprotonated Quinolizidine **51**

The compound, a clear viscous oil, showed no peak at 2500 cm^{-1} in the infrared, indicating that it had been deprotonated. The absorption maximum in UV was the same as that of the protonated mixture. EIMS indicated a molecular weight of m/z 347, the larger of the two compounds which had coeluted from the initial reverse

phase flash chromatography at 70% methanol. HRMS again gave the chemical formula $C_{22}H_{37}NO_2$, a compound with five sites of unsaturation. Taking into account the presence of an acetate and a diene, there remained two sites of unsaturation, implying a bicyclic ring system.

Structural assignments made with the protonated mixture were compared to the deprotonated species via ^1H NMR. In $CDCl_3$, a number of changes in chemical shifts was observed. All three alpha methines shifted upfield, as expected. The allylic methine shifted to δ 3.5, the methyl-bearing methine to δ 3.45 and the third alpha methine to δ 3.2. The acetate-bearing methine also experienced a shift of 0.3 ppm upfield, to δ 4.6. The three-proton multiplet at δ 6.01 remained unresolved.

Homodecoupling and difference decoupling of **1** in pyridine-d_5 (see Figure 9), performed on the 500 MHz NMR, resulted in the clear delineation of a major portion of the structure. The alpha methine at δ 3.4 was coupled to the methyl doublet, J=7.1 Hz, and its other vicinal neighbor, the methine at δ 4.6, J=3 Hz, which bears the acetate. Irradiation of this methine revealed coupling constants of 4.2 and 3.2 Hz to geminal protons at δ 1.74 and 1.83, respectively. The protons displayed a geminal coupling constant of 14.2 Hz. Irradiation of the

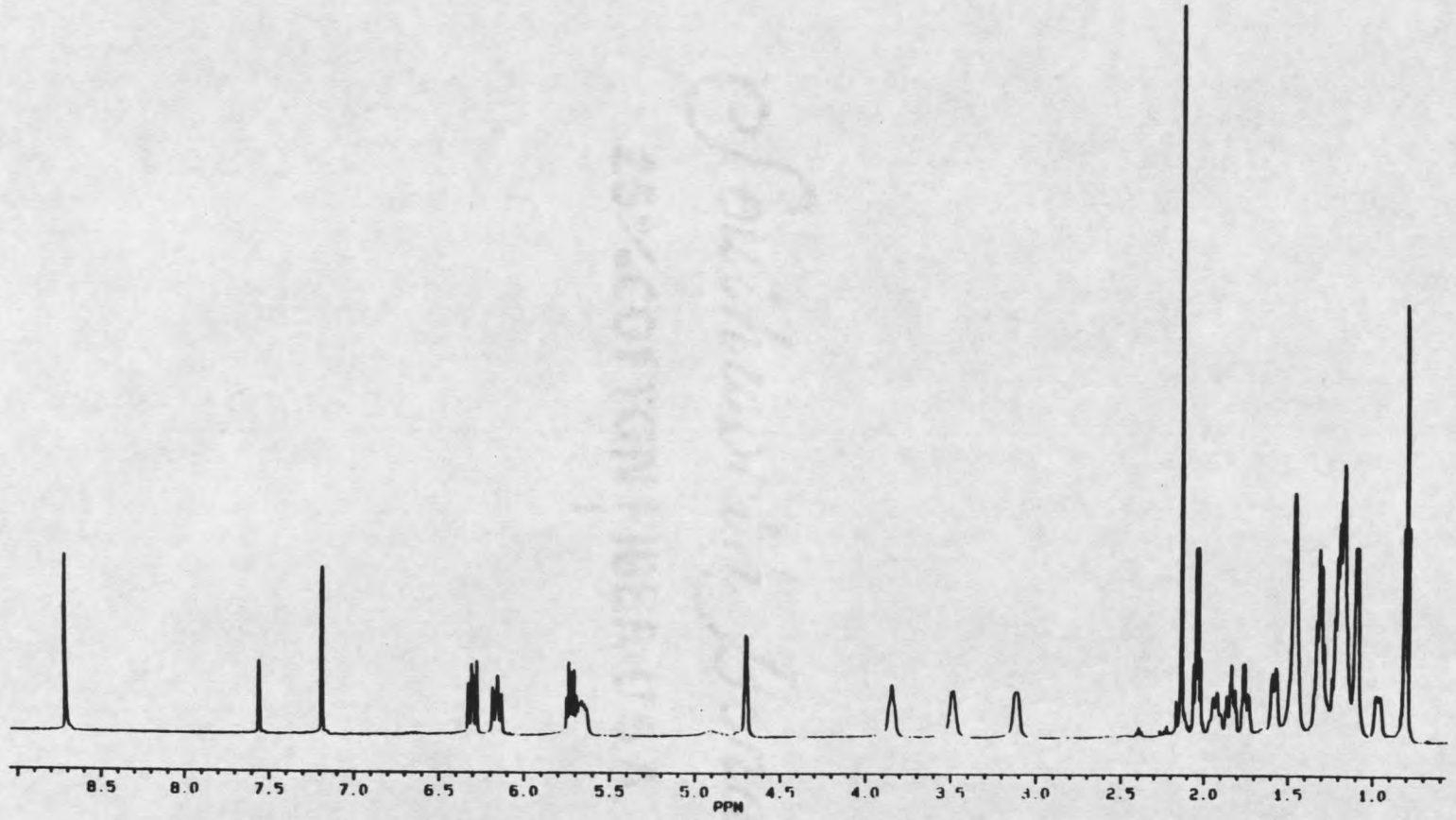
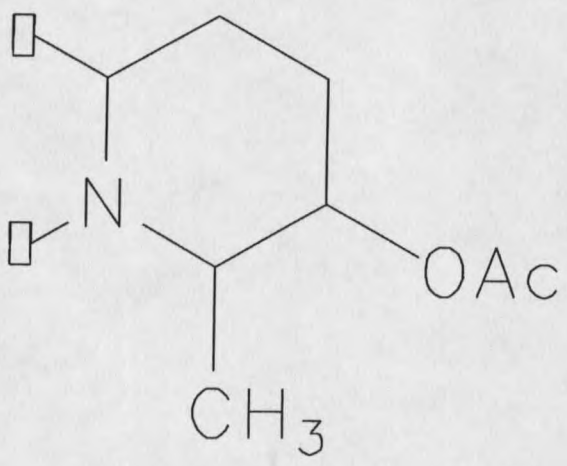


Figure 9. 500 MHz ^{1}H NMR Spectrum of **51** in Pyridine-d_5.

resonance at δ 1.73 revealed that its geminal partner apparently had another large coupling constant, on the order of 10 Hz, to one of a pair of geminal protons at δ 1.93 and 0.97. The value of 10 Hz was approximated, due to the interference of other couplings in that region of the spectrum. Irradiation of the signal at δ 1.93 led to a poorly defined multiplet centered at δ 1.83 and a broad, very poorly defined multiplet at δ 0.97. A coupling constant of 9.5 Hz , which is the geminal coupling constant, was measured previously for the signal at δ 0.97, to the best approximation. Irradiation of the signal at δ 0.97 led to the collapse of the geminal partner δ 1.93 to a broad triplet and a general broadening of the protons at δ 1.74 and 1.83. Abutting this methylene was an alpha methine, a broad doublet centered at δ 3.10. No coupling constants between the signal at δ 3.10 and the geminal protons at δ 0.97 and δ 1.93 could be determined. However, when the alpha methine was irradiated, both geminal protons appeared to sharpen slightly. These assignments enabled the elucidation of the six-membered heterocyclic ring, the piperidine, with substituents at the 2, 3 and 6 positions, **49**.



49

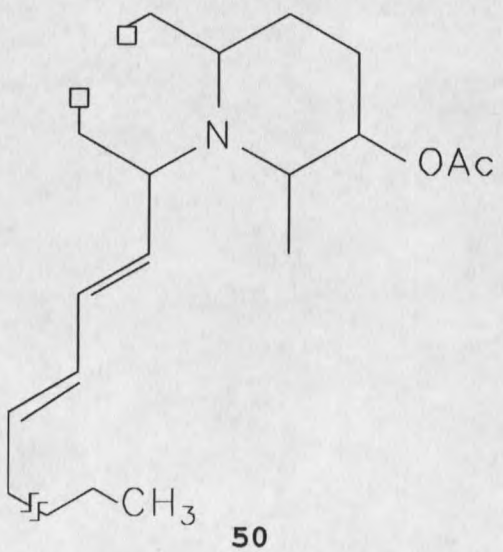
Irradiation of the alpha methine resonating at δ 3.85 revealed that it was coupled to a one-proton signal centered at δ 1.58, J=3 Hz. Difference decoupling of the methine at δ 3.85 also indicated a coupling to a signal centered approximately δ 1.3, suggesting the adjacent carbon was a methylene. A measured 12 Hz coupling constant from the signal at δ 1.58 to the signal centered at δ 1.3 was in accordance with a typical geminal coupling constant. Irradiating the signal at δ 1.3 also gave credence to the idea of a geminal proton, as the broad doublet of doublets at δ 1.58 appeared to collapse toward the center of the signal to form a broad singlet. Again, the center of the signal could only be approximated at δ 1.3, as it was completely concealed under other methylenes. Irradiation at δ 1.3 also

collapsed the triplet at δ 3.85 to a broad doublet, indicating coupling on the order of 7 Hz.

The proton envelope at δ 1.5 was irradiated to determine the presence of its adjacent protons. All that was observed was the sharpening of the broad doublet of the alpha methine, δ 3.10. This determined the position of at least one proton adjacent to the alpha methine, but, unfortunately, it left other connectivities in doubt.

Irradiation of the resonance at δ 3.85 also caused collapse of the vinyl doublet of doublets at δ5.65, J=7.7, 15.2 Hz, to a 15.2 Hz doublet. The smaller coupling constant was attributable to the signal at δ 3.85, now clearly an allylic methine alpha to nitrogen. The larger J value is typical for a trans disubstituted double bond[42]. Decoupling the vinyl signal at δ 5.65 led to the collapse of the doublet of doublets at δ 6.3, J=15.2, 10.2 Hz, to a sharp doublet. The signal at δ 6.3, when decoupled, caused the collapse of an adjacent vinyl proton, δ 6.15, to a doublet, J=14.9 Hz, once again indicative of a trans double bond. With the irradiation of the olefinic proton at δ 6.15, the multiplet resonating at δ 5.70 collapsed into a poorly resolved signal. Decoupling of the methylene quartet at δ 2.05 caused the collapse of the multiplet at δ 5.70 to a sharp

doublet, J=14.9 Hz, indicating the position of the allylic methylene. A multiplet, resonating at δ 1.3, also collapsed partially when the allylic methylene was decoupled. It was apparently a methylene adjacent to the allylic methylene. Finally, irradiation of the methylene envelope centered at approximately δ 1.2 caused the partial collapse of the methylene which resonated at δ 1.3. The last decoupling experiment also caused the methyl triplet, resonating at δ 0.85, to collapse to a singlet, lending supportive evidence of an extended alkyl chain with a terminal methyl group, as indicated earlier. Based upon the data obtained to this point in time, the structure of one ring was established, and the compound also bore a relatively long, unsaturated alkyl chain, as yet of undetermined length, **50**.



50

As the possibilities for structure elucidation with one-dimensional NMR were exhausted, it was hoped that two-dimensional H-H studies would clarify those parts of the compound which had escaped definition. A ^{1}H-^{1}H COSY[43] of the compound was performed using pyridine-d_5 as the solvent, due to its ability to disperse the signals. (see Figure 10). Those relationships which had already been assigned were confirmed by the COSY spectrum. The less well defined regions of the spectrum, between δ 1.5 and 1.2, did reveal more couplings. The one-proton resonance at δ 1.6 was clearly coupled into the envelope at δ 1.5 and a signal buried almost directly under the methylene envelope at δ 1.3. At least one resonance at δ 1.5 was also clearly coupled to a signal appearing to be the same one under the methylene at δ 1.3. Again, further definition of these signals left a reasonable assignment of the connectivities to C-6 and to the allylic methine wanting. The presence of only one other methyl group, that at the end of the unsaturated alkyl chain, did indicate that the compound should be enclosed, i.e., another ring was present, somehow connecting C-6 and the allylic methine. Also, the fifth site of unsaturation was still unaccounted for. This remained, after the COSY data was processed, the crux of the problem.

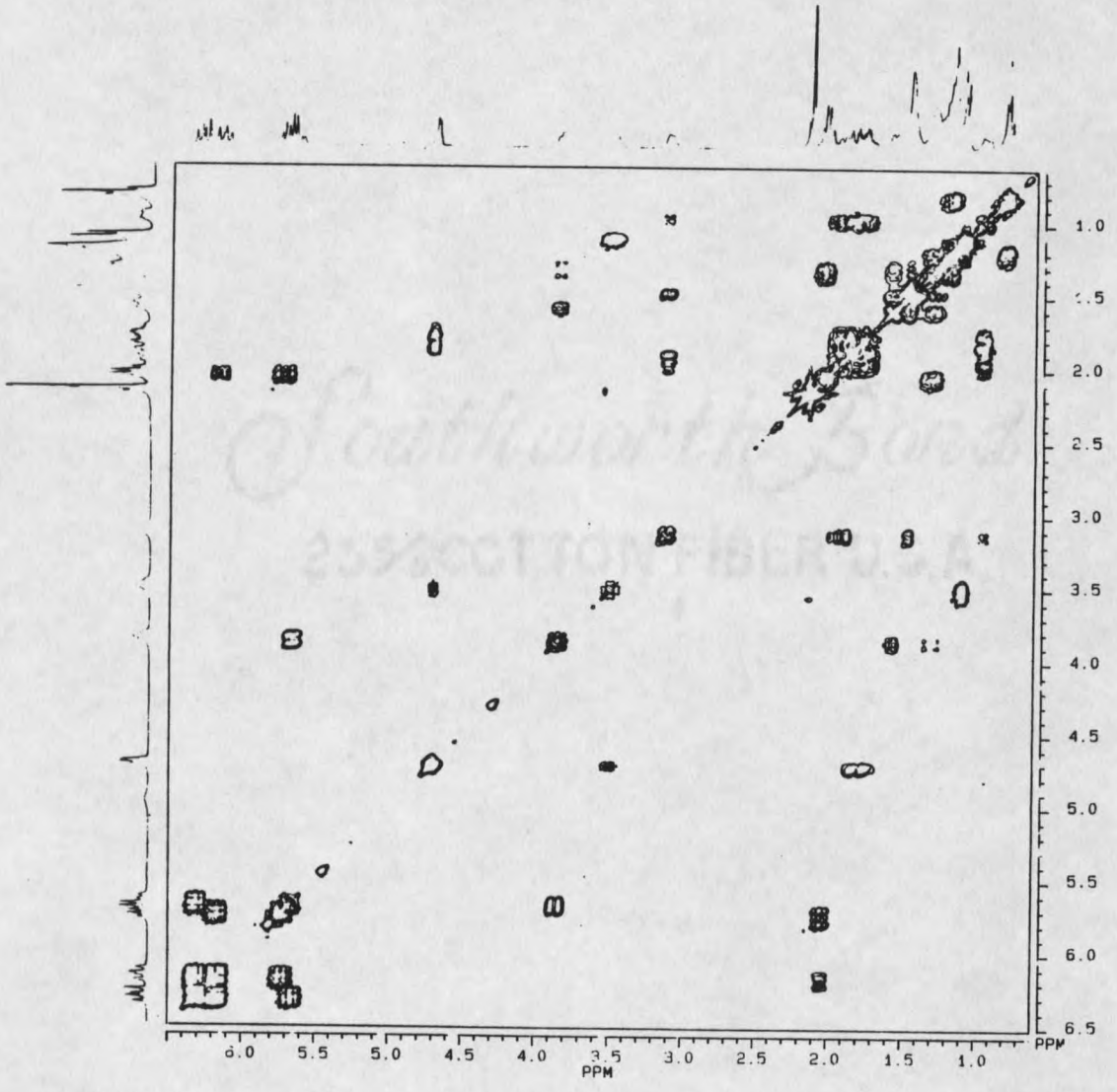


Figure 10. 300 MHz COSY Spectrum of **51** in Pyridine-d_5.

A series of various two-dimensional ^{1}H-^{1}H experiments was undertaken to help solve the remaining problem: 1) COSY 45[43]; 2) COSYRCT[44]; 3) DQCOSY[45]; 4) ZQCOSY[46]. Although all of the experiments were able to verify the structural assignments made with the one-dimensional decoupling experiments and the ^{1}H-^{1}H COSY experiment, better resolution of the envelope at δ 1.5 and 1.3 was not realized. For example, a COSYRCT, a ^{1}H-^{1}H relay coherency transfer experiment, was performed on the deprotonated compound (see Figure 11 and 12). Four-bond, H-H couplings were observed via this experiment, and it was postulated that the experiment could better delineate the unresolved portion of the structure. While a complete "walk-through", via observed four-bond coupling, was possible from C-1 through C-6, **49**, the signal at δ 1.5 proved to be enigmatic once again. The H-5 proton at δ 1.97 revealed a four-bond coupling to this signal, which apparently was the resonance of at least one H-7 proton. But the allylic methine, δ 3.8, was also four-bond coupled to the signal at δ 1.5.

Since homodecoupling could show a connectivity of the allylic methine to the adjacent geminal protons, δ 1.3 and 1.58, a five-membered heterocycle seemed plausible. An integration of the signal at δ 1.5 found a total of four or five protons. The integration yielded two

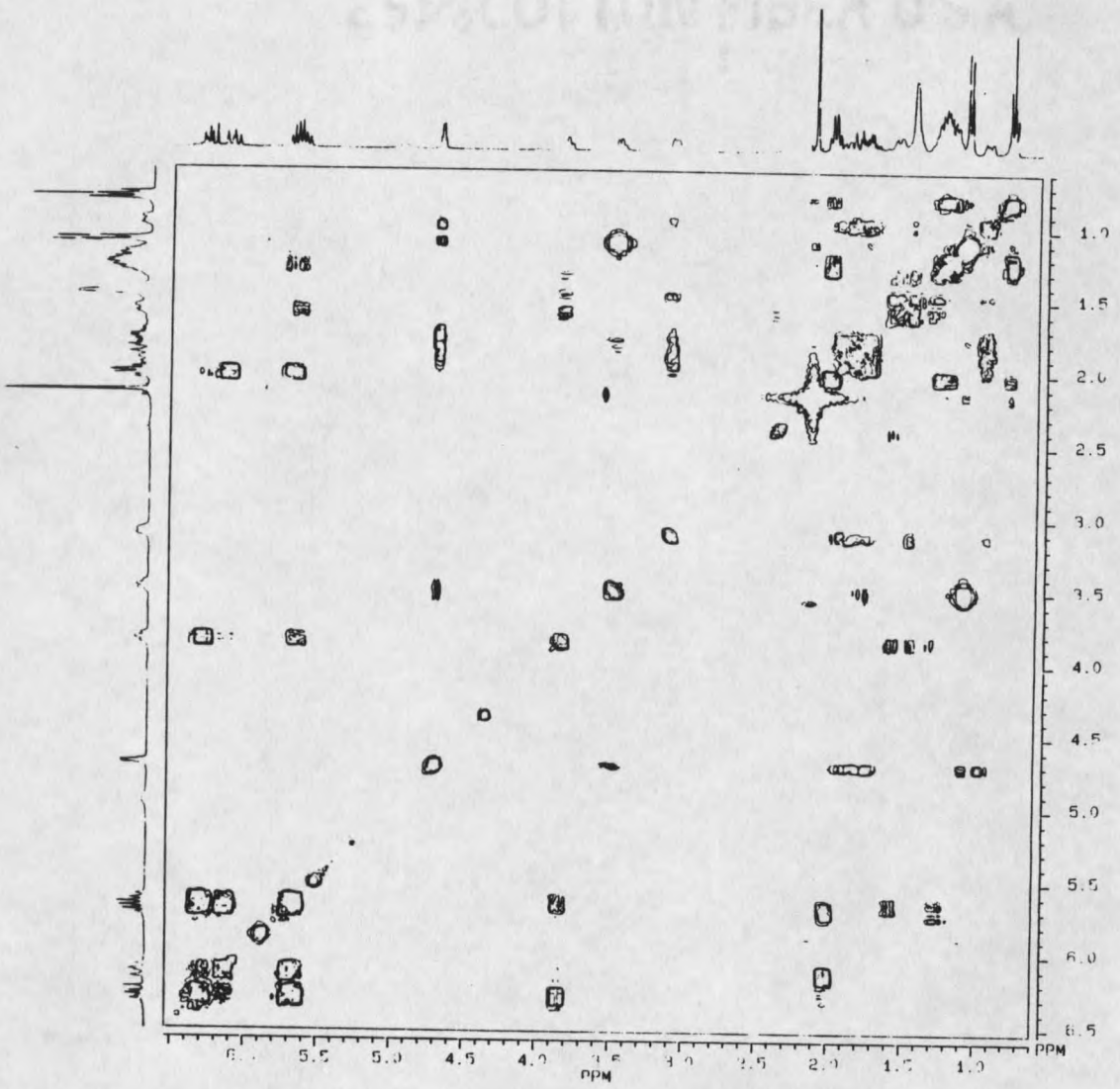


Figure 11. 300 MHz COSYRCT Spectrum of **51** in Pyridine-d_5.

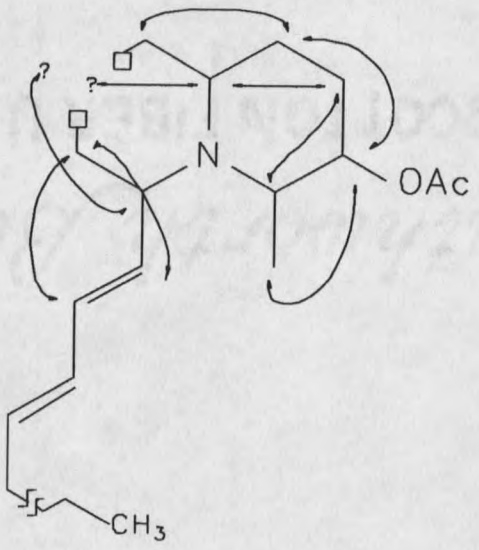


Figure 12. Four-bond Couplings Observed in COSYRCT.

possibilities: 1) the compound was an indolizidine, and the other protons at δ 1.5 were artifacts; and 2) the compound was a quinolizidine, where four protons on adjacent carbons coincidentally overlapped. Hence, the size of the second ring was still in doubt.

One-dimensional ^{13}C experiments were undertaken to verify the number of carbons and their multiplicities. A simple carbon-proton decoupled experiment (see Figure 13), in $CDCl_3$ yielded a spectrum with twenty-two readily observable carbons. As expected, four carbons in the midfield range at δ 49.1, 52.9, 57.9 and 73.4 were representative of the three alpha methines and the

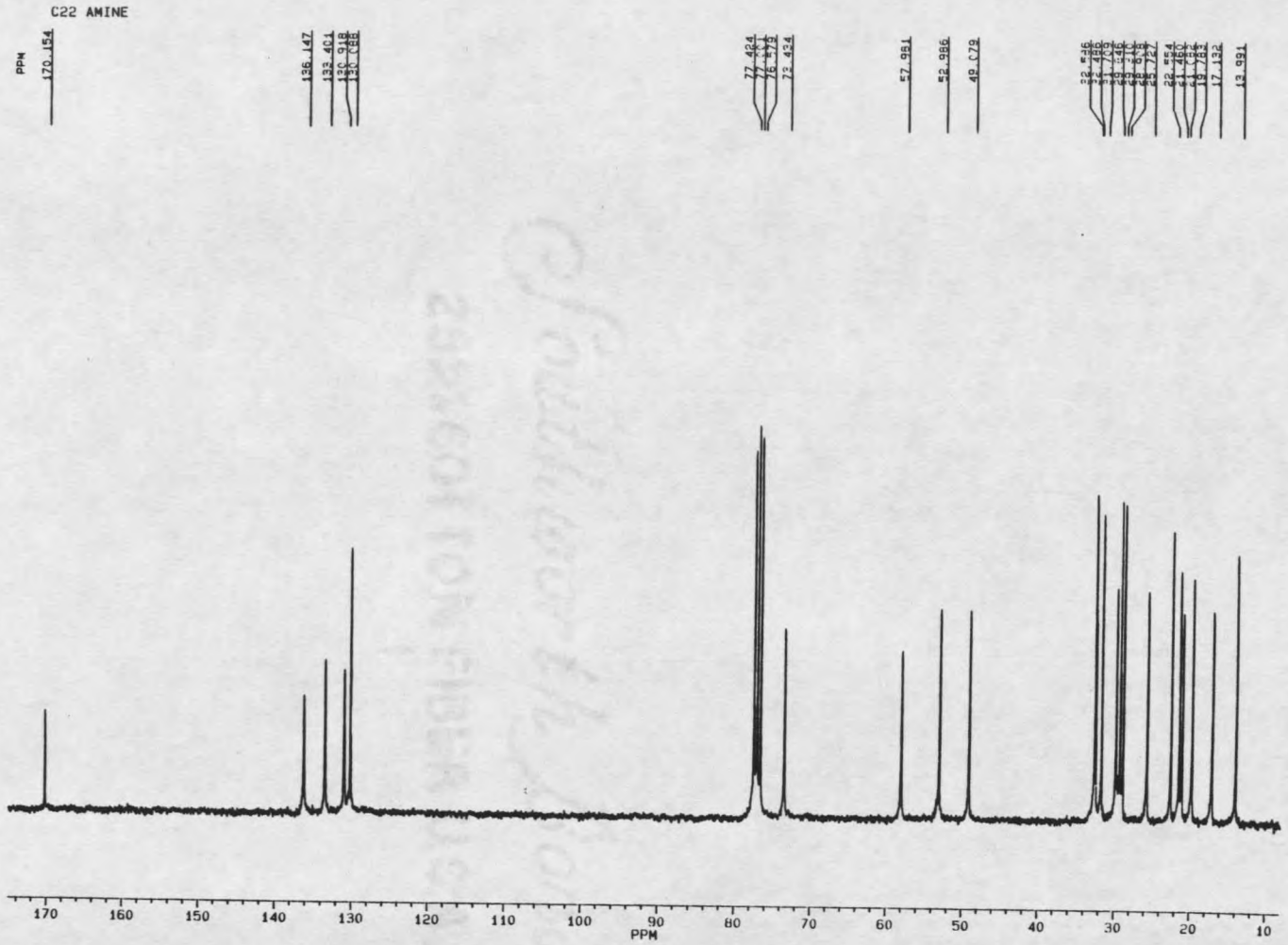


Figure 13. 75 MHz ^{13}C NMR Spectrum of **51** in Pyridine-d_5.

acetate-bearing methine. The four olefinic signals appeared at δ 136.1, 133.4, 130.9 and 130.1, respectively. The remaining sixteen carbons were spread out from δ 32.6 to 14.0. Multiplicities for methyls, methylenes and methines were determined via an INEPT[47] experiment (see Figure 14). Each of the four olefinic signals could be assigned as CH-, as was the case with the four midfield range carbons already mentioned. Three methyl groups were designated at δ 32.6, 21.5 and 14; the acetate methyl, the ring methyl and the terminal methyl. All other carbons were designated as methylenes. The data supported the part structure already proposed (65).

As none of the carbons were superimposed in the spectrum, a two-dimensional direct H-C correlation experiment, a HETCOR[48], was performed on the 125 MHz NMR (see Figure 15 and Table 2). Beginning with the midfield range of the spectrum, the signal at δ 73.4 correlated to the acetate-bearing carbon, C-3. The next three carbons at δ 57.9, 52.9 and 49.0 correlated to the allylic carbon, C-2 and C-6, respectively. Most gratifying was the assignment of three carbons to the envelope centered at δ 1.55 in the proton spectrum, δ32.6, 29.8 and 19.8. This was the first substantive evidence from NMR data that the second ring was comprised of five carbons; the three aforementioned carbons were the three carbons

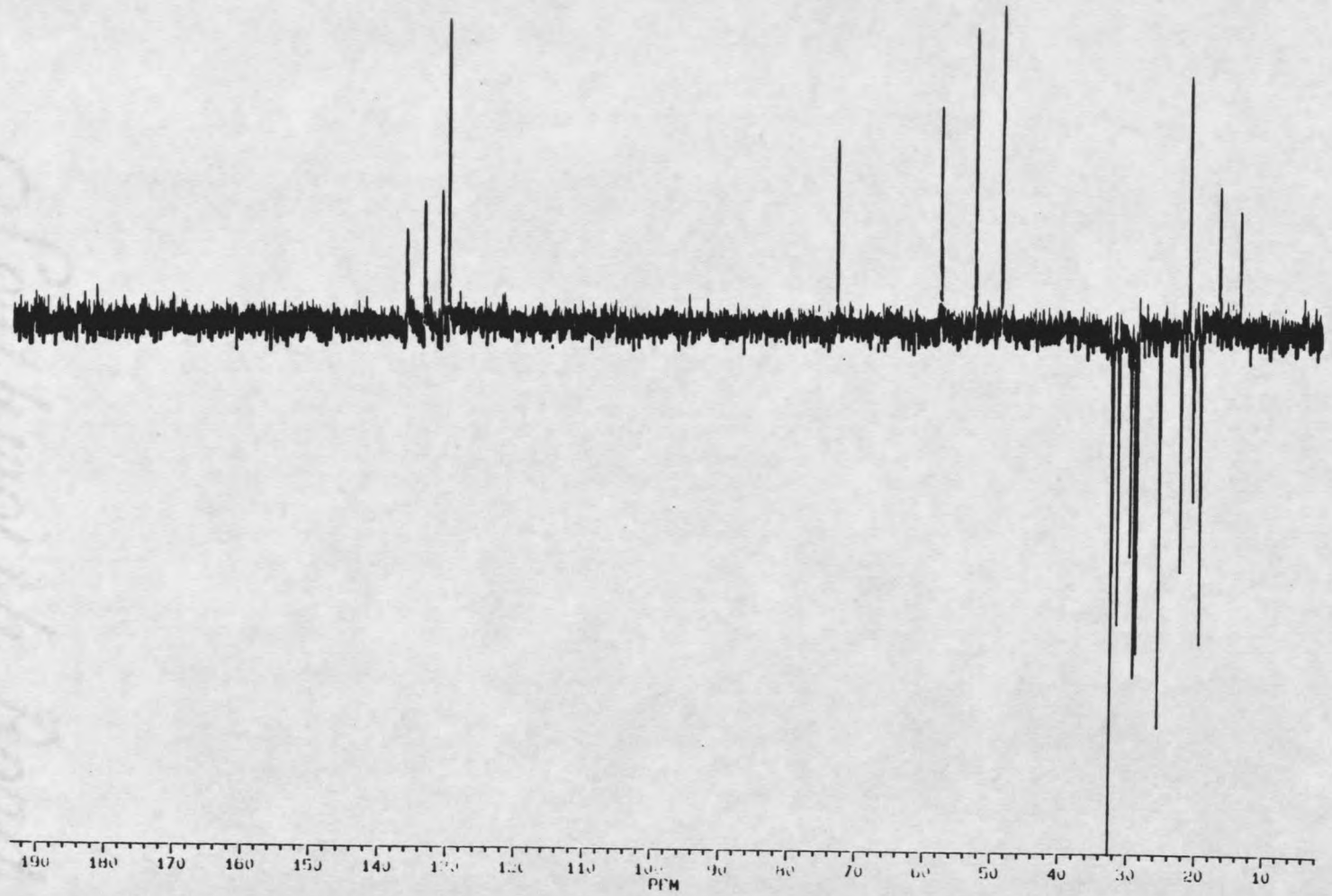


Figure 14. 125 MHz INEPT Spectrum of **51** in Pyridine-d_5.

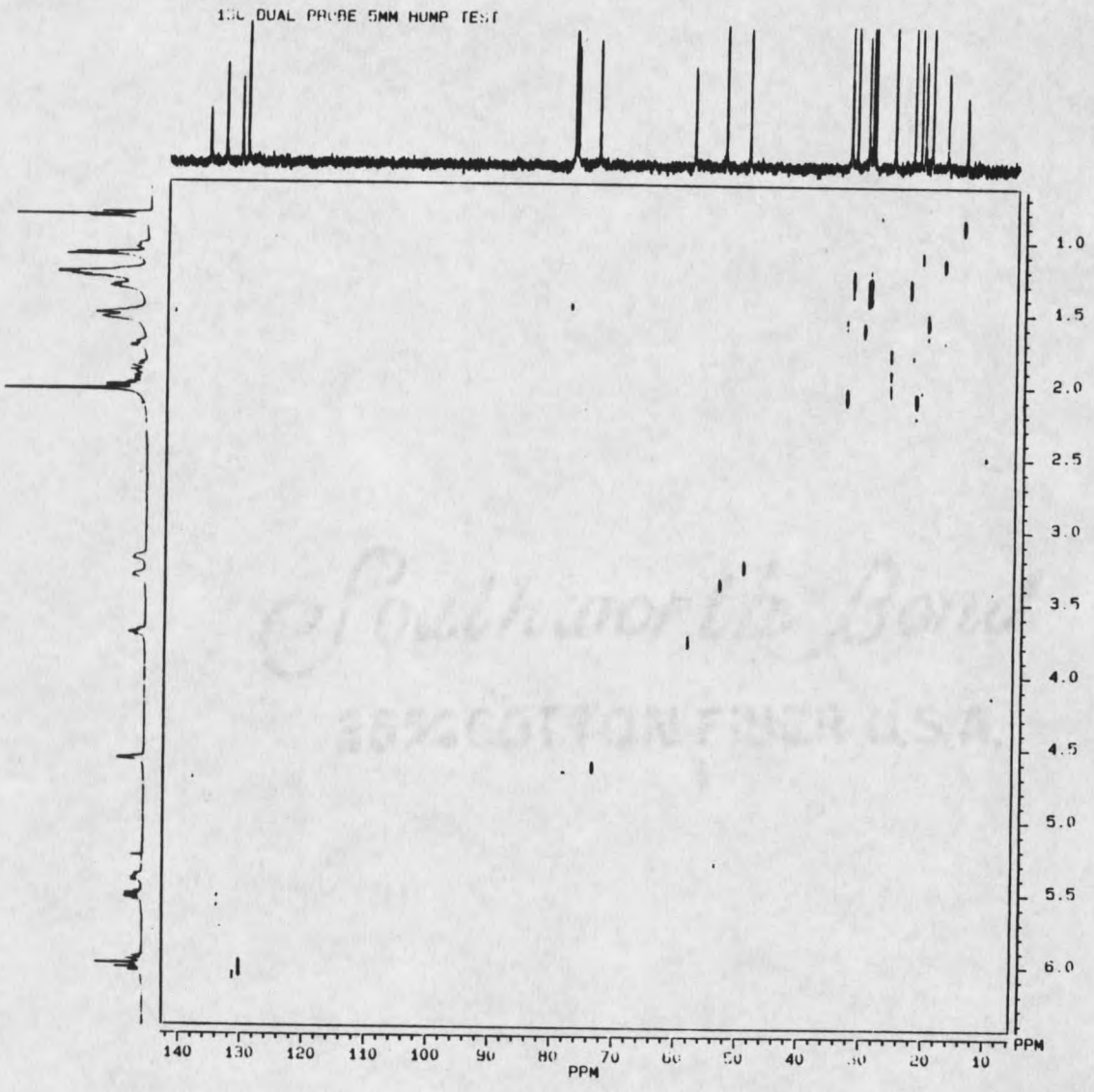


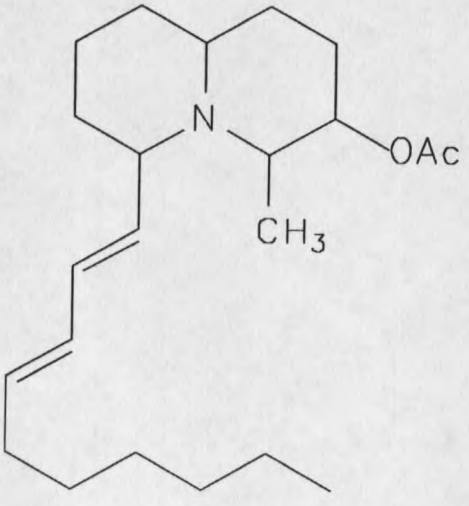
Figure 15. 125 MHz HETCOR Spectrum of **51** in Pyridine-d_5.

Table 2. NMR Data, **51**.[a]

Carbon #	$^{13}C(CDCl_3)$	$^{1}H(pyridine\text{-}d_5)$
1	14.0	1.09, t, 3H(J=7Hz)
2	53.0	3.50, dq, 1H(J=7,3Hz)
3	73.4	4.70, q, 1H(J=3Hz)
4	29.8	1.73, dq, 1H(J=14.4Hz)
		1.82, tt, 1H(J=14.4,11.1Hz)
5	22.6	0.97, m, 1H(J=10.2Hz)
		1.93, m, 1H(J=11.1,10.2Hz)
6	49.1	3.12, br d, 1H
7	32.6	1.47, m, 2H
8	21.5[b]	1.47, m, 2H
9	29.8[b]	1.58, m, 1H(J=7.3Hz)
		1.20, 1H
10	57.9	3.84, 1H(J=8.5,7.3,3Hz)
11	136.1	5.67, dd, 1H(J=15.3,3Hz)
12	130.9	6.31, dd, 1H(J=15.3, 10.5Hz)
13	133.4	6.15, dd, 1H(J=15.2, 10.5Hz)
14	130.1	5.73, m, 1H
15	32.6	1.31, m, 2H
16-18	29.3	1.24-1.12, 6H
	28.9	
	22.5	
19	31.7	1.24-1.12
20	17.1	0.85, t, 1H(J=6Hz)
21	170.1	
22	25.7	2.13, s, 3H

[a]recorded at 500 MHz (^{1}H), 125 MHz (^{13}C)
[b]interchangeable

linking from C-6 through to the carbon bearing the allylic methine. Further support of this assignment was the number of carbons correlated to the saturated portion of the alkyl chain, six. All of the supposed chain carbons correlated to that part of the proton spectrum which was attributable to the alkyl chain. The end methyl, C-20, was situated at δ 14.0, the allylic methylene, C-15, was located 32.4, and the four remaining carbons of the chain were assigned to δ 29.8, 29.3, 28.9 and 25.7. The remaining ring carbons, C-7, C-8 and C-9, were shifted to δ 21.1, 25.7 and 17.1. Due to the weak signal, only one correlation was discernible in the olefinic region, C-11; however, this was not critical for the structural assignments. UV and ^{1}H NMR data clearly supported the presence of a conjugated diene, to give **51**.



51

Some broadening of the ring methines had been observed in the ^{1}H spectra, and it was thought that this was attributable to a non-rigid ring structure. In order to determine if any signal broadening was observable in the carbon spectrum, a low temperature study of the molecule was undertaken. The C_{22} acetate was taken up in $CDCl_3$, and carbon spectra were obtained at 298 ^{0}K, 278 ^{0}K and 218 ^{0}K on the Bruker 125 MHz (^{13}C) instrument (see Figures 16, 17, and 18). Although very little broadening of the ring carbons had been observed at ambient temperature in a spectrum obtained with a 300 MHz instrument, ten signals, all the ring carbons and the ring methyl, were observed to clearly broaden even at 298 ^{0}K when the spectrum was obtained at 125 MHz. This phenomenon did not seem attributable to a long receiver delay, since the areas of the broadened peaks appeared to be comparable to the areas of the sharp peaks. This broadening phenomenon persisted even to some degree at 218 ^{0}K, although it was quite clear that the ring carbons had sharpened to a great extent. No second conformer was observed, and it was hypothesized that the non-rigid ring structure was possibly undergoing some form of rapid inversion due to a very low energy barrier for inversion. Further attempts to obtain spectra on carbon at a lower

temperature were not possible, as the sample froze below 218 ^{0}K.

Further supportive evidence for the quinolizidine structure was obtained by mass spectral analysis. As was mentioned previously, the HRMS gave an observable molecular ion, M^{+} 347. A close perusal of the spectrum revealed the presence of a minor fragment at m/z 210. This was suspected of being the mass of the fragment resulting from loss of the unsaturated alkyl chain, to give the intact bicyclic portion. However, the peak was of very low intensity, 1.2%, and was not considered significant enough by itself to be supportive evidence for the quinolizidine ring structure. The loss of the unsaturated alkyl chain is not facile. On the other hand, a saturated, branched alkyl chain is easily fragmented in the mass spectrometer source. Therefore, reducing the olefins should result in an increase in the peak at m/z 210. A 2 mg sample of the C_{22} acetate was taken up in EtOH and reduced under slight hydrogen pressure, with palladium on charcoal as a catalyst to give **52**. Not only did the fragment ion m/z 210 increase in **52**, it became the base peak in the spectrum. HRMS performed on the reduced compound established the

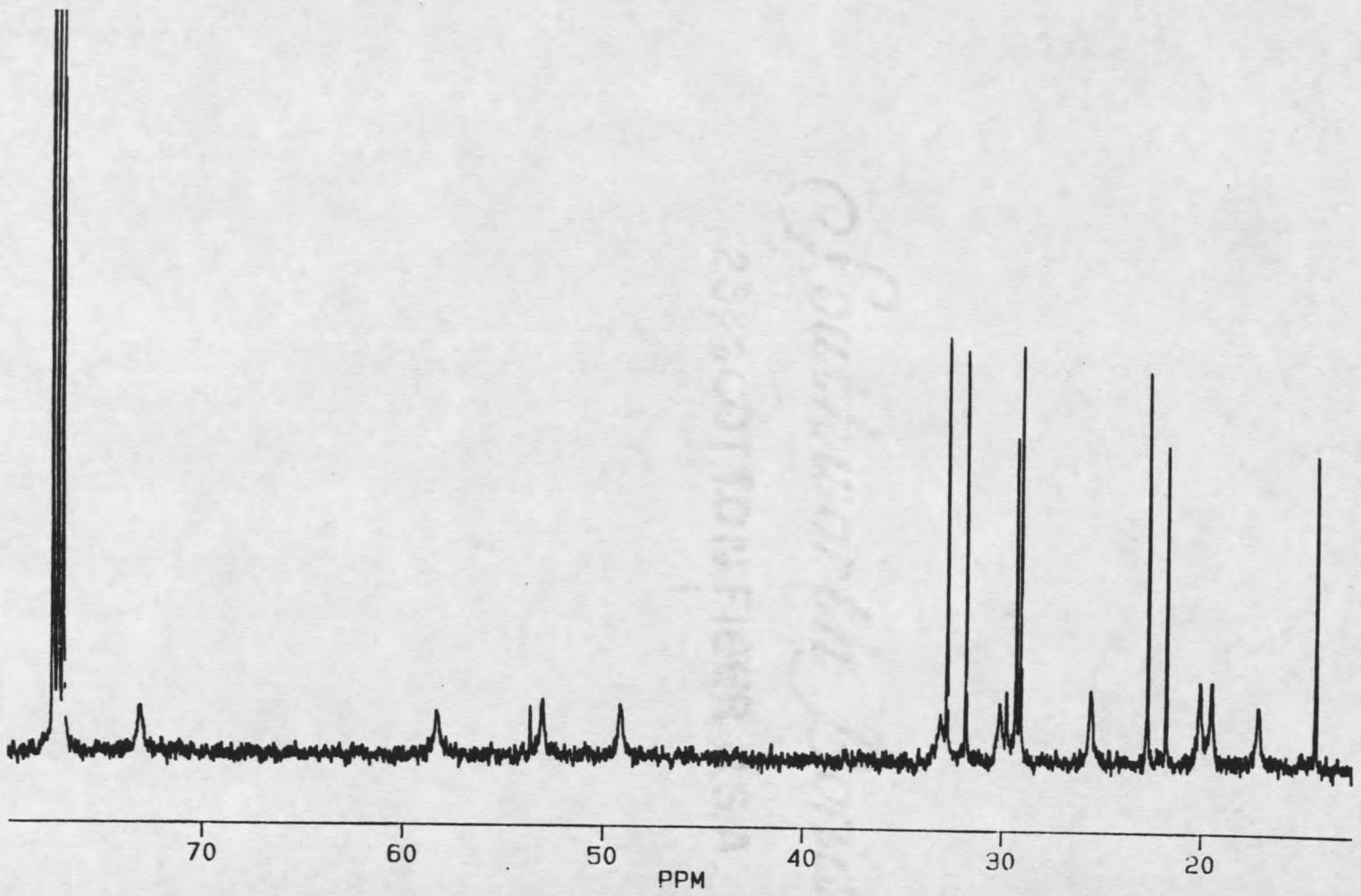


Figure 16. 125 MHz ^{13}C Spectrum of **51** at 298 ^{0}K in $CDCl_3$.

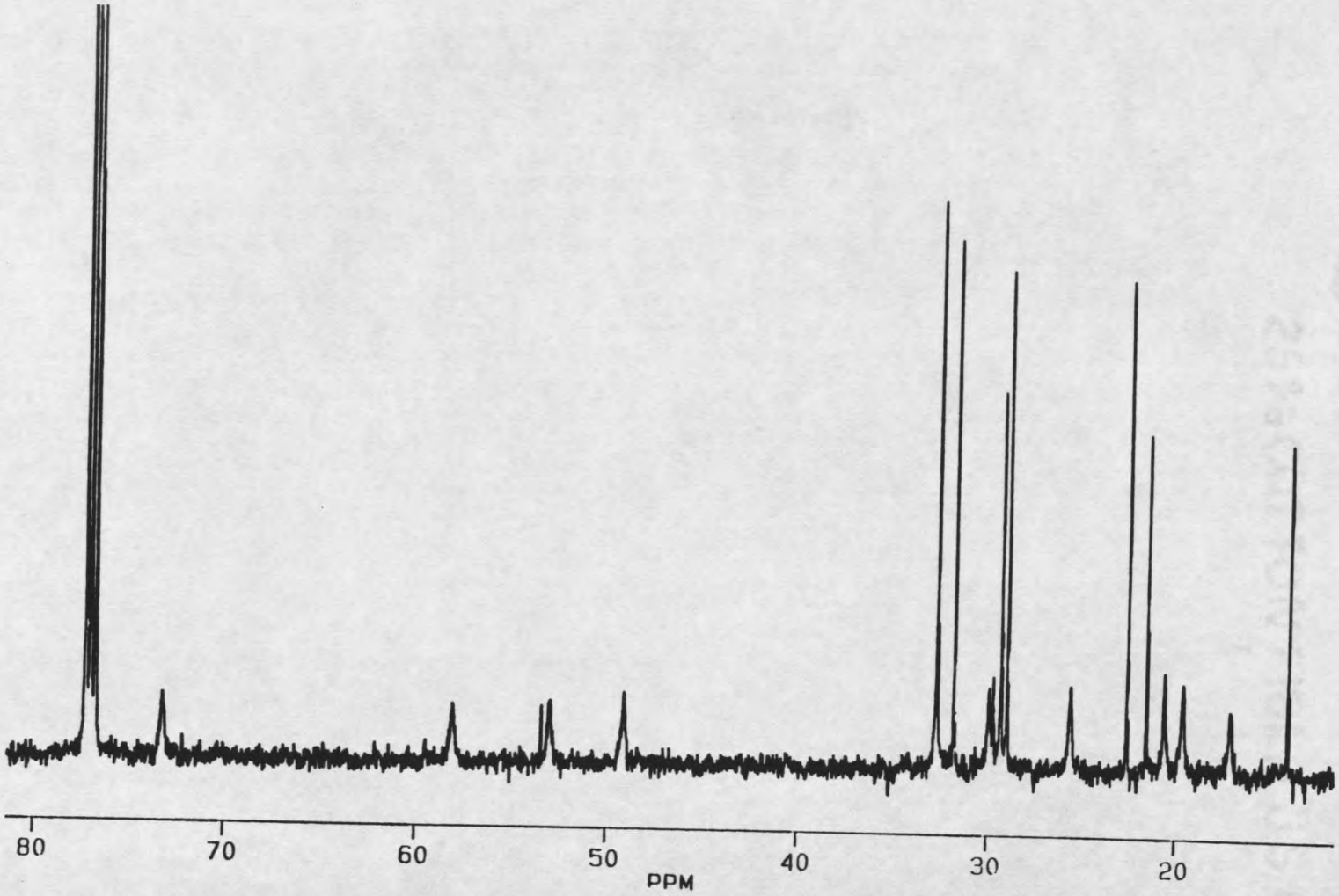


Figure 17. 125 MHz ^{13}C Spectrum of **51** at 278 ^{0}K in $CDCl_3$.

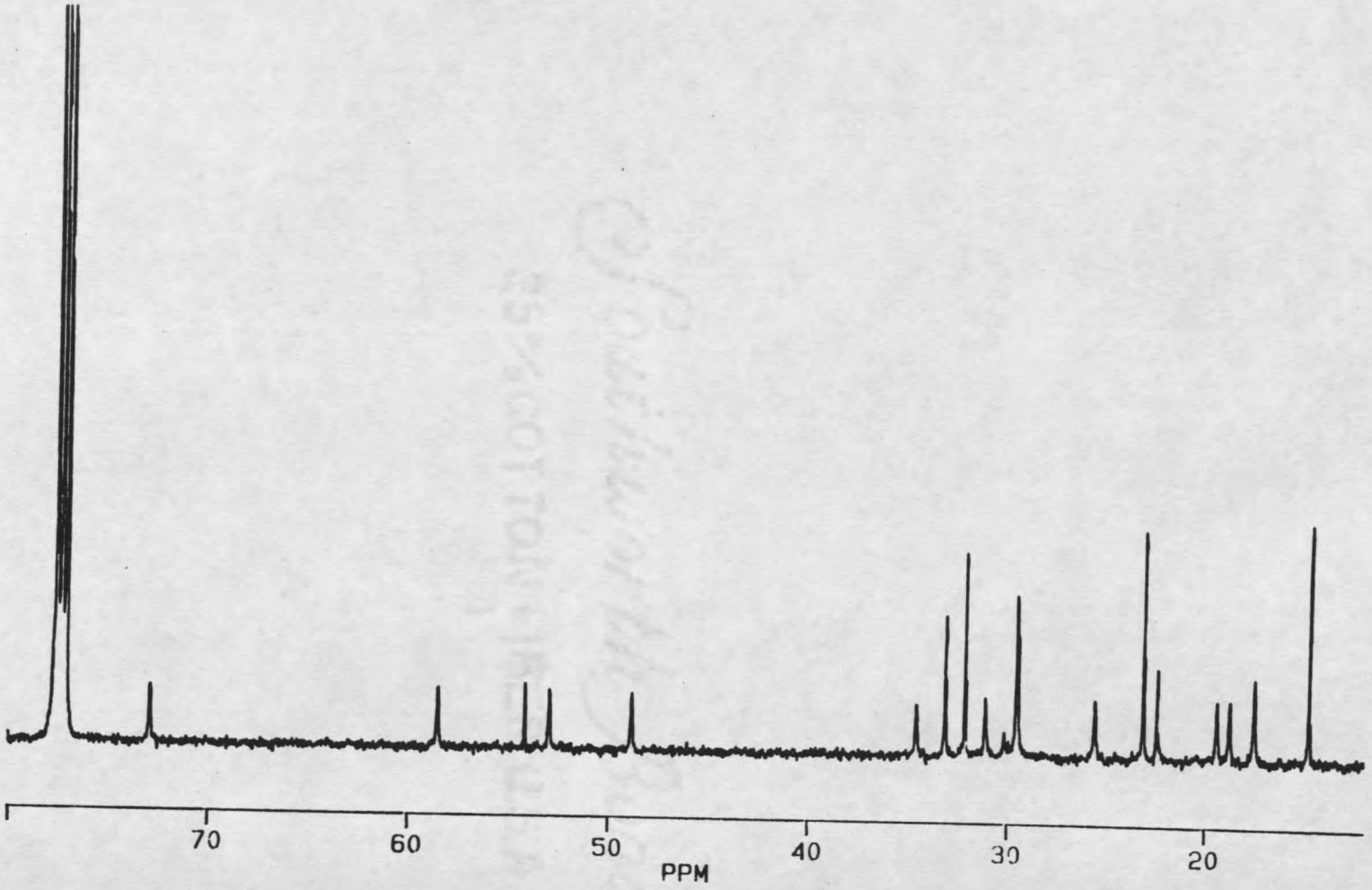
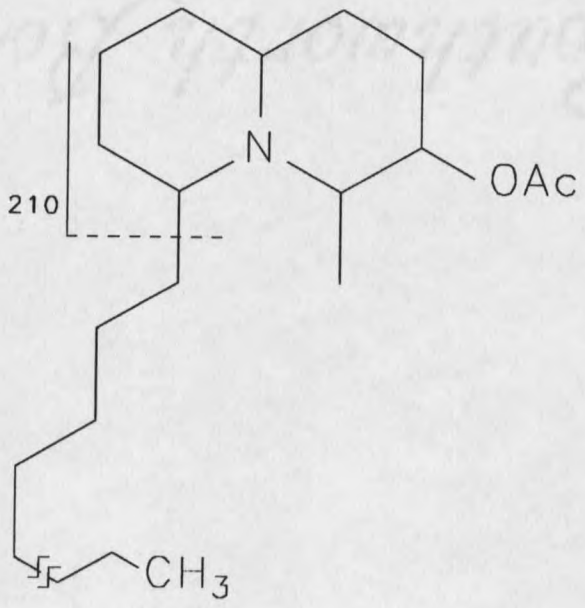
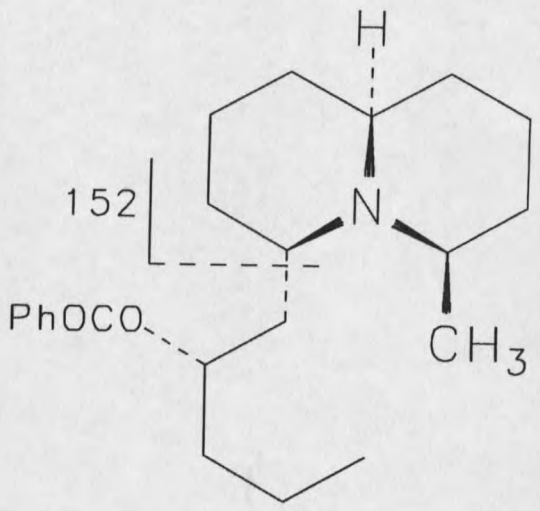


Figure 18. 125 MHz ^{13}C Spectrum of **51** at 218 ^{0}K in $CDCl_3$.

constitution of the m/z 210 fragment, $C_{12}H_{20}NO_2$, which is, then, the formula for the quinolizidine minus the alkyl chain. This fragmentation of the alkyl chain to yield the intact ring was also readily comparable to the fragmentation of a similar compound, porantherilidine, **53**[49]. The cleavage of the alkyl chain of porantherilidine gave a base peak at m/z 152, corresponding to the bicyclic ring structure with the methyl substituent.



52



53

Difference nOe[50] experiments were performed on the acetate to see if the relative stereochemistry could be determined. The methyl methine, H-2, revealed an nOe relationship to the attached methyl, C-1, 7%, the acetate-bearing methine, H-3, 7%, and the vinylic proton, H-11, 7.1%. Irradiation of the ring juncture methine, H-6, established the cis relationship to the ring methyl, C-1, with an observed nOe of 6%. A 2% nOe was also observed between H-6 and the proton envelope centered at approximately 1.42 ppm and apparently to the proton attached to C-5, at 0.97 ppm, 3%. The C-5 proton was irradiated in turn, giving an nOe to H-6 of the same amount, 3%. The only other nOe readily observable to the C-5 proton was to its geminal partner, approximately 20%;

crowding in the spectrum did not allow measurement of nOe to the C-4 methylene. For H-3, an nOe was established to H-2, 6%, and to a C-4 proton, approximated to be 4%. It did not appear to have nOe to any other ring protons. Finally, an nOe of 7.2% was also observed by irradiation of the allylic methine, H-10, to the vinylic proton, H-12.

With no further considerations of ring conformation, the nOe data would be consistent with the relative stereochemistry as in Figure 19.

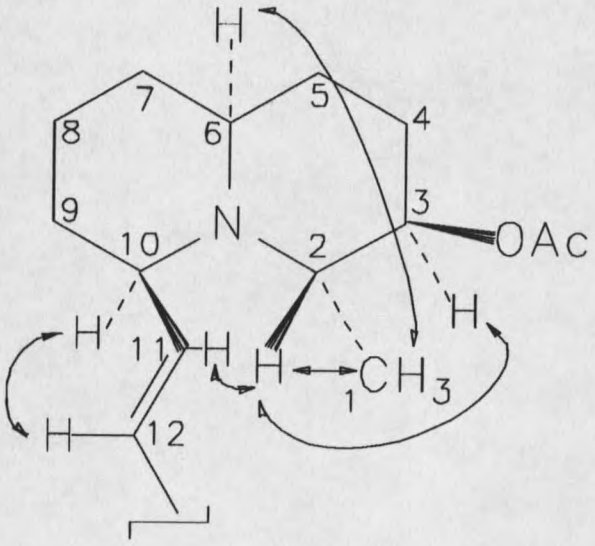


Figure 19. Relative Stereochemistry of **51**, as Suggested by nOe.

The nOe observed between H-1 and H-6 established their 1,3 diaxial relationship, and H-2 could be placed in the equatorial position. All J values measured for H-

3, 3 to 4 Hz, indicated that H-2 and H-3 could be either equatorial-equatorial or equatorial-axial. The small J value, however, also indicated that H-3 could not be trans-diaxial with the axial H-4. Hence, H-3 must be equatorial, and the acetate axial. Enhancement of the H-11 signal with the irradiation of H-2 suggested an equatorial placement for the chain substituent on the other ring. The chain also appeared to bisect the ring, since a strong nOe between H-12 and H-10 was observed. The only other nOe observed to H-10 was from protons on the ring containing the alkyl chain, indicating the presence of a cis-fused ring system, as in Figure 20.

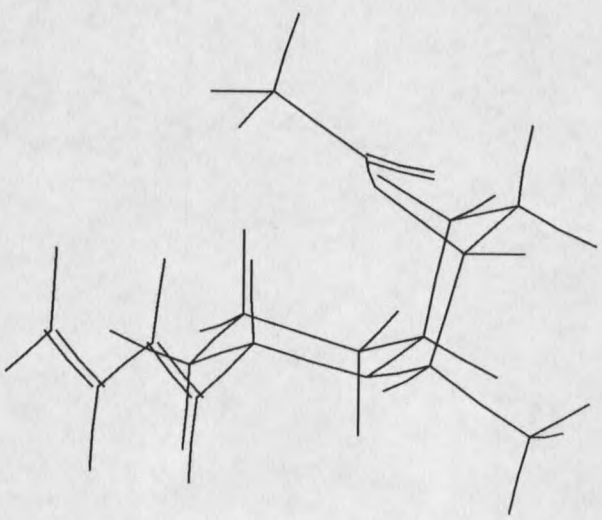
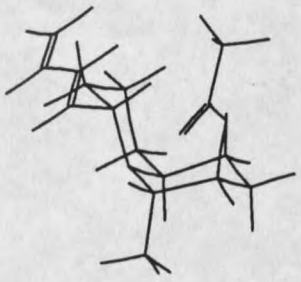
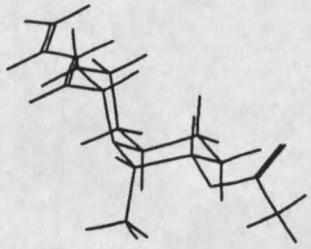


Figure 20. Alchemy II Generated stereochemistry of C_{22} Acetate.

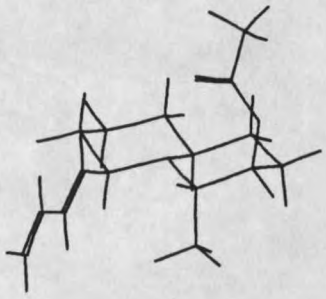
The presence of vicinal diaxial substituents on ring B at least suggested a conformation of relatively high energy. Since the naturally occurring product could not be crystallized for X-ray analysis, an indirect method for comparing different ring configurations was used. The heat of formation of the quinolizidine acetate, minus the extended alkyl chain, was evaluated with different ring conformations, including cis and trans, with the Alchemy II[51] program. Each of the structures were minimized using the program, whereafter the minimization file that had been created was converted to an MM2 file. The MM2 file for each structure was transferred to the TRIBL[52] program, which performed both MM2 and AMI calculations in order to determine the heat of formation for each possible structure, **54-64**. The results were a bit unexpected. The two cis ring conformations, **54** and **55**, not only had heats of formation comparable to the trans ring conformations, but they were even more stable. Also, placing the acetate in an equatorial position, **54** vs. **55**, gave a very similar heat of conformation for the respective compounds. According to this indirect method, the diaxial conformation, then, was practically as stable as any of the other conformations.



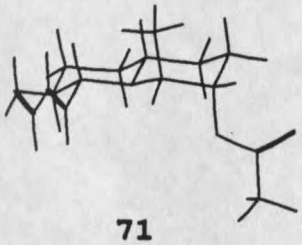
68
ΔH=-59.7



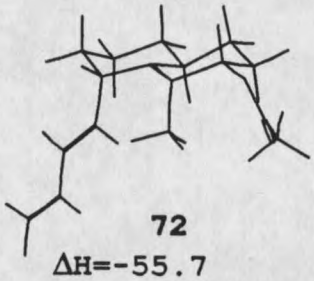
69
ΔH=-60.4



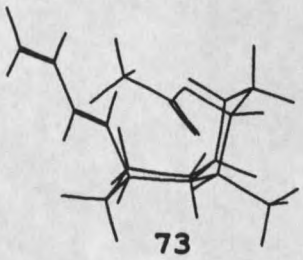
70
ΔH=-50.8



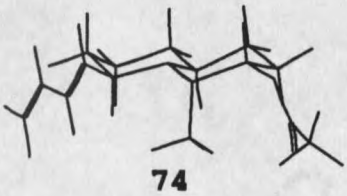
71
ΔH=-59.5



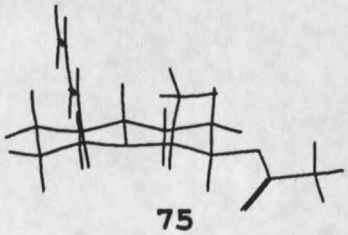
72
ΔH=-55.7



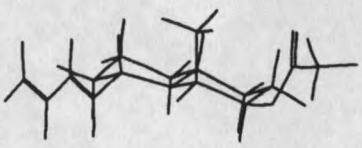
73
ΔH=-56.1



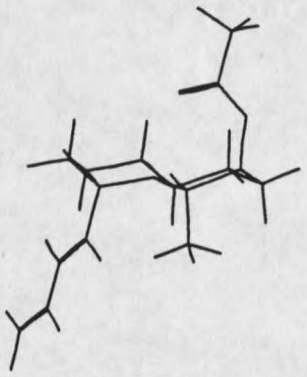
74
ΔH=-56.6



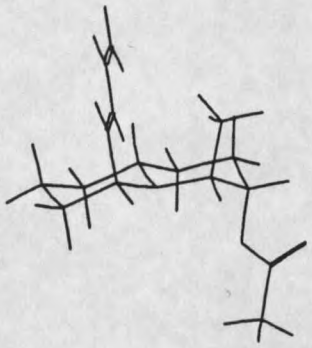
75
ΔH=-59.5



76
ΔH=-56.6



77
ΔH=-51.1



78
ΔH=-58.3

After the relative conformation of the quinolizidine had been suggested by nOe, efforts were undertaken to form a crystalline derivative for X-ray analysis. A variety of derivatizations were attempted. A triazoline derivative formed readily in 79% yield, and, according to ^{1}H NMR, the endo and exo products were formed in a 1:1 ratio (see Figure 21). Surprisingly, the derivative

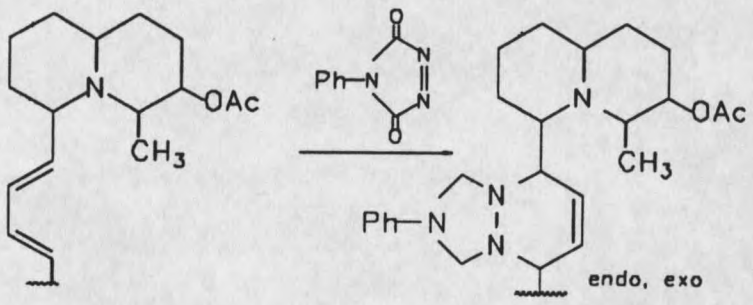


Figure 21. Formation of Triazolene Derivative.

apparently decomposed on the CCC column. The compound was hydrolyzed with $Ba(OH)_2$, and further derivatizations were attempted with the alcohol. A reaction with

bromobenzenesulfonyl chloride yielded no discernible derivative, nor did a quaternary salt form when the compound was reacted with methyl iodide. The alcohol did react with 2-chloro-4-nitrobenzoyl chloride quantitatively, but the heavy-atom product resisted all efforts to crystallize it. The alcohol was allowed to react with 4-bromophenyl isocyanate. According to TLC and EIMS, the product was being formed very slowly, and when the reaction mixture was allowed to stand for a 72 hour period, the product had decomposed.

The acetate was also reacted with H_2O_2 in hopes of forming a crystalline N-oxide. This reaction also led to the decomposition of the compound.

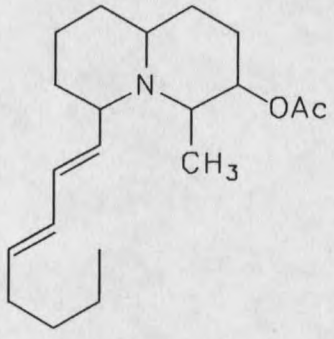
A modified Horeau's method for the determination of absolute stereochemistry of a secondary alcohol had been developed in this laboratory[53]. The method involved the reaction of 2 equivalents of racemic 2-phenylbutyryl chloride with the alcohol. The unreacted acid chloride was acidified, and an optical rotation measurement of it was taken. No optical rotation of the residual acid was observed. Racemization of the reagent was suspected, in light of the fact that the quinolizidine itself can act as a strong base. It was noted that the benzoyl derivative of porantherilidine, the compound used for comparison in mass spectral analysis, readily formed

crystals when the hydrogen bromide salt was made[49]. The crystals underwent a successful X-ray crystallographic analysis. Since **51** and **52** were very similar in structure, it was hypothesized that the hydrogen bromide salt of the Horeau derivative would crystallize. The amine hydrogen bromide salt was made, but, once again, crystallization could not be achieved.

Shorter Chain Quinolizidine Acetate Dienes **65, 67, 66**

The compound eluting with the C_{22} acetate diene, **51**, during the initial reverse phase flash chromatographies was found to have, according to HRMS, a chemical formula of $C_{20}H_{33}NO_2$. Since it differed from **51** by two supposed methylene units and eluted just prior to the C_{22} acetate during analysis via GC/MS, it was suspected of being a homologue of the C_{22} compound. The NMR profile of a mixture of the C_{22} acetate and its possible homologue appeared to have almost completely overlapping signals. The only way of distinguishing the two compounds was by virtue of the fact that their respective acetate resonances were not totally superimposable. As has been previously described, the isolation of the shorter chain compound was accomplished by the modification of the reverse phase flash chromatography with subsequent CCC to give pure **65**. A ^{13}C NMR spectrum (see Figure 22) of the

isolated compound revealed the presence of twenty carbons with chemical shifts corresponding to those of **51**. Infrared (see Figure 23) and uv analyses were also very similar to the spectral data for **51**. Homodecoupling and difference decoupling experiments performed on the 300 MHz NMR (pyridine-d_5, see Figure 24) determined that the only dissimilarity between this compound and **51** was the smaller number of resonances for the methylene chain. An integration of the region between δ 1.3 and δ 1.28 indicated only 6-7 protons instead of 10-11 protons, as was the case with **51**. Thus the structure was determined to be the C_{20} acetate diene, **65**.



65

During routine mass spectral analysis to determine the purity of fractions eluted from CCC or reverse phase flash chromatography, an occasional peak with an apparent

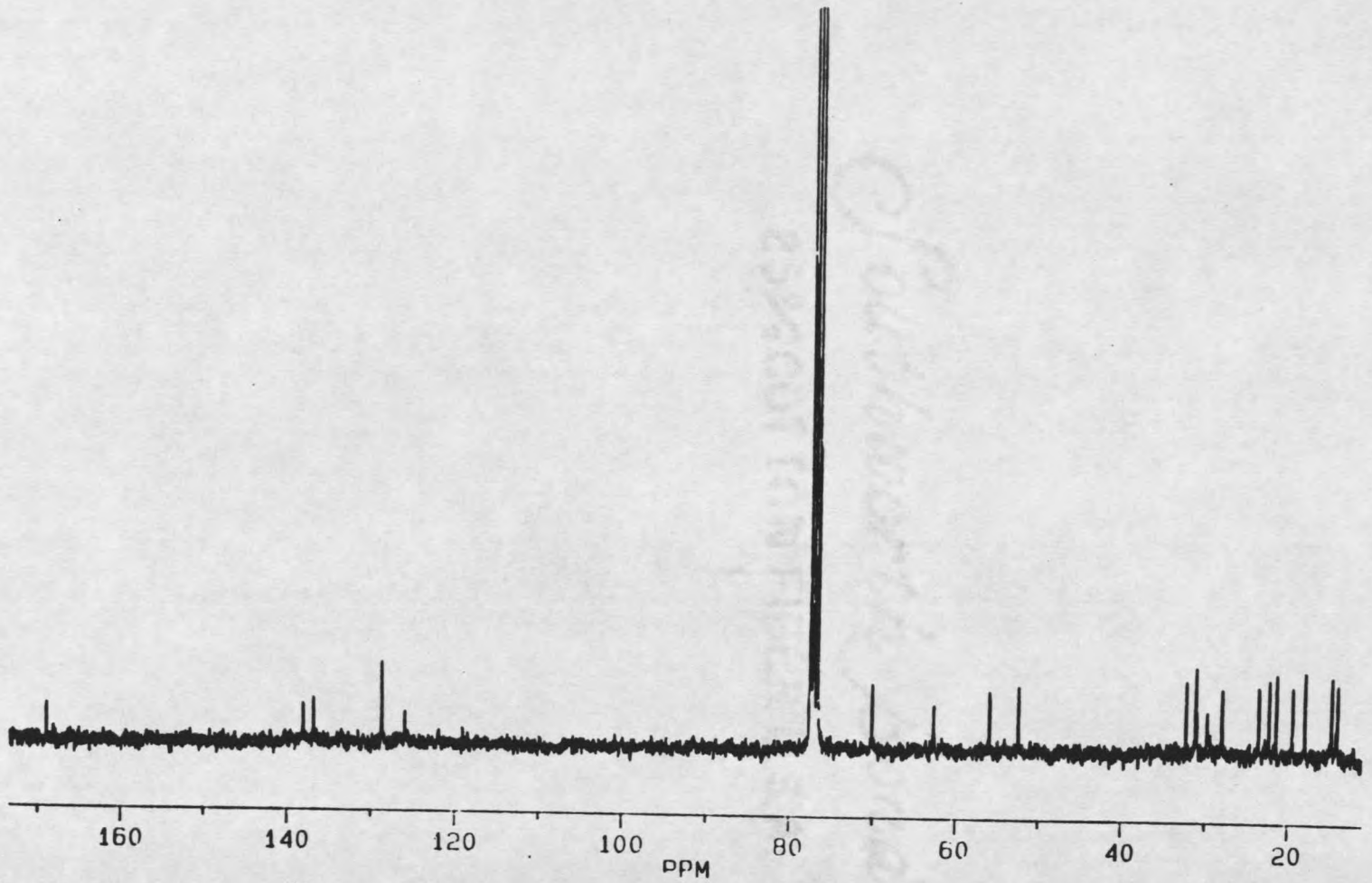


Figure 22. 75 MHz ^{13}C NMR Spectrum of **65** in $CDCl_3$.

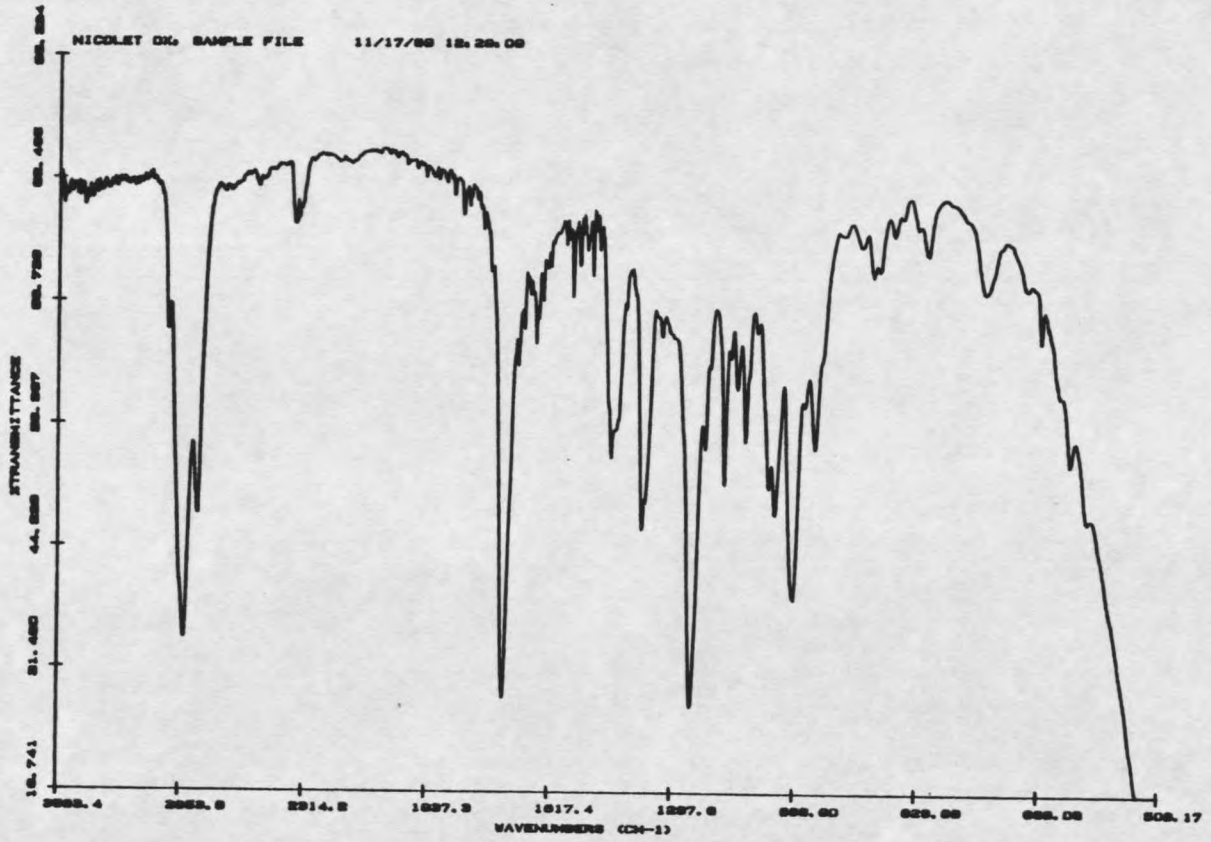


Figure 23. IR (Thin Film) of **65**.

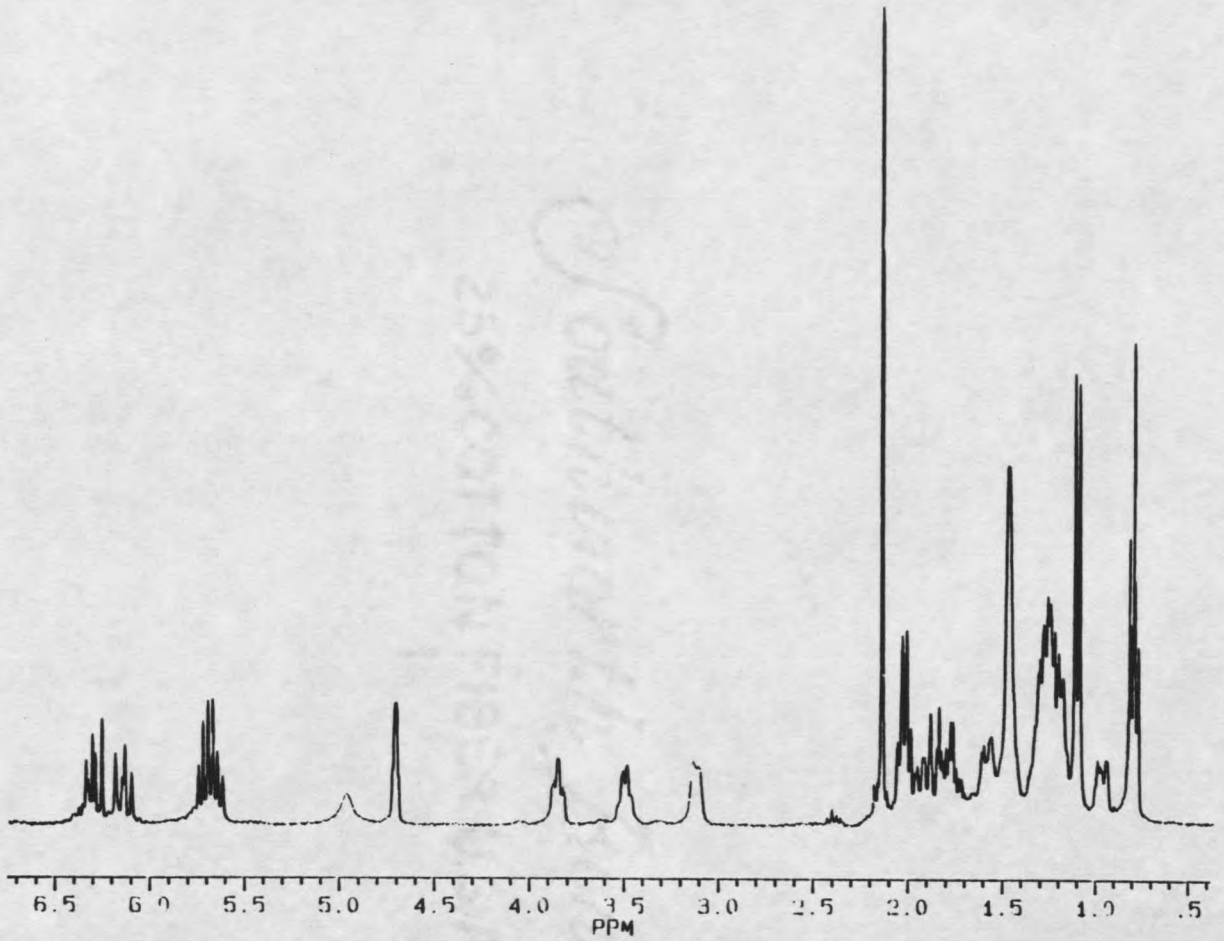
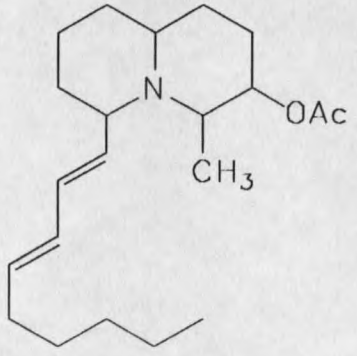


Figure 24. 300 MHz ^{1}H NMR Spectrum of **65** in Pyridine-d_5.

m/z 333 was noted. The mass observed was at first thought to be the result of a calibration error. It was believed that the mass was actually m/z 332, a loss of the methyl group from the C_{22} acetate, m/z 347. However, two peaks were observed on the GC trace. The first peak was m/z 333, whereas the second peak gave the parent mass of **51**. This could no longer be considered an anomaly, and the fraction was submitted for high resolution mass spectral analysis. HRMS supported the presence of a compound with a parent ion of m/z 333. When the fragmentation pattern of the smaller compound was compared to **51**, the same type of profile was noted (see Figure 24).

Although this compound was never isolated, it appeared to be another member of the family of quinolizidines. Based on the mass spectral data, which, unfortunately, has no supportive evidence, the structure **66** was assigned to this compound.



66

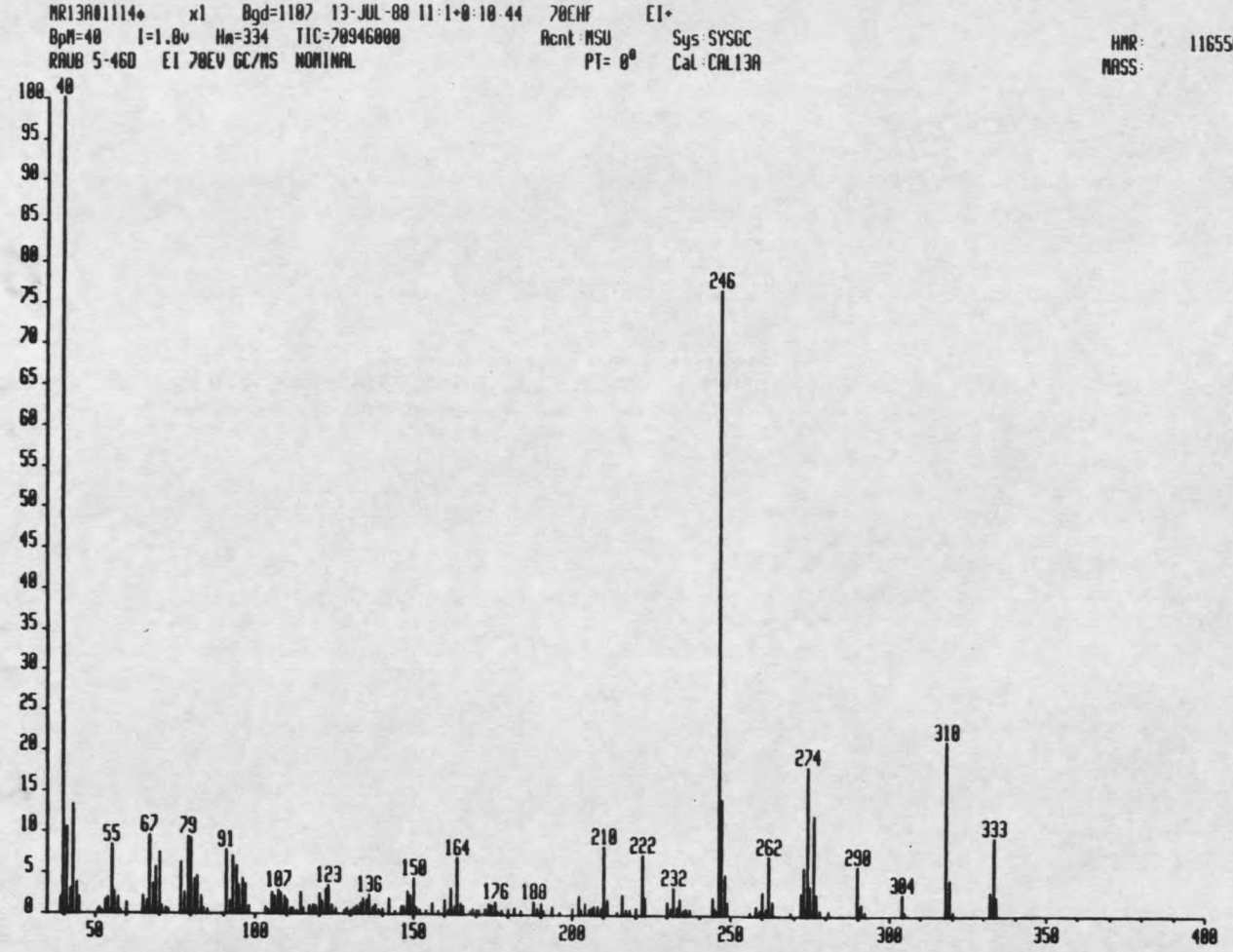
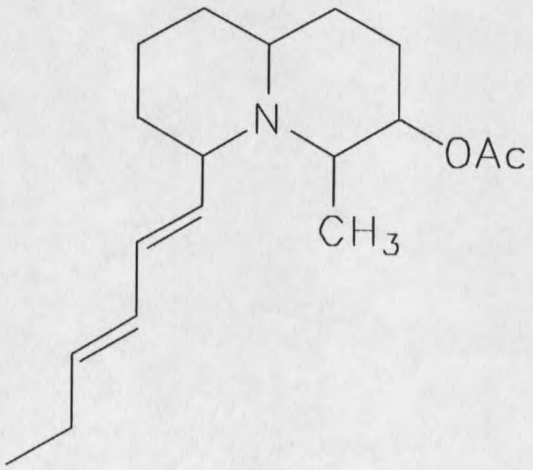


Figure 25. EIMS of **66**.

When the more polar fractions of the CCC runs were investigated, mass spectral analysis indicated the presence of yet another compound which had a profile very much like that of **51** and **65**. It had eluted within the first thirty minutes of the chromatography with other polar substituents. However, unlike the other polar substituents, which would not elute from a GC column on the mass spectrophotometer, it would always elute from the column. Efforts to isolate the compound, m/z 291, were unsuccessful. HRMS of the compound yielded a molecular formula of $C_{18}H_{29}NO_2$ (see Figure 26). Given the same number of sites of unsaturation, the similar fragmentation pattern and the ability to be eluted from the GC column, the compound was assigned the structure of **67**.



67

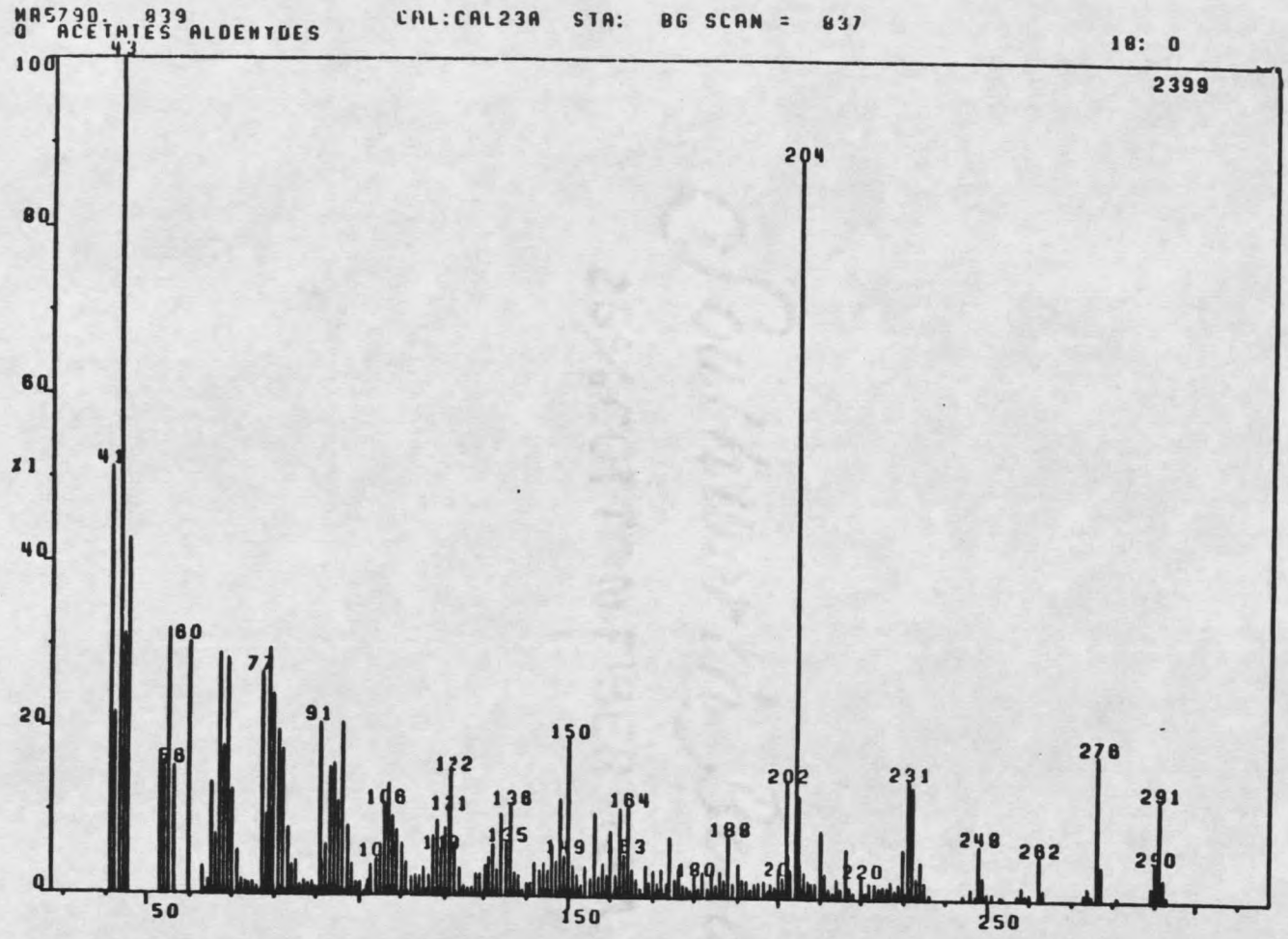


Figure 26. EIMS of 67.

Quinolizidine Acetate Trienes 69, 70

Compound **2**, which was isolated via the modified flash chromatography system and subsequent CCC, revealed a ^1H NMR in $CDCl_3$ which differed from **51** in two regions, the olefinic region and the region for alkyl chain methylenes (see Figure 27). A proton envelope at δ 6.02 appeared to be larger than the olefinic envelope of **51**, while the methylene envelope, centered around δ 1.08, seemed to contain fewer protons. While the ir measurements were very similar to those of **51**, a uv spectrum (EtOH) showed an absorbance maximum at 281 nm. This absorbance maximum was in good agreement for the calculated absorbance for a heteroannular conjugated triene[54]. EIMS yielded a parent peak at m/z 345 (see Figure 28). This molecular weight was supported by HRMS, which provided a formula of $C_{22}H_{35}NO_2$. The molecular formula was considered further evidence for the presence of a third double bond.

Homodecoupling and difference decoupling experiments were undertaken in pyridine-d_5, as the olefinic region could be resolved better with this solvent. A one proton multiplet, centered at δ 5.71, was overlapped on the downfield side by a broad, one-proton resonance. Also observed was a one proton triplet at δ 6.16 slightly overlapped with what appeared to be a signal that

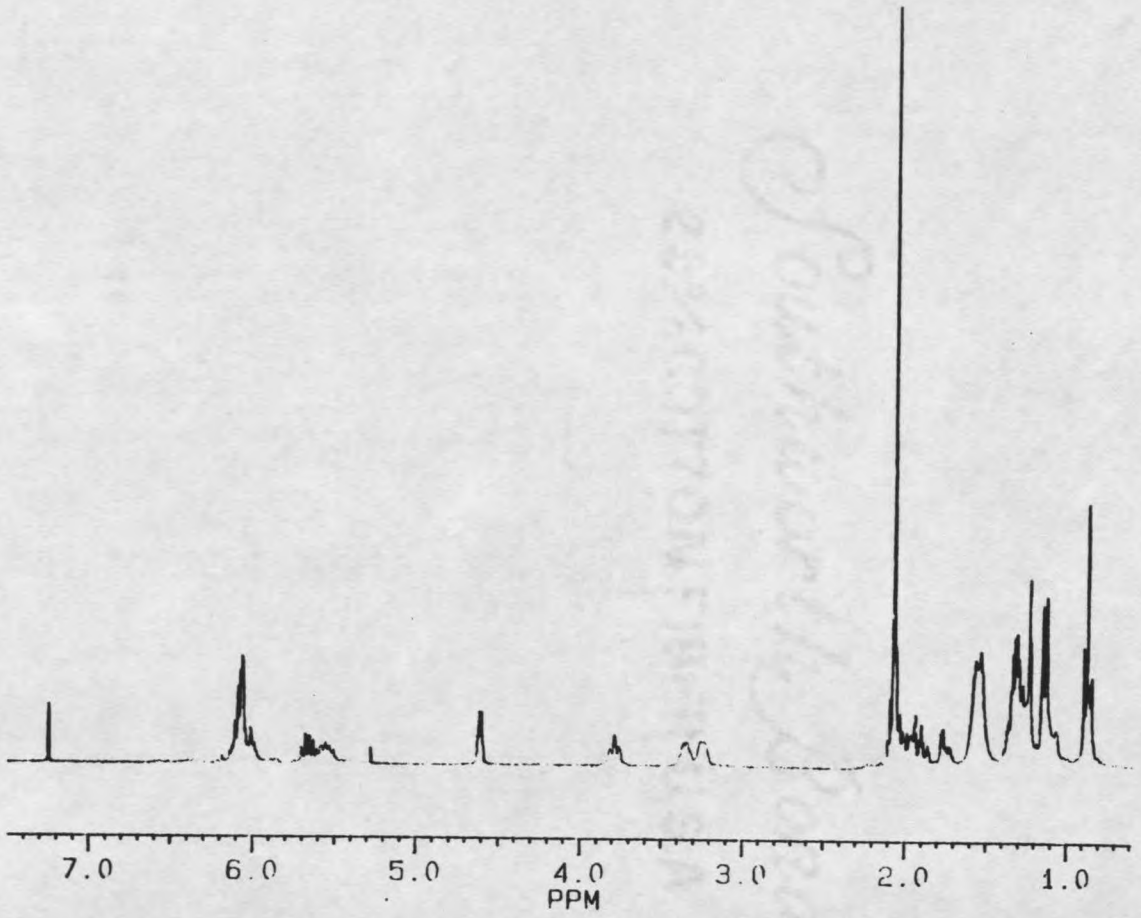


Figure 27. 300 MHz ^{1}H NMR Spectrum of **69** in $CDCl_3$

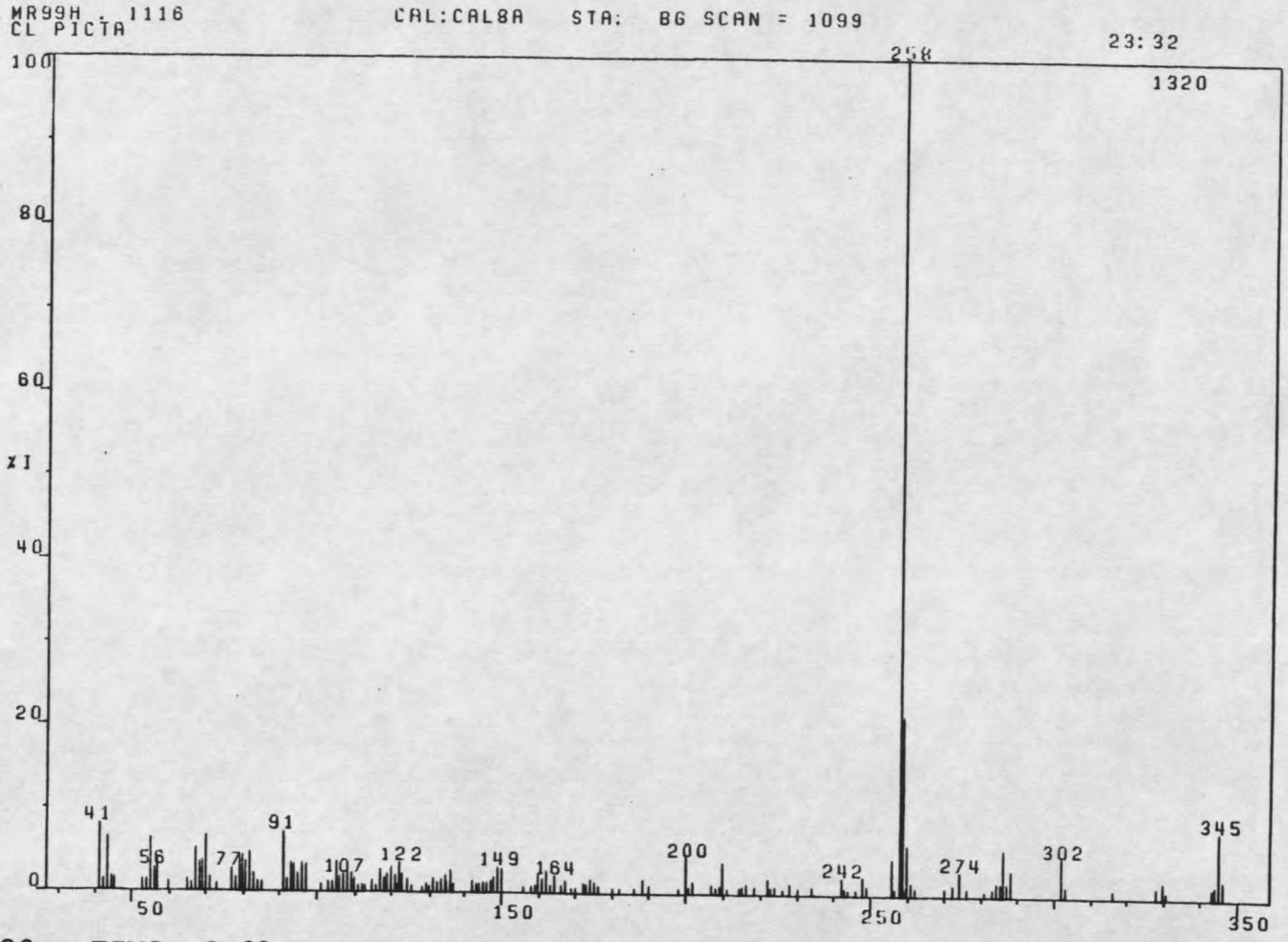
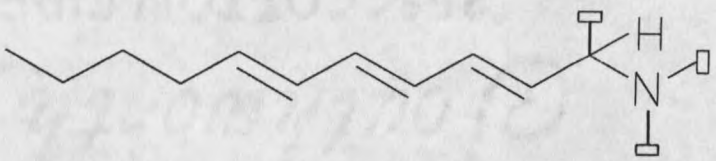


Figure 28. EIMS of **69**.

traversed the spectrum from δ 6.1 to 6.3. Another multiplet, two protons, was centered at approximately δ 6.3. When the methylene quartet at δ 2.04 was decoupled, the multiplet at δ 5.71 collapsed to a doublet, J=14.8 Hz. The coupling constant indicated trans-substitution, as was the case with **1**. Decoupling the olefinic resonance at δ 5.71 led to the collapse of the triplet at δ 6.16. The coupling constant of the signal at δ 6.16 could not be measured due to interference from the other overlapping resonance. However, measurements prior to decoupling indicated that the two coupling constants were approximately 12 and 14.8 Hz. Decoupling the doublet of doublets led to the partial collapse of the multiplet at δ 5.71 to a triplet. The coupling constant was not measured, because the broad one-proton signal overlapping that region of the spectrum interfered with a proper measurement. The furthermost downfield signal at δ 6.4 had changed ever so slightly when the triplet at δ 6.16 was decoupled. It was suspected that the other signal adjacent to the resonance at δ 6.16 was a signal which was partially superimposed on the triplet at δ 6.16 itself. When the signal centered at δ 6.3 was irradiated, the triplet at δ 6.16 coalesced to a poorly defined signal, and the broad, one-proton resonance centered at approximately δ 5.8

seemed to sharpen to a small degree. With the irradiation of the resonance centered at δ 5.8, the multiplet became partially resolved, and a doublet with a measured J value of 12 Hz was observed. The resonance at δ 3.9, the same chemical shift for the allylic methine of **51**, was also irradiated, and the broad signal at δ 5.8 sharpened. Again, no coupling constants were measured due to overlap of the signal at δ 5.71. Hence, although some signals did overlap, the decoupling data were consistent with a triene, and, in light of the other aforementioned spectral data collected, provided further evidence for a triene, as in **68**.



68

Finally, a ^{13}C NMR spectrum was obtained on the 300 MHz NMR in $CDCl_3$ (see Figure 29). Compared to the ^{13}C NMR spectrum of **51**, it was clear that there were two carbons less in the upfield, sp^3 region of the spectrum, while five, instead of four, signals resided in the olefinic region. Since the resonance at δ 130.32 was double the

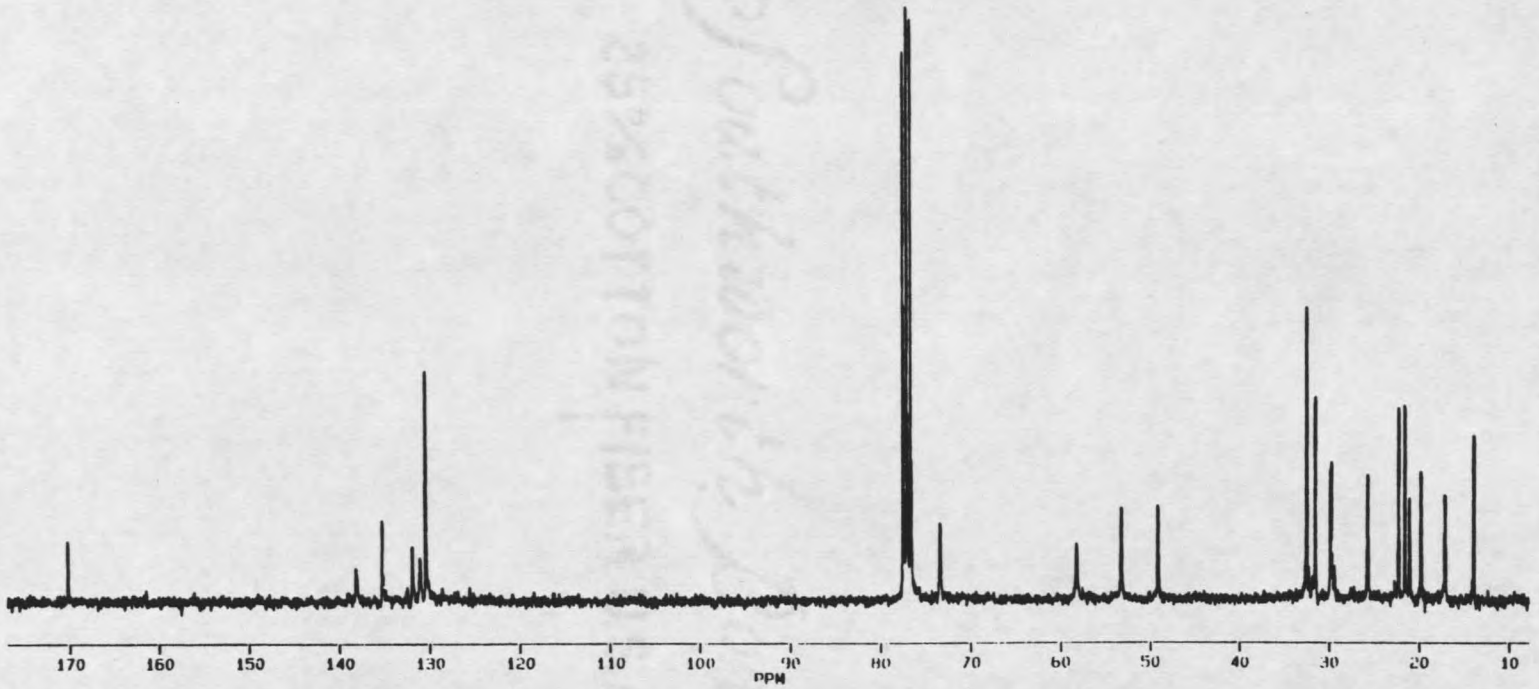
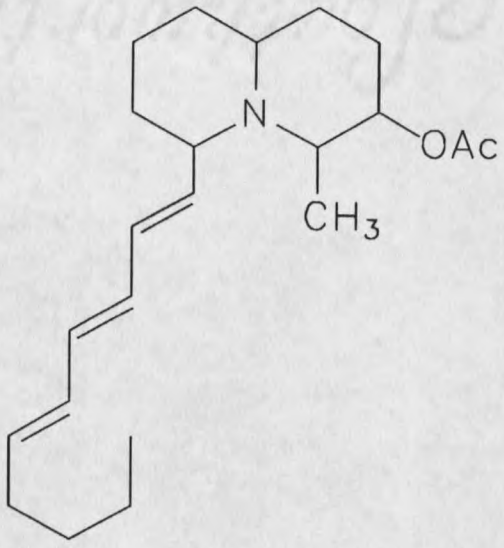


Figure 29. 75 MHz ^{13}C NMR Spectrum of **69** in $CDCl_3$.

height of the other olefinic signals, it apparently contained two carbons. The structural determinations indicated that the compound was the C_{22} quinolizidine acetate triene, **69**.



69

The CCC which resulted in the first successful isolation of **1** also yielded another triene, **4** (see Figure 4). Mass spectral analysis of the first fraction eluting from the column gave a spectrum characteristic of the other aforementioned quinolizidines but with a parent peak of m/z 317 (see Figure 30). This was two daltons less than the shorter chain diene, **65**. HRMS of the fraction yielded a chemical formula which indicated five sites of unsaturation, $C_{20}H_{31}NO_2$. Although the GC trace

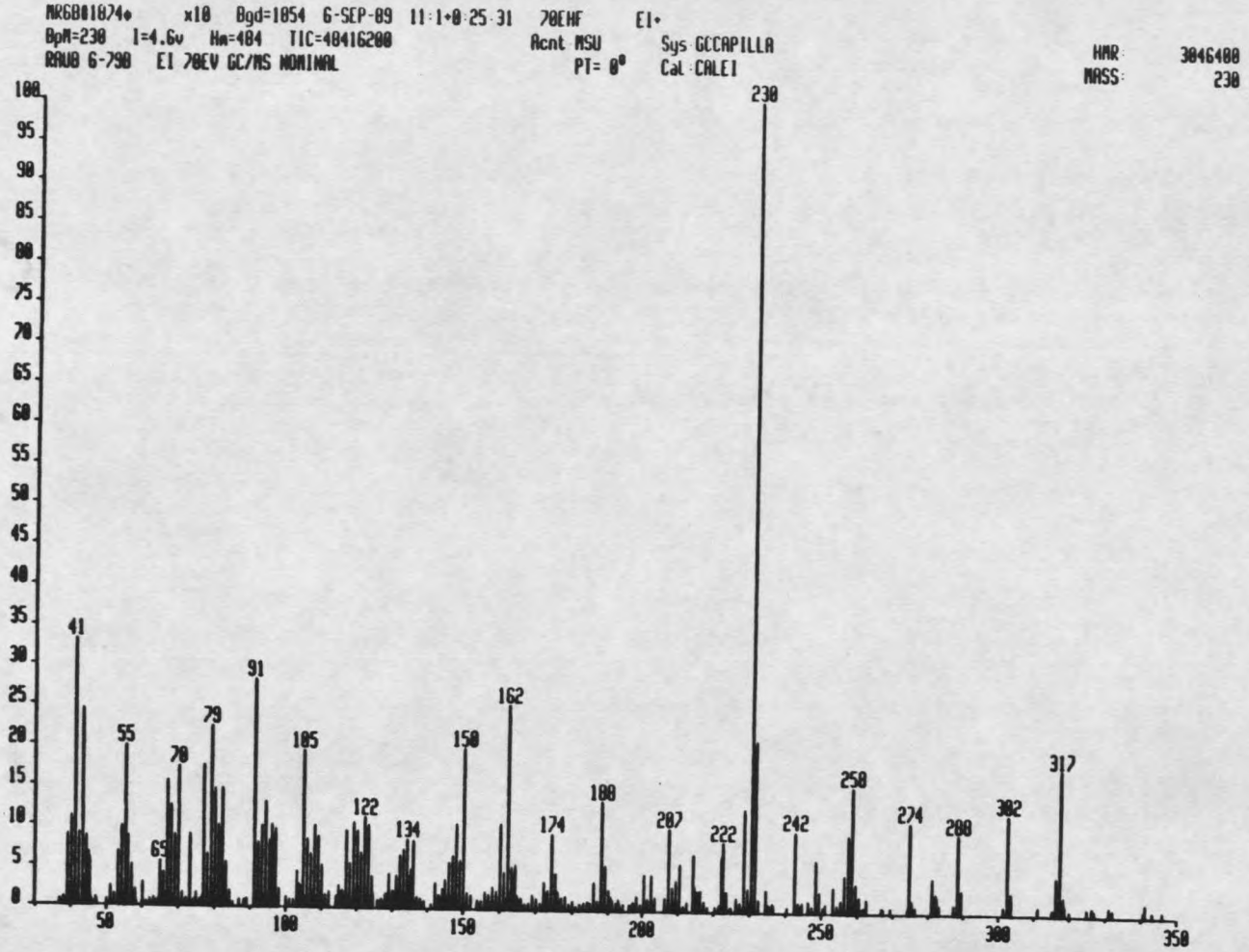
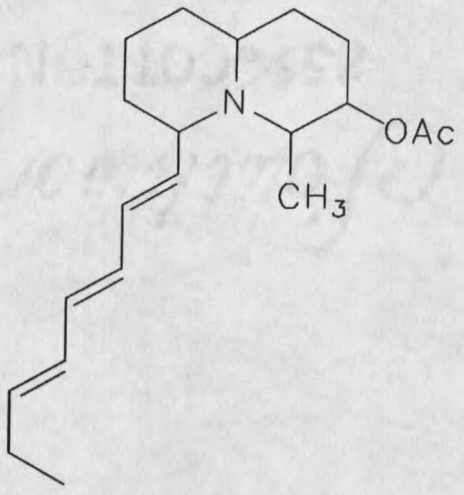


Figure 30. EIMS of **70**.

of the EIMS did not indicate any major contaminants in the fraction, the ^{1}H NMR did not yield a well-resolved spectrum. A profile typical of the quinolizidine of acetates was discernible, but decoupling experiments were unsatisfactory. The fraction was run on the CCC once again, but the apparent contaminants had not been removed from the compound. As a prior size exclusion chromatography had led to the loss of another fraction containing the apparent triene, no other cleanup attempts were undertaken. Curiously enough, the fraction did prove to be clean enough to collect very reasonable ir and uv spectra. The ir was quite similar to that of **69**, and the uv absorption maxima were almost identical to the maxima of **69**. The optical rotation was also in the same range of that of the other quinolizidine acetates. The evidence pointed once again to a quinolizidine acetate, in this case a C_{20} acetate triene, **70**.

As four quinolizidine dienes had been identified, it was deemed possible that other trienes of shorter or even longer bond length existed. A thorough search through the mass spectral data collected on all fractions did not turn up evidence for the presence of other trienes, however.



70

Alcohol Analogues of the Quinolizidine Acetates **74, 75**

Two compounds more polar than the longer chained acetates eluted from a CCC run at 1.3 and 1.75 hours, with baseline separation (see Figure 5). The ^{1}H NMR revealed the presence of two compounds, differing in the size of the methylene envelope centered around δ 1.2 (see Figures 31 and 32). This again suggested two similar compounds with alkyl chains of different length. Characteristic of the ^{1}H NMR was a methyl triplet at δ 0.85, with the aforementioned envelope centered at δ 1.2. A methyl doublet was shifted to δ 1.1, as with the methyl doublet of the C_{22} acetate. Many signals were compressed between δ 1.13 and 1.92, while a methylene quartet was isolated at δ 2.1. Two two-proton signals

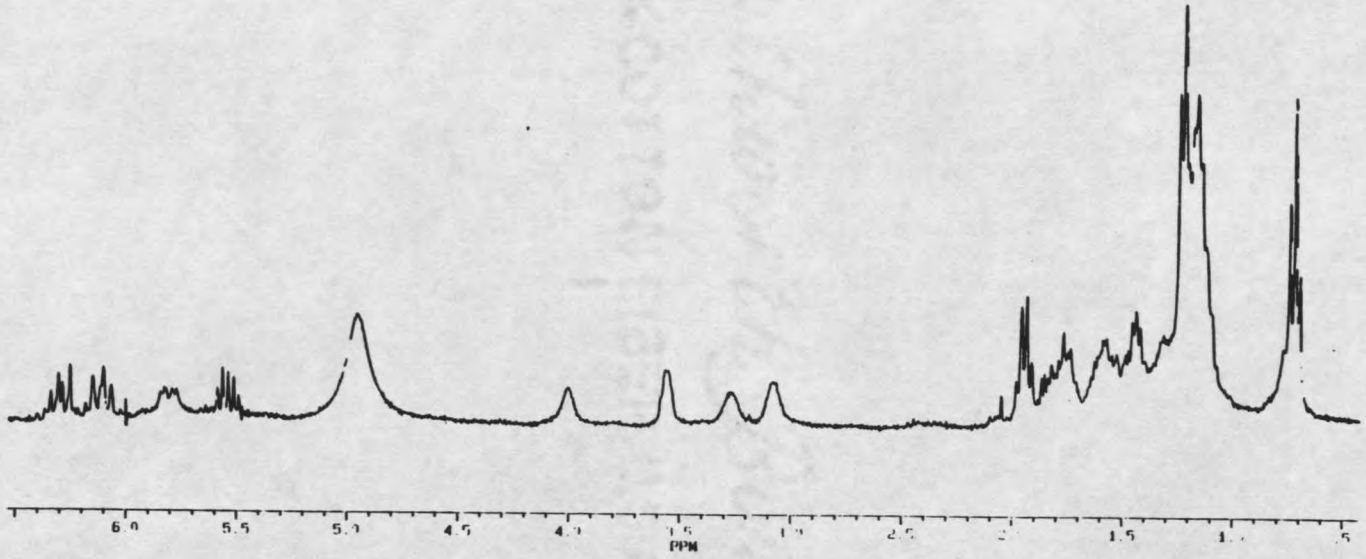


Figure 31. 300 MHz ^{1}H NMR Spectrum of **75** in Pyridine-d_5.

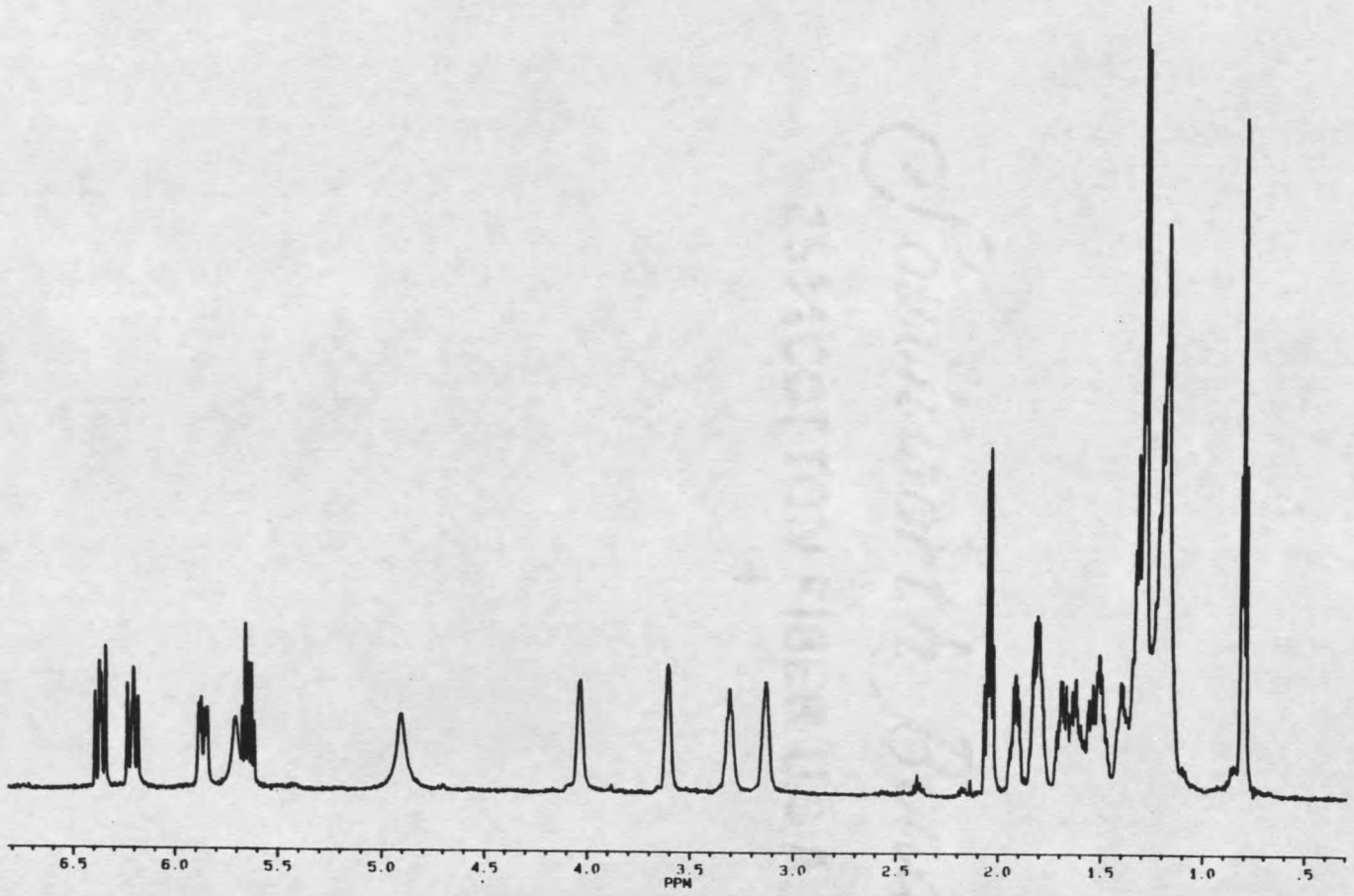


Figure 32. 500 MHz ^{1}H NMR Spectrum of **74** in Pyridine-d_5.

were shifted to δ 3.0 and 3.5, respectively, and a proton profile in the olefinic region similar to that of the C_{22} acetate was observed. Low resolution mass spectral analysis of the two compounds yielded molecular ions at m/z 277 for the smaller, and m/z 305 for the larger compound (see Figures 33 and 34). HRMS supported the EIMS data and suggested formulas of $C_{18}H_{31}NO$ and $C_{20}H_{35}NO$, respectively. The unsaturation number for both compounds was four, and there was no indication for the presence of an acetate in the mass spectrum or in the ^1H NMR spectrum. The uv (EtOH) absorption maximum was the same as that of **1**, but the ir spectrum showed a profile unlike that of **1**. No absorbance for an acetate was present. Instead, a broad peak centered at 3200 cm^{-1}, typical of an alcohol moiety, was observed (see Figure 35).

A proton spectrum (500 MHz NMR, $CDCl_3$) of the C_{20} compound in pyridine-d_5 resolved all four midrange protons and the four olefinic protons to eight distinct signals. The higher field instrument also served to disperse the upfield region of the spectrum. Difference decoupling and homodecoupling experiments generated a familiar structural pattern, reminiscent of the quinolizidine profile.

When the broad quartet at δ 4.03, J=6.2 Hz, was irradiated the vinylic doublet of doublets at δ 5.87,

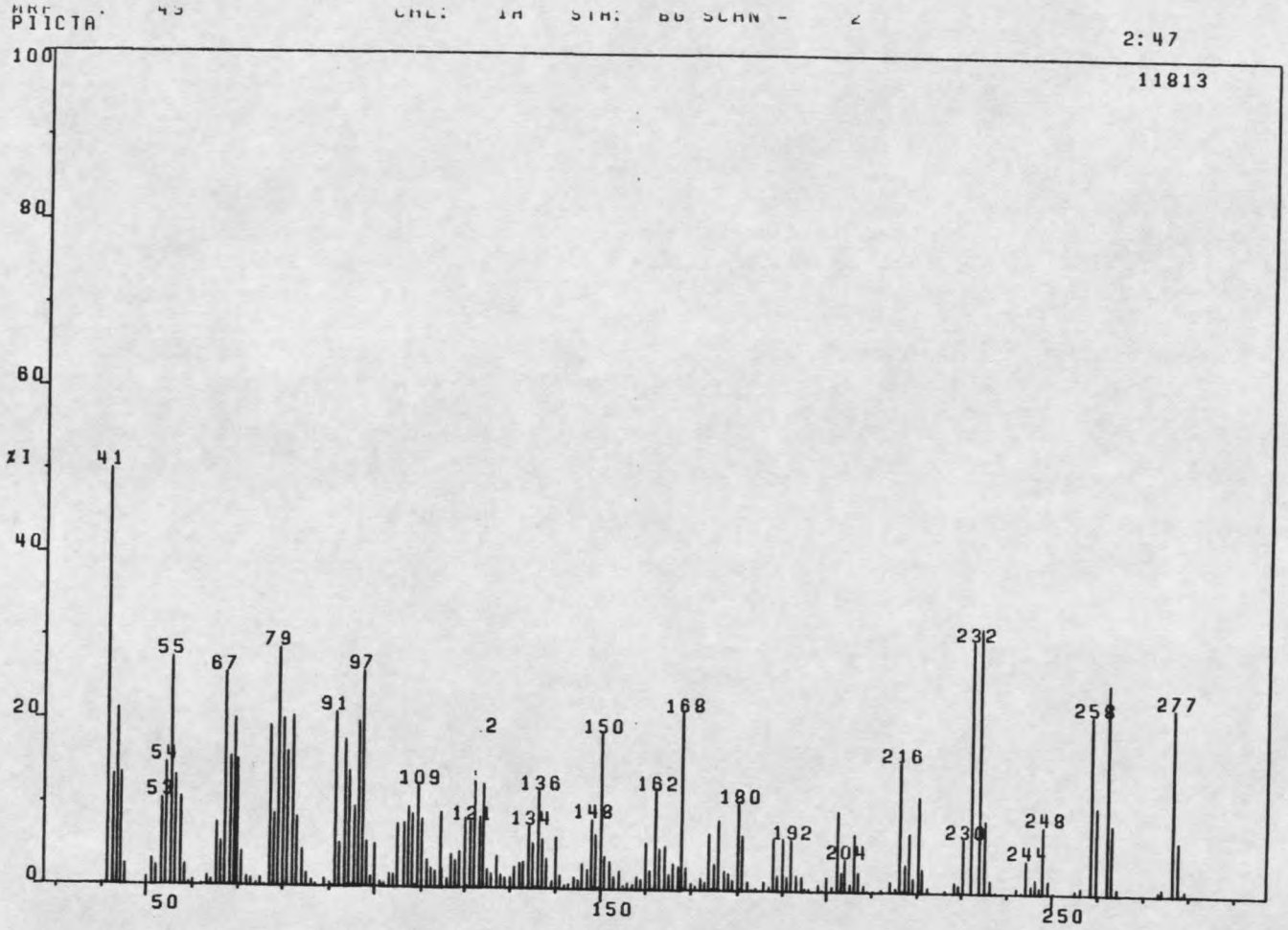


Figure 33. EIMS of 75.

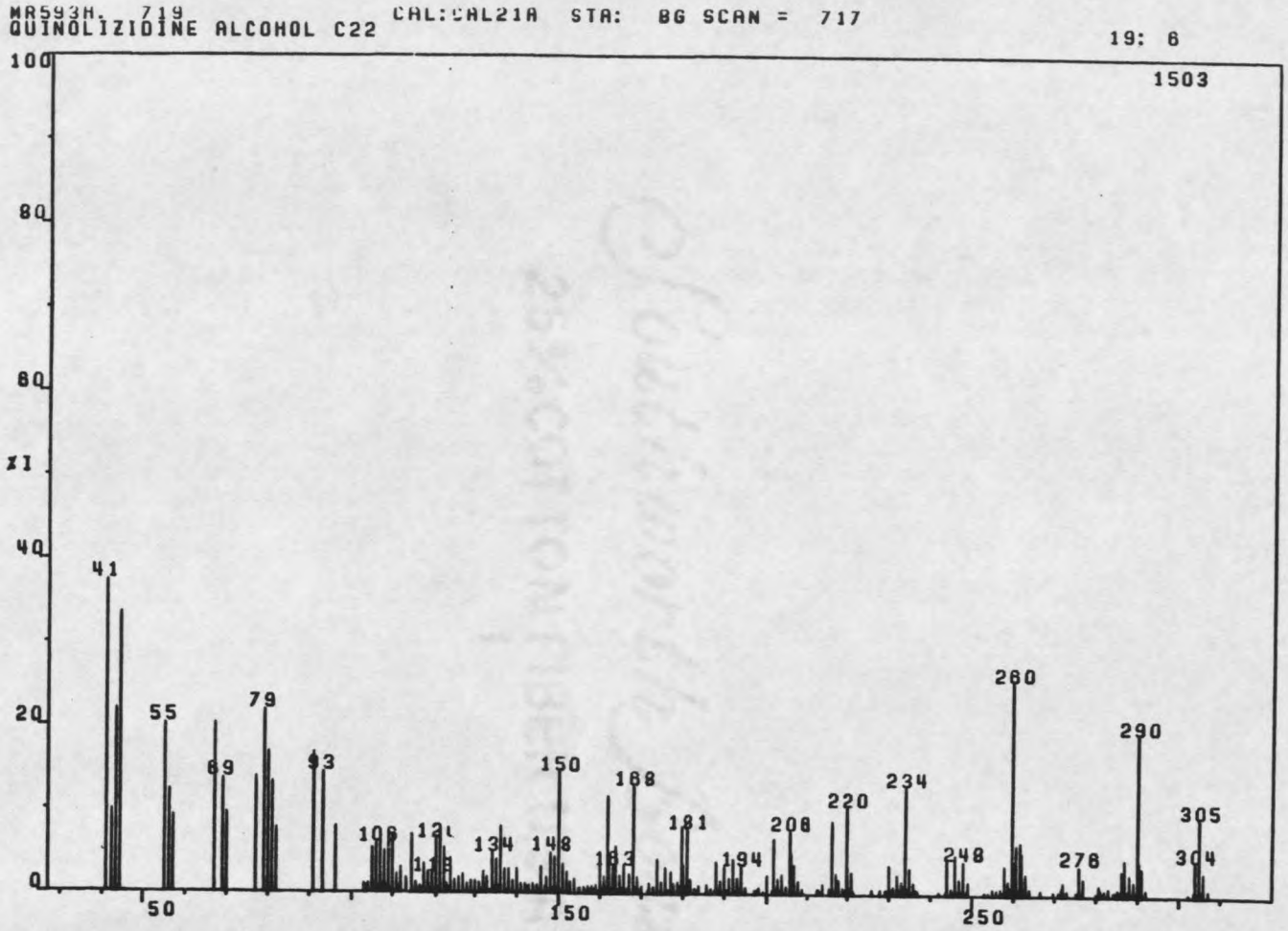


Figure 34. EIMS of 74.

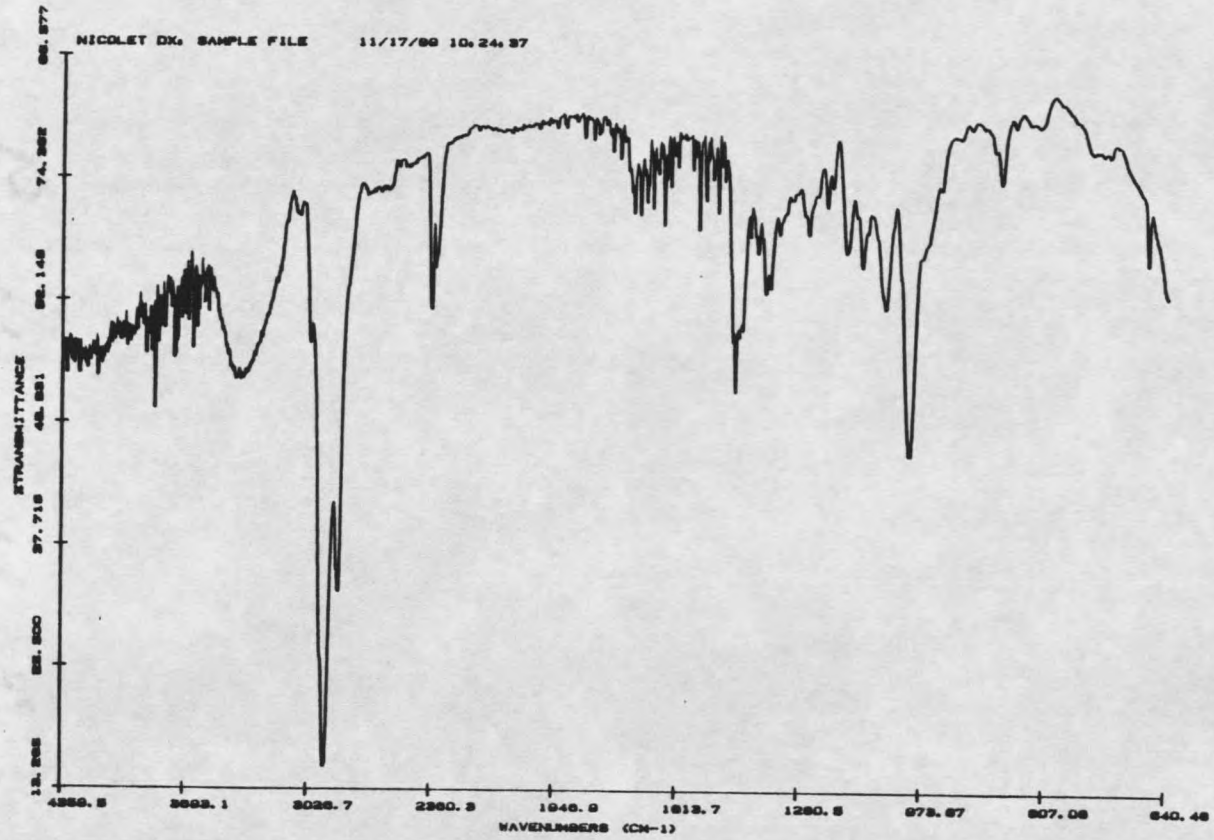
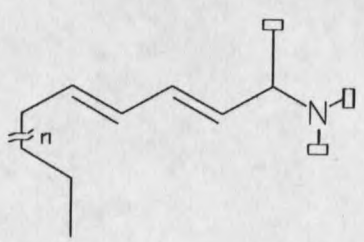


Figure 35. IR (Thin Film) of **74**.

J=6.2, 15.3 Hz, collapsed to a sharp doublet, J= 15.3 Hz. Irradiation of the vinylic proton collapsed the doublet of doublets at δ 6.38 (J=10.5, 15.3 Hz) to a doublet, with the remaining coupling constant measured at 10.5 Hz. This would indicate that the first double bond is trans substituted, owing to the large coupling constant. The resonance at δ 6.38 was irradiated next, and the adjacent upfield doublet of doublets at δ 6.21, J=10.5, 15 Hz, coalesced to a doublet, J=15 Hz. This, again, was indicative of a trans-substituted olefin. The remaining multiplet collapsed to a less well defined signal when the signal at δ 6.21 was irradiated, suggesting its placement at the other end of the second olefin. The adjacency of the allylic methylene quartet at δ 2.0 was shown by irradiating the olefinic proton at δ 5.63; it collapsed to a broad triplet. The structure of two conjugated double bonds, both trans disubstituted, was thus established, **71**.

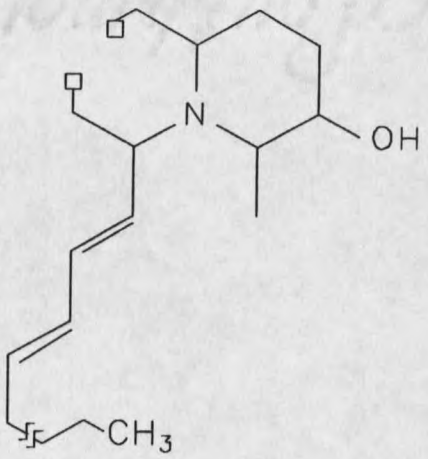


71

The irradiation of the broad singlet at δ 3.6 caused the adjacent upfield broad methine multiplet to collapse to a broad quartet, and two signals, δ 1.9 and 1.8, appeared to lose some small coupling constants. Further upfield the broad methine multiplet at δ 3.3 was irradiated and was determined to be a methyl-bearing methine; the methyl doublet at δ 1.1 collapsed to a singlet. Two signals were observed to collapse between the range of δ 1.6-1.65 and 1.35-1.45 when the broad one-proton signal at δ 3.12 was irradiated.

Before irradiation of the upfield signals was attempted, difference decoupling experiments were performed on the three methine signals at δ 3.6, 4.0 and 3.1, respectively. The resonance at δ 3.6 was confirmed to be coupled to two signals, centered at about δ 1.9 and 1.8. With difference decoupling, three signals were observed to be coupled to the one-proton signal at δ 3.1; homodecoupling had shown only two. The two signals previously observed were centered at approximately δ 1.6 and 1.4, whereas the other signal was centered at δ 1.8. The third signal overlapped with one of the protons adjacent to the supposed alcohol-bearing methine. Coupling of the allylic methine at δ 4.0 to a signal or signals spread across the range from δ 1.65 to 1.73 was also demonstrated. To that point in the structure

elucidation, the position of the alcohol, the methyl group and the unsaturated chain, was established. Also, portions of both rings were also determined, **72**.

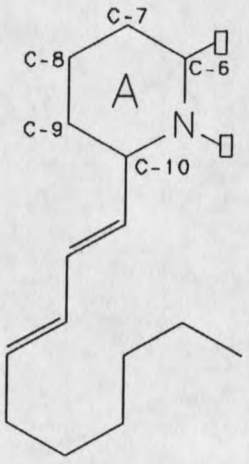


72

Difference decoupling had revealed a very important piece of information by not showing any coupling to the region between δ 1.45 and 1.6. Homodecoupling experiments were then undertaken in the upfield region to see if any connectivities could be discerned. Irradiation of the resonance at δ 1.4 decoupled the one-proton signal at δ 1.9 and two protons seemingly superimposed on one another at δ 1.8. Irradiation of the signals at δ 1.8 confirmed the previous result; they were all coupled to each other. When the signal at δ 1.6-1.65 was irradiated with a low decoupling power, it caused a

partial collapse of only the signals between the range of δ 1.45-1.55. Low power irradiation of the latter signal simplified the signal spread from δ 1.6 to δ 1.65, and more importantly, the signal from δ 1.65-1.75 (see Figure 36).

If the hypothesis that the molecule was the C_{20} analogue of the C_{22} acetate, **51**, were correct, the decoupling data demonstrated the connectivity between C-7, C-8 and C-9, thus establishing the structure of the left, or A, ring of the quinolizidine, **73**.



73

It had not been possible to make a clear connection between these three units of the A ring skeleton with the quinolizidine acetates by simple one-dimensional experiments alone.

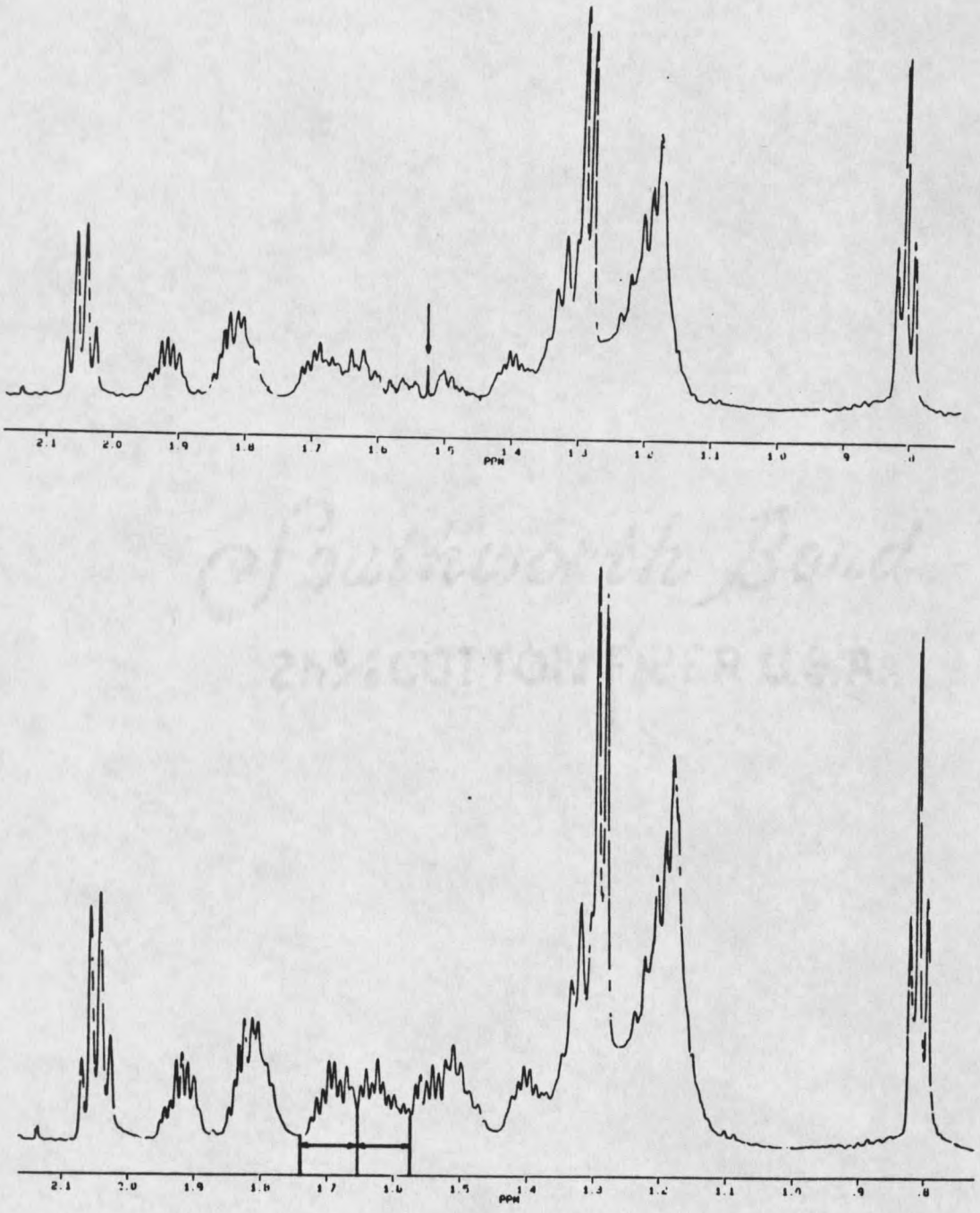


Figure 36. 500 MHz ^{1}H NMR Spectrum of **74** in Pyridine-d_5. A. Standard Spectrum. B. Irradiated Spectrum. Irradiation at Vertical Arrow.

Confirmation of the structure of the alcohol was gleaned from the hydrolysis of the C_{22} acetate, **1**. A 10 mg aliquot of **51** was hydrolyzed with barium hydroxide, giving a quantitative yield of the alcohol, which crystallized upon removal of the solvent. All spectral data collected for the hydrolyzed compound were identical to the data for the naturally occurring C_{20} alcohol. A ^{13}C NMR experiment (pyridine-d_5) of the hydrolyzed compound (see Figure 37) delineated every carbon, as was the case with the acetate. The four olefinic signals were determined to be at δ 134.4, 133.6, 130 and 129.9, respectively. The chemical shift of a carbon at δ 70.6 was typical of the alcohol-bearing carbon. A probable assignment for the allylic carbon was the resonance at δ 60.8, whereas the ring juncture carbon and the methyl-bearing carbon probably shifted to δ 58.8 and 47.2, respectively. The remainder of the carbon shifts were spread between δ 32.6 and 13.9. A HETCOR experiment (see Figure 38 and Table 4) substantiated the assignments of the midrange carbons and also provided a much clearer determination of the individual carbons of the A ring. The resonances at δ 28.3, 20.6 and 28.1 correlated to C-7, C-8 and C-9, respectively. The remaining two ring carbons, C-4 and C-5, could be assigned chemical shifts of δ 29.3 and 26.2. The ring methyl was assigned to the

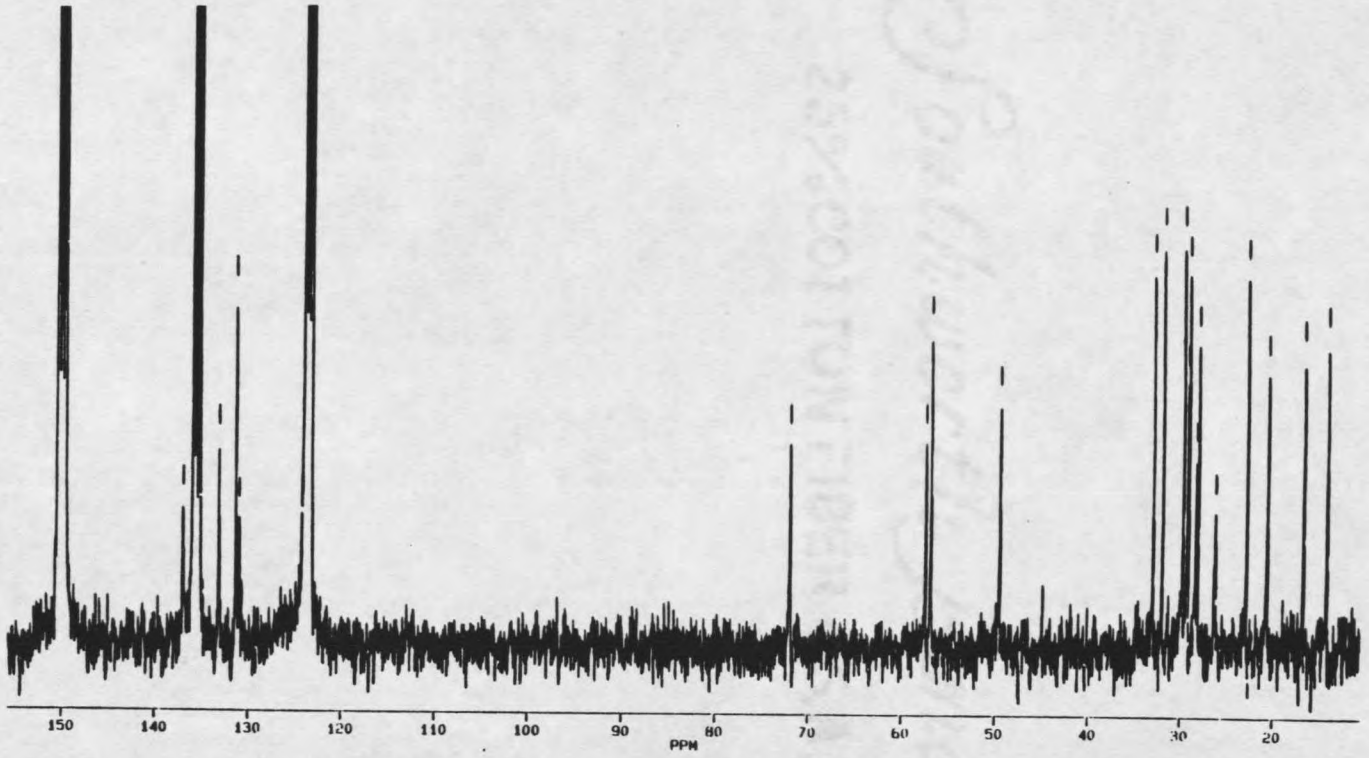


Figure 37. 75 MHz ^{13}C NMR Spectrum of **74** in Pyridine-d_5.

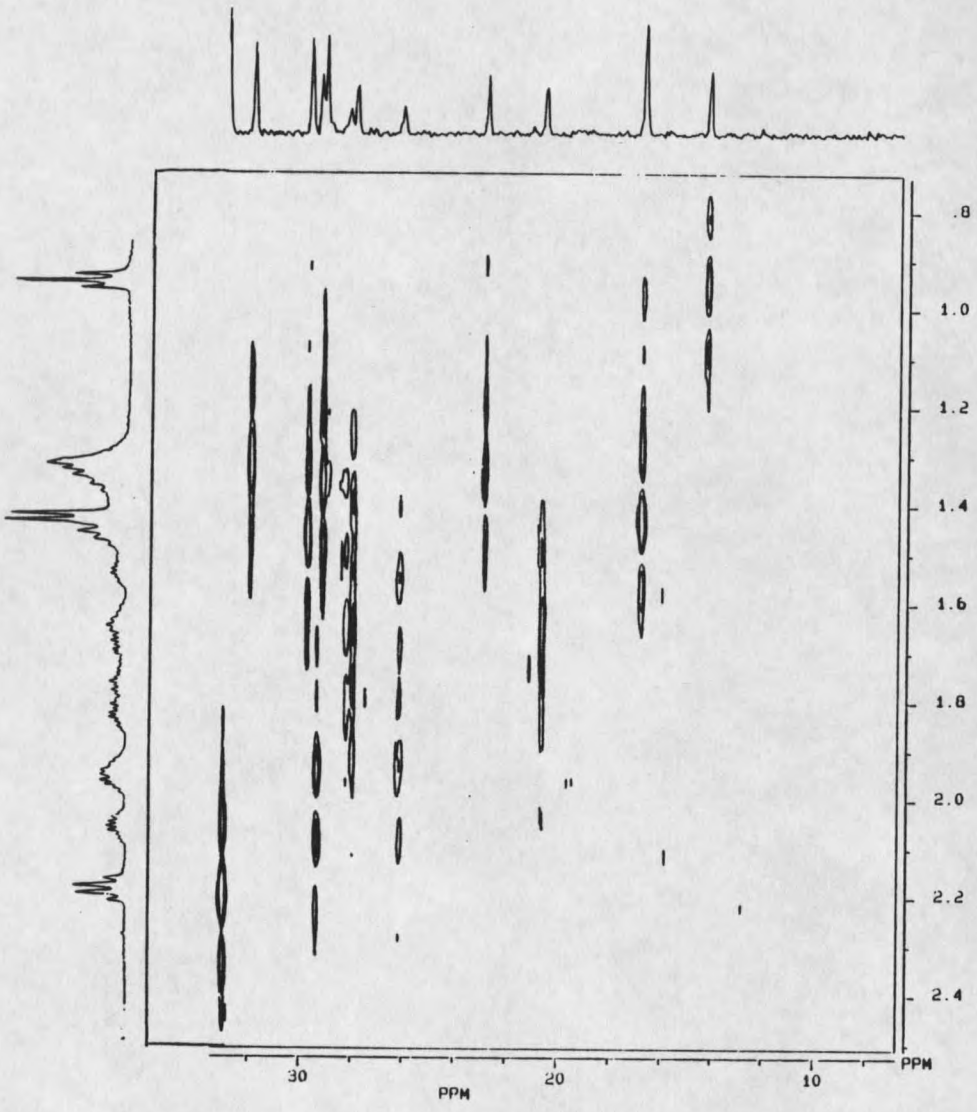


Figure 38. 125 MHz HETCOR Spectrum of **74** in Pyridine-d_5.

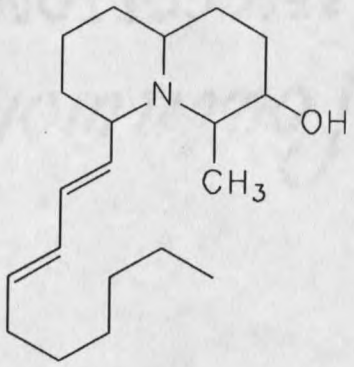
Table 3. NMR Data, **74**.[a]

Carbon #	^{13}C(pyridine-d_5)	^{1}H(pyridine-d_5)
1	16.7	1.3, d, 3H(J=6Hz)
2	58.8	3.3, br t, 1H(J=6Hz)
3	70.6	3.6, br s, 1H
4	29.3	a)1.8, m, 1H b)1.9, m, 1H
5	26.2	a)1.8, m, 1H b)1.5, m, 1H
6	47.2	3.13, br m, 1H
7	28.3	a,b)1.6-1.65, m, 2H
8	20.6	a,b)1.45-1.5, m, 2H
9	28.1	a,b)1.65-1.73, m, 2H
10	60.8	4.03, br q, 1H(J=6Hz)
11	135.1	5.9, dd, 1H(J=15.3,6Hz)
12	130.9[b]	6.4, dd, 1H(J=15.3, 10.5Hz)
13	131.2[b]	6.2, dd, 1H(J=15, 10.5Hz)
14	136.9	5.63, m, 1H(J=15Hz)
15	32.9	2.1, q, 2H
16	26.7	1.44, m, 2H
17-19	32.9, 29.1, 22.8	1.1.3-1.4, m, 6H
20	14.2	0.81, t, 3H(J=7Hz)

[a]300 MHz (^{1}H), 75 MHz (^{13}C)
[b]interchangeable

peak at δ 16.2, whereas the chain carbons were found at δ 32.9 for the allylic methylene, δ 29.7, 29.1, 22.3, 31.9 and 14.8 for the remaining carbons.

The structural pieces suggested the structure of **74**, and it was hoped that a heavy atom derivative could be made to determine the absolute stereochemistry of the molecule, **74**.



74

As was explained in the section on derivatization, the formation of a crystalline product remained elusive. Very small crystals of the alcohol itself were sent to the laboratory of Dr. Jon Clardy, were successful X-ray crystallographic analysis was performed (see Figure 39).

It was satisfying to note that the structure of the alcohol agreed with both the structure elucidation of the quinolizidines and the relative stereochemistry defined by analysis of nOe measurements taken of the acetate, **51**.

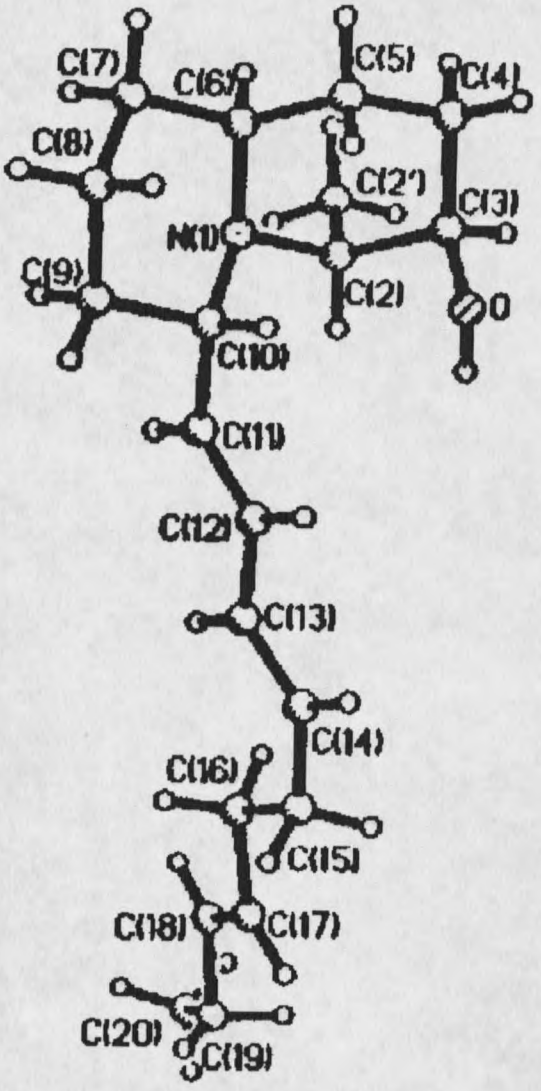


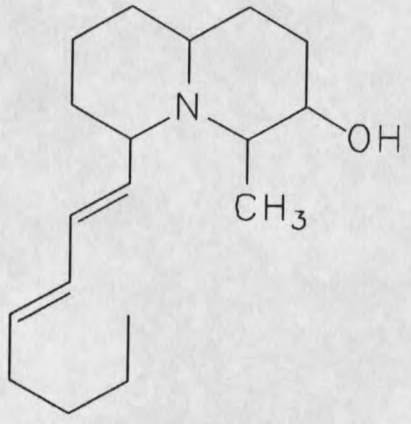
Figure 39. X-ray of **74**.

The diaxial relationship of the substituents at C-2 and C-3 seemed to imply a strained ring system. However, when the X-ray coordinates were entered into the Alchemy II program, the chair-chair conformation

generated by the program demonstrated only a very slight twist of the B ring. Also, the free electron pairs of the two heteroatoms described essentially a 180^0 arc, that is, they were directly opposite to one another. The X-ray coordinates were also used to run MM2 and AMI calculations with the TRIBL program in order to calculate the heat of formation. The value determined was -65 kcal, a value which compared very favorably with that of the acetate model compound, -59 kcal, which had been calculated previously. Although the model compound did not bear the complete alkyl chain, the differences in ΔH would not be expected to be very large. Since the heat of formation of the alcohol compared very favorably with those of the model compounds of differing stereochemistries, it was unlikely that the stereochemistry of the alcohol caused any large ring strain.

The other compound which had eluted just prior to **74** in the CCC run seemed quite similar to the alcohol in the ^{1}H NMR spectrum. The only obvious difference was in the region of the alkyl methylenes; the methylene envelop was visibly smaller. The HRMS, previously determined, also indicated the probable presence of a shorter chain alcohol. Both ir and uv spectra were identical to that of **74**. Homodecoupling and difference decoupling experiments also were unable to determine any differences

in the structure of the smaller compound. When the solvent of the fraction containing the compound was removed, crystal formation appeared to take place. A 5 mg amount of the C_{20} acetate, **65**, was hydrolyzed, yielding a compound identical to the C_{18} alcohol, **75**.



75

Chain Alcohols **77, 78**

Among the more polar fractions eluting from the CCC column within the first hour of the run was a fraction whose major component was apparently adsorbed to the GC column of the mass spectrometer. A DIP/EIMS run on the MM16 was successful, yielding what was first thought to be the M^+ peak, m/z 363. Loss of an acetate and water was observed, an indication of the polar nature of the

compound. However, the elution of the alcohols **74** and **75** from the GC column suggested that perhaps the compound in question contained more than the one alcohol moiety. A low resolution DIP/EIMS run on the VG-7070 gave m/z 379 (M^+) (see Figure 40), and HRMS supplied a formula of $C_{22}H_{37}NO_4$, with five sites of unsaturation. Since the highly polar nature of the compound had already been established, the possibility of more than one alcohol unit was likely.

The mixture of compounds in the fraction gave confusing results when homodecoupling and difference decoupling experiments were attempted. A ^1H-^1H COSY also was not very helpful in resolving the structure of the major compound. Therefore the compound was reacted with acetic anhydride to give a triacetate. The three acetate signals were readily observable in the ^1H NMR spectrum, and DIP/EIMS gave the expected m/z 463 (M^+). HRMS confirmed the expected chemical formula, $C_{26}H_{41}NO_6$.

A 500 MHz ^1H NMR spectrum (see Figure 41) indicated that the compound had been protonated during the reaction due to the formation of acid. Thus the spectrum revealed an upfield region with a pattern essentially identical to the other protonated quinolizidine acetates, from the

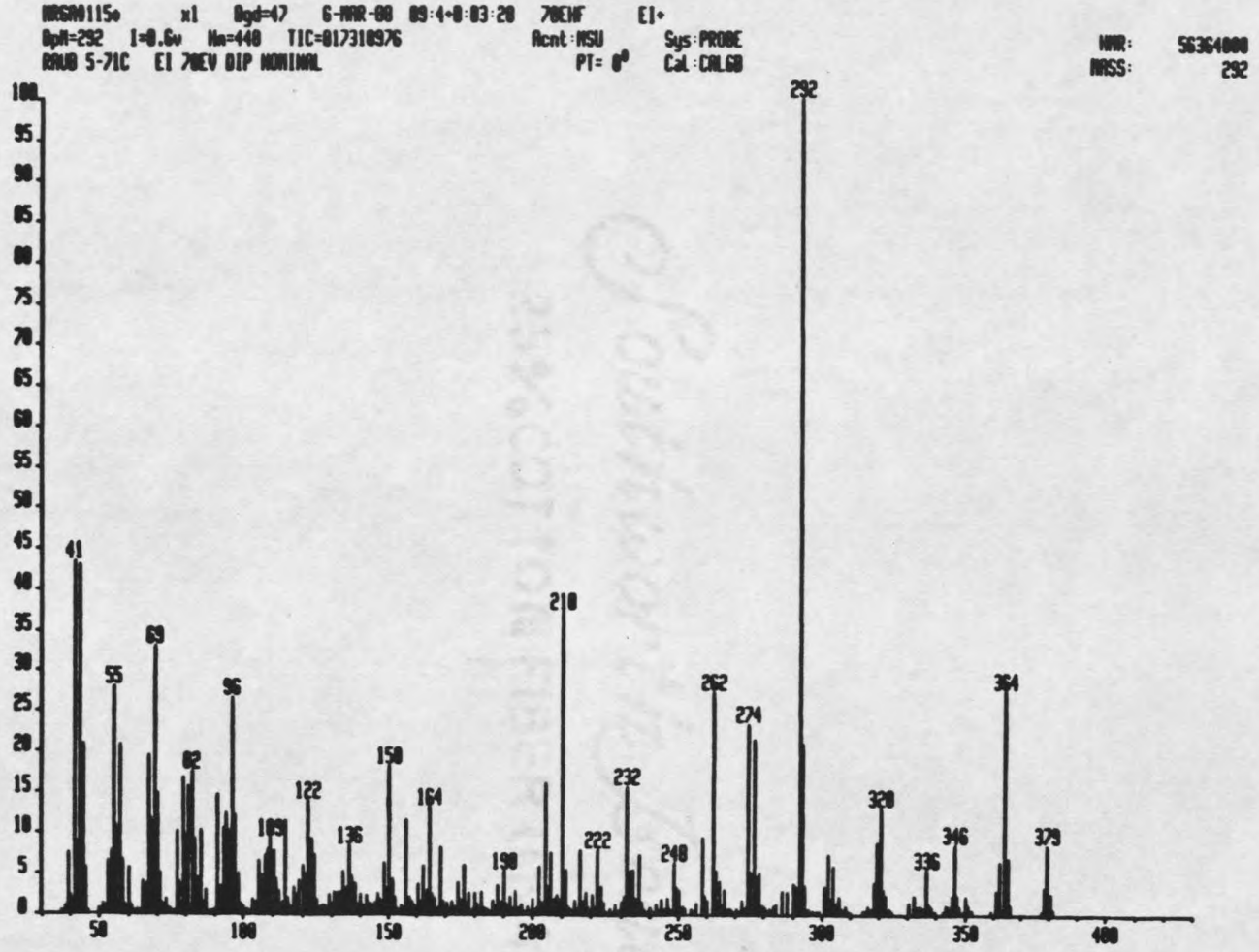


Figure 40. EIMS of 77.

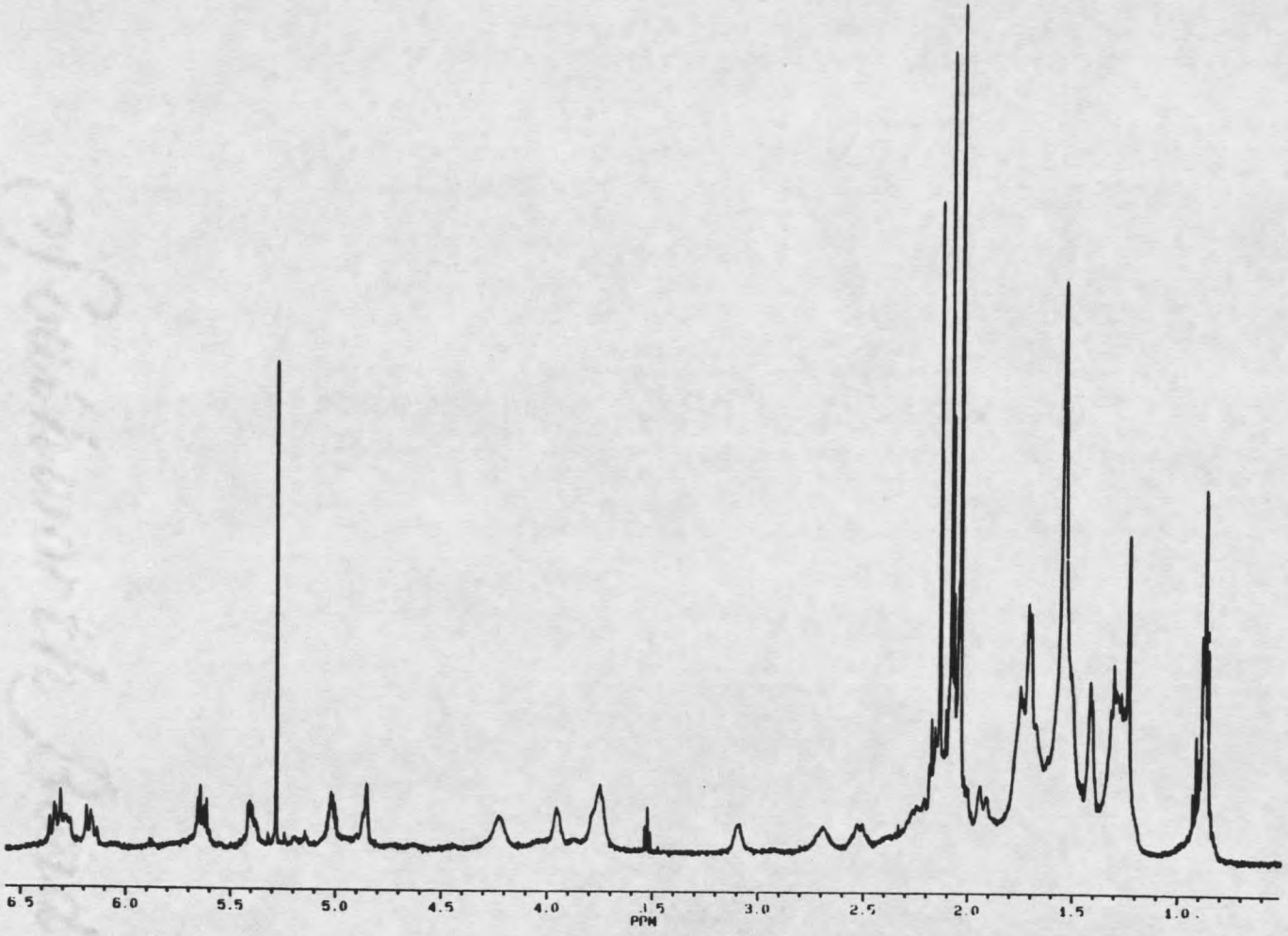
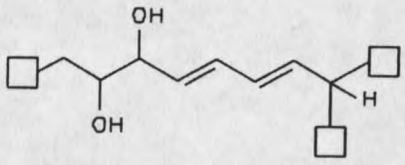


Figure 41. 500 MHz ^{1}H NMR Spectrum of Triacetate of 77 in Pyridine-d_5.

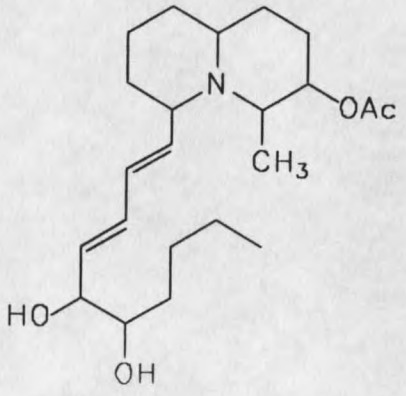
methyl triplet at δ 0.88 to the acetate-bearing methine at δ 4.85. The signal downfield from the methine at δ 4.85 was a one-proton multiplet at δ 5.03. Irradiation of the multiplet revealed coupling to the doublet of doublets at δ 5.4, which had collapsed to a doublet, J=6.7 Hz. Decoupling the resonance at δ 5.4 led to the collapse of the doublet of doublets at δ 5.64, J=6.7 Hz, to a doublet with a large J value, 15.7 Hz. The large coupling constant suggested that the signal at δ 5.64 was olefinic, attached to a trans-double bond. When the signal at δ 5.64 was decoupled, the doublet of doublets at δ 6.35 also collapsed to a doublet. The large coupling constant, 15.7 Hz, was removed, leaving a J value of 12 Hz. When the proton signal at δ 6.35 was decoupled, the doublet of doublets δ 6.15 became a doublet, J=15 Hz. The J value was, again, consistent with that of a trans-disubstituted olefin. The olefin proton next to that at δ 6.15 was the doublet of doublets at δ 6.25, J=15, 10 Hz. The proton at δ 6.25 shared the 10 Hz coupling constant with the broad doublet at δ 4.2; the signal at δ 4.2 was decoupled, leaving the signal at δ 6.25 a doublet, J=15 Hz. The methine at δ 4.2 was only slightly downfield from the resonance of the allylic methine of the protonated quinolizidine acetates and was thus assigned the allylic position. The methine at

δ 4.0 was in agreement with the chemical shift of the acetate-bearing methine of the other acetates. The decoupling experiments yielded the part structure, **76**.



76

A ^{1}H-^{1}H COSY confirmed the connectivities determined from the decoupling experiments (see Figure 42). Also, the one-proton resonance at δ 5.03 was coupled to the region upfield, at δ 1.5-1.6. The signal at δ 1.5-1.6 appeared to be coupled further upfield, to the alkyl chain, ending with the terminal methyl. Thus, the data yielded a compound, **77**, with the quinolizidine skeleton and with two chain substituents vicinal to each other, two alcohols.



77

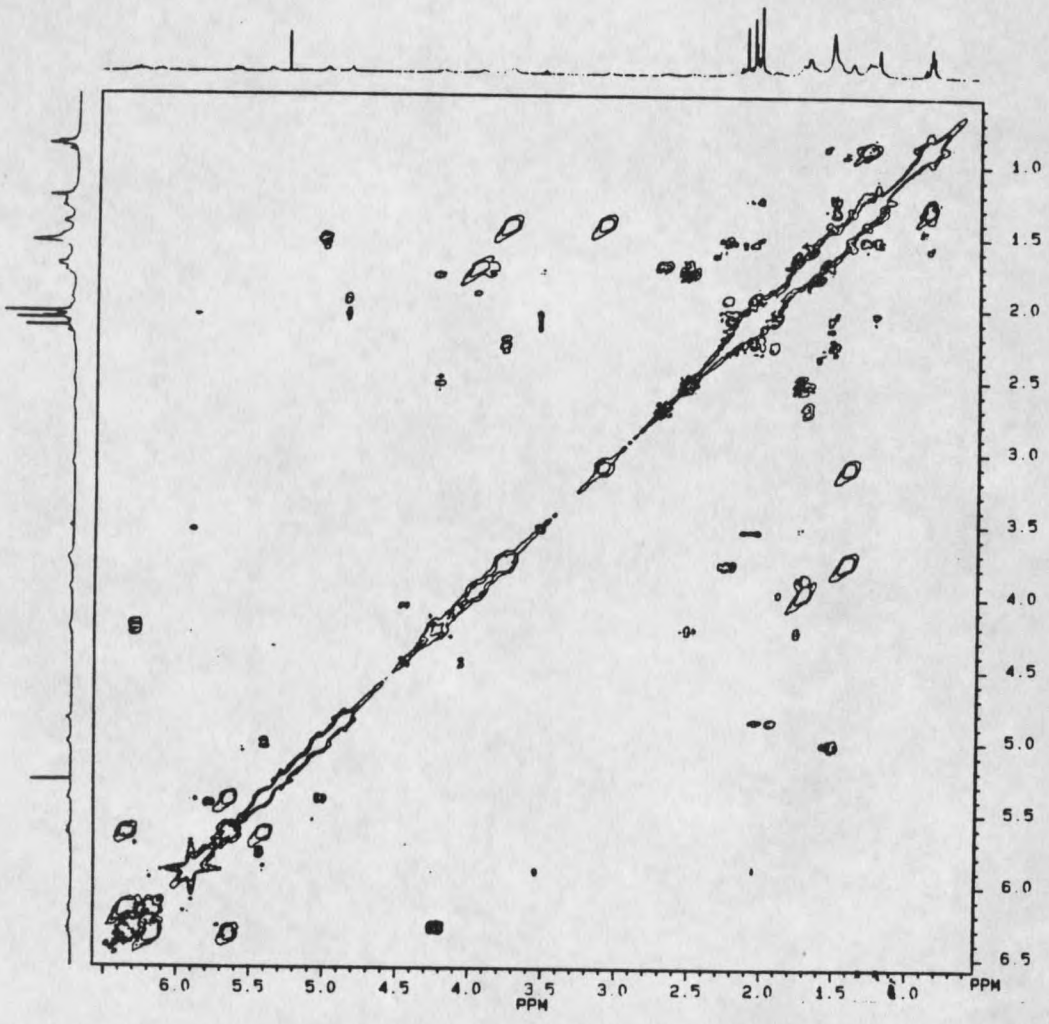


Figure 42. 500 MHz COSY Spectrum of the Diacetate of **77** in Pyridine-d_5.

A compound with a ^1H NMR spectrum very much alike that of **77** had eluted from a reverse phase flash chromatography (see Figure 43). Low resolution mass spectral analysis was performed on the fraction containing the compound, giving m/z 363 (M^+) (see Figure 44). HRMS gave a molecular formula of $C_{22}H_{37}NO_3$. A very disappointing event occurred with the fraction when it was applied to Bio-Beads S-X8 gel in methylene chloride-acetonitrile (4:1) to remove any trace contaminants. Only a very small portion of the original fraction was recovered, and it was greatly contaminated by material left on the column from a previous chromatography. Subsequent attempts to remove the contaminants from the original fraction were unsuccessful, and it was not possible to carry out further experiments to determine the structure of the compound. What could be inferred from mass spectral analysis did indicate the presence of one alcohol moiety. The supposed alcohol was most likely located on the alkyl chain, owing to the similarities in the mass spectral fragmentation patterns. The chemical shift of the olefinic protons was similar enough to those of **77** that the probable site of attachment of the alcohol moiety was at C-14, to give compound **78**.

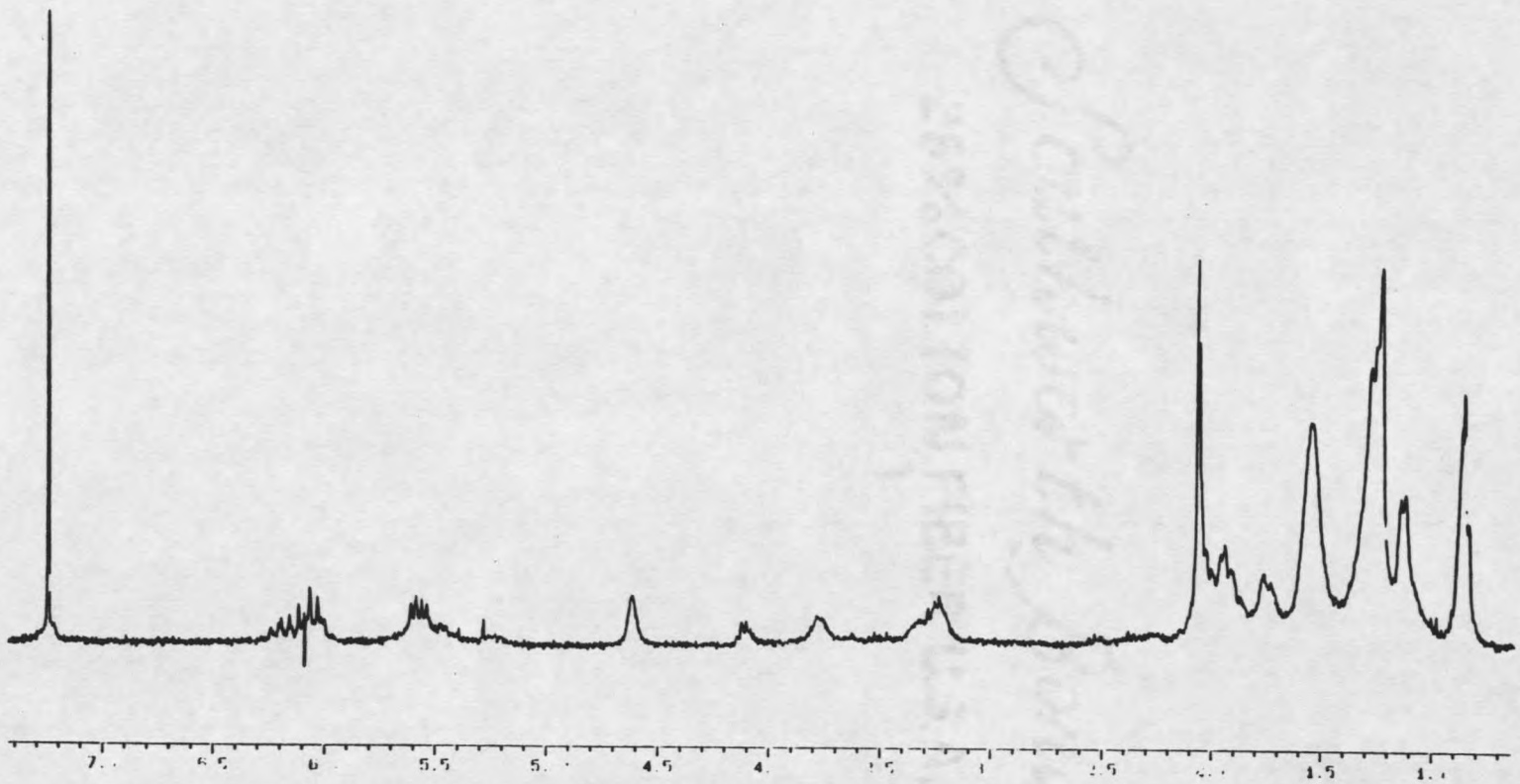


Figure 43. 300 MHz ^{1}H NMR Spectrum of **78** in $CDCl_3$.

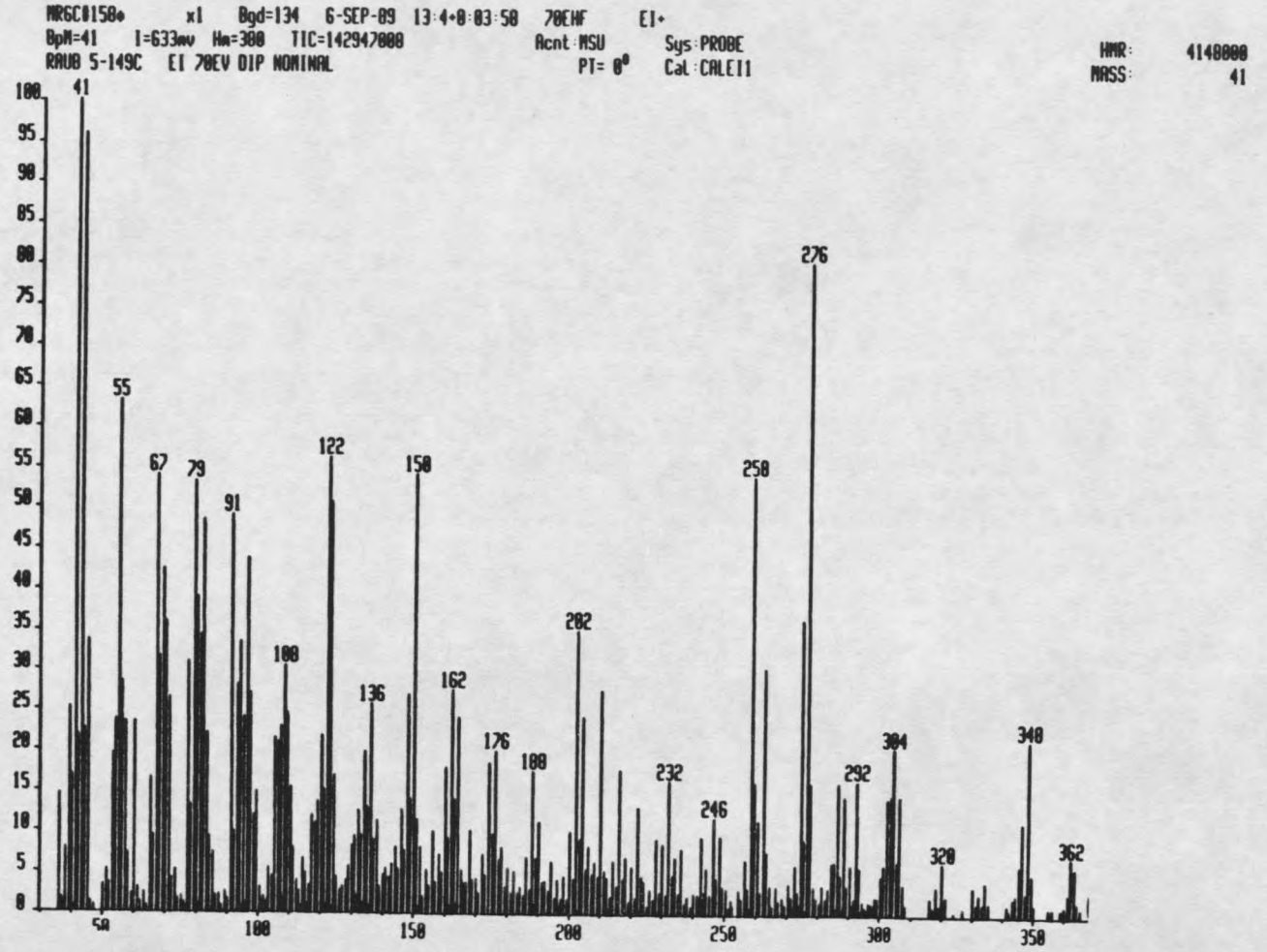
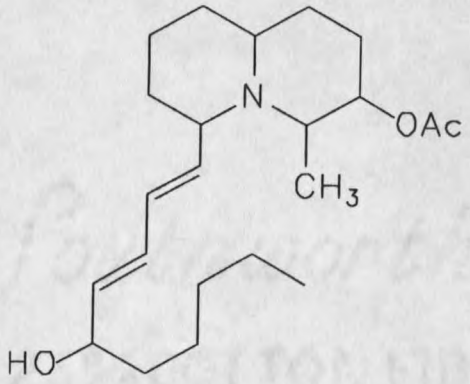


Figure 44. EIMS of **78**.



78

Aldehydes 80, 81

The two compounds which appeared to contain an aldehyde moiety displayed very similar profiles in the upfield region of the ^{1}H NMR spectra (see Figures 45 and 46). Enough chromatographies with CCC had been performed that some fractions contained one or the other aldehyde as the major compound, with ratios on the order of 9:1. Since the ratio was so large, assignments for structural determinations were not difficult. Indeed, one two-dimensional experiment was carried out with a fraction containing a mixture of both compounds with a ratio of approximately 1:1, but the connectivities were fairly straightforward.

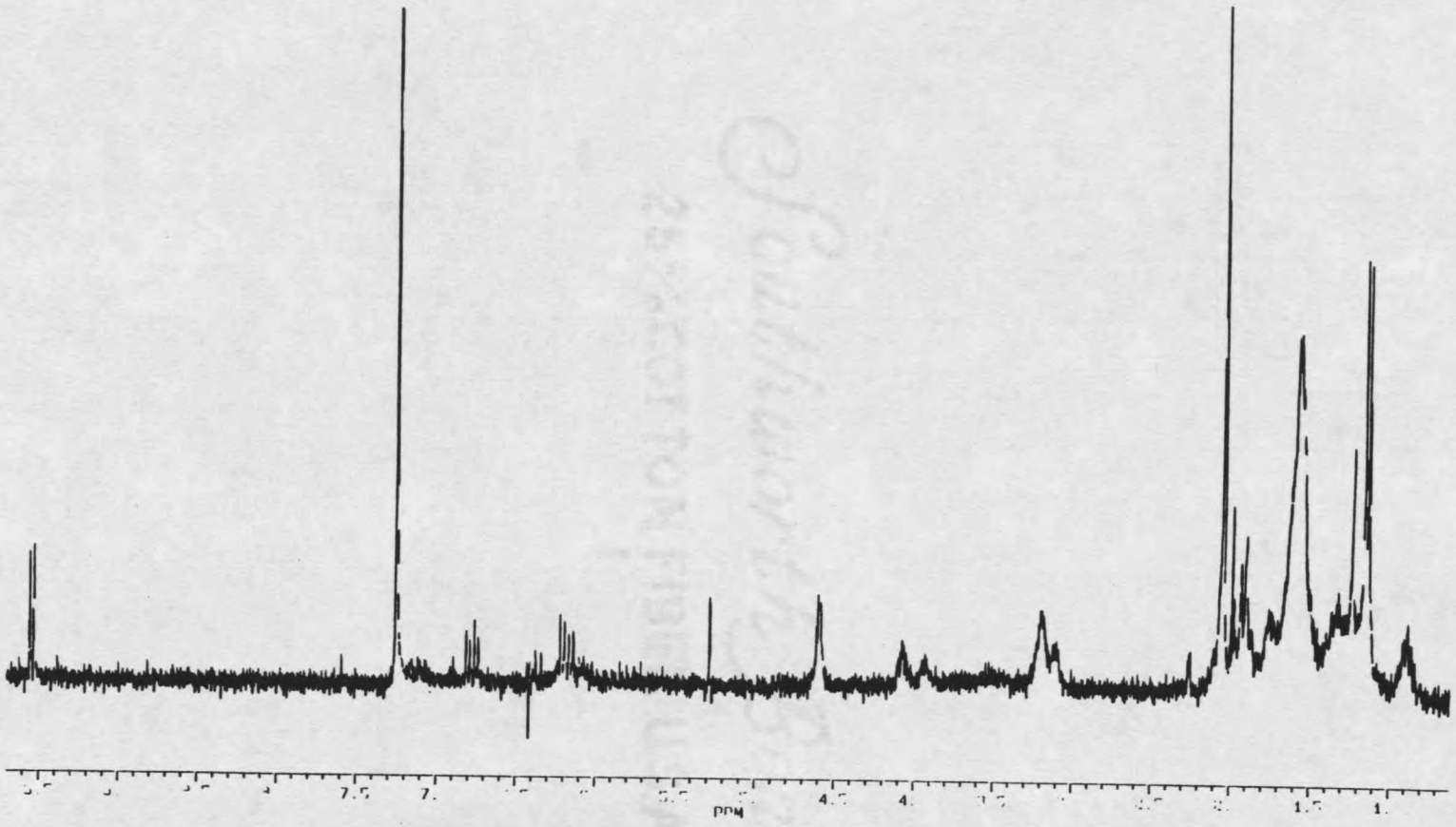


Figure 45. 300 MHz ^{1}H NMR Spectrum of **81** in $CDCl_3$.

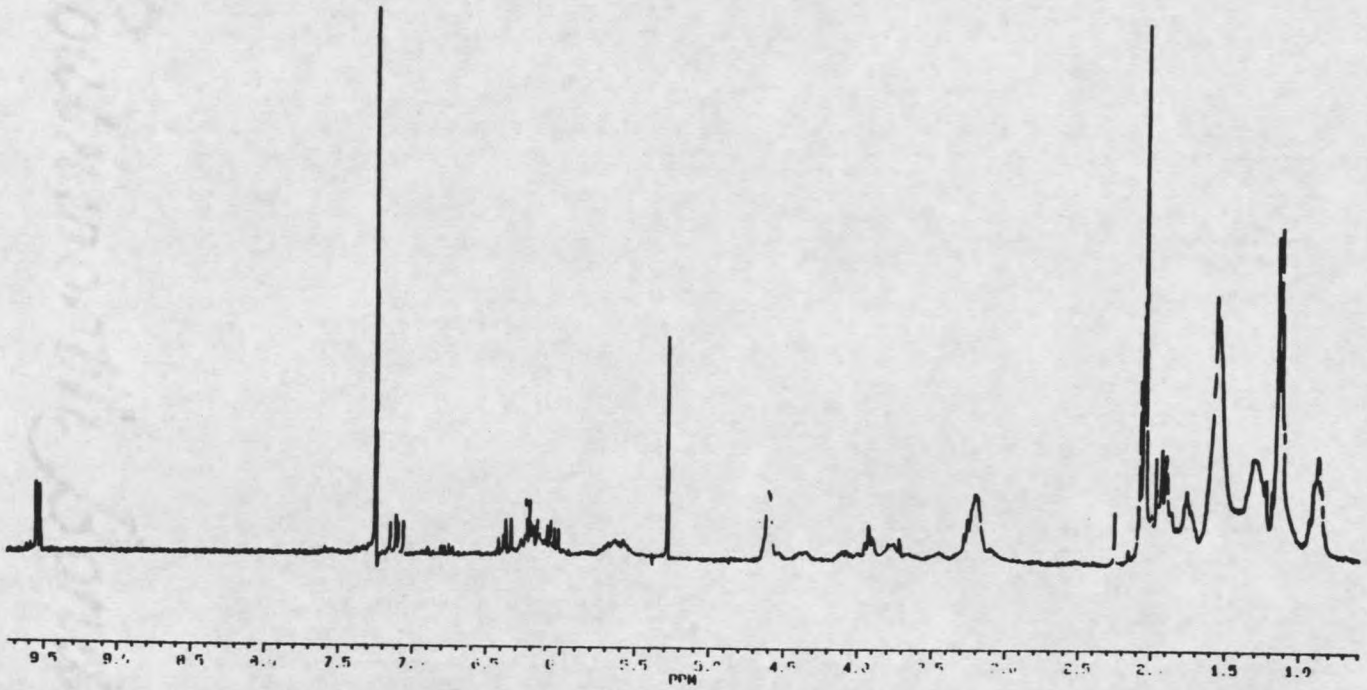


Figure 46. 300 MHz ^{1}H NMR Spectrum of **81** in $CDCl_3$.

An EIMS indicated that the two compounds differed in molecular weight, m/z 265 (M^+) (see Figure 47) and m/z 295 (M^+) (see Figure 48), respectively. HRMS of the compounds, $C_{15}H_{23}NO_3$ and $C_{16}H_{25}NO_4$, revealed the difference to be CH_2O. An ir of the individual compounds was not successful, apparently due to the scant amount of material. An ir of a mixture of the two compounds showed the typical absorbance of the acetate carbonyl at 1735 cm^{-1} and at 1685 cm^{-1} the absorption for an α,β-unsaturated aldehyde (see Figure 49). Also readily observable was a peak indicating an alcohol, a moderately strong, broad peak at 3380 cm^{-1}. A uv absorption maximum (EtOH) was measured at 232 nm, a higher than expected absorption of an α,β unsaturated aldehyde[55].

The only observable difference in the ^1H NMR spectra from that of the quinolizidine acetate dienes was in the region below δ 5. Homodecoupling was used to elucidate the area of the spectrum below δ 5. The furthermost downfield signal, the doublet centered δ 9.4, J=7.5 Hz, was irradiated. This led to the collapse of the doublet of doublets at δ 6.17 to a doublet, where J=17 Hz. The large coupling constant was, once more, in good agreement with the J value for a trans-substituted olefin. When the signal at δ 6.17 was decoupled, it revealed coupling

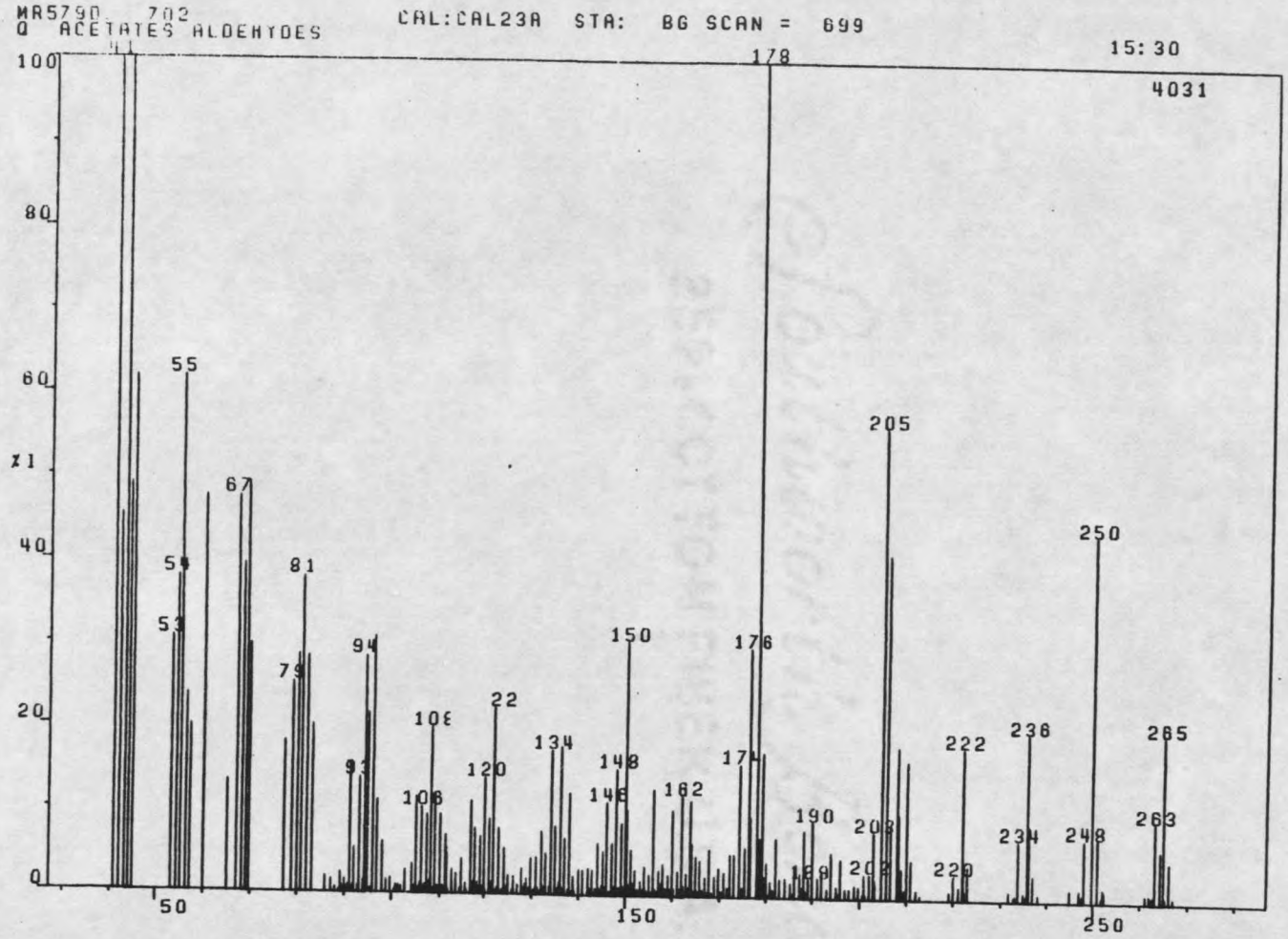


Figure 47. EIMS of 80.

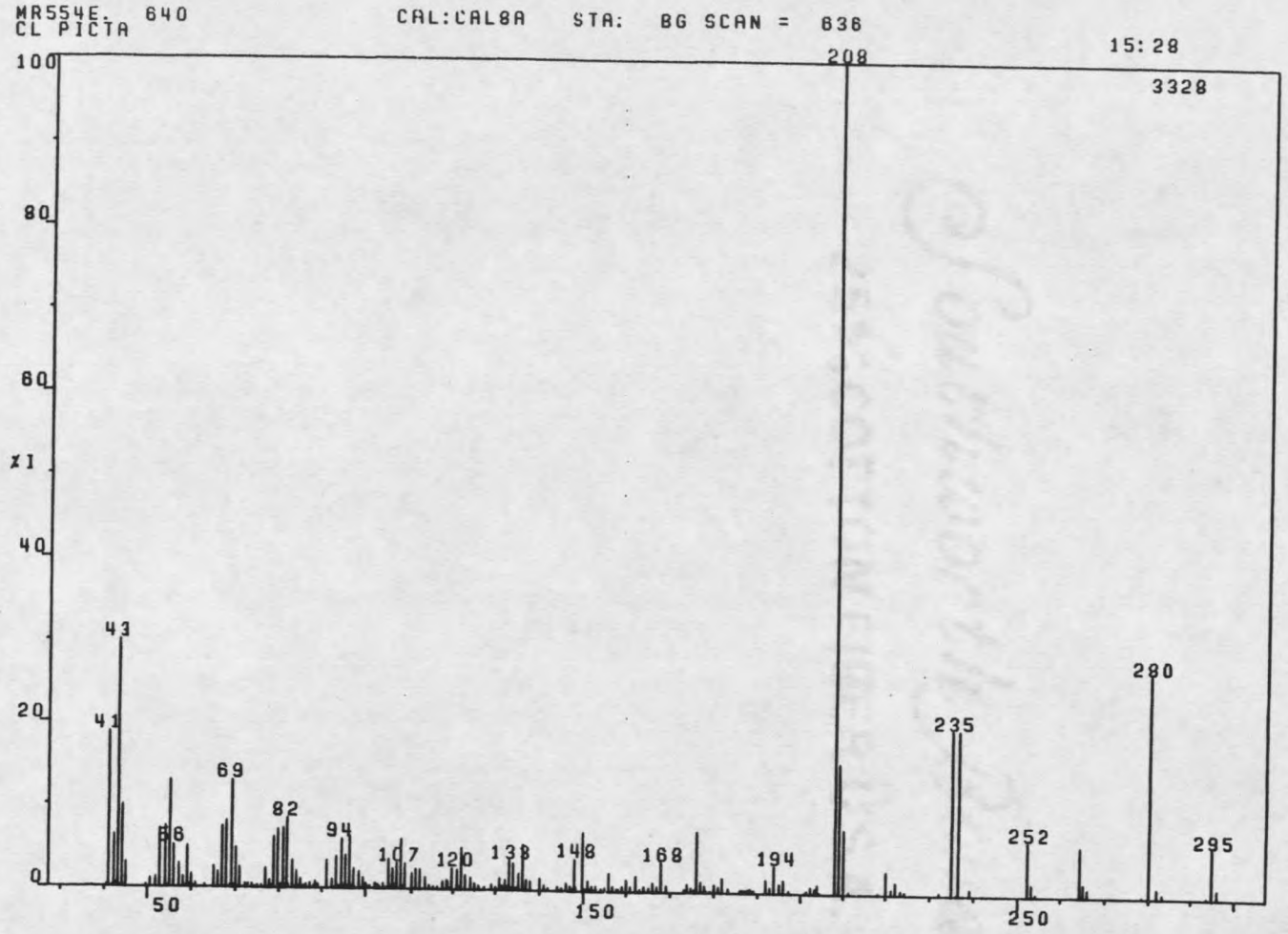


Figure 48. EIMS of **81**.

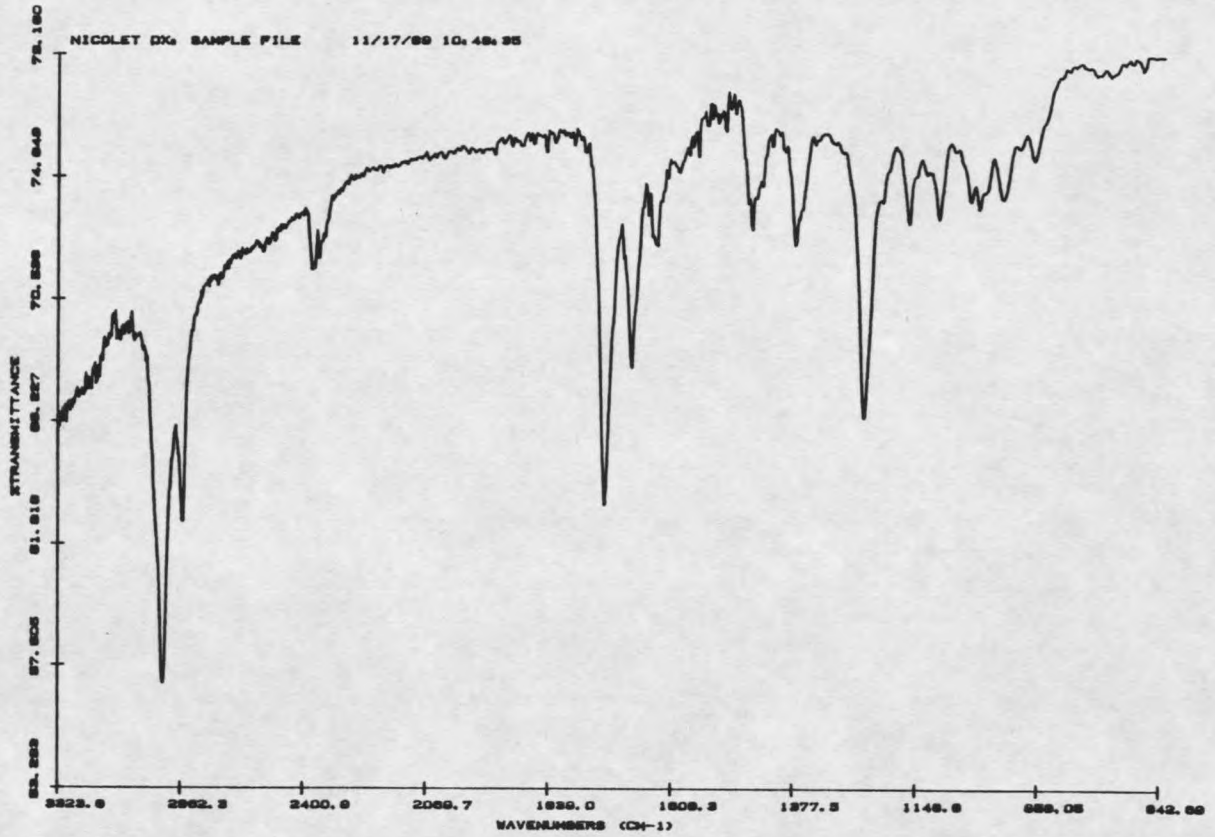
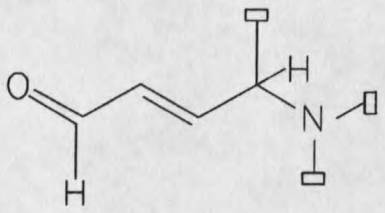


Figure 49. IR (Thin Film) of **80** and **81**.

to the doublet of doublets at δ 6.75. The doublet of doublets coalesced to a doublet, with J=7.3 Hz. Irradiation of the resonance at δ 6.75 not only caused the collapse of the signal at δ 6.17 to a doublet, but also sharpened the signal at δ 4.08, which could be defined as an allylic methine, alpha to nitrogen, **79**.

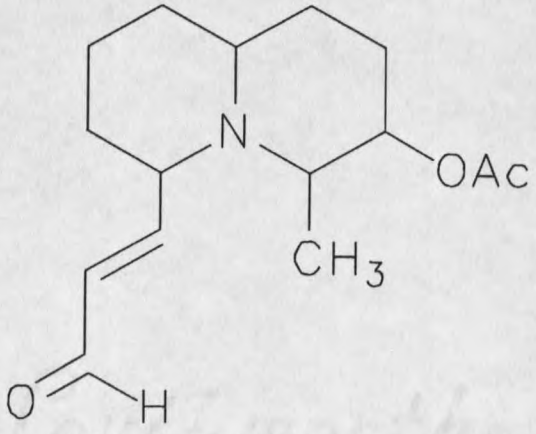


79

Some upfield connectivities were also readily determined. For example, irradiation of the signal at δ 4.6 sharpened the one-proton signal at δ 3.1 slightly. Decoupling of the resonance at δ 3.1 collapsed the methyl doublet at δ 1.15. The irradiation of the last two signals would again support the part structure of a methyl-bearing methine alpha to a nitrogen, with an acetate-bearing methine vicinal to it.

A ^{1}H-^{1}H COSY of the downfield region showed connectivities consistent with those determined by homodecoupling. Some assignments in the upfield region could also be made. The acetate-bearing methine was

coupled into the cluster of protons between δ 1.75 and 1.95. The broad singlet centered at δ 3 was found to be connected to three regions upfield; δ 1.9, 1.8 and 1.5. The resonance at δ 3 was apparently the ring juncture methine. The structure most consistent with the data was, again, a quinolizidine acetate with an acrylaldehyde moiety at C-10, **80**.



80

The structure of the second aldehyde remained enigmatic. As mentioned, the HRMS indicated a compound with five sites of unsaturation. A comparison of the ^{1}H NMR of the second aldehyde (see Figure 46) with that of the quinolizidine acetates did not show a shift of any of the ring protons upfield, as would have been the case of placing a fourth substituent on the ring system. Hence

it was very likely that any variances in the structure were limited to the exocyclic chain.

The fraction containing **80** that had been used for the H-H COSY also contained the other aldehyde. An examination of the downfield signals indicated a similar construction of the exocyclic piece of the molecule. The aldehyde signal at δ 9.4 was coupled to the one-proton resonance at δ 6.2. The signal at δ 6.2 was, in turn, coupled to what appeared to be a doublet of doublets abutting the $CDCl_3$ signal, centered at δ 7.1. Coupled to the signal at δ 7.1 was a signal slightly upfield, at δ 6.4. Any coupling from the signal at δ 6.4 to further upfield signals was not observed; the weak signal strength was attributable to the fact that the fraction contained probably less than 0.5 mg of the aldehyde. A ^{13}C NMR spectrum (see Figure 50) of a fraction containing the aldehyde and **11** in a 5:1 mixture clearly showed four midrange signals, attributable to the four ring methines, and an aldehyde resonance at δ 193.7, typical of an α,β-unsaturated aldehyde[56]. The aldehydes of the two compounds apparently overlapped, whereas the olefinic signals did not. It was not possible to determine which of the olefinic signals were attributable to the individual aldehydes.

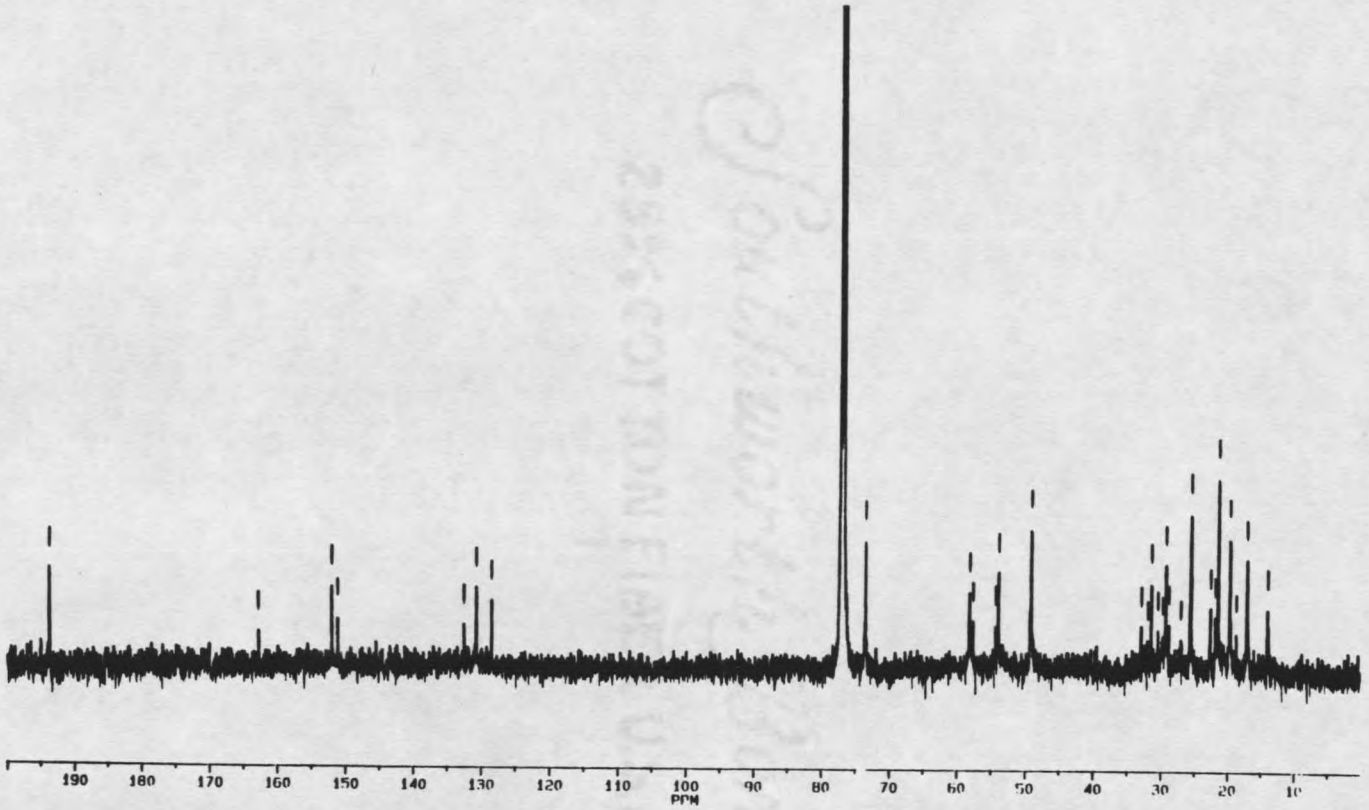
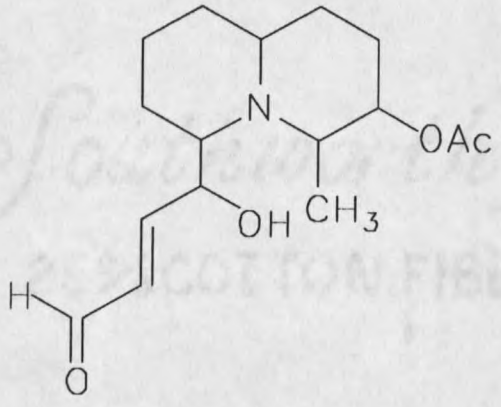


Figure 50. 75 MHz ^{13}C NMR Spectrum of **80** and **81** in $CDCl_3$.

When it became clear that a further structural determination was hampered by the presence of the other aldehyde, all of the fractions containing the compound were pooled together and subjected once again to size exclusion chromatography, in hopes of separating the two compounds and removing any traces of contaminants. The result of that chromatography was what can be called an unmitigated disaster. Only one fraction appeared to contain the aldehyde, it contained a large amount of contaminants and the fraction weighed only 1.5 mg, 20% of the original fraction.

Further structural analysis of the compound was limited to what could be inferred from the data already collected. When **80** and the other aldehyde were compared, there were two obvious differences: 1) the downfield chemical shifts; 2) the additional $-CH_2O$. Since the ir of a mixture of the two aldehydes clearly indicated an alcohol, the larger compound most likely contained the -OH group. This still left a -CH to be added to the compound. The upfield portion of **80** looked very similar to that of the larger aldehyde, as was mentioned previously. The only site for the alcohol moiety was, by default, the exocyclic portion of the compound. The ^{13}C at least suggested four olefins, but the sites of unsaturation would then have been six, in contradiction

of the HRMS. Thus the alcohol moiety probably consisted of a H-C-OH unit and was at C-11, an alcohol bonded vicinally to the nitrogen-bearing methine, C-10, and to the olefinic carbon, C-12. Mass spectral analysis did not indicate a very facile cleavage at C-10 , as would be expected if the alcohol were attached to C-11. A peak at m/z 210 was observed, but the intensity of the peak was on the order of that of compounds **51** and **65**. On the other hand, the base peak of m/z 208 was typical of the loss of a vinyl acetate, lending support to a structure similar in its substitution pattern to the other quinolizidines. A further loss of the chain containing the proposed aldehyde to give m/z 123 also was not observed. The "best determined fit" for the aldehyde, in light of the data that could be gathered, was **81**.



81

STRUCTURE ELUCIDATION OF THE INDOLIZIDINES

Indolizidine Monoenes **86**

The ^{1}H NMR ($CDCl_3$) of fraction A (see Figure 51) was characterized by what appeared to be a monoene, with one-proton signals centered at δ 5.25 and 5.4. The upfield region of the spectrum revealed one-proton multiplets at δ 3.0, 2.8, 2.65, 2.4 and 2.2, respectively. Multiple-proton signals were grouped at δ 2.0, 1.8 and 1.6. A methyl triplet, δ 0.87, was characteristic for a terminal methyl group of an alkyl chain.

A contrasting picture was presented by the ^{13}C NMR spectrum of this material (see Figure 52). Whereas the proton spectrum indicated a monoene, four distinct olefinic signals of approximately the same intensity were observed at δ 136, 134, 123 and 122. Three midrange signals, δ 59, 58 and 50 ppm, appeared to be doublets. The upfield region from δ 33 to 14 was comprised of at least twenty-two carbons, an unlikely number of carbons compared to the number of signals in the ^{1}H NMR spectrum. GC/EIMS (PCI/NH_3) of the fraction resulted in the elution of a series of peaks within a narrow temperature range, all of the eluants yielding the same molecular weight, m/z 264 (MH^+) (see Figure 53). The GC trace (see Figure 54) for the HRMS of A displayed a similar profile, and

the molecular weights found in the low resolution mass spectrum were determined to be correct. Since all four peaks in the trace were of the same molecular weight and formula, $C_{18}H_{33}N$, four isomeric compounds were likely. Also, the last two peaks to elute, clearly the major compounds, eluted from the GC column over a very narrow temperature range, indicating the possibility of geometrical isomers. The existence of different isomers could then explain the appearance of four olefinic carbons and the three midrange doubled signals found in the ^{13}C NMR spectrum. With an olefin comprising one of the three sites of unsaturation, the two remaining sites suggested the possibility of a bicyclic ring system. It was readily noted, however, that major differences from the spectra from other compounds collected to that point were observed. The ^{13}C NMR spectrum indicated three, not four, midfield carbons. The fragmentation pattern in the mass spectrum did not exhibit the same profile as the quinolizidines. A fragment of 124 a.m.u., HRMS $C_8H_{14}N$, 15%, was characteristic for all the supposed isomers, and it is a typical fragment of a monosubstituted indolizidine[57]. The ^{13}C NMR chemical shifts of the midfield carbons also compared favorably with those of the three alpha carbons of the *Dedrobates* alkaloids[58],

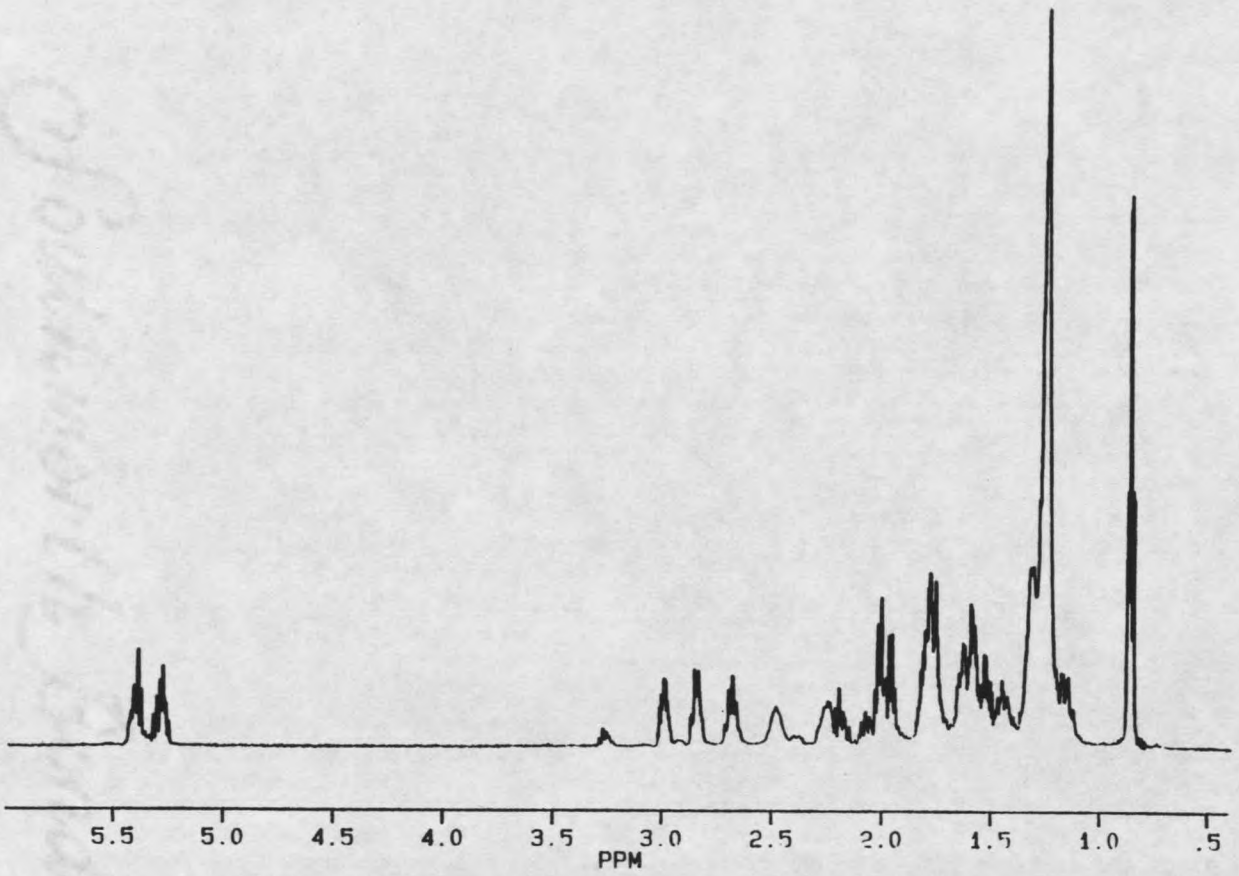


Figure 51. 500 MHz ^{1}H NMR Spectrum of **86** in $CDCl_3$.

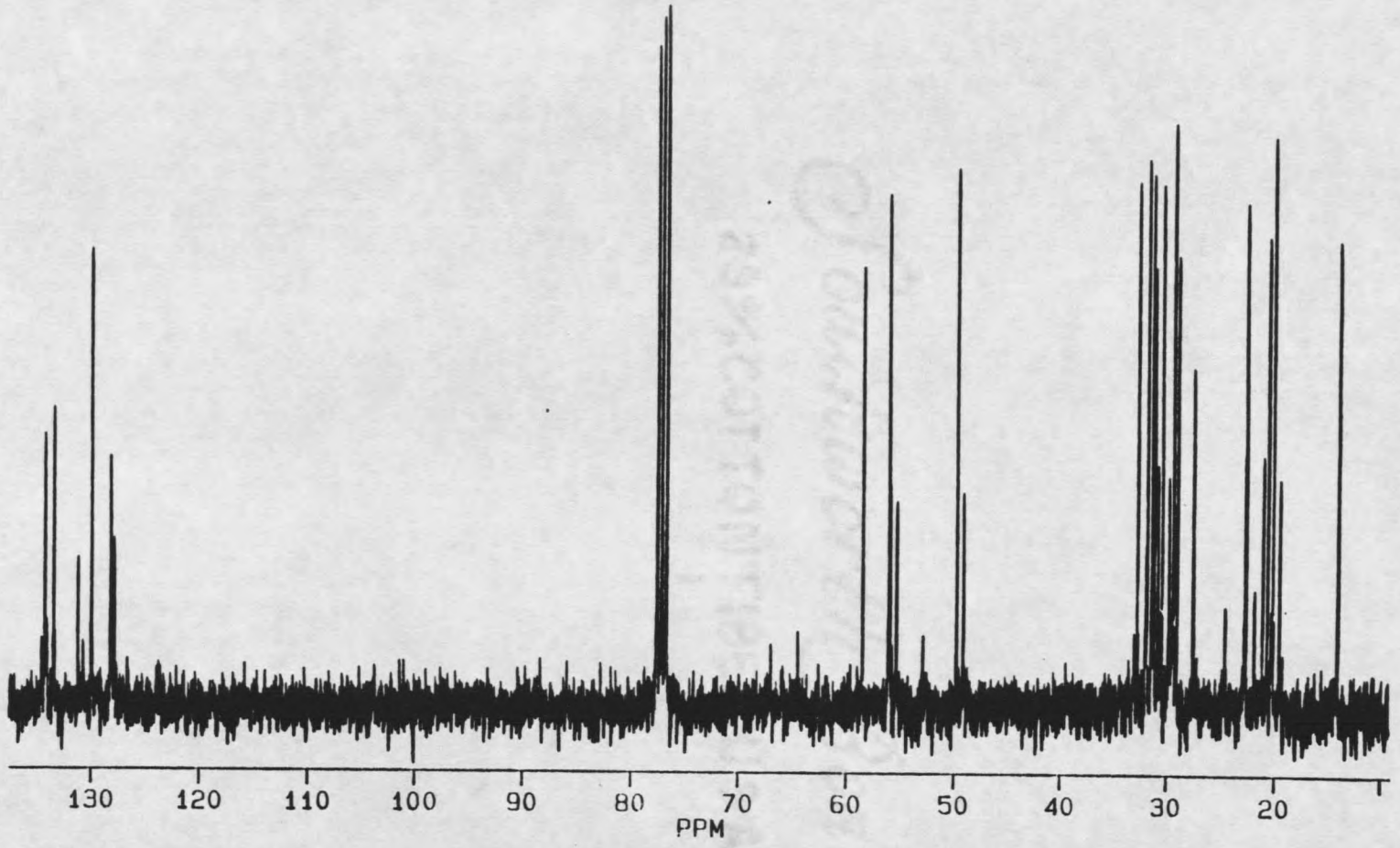


Figure 52. 75 MHz ^{13}C NMR Spectrum of **86** in $CDCl_3$.

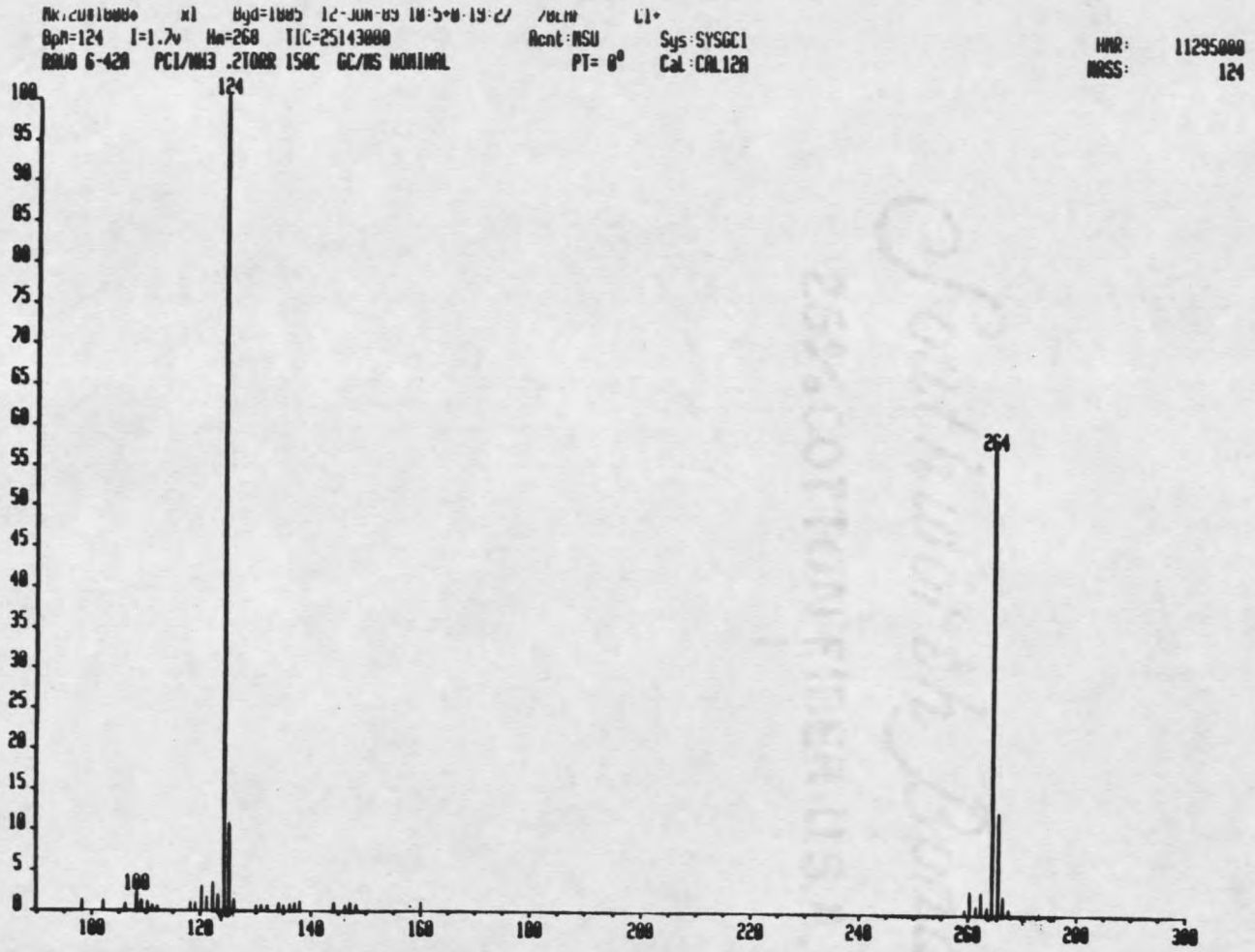


Figure 53. CIMS of **86**.

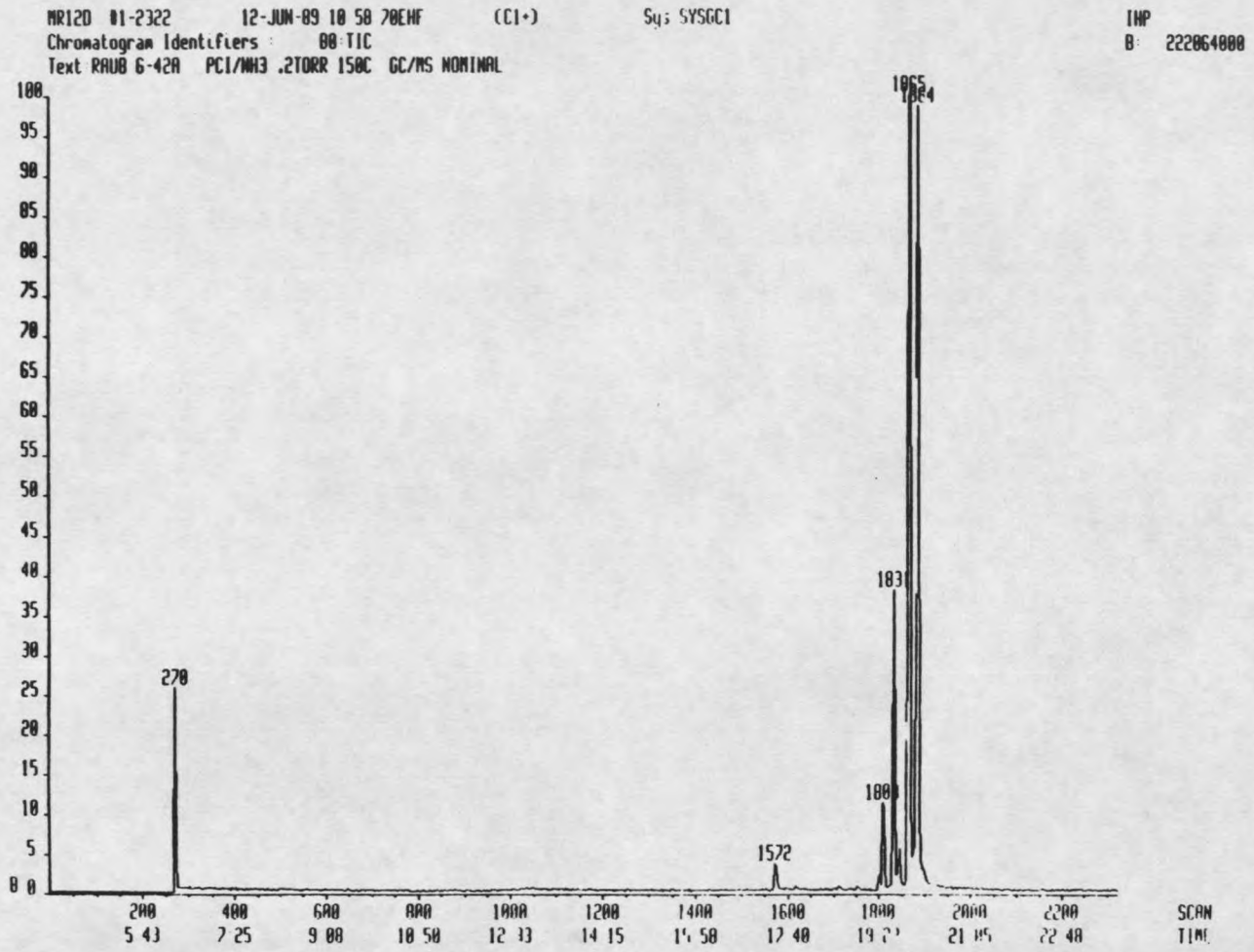


Figure 54. GC Trace of GC/MS of **86**.

indolizidines characterized by alkyl substituents. A ^{13}C INEPT was carried out on the fraction, indicating that the three midfield signals at 47, 57 and 59 ppm were a methylene, a methine and a methine, respectively (see Figure 55). Once again, the shifts of all three carbons were similar to those of the aforementioned compounds. The INEPT experiment also revealed the presence of only one methyl group, the terminal chain methyl. Each of the observed olefinic carbons was shown to be bonded to a single proton.

Further structural analysis was carried out via homodecoupling and difference decoupling experiments in $CDCl_3$. Irradiation of the one-proton signal at δ 2.98 partially collapsed the multiplet at δ 2.19. Homodecoupling of the next multiplet upfield, δ 2.8, partially collapsed the adjacent signal at δ 2.62 and an upfield signal at δ 1.75. The multiplet at δ 2.62 showed coupling to its neighbor at δ 2.8 and also appeared to be coupled to the signal centered at approximately δ 1.75. A less well defined multiplet, δ 2.4, seemingly a broad quintet, was irradiated, leading to partial collapse of the multiple-proton signal at δ 1.75. The poorly resolved one-proton signal at δ 2.2 appeared to be coupled to the signal at δ 2.98 and the vinyl proton at δ 5.25; upon irradiation, the upfield

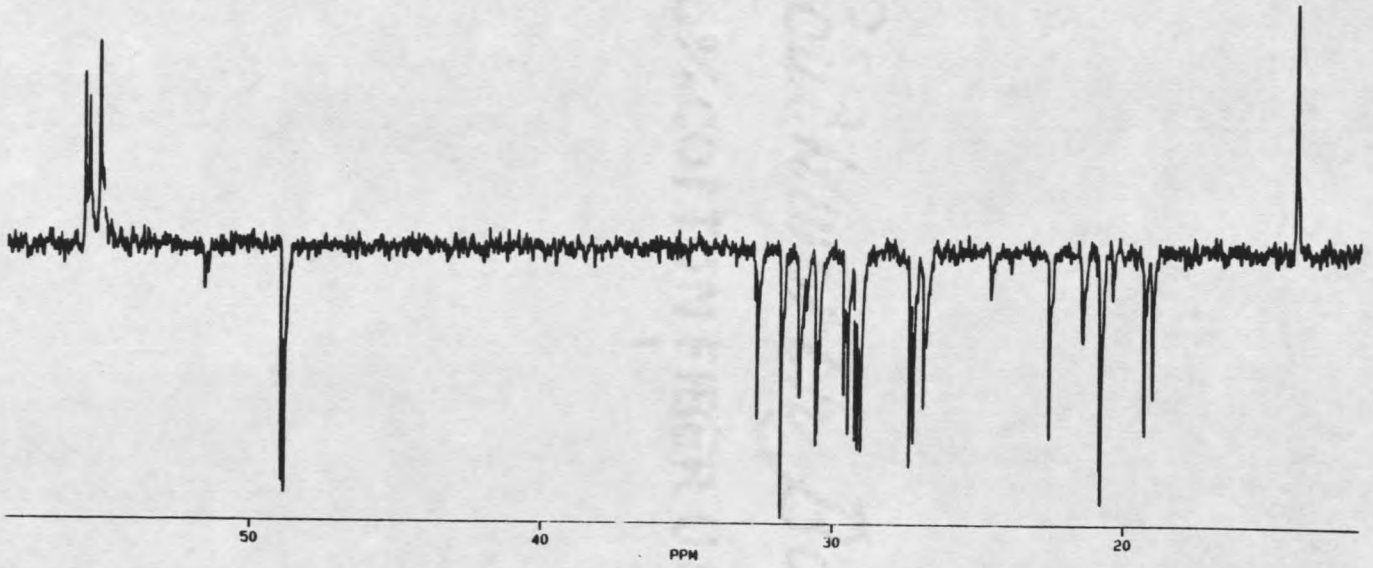
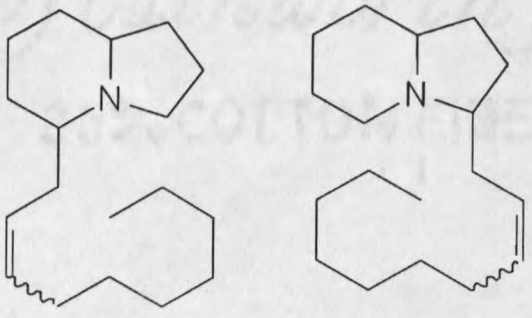


Figure 55. 75 MHz INEPT Spectrum of **86** in $CDCl_3$.

signal collapsed to a broad singlet, and the vinyl signal became less well defined. The multiplet at δ 2.0, a typical chemical shift for an allylic methylene, was irradiated, leading to the collapse of the vinyl proton, δ 5.35, to a doublet. The couplings to the signal at δ 1.75 were substantiated by irradiation of that multiplet, resulting in the partial collapse of the three signals at δ 2.8, 2.62 and 2.4. Difference decoupling pointed out further connectivities to the signal at δ 2.4, indicating that it was also coupled to a signal within the large methylene envelope, circa δ 1.3, and to a one-proton signal centered at approximately δ 1.12.

With the information at hand, some part structures could be determined. There did not appear to be an allylic methine α to nitrogen, as in the case of the quinolizidines from *C. picta*. The chemical shift of the olefin and its couplings to signals at δ 2.0 and 2.2 suggested an unsaturated alkyl chain. Since the vinyl signal at δ 5.25 and the one-proton signal at δ 2.98 were mutually coupled to the signal at δ2.2, a slightly different pattern emerged. The one-proton signal at δ 2.98 is fairly consistent with the chemical shift of a methine alpha to nitrogen. It was not allylic, and it appeared that the olefin for the major compound was not situated on the carbons α and β to the ring carbon, but

on the β and γ carbons. The coupling of the vinyl proton at δ 5.35 to the multiplet at δ 2.0 and its coupling into the upfield region centered at the methylene envelope further suggested the existence of the unsaturated chain. The chemical shift of the protons at δ 2.62 and 2.8 is indicative of a methylene alpha to nitrogen. They were mutually coupled to each other and to the same upfield signal at δ 1.75. The candidate for the third alpha substituent was the one-proton signal at δ 2.4. Difference decoupling of the signal produced a profile of an irradiated signal with a very complex coupling pattern, reminiscent of the ring juncture methine of the quinolizidines. Also, it was coupled to at least three readily distinguishable signals in the difference decoupled spectrum. Based on the data collected, the structure of the compound seemed to be one of the two possibilities, **82** and **83**.

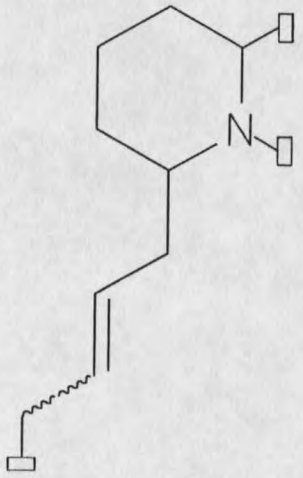


82 **83**

It was not apparent from the spectroscopic data obtained which of the two rings bore the alkyl chain. One could postulate that structure **83** was the more viable alternative. The supposed ring juncture methine was coupled to the signal at δ 1.12, which, in turn, was coupled to a signal at δ 1.75. A five-membered ring could be built based on the fact that the apparent methylene doublet, δ 2.62 and 2.8, was clearly coupled into the signal at δ 1.75. Unfortunately, the multiplet at δ 1.75 also displayed a coupling to the signal at δ 2.4, indicating the possibility that it is the geminal partner of the signal at δ 1.12. This would even invoke another type of dual ring system, a 7,4 system! However, the chemical shift of the protons β on such a four-membered ring require a chemical shift of δ 2.4, not δ 1.75; this rendered the possibility of an azacyclobutane ring moot.

A ^{1}H-^{1}H COSY experiment was performed on the fraction in $CDCl_3$, utilizing the 500 MHz NMR (see Figure 56). The connectivities which had already been established by the one-dimensional experiments were found to be in agreement with the COSY data. The allylic methylene was somewhat better delineated, revealing the two geminal protons to be centered at δ 2.2 and 2.1, respectively. More importantly, the chain-bearing methine was shown to be

coupled to a signal in the upfield region, at approximately δ 1.59. The signal at δ 1.59 was apparently a ring proton, and it displayed a correlation to the multiplet centered at δ 1.5 and 1.45. Both signals were coupled into a signal centered at approximately δ 1.75, which, in turn, was coupled to the signal at δ 1.15. Neither the signal at δ 1.75 nor the signal δ 1.15 displayed any correlation to the signal at δ 1.59. The correlation of the resonance at δ 1.15 to the broad singlet at δ 2.48 was designated as the alpha methine, the pattern which emerged from the investigation of the aforementioned signals was that of a six-membered ring bearing the unsaturated alkyl chain, **84**.



84

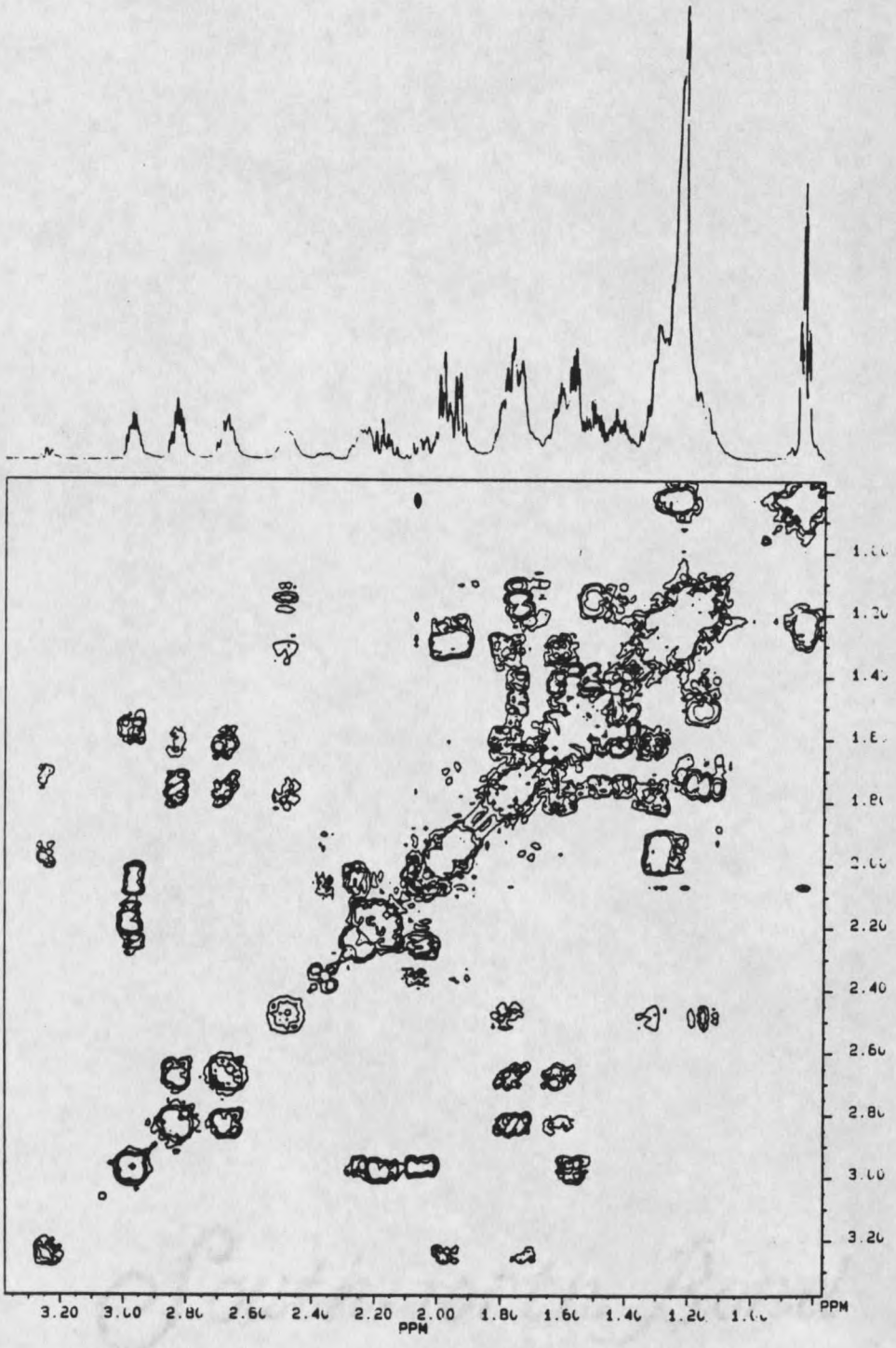
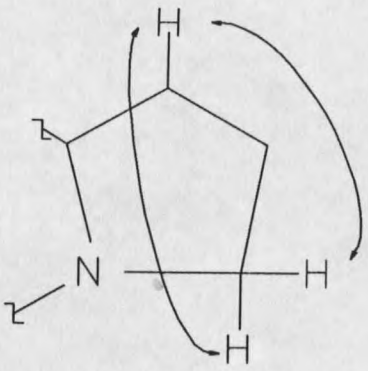


Figure 56. 500 MHz COSY Spectrum of **86**, Upfield Region, in $CDCl_3$.

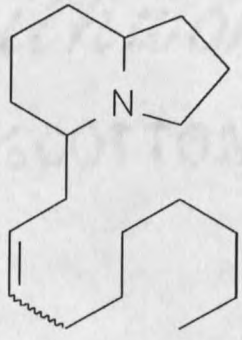
A further coupling observed was that of the singlet at δ 2.48 to a signal centered at δ 1.8 and 1.3. The connectivities could be followed next from δ 1.3 to two mutually coupled signals at δ 1.61 and δ 1.80. Both signals were also coupled to the supposed geminal protons, alpha to the nitrogen, at δ 2.68 and 2.82, respectively. The coupling profile supported the existence of a five-membered ring with no substituent. A HETCOR experiment served to substantiate the suggestion that the signals at δ 2.68 and 2.82 were indeed geminal; the carbon at δ 49 correlated to both proton signals (see Figure 57).

In order to obtain more evidence for the structural assignments made, a COSYRCT experiment was undertaken. Three four-bond couplings were observed (see Figure 58). First, a vinyl proton showed coupling to the ring methine slightly above δ 2.98. Secondly, the methylene situated between the methine and the olefin yielded a four-bond correlation to the signal at δ 1.59, supporting the assignment made previously. The third correlation gleaned from the spectrum was that of both alpha methylenes, δ 2.68 and 2.82, to the signal at 1.3. The last correlation lent credence to the five-membered ring, **85**.



85

The best fit for the data was an indolizidine, **86**, with one substituent group, a monounsaturated alkyl chain, attached to the ring at the C-8 position. The position of the double bond for the major isomers, the supposed geometrical isomers, was from C-10 to C-11. Since mass spectral analysis indicated that the other two compounds eluted via CCC were also of the same molecular weight, they could also be considered positional isomers with respect to the double bond.



86

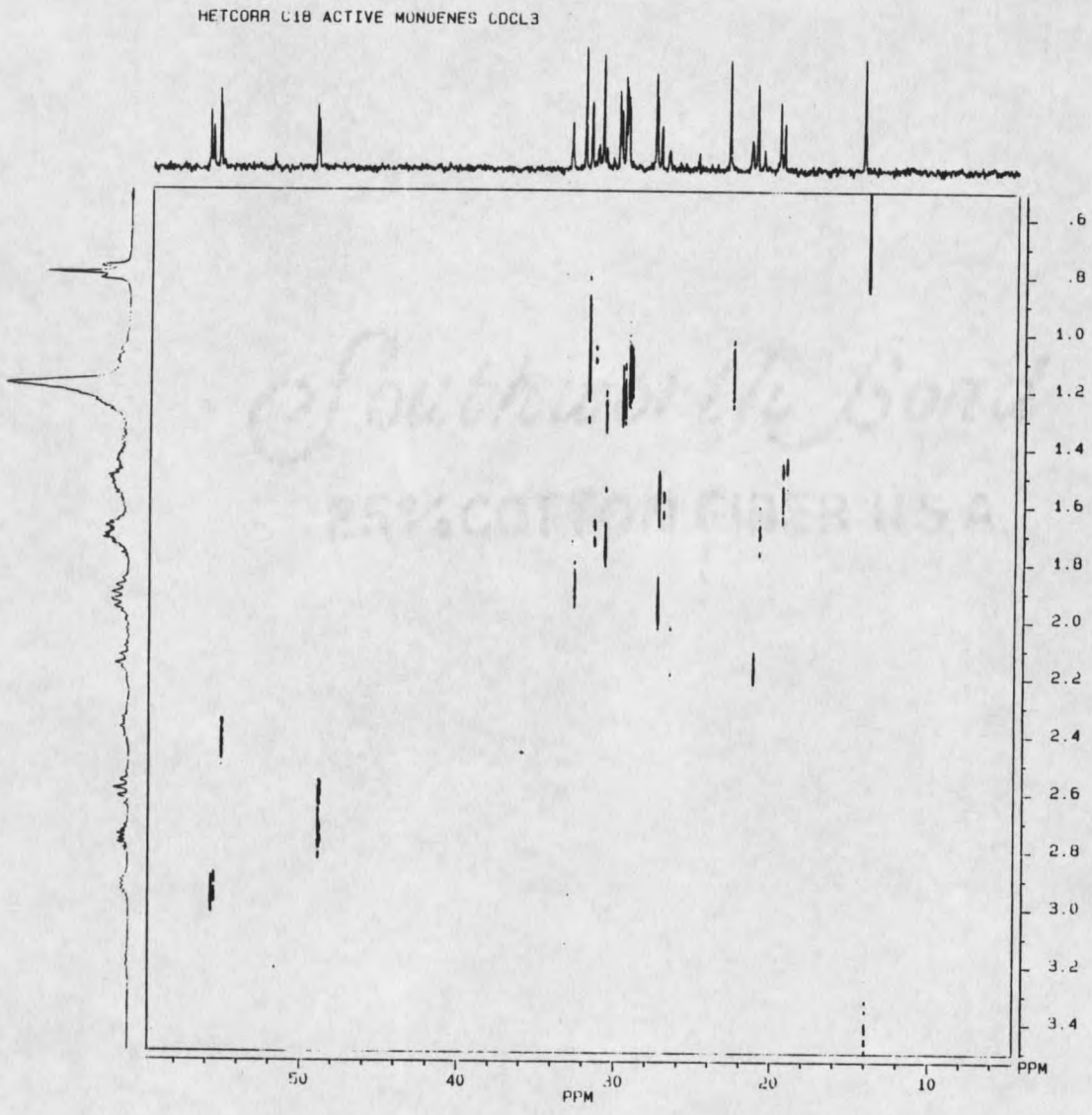


Figure 57. 75 MHz HETCOR Spectrum of **86** in $CDCl_3$.

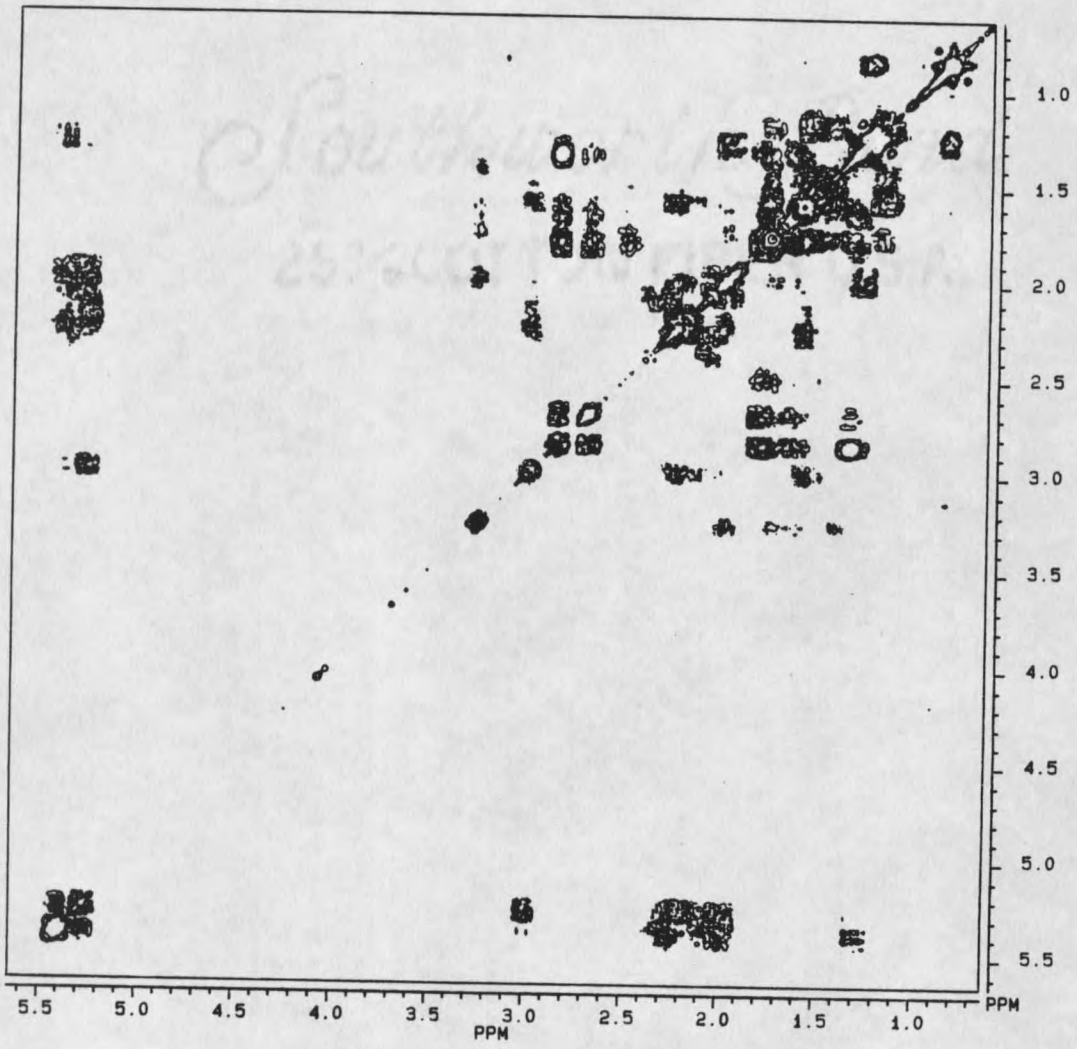


Figure 58. 500 MHz COSYRCT Spectrum of **86** in $CDCl_3$.

An attempt was made to cleave the double bond with osmium tetroxide. Unfortunately, when periodate was added to the reaction mixture, ^{1}H NMR indicated that the compound had decomposed, leaving only a trace of the expected aldehyde, a very weak signal at δ 9.5.

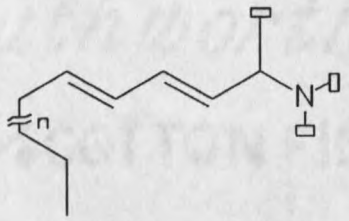
Indolizidine Dienes **88**

According to CIMS, the second fraction to elute from the CCC also had a series of compounds, all with m/z 261, including apparently another positional isomer of the indolizidine monoene, m/z 263 (see Figure 59). HRMS gave a molecular formula of $C_{18}H_{31}N$. The ^{1}H NMR profile showed an olefinic region with two proton signals resonating at the same position of the monoenes of **70**, and also to that of the quinolizidine alcohol (see Figure 60). An observed uv maximum (EtOH) at 232 nm was, again, typical of a heteroannular conjugated diene. The double bond accounted for the fourth site of unsaturation, versus three for **86**.

Although the isomeric mixture and the presence of one of the monoenes rendered an exhaustive structural elucidation via NMR techniques impossible, some connectivities were established. The irradiation of the resonance at δ 3.5 caused the collapse of the apparent

doublet of doublets at δ 5.8 to a doublet. When the signal at δ 5.8 was decoupled, the two-proton multiplet centered at approximately δ 6.1 coalesced partially. Decoupling the two-proton signal caused the multiplet at δ 5.6 to collapse partially. By irradiating the resonance at δ 5.6, its position next to an upfield methylene was demonstrated; a multiplet centered just below δ 2.0 was observed to lose some couplings. The two proton multiplet at δ 6.1 also became less complicated.

This decoupling data established the structure of the juncture of the chain and the ring, **87**. The signal at δ



87

3.5 could be designated as a methine alpha to nitrogen. It was allylic and shifted downfield to δ 3.5, versus δ 2.98 for **86**, where the methine was not allylic. Further decoupling experiments provided no more information, due to overlapping signals from the other isomers and the monoene. A ^{1}H-^{1}H COSY experiment was performed, in spite of the mixture of compounds. Many similarities to the

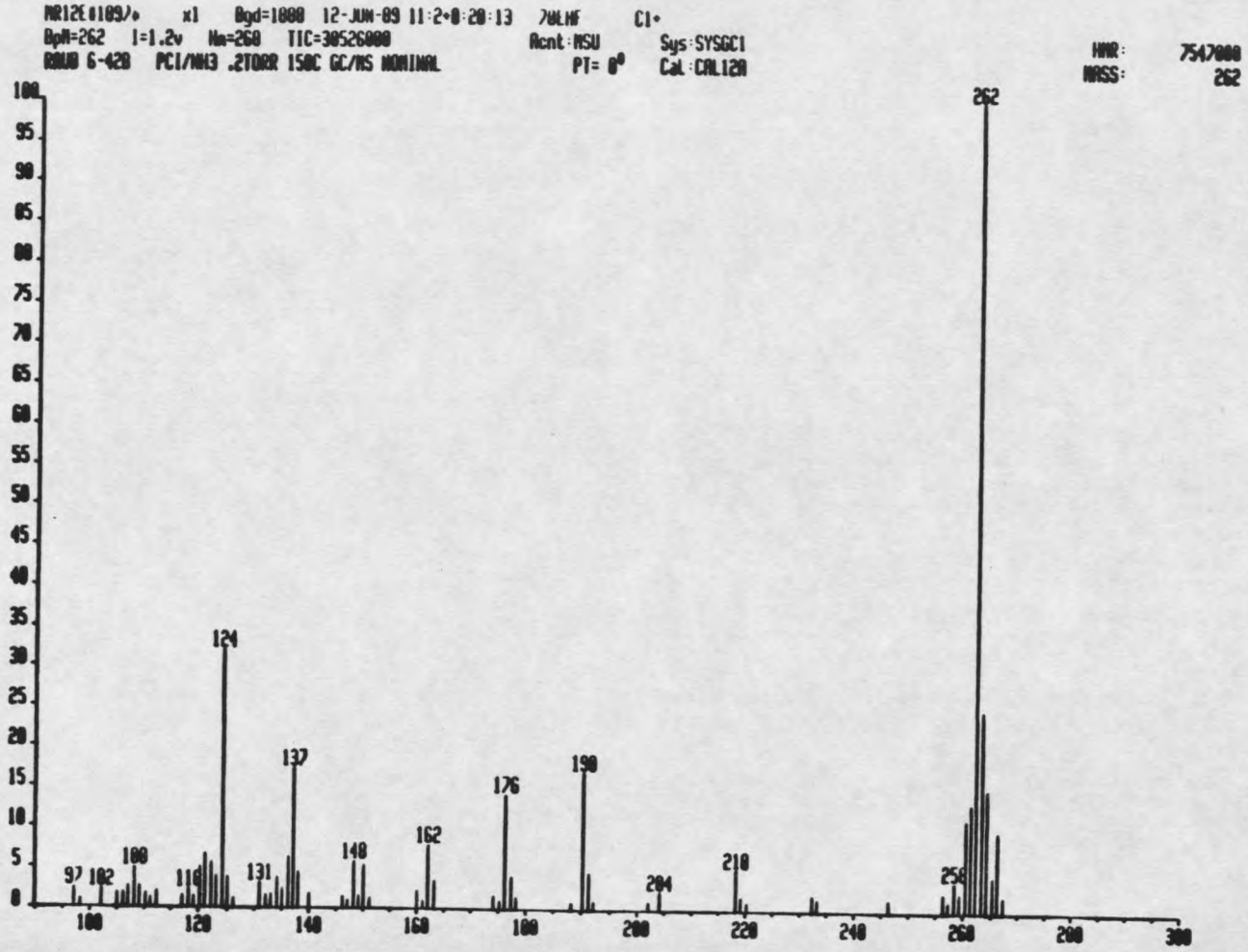


Figure 59. CIMS of **88**.

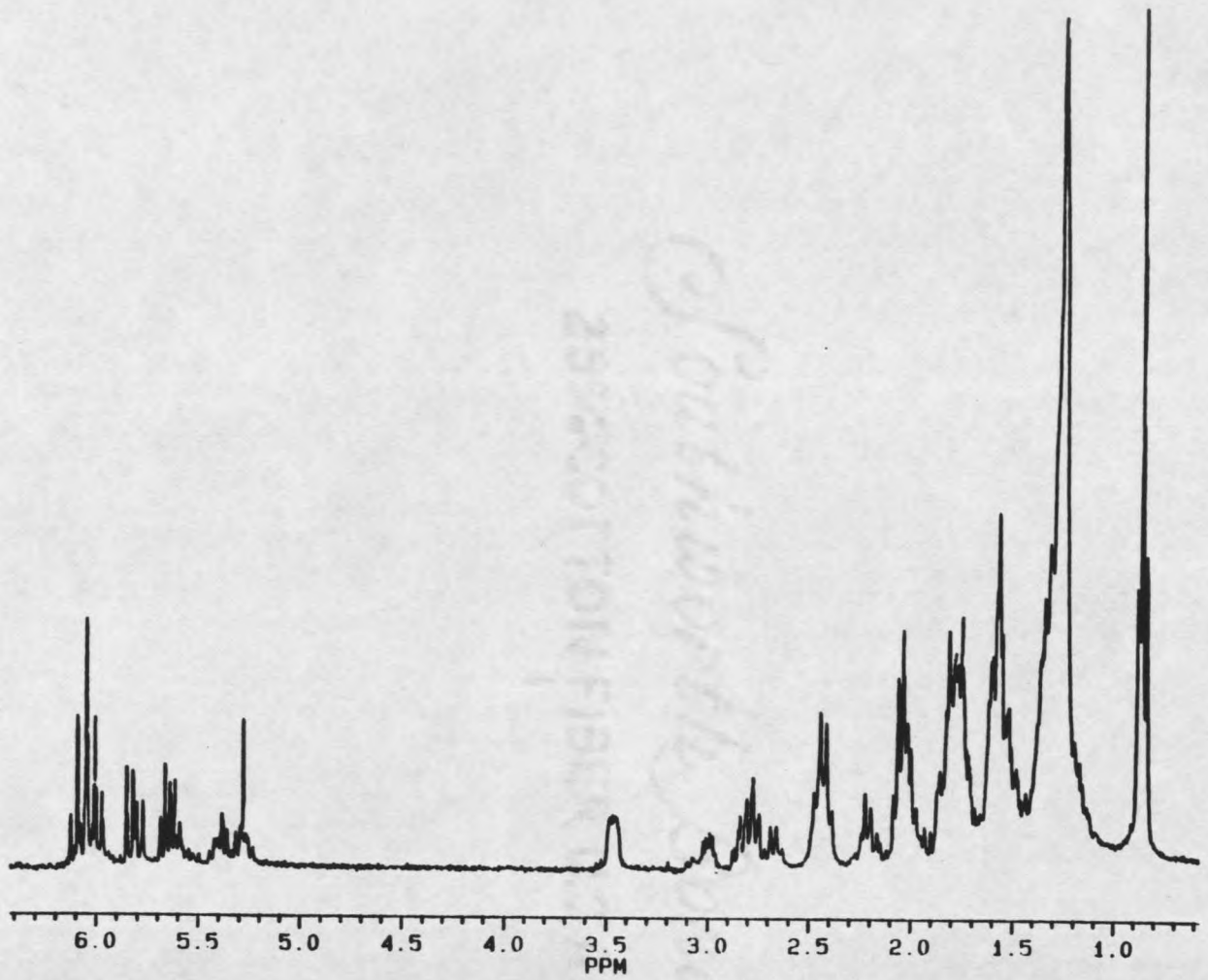
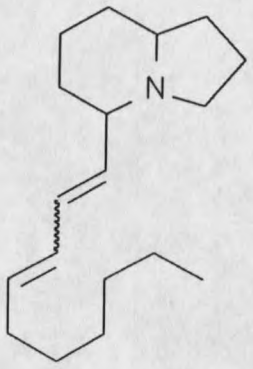


Figure 60. 300 MHz ^{1}H NMR Spectrum of **88** in $CDCl_3$.

monoenes could be observed, but further structural determinations were too ambiguous. The most likely structure for the compounds could be constructed as **88**.



88

Indolizidine Trienes **89**

The third fraction to elute from the CCC run in normal phase mode was another mixture of apparent isomers, EIMS m/z 259 (see Figure 61). HRMS gave the formula of $C_{18}H_{29}N$, indicating five sites of unsaturation. Absorption maxima in the uv (EtOH) were identical to those of the quinolizidine trienes, supporting the presence of a conjugated triene. The olefinic region of the ^{1}H NMR spectrum (see Figure 62) displayed a profile quite like the quinolizidine trienes as well. Since the fraction also contained a mixture of isomers, it was thought that the quaternary salt of each might be separable on CCC. The methylation with methyl iodide

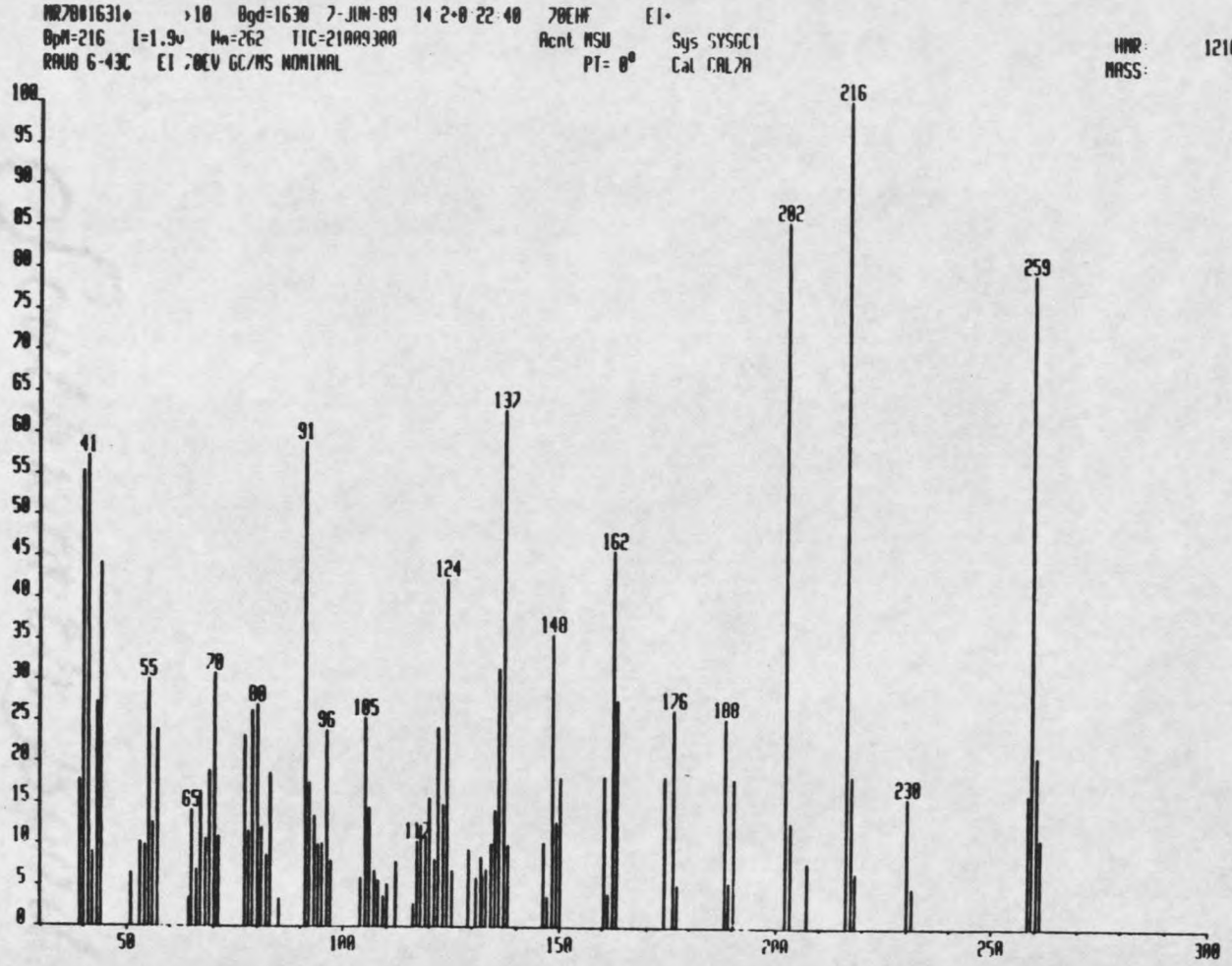


Figure 61. EIMS of **89**.

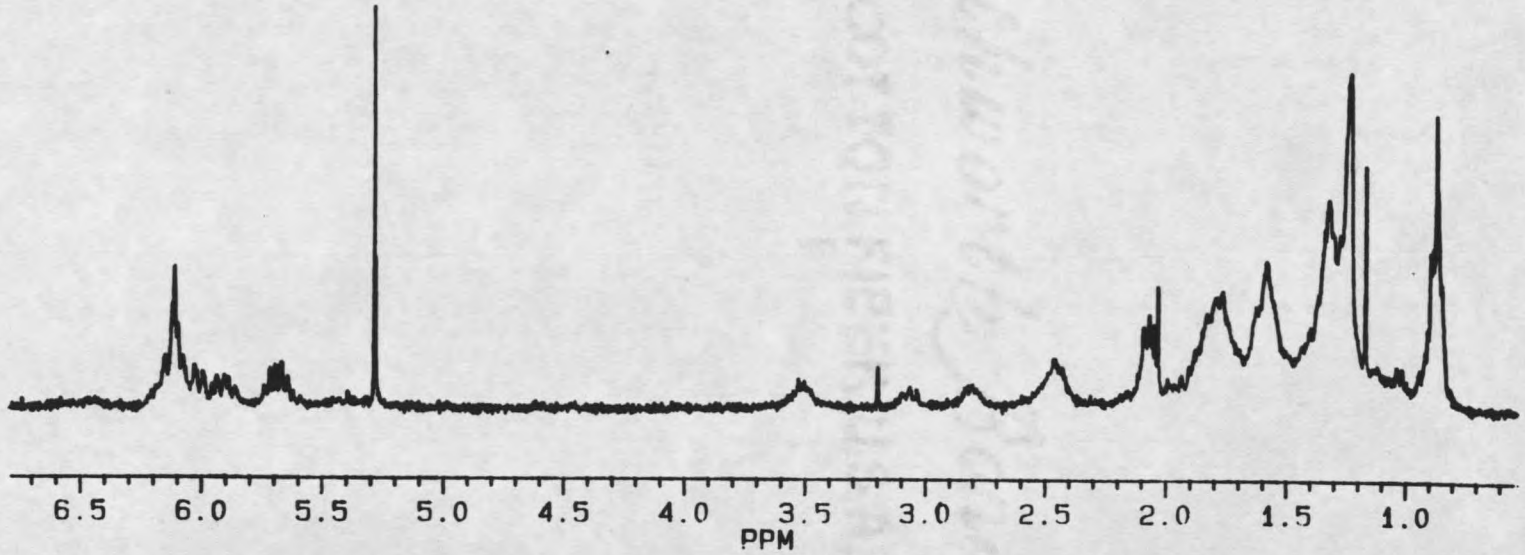
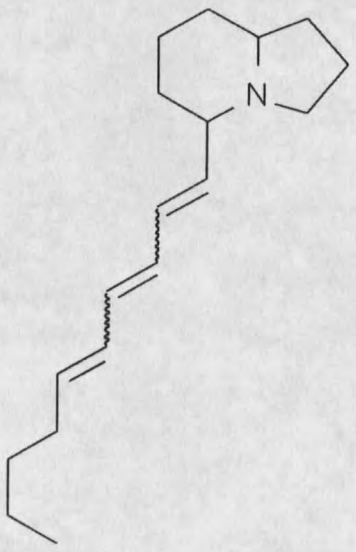


Figure 62. 300 MHz ^{1}H NMR Spectrum of **89** in $CDCl_3$.

proceeded readily, but the individual isomers could not be separated. The ^{1}H NMR of the quaternary amine salt had also become much more complex.

Only one reliable connectivity was established with homodecoupling. Decoupling the multiplet at δ 6.0 demonstrated that the signal at δ 3.5 was, again the allylic methine of C-9.

It was thought, that, by virtue of the larger number of olefins, the upfield region would appear less cluttered, allowing for structural elucidation. This was not the case. The signals were overlapped to a greater degree than with the diene mixture, **88**. Based on the spectral data, then, **89** was constructed.



89

BIOLOGICAL ACTIVITY RESULTS

Brine shrimp assays were utilized primarily for bioassay-guided fractionation, but no LD_{50} values were determined. Antimicrobial assays were also utilized in this fashion, and the pure compounds were tested for their efficacy in this assay. The C_{22} acetate, **51**, and its alcohol analogue, **74**, also were tested by Dr. Michael Alley at the National Cancer Institute for anti-cancer activity. The indolizidines were also subjected to in-house brine shrimp and antimicrobial assays. Since the organic crude extract had exhibited very little insect antifeedant and no phytotoxicity, no tests were carried out with the pure compounds in insect or plant growth assays.

Antimicrobial Assay

Indolizidines

The compounds demonstrating the most potent activity were the indolizidine mixtures, **86**, **88**, and **89** (see Table 5). Since each mixture contained isomers that possibly differed in the position of the double bond, it was not possible to determine if one isomer was active and the others inactive or if they were acting in a synergistic fashion. This also created the question as to whether a

diene or dienes in the mixture of **88** was the active species or if it was the one monoene in the mixture. The trienes also demonstrated antimicrobial activity, but on a lesser scale than the monoenes and dienes. The same argument for the activity of the monoenes could be used here, that is, perhaps only one of the trienes is active and is present in minor amounts in the mixture.

The difference in antibacterial activity versus antifungal activity was appreciable. In the cases of all three mixtures tested, antifungal activity was 40% to 66% greater than antibacterial activity.

Quinolizidines

In the antimicrobial tests, the quinolizidines expressed much less antimicrobial activity at the same concentration as the indolizidines, 100 μg (see Table 6). In comparison to the indolizidines, the dienes appear to be less active, and the triene acetate, **69**, does not express any activity at 100 μg. The two alcohols display activity comparable to the acetates, except in the case of *Geotrichum candidum*, where **74** shows a much larger zone of inhibition.

Table 4. Antimicrobial Assays, Indolizidines[a]

Fungi	**86**	**88**	**89**
Candida albicans	9	9	6
Geotrichum candidum	12	10	7
Aspergillus terreus	12	10	2
Pythium ultimum	0	0	NT[b]
Rhizoctonia solani	NT[b]	NT[b]	0
Helminthosporium sativum	0	0	NT[b]
Bacteria			
Staphylococcus aureus	4	3	2
Bacillus cereus	5	5	3
Corynebacterium michiganensis	7	6	3
Xanthomonas campestris	NT[b]	NT[b]	NT[b]
Escherichia coli	0	0	0
Pseudomonas aeruginosa	0	0	0

[a]zones of inhibition in mm using impregnated disk
[b]not tested

Table 5. Antimicrobial Assays, Quinolizidines[a]

Fungi	**51**	**69**	**65**	**74**	**75**
Candida albicans	2	0	0	1	2
Geotrichum candidum	2	0	0	7	3
Aspergillus terreus	4	0	0	3	2
Pythium ultimum	0	0	0	3	NT[c]
Rhizoctonia solani	NT[c]	1	0	b	0
Helminthosporium sativum	0	1	NT[c]	3	NT[c]
Bacteria					
Staphylococcus aureus	1	0	1	1	1
Bacillus cereus	3	1	2	2	1
Corynebacterium michiganensis	3	b	1	0	1
Escherichia coli	0	NT[c]	NT[c]	0	0
Pseudomonas aeruginosa	0	NT[c]	d	0	d

[a]zones of inhibition in mm using impregnated disk
[b]contaminated plate.
[c]not tested.

NCI Assays

Quinolizidines

The C_{22} acetate and its alcohol analogue, **51** and **74**, were submitted to the National Cancer Institute, where a preliminary test in an AIDS screen demonstrated cytotoxicity but no anti-HIV activity for the compounds. In further tests against murine leukemia (P-388) and renal cell carcinoma (SN12K1) cell lines, both compounds displayed quite comparable activity (see Table 7). The GI_{50} was of the same order of magnitude for each compound in both tests. Growth inhibition persisted to the end of the test, seven days.

Table 6. NCI Bioassay Results[a].

	Acetate **51**	Alcohol **74**
Cell Line	GI_{50}	GI_{50}
Renal Cell Carcinoma		
Day 4	4.23	6.33
Day 7	6.64	6.72
Murine Leukemia		
Day 4	1.78	1.70
Day 7	1.76	1.81

[a]μg/ml concentration

DISCUSSION

Discovery, Isolation and Activity

The novel nitrogenous heterocycles from *C. picta* represent an important contribution to the chemistry of tunicates. Both the indolizidines and the quinolizidines are the first ever reported from a tunicate source. The substitution pattern on the quinolizidines is also unprecedented in marine natural products.

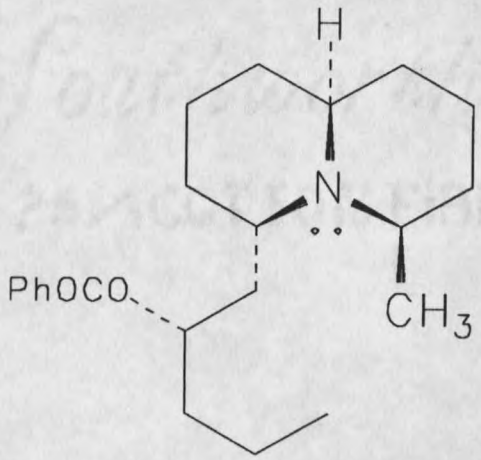
The demonstrated ability of countercurrent chromatography to separate mixtures of structurally similar tertiary amines is an indication of the versatility and power of this chromatographic methodology. CCC proved itself to be a viable alternative for isolation when other more traditional methods failed.

Another important facet of the research is the biological activity of a number of the compounds. The cytotoxic activity displayed by **51** and **74** in the tests at NCI should be enough impetus for further investigation of the properties of the molecules, such as the determination of structure-activity relationships. Although only preliminary in-house antimicrobial assays of the indolizidines have been performed, the potent

activity displayed by them warrants a more detailed investigation of their potential.

Biogenesis and Significance

The determination of an unequivocal biogenetic pathway for both the quinolizidines and indolizidines would be incomplete without labeling experiments. It is possible, however, to compare the biogenesis of the compounds with those of other metabolites. Although the quinolizidines are unique in their substitution pattern, porantherilidine, **90**, does bear the closest resemblance to the marine metabolite in nature.

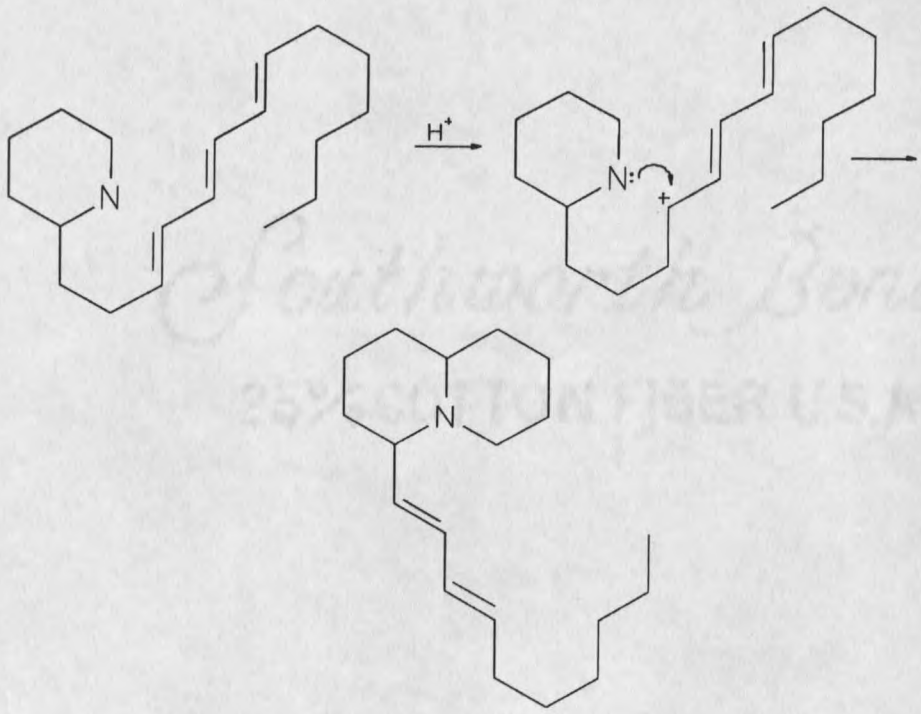


90

The biogenesis suggested by the authors was via a polyketide and a piperidine:

> The biogenesis of the porantherilidine alkaloids is suggested to be from the condensation of a C-16 polyketide chain with NH_3, followed by appropriate cyclizations and loss of the terminal carboxy group. The implication of the suggestion is that 2,6-disubstituted piperidines, themselves well known as alkaloids, are biosynthetic intermediates[49].

The existence of the pseudodistomins, isolated from a Japanese tunicate, **20,21**[12], indicates that piperidines can be made by at least one marine invertebrate. Indeed, one could envision a piperidine intermediate similar to this compound undergoing cyclization to form the quinolizidine skeleton:



Scheme 1. Speculative Biogenetic Pathway for the Formation of Quinolizidine via a Piperidine Intermediate.

The source of such a metabolite is also the object of study. It has now been shown that microbial symbionts of marine invertebrates are the true producers of some of the compounds attributed to the invertebrates themselves[37]. Since nothing about possible *C. picta* symbionts is known, the origin of the compounds remains undefined.

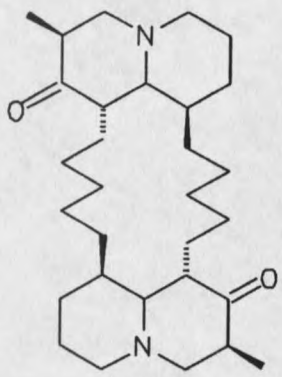
As has been discussed, a number of both the quinolizidines and indolizidines expressed antimicrobial and cytotoxic activity in the bioassays. At this point, however, no direct relationship between the bioactivity results and the tunicate's use of these compounds can be determined. The ability of these lipophilic substances to extract a proton from its environment could be just as well to serve as an ionophore or to be a proton source for a regulatory mechanism, or this ability could just as easily be coincidental. The nonpolar nature of the compounds does imply the ability to traverse cell membranes. Therefore, any activity could conceivably occur in the intracellular matrix.

Since marine as well as terrestrial organisms are prone to predation on a macroscopic or a microscopic scale, a built-in defense mechanism is a handy thing to have. Both the quinolizidines and the indolizidines could serve the tunicate in the same fashion as the tunic

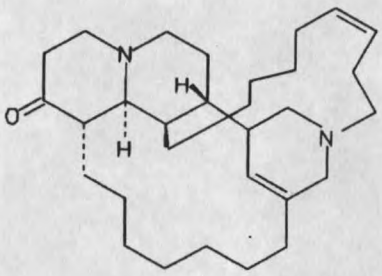
of the organism, as a water-insoluble prophylactic, i.e., as an antifouling agent or antifeedant. To prove this hypothesis would involve on-site testing.

CONCLUSION

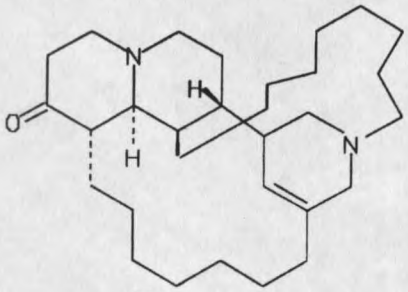
Clavelina picta has been shown to harbor an array of novel compounds bearing as the basic skeletal structure either a quinolizidine or an indolizidine. While many examples of quinolizidines are known to exist in the terrestrial sphere, few have, to date, been found in the marine environment. Petrosin, **91**[58], and nine very closely related compounds[59] were isolated from sponges. Sponges were also the source for sarain-1, **92**, sarain-2, **93**[60] and isosarain-1, **94**[61].



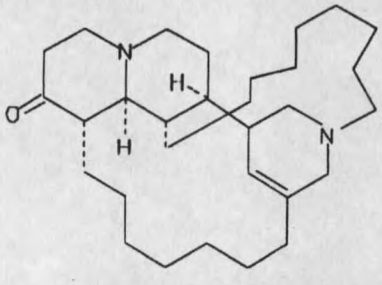
91



92



93



94

Twelve novel quinolizidines have now been added to the list of known compounds. Both the uniqueness of the substitution pattern and the activity of these compounds has made them worthy objects for isolation and identification. The indolizidines are comprised of mixtures of three new compounds. They bear closest resemblance to the *Dendrobates* alkaloids, differing in alkyl chain length and unsaturation number.

The fact that many of the compounds from *C. picta* differed only slightly in size and unsaturation proved to be a major hurdle for their separation. Indeed, isolating the mixtures was facile, whereas the separation of the individual homologues and analogues required a lengthy trial-and-error method of chromatography. It was not until solvent systems were developed for counter current chromatography that the first quinolizidine was isolated as a pure compound. The fact that the deprotonated form of the compounds was very amenable to counter current chromatography led to the isolation of most of the quinolizidines. The data collected on the pure compounds enabled the identification of other compounds present only in trace amounts and those which eluded complete separation, such as the aldehydes. With the exception of the one monoene isomer, the indolizidines could be separated via normal phase CCC

according to the number of double bonds on the alkyl chain. No further separation of the individual isomers was achieved.

The last goal of this research was to determine the specific activity of the compounds. The preliminary brine shrimp assays indicated biological activity for the indolizidines but not for the quinolizidines. In the anti-HIV assay performed under the auspices of the National Cancer Institute by Dr. James McMahon, both the C_{22} acetate, **51**, and the C_{20} alcohol analogue, **74**, displayed cytotoxic activity. Further NCI tests revealed that **51** and **74** were active in the renal cell and murine leukemia cell lines. Both the indolizidines and the quinolizidines tested displayed antimicrobial activity, with a clear tendency to be more potent antifungal agents than antibacterial agents.

EXPERIMENTAL

General

All NMR spectra were recorded on a Bruker WM-250 spectrometer utilizing an Aspect 2000 computer which was later replaced with an Aspect 3000 computer, a Bruker AC-300 or a Bruker AM-500 spectrometer utilizing an Aspect 3000 computer. Chemical shifts are reported in ppm units, δ, relative to trimethylsilane, δ=0, with $CDCl_3$ or pyridine-d_5 as the solvent and internal standard as indicated in the text and NMR tables. Mass spectra were obtained with a VG-MM16 or a VG-7070 EHF mass spectrometer. The GC column utilized for GC/MS was a DB-5 column (JMW Scientific), 30m x 0.25mm (i.d.), 0.25 μ film thickness. IR data was obtained with a Nicolet NX-FT spectrophotometer. UV measurements were taken with a Varian G34 uv-visible spectrophotometer. Optical rotations were performed on a Perkin-Elmer 241 MC Polarimeter. All chromatographies were monitored with an ISCO V^4 variable wavelength uv absorbance detector or with an ISCO UA-5 monitor operating at 254 nm. HPLC experiments were performed with a Beckman Altex model 112 pump or a Shimadzu LC-6A liquid chromatograph system. Separations with CCC were via a PC Inc. Multi-Layer Coil

Separator-Extractor. Melting points were determined on a Fisher-Johns apparatus and are not corrected.

Brine Shrimp Assay

The brine shrimp assay, utilized to determine cytotoxicity, was performed according to the method described by Meyer, et. al.[62], in a somewhat modified fashion. A small number of brine shrimp eggs, approximately thirty, were transferred to 6 ml glass tubes containing Instant Ocean, 2g/ml in distilled H_2O. the eggs were allowed to stand under fluorescent light for 48 hours, by which time they had hatched. At the end of that period, 3 mg of baker's yeast were introduced to each tube as food. Aliquots of the test samples were taken up in H_2O or acetone and injected into the tubes. In order to create as homogeneous a mixture as possible, the tubes were shaken for about thirty seconds and then placed in a stand under the fluorescent lamp. Deaths were recorded at different intervals over a period of twenty-four hours. A brine shrimp was considered deceased if it was lying at the bottom of the tube and exhibited no movement during the time the brine shrimp deaths were being counted.

Antimicrobial Assays

Both the organic crude extract and later individual, pure compounds were tested against Gram-positive and Gram-negative bacteria and fungi. The extract or compound to be tested was taken up in CH_2Cl_2 and then applied to a sterile paper disk and allowed to dry in air. Fungi were grown on DIFCO mycological agar plates, and bacteria were grown on DIFCO tryptic soy agar plates. The test organism was suspended in sterile H_2O and spread across the surface on the agar plate to form a smooth plain. The test disks which had been inoculated were applied to the surface of the agar and left standing for 24-48 hours. The size of zones of inhibition produced were determined by measuring in mm the radius of the zone of inhibition.

ISOLATION AND CHARACTERIZATION OF QUINOLIZIDINES AND INDOLIZIDINES

Collection and Extraction

Clavelina picta was collected in Bermuda in the early summer of 1984 and in midsummer, 1987, from bridge pilings at depths of up to three meters. Both collections were immediately transferred to polyethylene containers and stored in acetone at -5^{0} C for transport to Bozeman. Upon arrival, the containers were placed in a freezer at -10^{0} C prior to extraction. Processing the tunicates proceeded by removal of solvent and sea water via vacuum filtration, followed by homogenization in methanol in a one gallon Waring blender. The resulting slurry was filtered, and the marc was steeped in methylene chloride for approximately ten hours. After soaking, the organic solvent was filtered via vacuum filtration. The soaking in solvent with subsequent filtration was repeated twice more. The acetone and methanol solubles were combined and were reduced *in vacuo*, giving a thick, dark green aqueous suspension containing both organic and water solubles. This material was then equilibrated with the methylene chloride extract. Removal of the methylene chloride

yielded a combined total for both collections of 19.81 g of organic solubles, 6.1% dry weight.

Quinolizidine Acetate Diene **51**

To a Bio-Beads S-X4 gel, (90 x 4 cm), hexane-methylene chloride-ethyl acetate (4:3:1), were applied 1.143 grams of the hexane solubles of the organic crude extract. The chromatography resulted in the elution of seven fractions. Fraction F, 35.6 mg, was chromatographed via countercurrent chromatography, utilizing as the solvent system hexane-methylene chloride-acetonitrile (50:15:35). The CCC was run in reverse phase mode for two hours with the lower phase acting as the mobile phase. Thirteen fractions were eluted, including the constituents of the hexane solubles that had not eluted with the mobile phase. This last fraction, M, contained **51** and a mixture of other compounds. Fraction M, 8.3 mg, was run via CCC once more for a period of three hours. This resulted in the elution of pure C_{22} quinolizidine acetate at 2.5 hours, 5 mg, a clear oil, $[\alpha]_D$ -75.6° (c 7.0, CH_2Cl_2); ir ν_{max} (neat) 3015, 2932, 2861, 1735, 1453, 1375, 1252, 1163, 1130, 1105, 1039, 992 cm^{-1}; uv λ_{max} (EtOH) 230 nm (ε=19,000); ^{13}C-NMR, ^{1}H-NMR, (see table 2, p. 62); HRMS: m/z 347.2831 ($C_{22}H_{37}NO_2$ requires 347.2824); EIMS: m\z (relative

intensity) 347 (M^+, 6), 332 (9), 288 (9), 260 (100), 210 (4), 150 (7).

Quinolizidine Acetate Triene **69**

The $CHCl_3$ solubles of the organic crude extract, 5.87 grams were applied to a Bio-Beads S-X4 column, (90 x 4 cm), and eluted with hexane-methylene chloride-ethyl acetate (4:3:1), yielding four fractions. A 0.5 gram aliquot of fraction D, 1.064 grams, was subjected to step gradient elution on a reverse phase C_{18} gel, (22 x 2.2 cm): step 1)70% methanol in water; step 2)80% methanol; step 3)100% methanol. From the five fractions eluted, a 140 mg aliquot of fraction A was again subjected to reverse phase flash chromatography using a step gradient: step 1)50% methanol in water; step 2)70% methanol; step 3)90% methanol; step 4)100% methanol. The chromatography yielded six fractions. A small portion, 10 mg, of fraction E, 90 mg, was taken up in 3 ml of methanol and spiked with 3 µl of triethylamine and subjected once again to reverse phase flash chromatography, step gradient: step 1)90% methanol in water; step 2)100% methanol. Eluted with 90% methanol was the triene, **69**, 4 mg, a yellow viscous oil, $[\alpha]_D$ -49.6° (c 2.0, CH_2Cl_2); ir ν_{max} 3014, 2932, 2862, 1736, 1457, 1375, 1255, 1217, 1160, 1116, 1040, 995 cm^{-1}; uv λ_{max} (EtOH) 282 nm (ε=33,000), 271

nm (43,000), 262 nm (37,000); ^{13}C-NMR (300 MHz, $CDCl_3$): δ 170.14, 138.04, 135.11, 131.81, 130.96, 130.32 (two carbons), 73.35, 58.18, 53.21, 49.14, 32.43, 31.48, 29.79, 29.69, 25.64, 22.28, 22.18, 21.50, 19.70, 17.05, 13.85; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.87 (3H, t, H-20), 1.09 (1H, m, H-5_a), 1.13 (3H, d, J=7Hz, H-1), HRMS: m/z 345.2668 ($C_{22}H_{35}NO_2$ requires 345.2668); EIMS: m/z (relative intensity) 345 (M^+, 6), 302 (5), 285 (3), 258 (100), 210 (6), 150 (6).

Quinolizidine Acetate Diene **65**

From the modified flash chromatography used for **69**, the fourth fraction, D, 14 mg, was deprotonated with 5 µl of triethylamine and fractionated once again on the C_{18} gel. Eluting from the column at 90% methanol in water was **65**, a pale yellow viscous oil, $[\alpha]_D$ -56° (c 1.4, CH_2Cl_2); ir ν_{max} (neat) 3015, 2930, 2864, 1736, 1250, 1164, 1129, 1105, 1039, 991, 962 cm^{-1}; uv λ_{max} (EtOH) 229 nm (ε=18,000); ^{13}C-NMR (300 MHz, C_5D_5N): δ 170.05, 137.06, 133.38, 131.22, 130.87, 73.45, 58.35, 53.37, 49.10, 32.95, 32.51, 31.70, 30.09, 25.97, 22.46, 21.46, 21.35, 20.17, 17.21, 14.04; ^{1}H-NMR (500 MHz, C_5D_5N): δ 0.79 (3H, t, 6Hz, H-20), 0.97 (1H, bd, J=10.2Hz, H-5_b), 1.09 (3H, t, H-1), 1.17-1.3 (8H, m, H-16,17,18,19), 1.31 (2H, q, H-15), 1.2 (1H, H-9_a) 1.47 (4H, bs, H-7,8), 1.58

(1H, m, J=7.3, 3, H-9_b), 1.74 (1H, dq, J=14.4, 4.3, 4.2, H-4_a), 1.82 (1H, tt, J=14.4, 11.1, H-4_b), 1.92 (1H, bq, J=11.1, 10.2, H-5_b), 2.13 (3H, s, H-22), 3.12 (1H, bd, H-6), 3.50 (1H, dq, J=7, 3, H-2), 3.84 (1H, bt, J=8.5, 7.3, 3), 4.70 (1H, q, J=3, H3), 5.67 (1H, dd, J=15.3, 3, H=11), 5.73 (1H, m, H=14), 6.15 (1H, dd, J=15.3, 10.5, H-13), 6.31 (1H, dd, J=15.3, 10.5, H-12); HRMS: m/z 319.2153 ($C_{20}H_{33}NO_2$ requires 319.2511); EIMS: m/z (relative intensity) 319 (M^+, 4), 304 (6), 276 (7), 260 (7), 232 (27), 210 (3), 150 (3), 44 (100).

Quinolizidine Acetate Triene **70**

From the same fractionation with CCC that yielded fraction M from the isolation of **51**, was eluted fraction K, 1.5 mg, containing the triene, **74**, a dark yellow film, $[\alpha]_D$ -33.3° (c 3.0, CH_2Cl_2); ir ν_{max} (neat) 3014, 2928, 2863, 1735, 1458, 1374, 1257, 1217, 1162, 1115, 1039, 995 cm^{-1}; uv λ_{max} (EtOH) 281 nm (ε=21,300), 271 (27,200), 262 (21,200); HRMS: m/z 317.2355 ($C_{20}H_{31}NO_2$ requires 317.2355); EIMS: m/z (relative intensity) 317 (M^+, 17), 302 (14), 258 (18), 230 (89), 210 (7), 43 (100).

Quinolizidine Acetate Diene **67**

The same fraction, K, that contained **70**, contained a trace amount of **67**. HRMS: m/z 291.2211 ($C_{18}H_{29}NO_2$

requires 291.2210); EIMS: m/z (relative intensity) 291 (M^+, 26), 276 (36), 231 (17), 210 (13), 204 (100), 150 (15).

Quinolizidine Acetate Diene **66**

From fraction E of the reverse phase chromatography which contained **69** were found trace amounts of **66** via mass spectral analysis. HRMS: m/z 333.2649 ($C_{21}H_{35}NO_2$ requires 333.2647); EIMS: m/z (relative intensity) 333 (M^+, 13), 318 (24), 273 (58), 246 (100), 210 (13), 150 (93).

Quinolizidine Alcohol **74**

A 357 mg aliquot of the methanol/water solubles of the organic crude extract was fractionated on a Sephadex LH-20 gel (178 x 2.5 cm) with methylene chloride-methanol (1:1) to yield ten fractions. Fraction C, 143 mg, was leached with the lower phase of a CCC binary phase solvent system, hexane-methylene chloride-acetonitrile (50:15:35), to give 44 mg of a mixture of quinolizidines. The mixture was fractionated on Sephadex LH-20 gel (110 x 2.2 cm) with methylene chloride-methyl t-butyl ether-isopropanol (1:1:1), yielding five fractions. Fraction B, 26 mg, was spiked with 5 µl of triethylamine immediately prior to chromatography via CCC, lower phase

as the mobile phase, utilizing the same solvent system as the one used for the isolation of **51**. Nine fractions were eluted. Fraction H contained the alcohol, **74**, 1 mg, a clear crystalline solid, m.p. 72-74 ^{0}C, $[\alpha]_D$ +27.1° (c 3, CH_2Cl_2); ir ν_{max} (neat) 3408, 3015, 2928, 2855, 1715, 1454, 1374, 1257, 1156, 1112, 1047, 989 cm^{-1}; uv λ_{max} (EtOH) 230 nm (ε=18,000); ^{1}H-NMR, ^{13}C-NMR (see table 3, p. 111); HRMS: m/z 305.2720 ($C_{20}H_{30}NO$ requires 305.2119); EIMS: m/z (relative intensity) 305 (M^+, 24), 290 (51), 260 (68), 234 (34), 168 (34), 150 (38), 41 (100).

X-ray crystallographic data:

Atomic coordinates (x10^4).

	x	y	z
N(1)	5048(3)	4599	2108(2)
C(2)	5431(4)	3337(10)	1307(3)
C(2')	6379(4)	4637(12)	947(3)
C(3)	5776(4)	852(11)	1542(3)
C(4)	6579(4)	652(11)	2479(3)
C(5)	6100(5)	1830(11)	3290(3)
C(6)	5833(4)	4323(11)	3022(3)
C(7)	5448(5)	5657(12)	3842(3)
C(8)	4259(5)	5099(13)	3946(4)
C(9)	3497(4)	5480(13)	2999(3)
C(10)	3857(4)	4054(11)	2196(3)
C(11)	3101(4)	4591(13)	1270(3)
C(12)	2379(4)	3119(14)	792(4)
C(13)	1599(4)	3614(16)	-81(4)
C(14)	905(5)	2035(19)	-525(5)
C(15)	107(8)	2301(25)	-1395(6)
C(16)	145(6)	4331(26)	-1949(6)
C(17)	-754(7)	4454(31)	-2873(6)
C(18)	-683(7)	6094(25)	-3520(6)
C(19)	-1496(8)	6239(30)	-4442(7)
C(20A)	-1494(13)	7784(46)	-5061(11)

C(20B)	-1225(43)	5626(109)	-5387(36)
O	4801(3)	-480(9)	1580(2)

Bond lengths (A).

N(1)-C(2)	1.485	(6)	N(1)-C(6)	1.479	(5)
N(1)-C(10)	1.501	(6)	C(2)-C(2′)	1.529	(8)
C(2)-C(3)	1.536	(8)	C(3)-C(4)	1.511	(6)
C(3)-O	1.422	(6)	C(4)-C(5)	1.527	(8)
C(5)-C(6)	1.530	(9)	C(6)-C(7)	1.529	(8)
C(7)-C(8)	1.504	(9)	C(8)-C(9)	1.509	(7)
C(9)-C(10)	1.525	(8)	C(10)-C(11)	1.499	(6)
C(11)-C(12)	1.330	(9)	C(12)-C(13)	1.453	(7)
C(13)-C(14)	1.335	(11)	C(14)-C(15)	1.440	(10)
C(15)-C(16)	1.427	(19)	C(16)-C(17)	1.556	(11)
C(17)-C(18)	1.338	(19)	C(18)-C(19)	1.497	(12)
C(19)-C(20A)	1.301	(27)	C(19)-C(20B)	1.453	(55)

Bond angles (°).

C(2)-N(1)-C(6)	111.8	(3)
C(6)-N(1)-C(10)	113.2	(3)
N(1)-C(2)-C(3)	114.6	(4)
C(2)-C(3)-C(4)	112.1	(5)
C(4)-C(3)-O	110.1	(4)
C(4)-C(5)-C(6)	109.7	(4)
N(1)-C(6)-C(7)	111.7	(4)
C(6)-C(7)-C(8)	112.1	(5)
C(8)-C(9)-C(10)	111.6	(5)
N(1)-C(10)-C(11)	110.0	(4)
C(10)-C(11)-C(12)	124.2	(6)
C(12)-C(13)-C(14)	122.1	(8)
C(14)-C(15)-C(16)	118.6	(10)
C(16)-C(17)-C(18)	119.4	(11)
C(18)-C(19)-C(20A)	121.5	(14)
C(2)-N(1)-C(10)	112.3	(3)
N(1)-C(2)-C(2′)	110.3	(4)
C(2′)-C(2)-C(3)	110.6	(4)
C(2)-C(3)-O	109.3	(4)
C(3)-C(4)-C(5)	110.5	(4)
N(1)-C(6)-C(5)	113.5	(4)
C(5)-C(6)-C(7)	112.3	(4)
C(7)-C(8)-C(9)	109.8	(5)
N(1)-C(10)-C(9)	109.7	(4)
C(9)-C(10)-C(11)	108.9	(4)
C(11)-C(12)-C(13)	125.6	(7)
C(13)-C(14)-C(15)	127.4	(10)
C(15)-C(16)-C(17)	114.6	(10)
C(17)-C(18)-C(19)	119.9	(11)

C(18)-C(19)-C(20B) 125.2 (21)

Quinolizidine Alcohol **75**

From the same chromatography used to isolate **74** eluted fraction G, 0.3 mg, the C_{18} alcohol **75**, a white amorphous solid, mp 72-74°C $[\alpha]_D$ +21.7° (c 1.0, CH_2Cl_2); ir ν_{max} (neat) 3404, 3015, 2928, 2855, 1715, 1454, 1374, 1257, 1156, 1112, 1047, 989 cm^{-1}; uv λ_{max} (EtOH) 231 nm (ε=18,500); ^{1}H-NMR (300 MHz, C_5D_5N): 0.81 (3H, t, J=6Hz, H-18), 1.27-1.4 (4H, m, H-16,17), 1.29 (3H, d, J=6, H-1), 2.04 (2H, q, H-15), 3.13 (1H, bm, H-6), 3.31 (1H, bt, J=6, H-2), 3.61 (1H, bs, H-3), 4.03 (1H, bq, J=6.2, H-10), 5.63 (1H, m, J=15, H-14), 5.87 (1H, dd, J=15.3, 6.2, H-11), 6.2 (1H, dd, J=15, 10.5, H-13), 6.38 (1H, dd, J=15.3, 10.5, H-12); HRMS: m/z 277.2407 ($C_{18}H_{31}NO$ requires 277.2407); EIMS: m/z (relative intensity) 277 (M^+, 24), 262 (44), 232 (62), 168 (25), 41 (100).

Quinolizidine Acetate Diol **77**

Another 350 mg aliquot of the methanol/water solubles of the organic crude extract was fractionated on Sephadex LH-20 gel (178 X 2.5 cm) with methylene chloride-methanol (1:1), to yield 10 fractions. Fraction C, 144 mg, was washed through a plug (2 X 10 cm) containing Sephadex LH-20 gel with methylene chloride-methyl t-butyl ether-

isopropanol (1:1:1), to give 65 mg of a quinolizidine mixture. The mixture was applied to a Sephadex LH-20 column (115 X 2.2 cm) of the same solvent mixture, and three fractions were eluted. Fraction C, 28 mg, was chromatographed via CCC with the same solvent system used to isolate **51**, again in reverse phase mode. The first fraction to elute, 9 mg, was spiked with 3 µl of triethylamine and run under the same conditions once more with CCC. Five fractions eluted, with fraction C, 1.7 mg, containing a mixture of compounds including **77**. Fraction C was run once more on CCC under the same conditions but at a slower chart speed. The first fraction eluted, 0.9 mg, was acetylated. The reaction mixture was chromatographed via CCC to yield 0.7 mg of the protonated triacetate, a clear solid, $[\alpha]_D$ -40° (c 1.0, CH_2Cl_2), ir ν_{max} (thin film) 2956, 2928, 2855, 2548, 1739, 1460, 1432, 1371, 1237, 1041, 1025, 997 cm^{-1}; uv λ_{max} (EtOH) 233 nm (ε=18,900); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.83 (3H, t, H-20), 1.70 (3H, d, J=7Hz, H-1), 2.03 (3H, s, H-22), 2.07 (3H, s, H-24, interchangeable with H-26), 2.11 (3H, s, H-26, interchangeable with H-24), 3.72 (1H, bs, H-6), 3.93 (1H, bs, H-2), 4.2 (1H, br d, J=10, H-10), 4.83 (1H, bs, H-3), 5.03 (1H, m, J=4, H-16), 5.4 (1H, dd, J=4, 6.7, H-15), 5.64 (1H, m, J=15.7, 6.7, H-14), 6.15 (1H, m, J=15, 12, H-12), 6.25 (1H, m,

J=15, 10, H-11), 6.35 (1H, dd, J=15.7, 12, H-13); HRMS: m/z 463.2917 ($C_{22}H_{37}NO_4$ requires 463.2916); EIMS: (PCI/NH_3) m/z (relative intensity) 464 (M^++1, 100), 404 (6).

Quinolizidine Acetate Monool **78**

From the same modified flash chromatography for **69**, the third fraction, C, 10 mg, was spiked with 5 µl of triethylamine and run at 90% methanol in water. The monool eluted as the third fraction, 2.7 mg, a light yellow film, ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.85 (3H, br s), 1.33 (2H, br d), 1.2-1.2 (m), 1.5-1.65 (m), 1.7-1.8 (m), 1.8-2.05 (m), 2.1 (s), 3.25 (br), 3.8 (br), 4.12 (m), 4.62 (br s), 5.6 (dd), 6.1 (m), 6.2); HRMS: m/z 363.2800 ($C_{22}H_{37}NO_3$ requires 363.2873); EIMS: m/z (relative intensity) 363 (M^+, 15), 348 (45), 304 (35), 276 (100), 210 (48), 150 (36).

Quinolizidine Aldehyde 80, 81

The fractionation of the $CHCl_3$ solubles on Bio-Beads S-X4 gel (90 x 4 cm), hexane-methylene chloride-ethyl acetate (4:3:1), yielded four fractions. A 375 mg aliquot of fraction D was chromatographed on Sephadex LH-20 gel (178 x 2.5 cm) with methylene chloride-methanol (1:1), to give four fractions. A 100 mg aliquot of

fraction B was subjected to reverse phase flash chromatography using C_{18} gel (22 x 2.2 cm), step gradient: step 1)70% methanol in water; step 2)90% methanol; step 3)100% methanol. Eluted at 70% methanol was fraction B, 67 mg. A 30 mg portion of fraction B was spiked with 10 µl of triethylamine and chromatographed via CCC, using the same solvent system as for the isolation of **51**, reverse phase mode. Fraction C, 5.3 mg, contained a mixture of **80** and **81**. Fractions from other chromatographies containing the aldehydes were chromatographed separately via CCC, reverse phase mode. In the best cases ratios of **80**:**81** were on the order of 8:1 or 1:8. Aldehyde **80** was a yellow film, $[\alpha]_D$ -56.2° (c 1.3, CH_2Cl_2); ir ν_{max} (neat) 2939, 2867, 2464, 1736, 1685, 1642, 1599, 1448, 1375, 1240, 1166, 1138, 1102, 1037, 950 cm^{-1}; uv λ_{max} (EtOH) 252 nm (ε=16,800), 228 (18,300); ^{1}H-NMR (300 MHz, C_5D_5N): 1.14 (3H, d, J=7Hz, H-1), 3.10 (1H, bm, J=7, H-2), 4.59 (1H, bs, 1H, H-3), 4.08 (1H, dt, J=7.3, H-10), 6.17 (1H, dd, J=17, 7.5, H-12), 6.75 (1H, dd, J=17, 7.3, H-11), 9.53 (1H, d, J=7.5); HRMS: m/z 265.1677 ($C_{15}H_{23}N$ requires 265.1677); EIMS: m/z (relative intensity) 265 (M^+, 7), 247 (100), 232 (33), 206 (14), 187 (22), 160 (25), 43 (36).

The aldehyde **81** was also a yellow film, $[\alpha]_D$ -34.9° (c 3, CH_2Cl_2); ir ν_{max} (neat) 2926, 2853, 1734, 1684,

1637, 1453, 1370, 1244, 1162, 1104, 992cm^{-1}; uv λ_{max} (EtOH) 232 nm (ε=28,300); ^{13}C NMR (300 MHz, $CDCl_3$): δ 193.65, 151.99/151.15 indeterminable due to similar shift of olefins of **11**), 130.67/128.41 (indeterminable due to similar shift of olefins of **11**), 73.51, 58.26, 53.95, 49.15, 31.42, 29.25, 25.53 (two C), 21.43, 19.69, 17.06, 13.98; HRMS: m/z 295.1749 ($C_{16}H_{25}NO_3$ requires 295.1746); ms: m/z (relative intensity) 295 (M^+, 6.3), 280 (26.9), 235 (20), 208 (100), 150 (7.5).

Indolizidine Monoenes **86**

From the third fraction of the Bio-Beads S-X4 separation described in the isolation of **80**, a 525 mg aliquot was chromatographed on Sephadex LH-20 gel (178 x 2.5 cm) with methylene chloride-methanol (1:1), yielding five fractions. Fraction C was subjected to reverse phase flash chromatography, C_{18}, (25 x 2.2 cm), step gradient elution: step 1) 70% methanol in water; step 2) 90% methanol; step 3) 100% methanol. Fraction C, 167 mg, was subjected once more to reverse phase flash chromatography, a step gradient beginning with 50% methanol in water. This gave three fractions at the 1:1 solvent mixture. From the fourth fraction, 359 mg, eluting at 100% methanol, was partitioned between aqueous triethylamine (5%) and methyl-t-butyl ether. From the

organic layer, 215 mg of a deprotonated quinolizidine mixture was recovered. A 60 mg aliquot of the deprotonated mixture was fractionated via CCC, with the same solvent system used for the isolation of **51**, with the upper phase as the mobile phase. The first fraction to elute, 19.5 mg, was **86**, the monoene mixture, a clear oil, $[\alpha]_D$ +15° (c 5.0, CH_2Cl_2); ir ν_{max} ($CDCl_3$) 3005, 2961, 2911, 2856, 2796, 1456, 1442, 1379, 1346, 1263, 1241, 1163, 1131, 1093, 1065, 966 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), δ 0.87 (3H, t, H-18), 2.4 (1H, m, H-4), 2.65 (1H, m, H-1_a), 2.8 (1H, m, H-1_b), 3.0 (1H, m, H-8), 5.25 (1H, m, H-10 or H-11), 5.4 (1H, m, H-10 or H-11); ^{13}C NMR (125 MHz, $CDCl_3$), δ 59 (C-8), 58 (C-1), 50 (C-4); HRMS: (PCI/NH_3) m/z 264.2690 ($C_{18}H_{34}N$ requires 264.2690); EIMS: m/z (relative intensity) 263 (M^+, 3), 124 (100), 96 (10).

Indolizidine Dienes **88**

From the chromatography described in the isolation of **86**, the second fraction to elute was the diene mixture, **88**, 4 mg, a clear oil, $[\alpha]_D$ +33.5° (c 1.0, CH_2Cl_2); ir ν_{max} 3013, 2928, 2854, 1462, 1381, 1243, 1163, 1126, 1054, 1005 cm^{-1}; uv λ_{max} (EtOH) 232 (12,500); ^1H-NMR (300 MHz, $CDCl_3$), δ 0.86 (3H, t, H-18), 3.5 (1H, m, H-8), 5.8 (1H, dd, H-9); HRMS: (PCI/NH_3) m/z 262.2519 ($C_{18}H_{32}N$ requires

262.2504); EIMS: m/z (relative intensity) 261 (M^+, 50), 190 (89), 124 (70), 96 (40), 41 (100).

Indolizidine Trienes **89**

From the same chromatography used to isolate **86** and **88** was isolated **89** as the third fraction eluted, 1.4 mg, a light yellow film, $[\alpha]_D$ +36° (c 5.0, CH_2Cl_2); ir ν_{max} 3013, 2928, 2854, 1463, 1382, 1242, 1163, 1126, 1052, 996 cm^{-1}; HRMS: m/z 259.2280 ($C_{18}H_{29}N$ requires 259.2278); ms: m/z (relative intensity) 259 (M^+, 58), 216 (100), 124 (39), 96 (23).

REACTIONS

Reduction of a Mixture of **51** and **65**

To a 50 ml round bottom flask were added 7 ml MeOH containing 6 mg of a mixture of **51** and **65**. To the contents were added 10 mg of palladium on charcoal. The reactants were stirred at room temperature under slight H_2 pressure for 7 hours. The palladium catalyst was removed via vacuum filtration, and the solvent was removed in vacuo, leaving a clear film. EIMS yielded M^+ of 349 and 321 for the reduced products of **51** and **65**, respectively.

Reaction of Quinolizidine Acetate, **51**, with 4-Phenyl-1,2,4-triazoline-3,5-dione

To 10 mg of the quinolizidine acetate, **51**, were added 0.5 ml CH_2Cl_2 in a 7 dram vial. The vial was flushed with nitrogen, sealed with a septum and placed in an acetone dry ice bath. To the reaction mixture were added dropwise 1.5 eq. of the triazoline in 0.5 ml CH_2Cl_2 via a syringe with continual stirring. The reagent was added until the solution remained a permanent pink, the indication for saturation. The contents were left in the ice bath for one hour, removed and allowed to warm up to room temperature. After sitting for five hours at room

temperature, the solvent was blown down with nitrogen. The reaction mixture was permeated through Sephadex LH-20 gel with MeOH-CH_3CN (4:1), giving three fractions. The third fraction, 11.9 mg, was demonstrated to be the derivative, according to ^1H NMR analysis. The spectrum revealed a doubling of signals both in the olefinic region and in the acetate region, indicating the possible formation of both endo and exo products. The fraction was then chromatographed via centrifugal counter-current chromatography with n-hexane-CH_2Cl_2-CH_3CN (10:3:7). The CCC was run in the reverse phase mode, using the lower phase as the mobile phase. One fraction eluted with the mobile phase, and other material was recovered from the stationary phase. The solvent was removed *in vacuo*. ^1H NMR analysis of the two fraction revealed that the fraction which had eluted with the mobile phase to be the reagent or a decomposed form of the reagent. The fraction which remained in the stationary phase, upon analysis, appeared to be decomposed starting material. There was no trace of the derivative.

Reaction of Quinolizidine Acetate, **51**, with Methyl Iodide

To 3 mg of the deprotonated quinolizidine acetate, **51**, were added 0.75 ml of benzene-d_6 in a ^1H NMR tube.

Into the tube were injected 0.7 µl of CH_3I via syringe, and the reaction was followed via ^1H NMR. ^1H NMR spectra were taken at 30 minute intervals for 2 hours, but no reaction was observed.

Attempted N-oxide Formation of the Quinolizidine Acetate, **51**

To 4.2 mg of the quinolizidine acetate, **51**, were added 0.5 ml of 30% H_2O_2 in 0.5 ml MeOH in a 7 dram vial. With the addition of the peroxide, the clear solution became opaque. After a period of 2 hours, the solution again became clear. The contents were constantly stirred for 30 hours at room temperature. The progress of the reaction was followed via thin layer chromatography, using ODS plates. At 24 hours there still appeared to be a small amount of unreacted material. A more polar component, the supposed n-oxide, was observed at that time. A third component, which had an Rf less than the starting material, was also observed. At 32 hours, there was no change in the tlc plate. The contents of the vial were transferred to a 50 ml round bottom flask, and the solvents were removed *in vacuo*. A ^1H NMR of the contents of the round bottom flask revealed the presence of a very complex mixture of compounds. Subsequent permeation of the reaction mixture through Bio-Beads S-X8 gel with

CH_2Cl_2 (3:2), resulted in four fractions, none of them having any identifiable compounds.

Formation of the Hydrogen Bromide Salt of the 2-phenyl-butyryl Derivative of **51**

To 5 mg of the deprotonated 2-phenyl-butyryl derivative of the quinolizidine acetate, **51**, were added 2 ml dilute HBr, pH4, in a 7 dram vial. The vial was sealed and shaken to mix the solvents. The organic layer was removed, and the vial was washed five times with methylene chloride. The organic solvent containing the compound was reduced *in vacuo* and taken up in $CDCl_3$. ^1H NMR of the compound revealed shifts indicative of a protonated tertiary amine but not exactly the same as the naturally occurring HCl amine salt. Attempts to crystallize the hydrobromide salt were unsuccessful.

Hydrolysis of the Quinolizidine Acetate, **51**

To 20 mg of the quinolizidine acetate, **51**, were added 1 eq. of $Ba(OH)_2 \cdot 8H_2O$ in 2m 90% EtOH in a 15 ml pear-shaped round bottom flask. The contents were heated to 60 ^0C for 5 hours with stirring, whereafter the contents were allowed to stand at room temperature overnight with stirring. The solvent was removed *in vacuo*, and solids were removed via vacuum filtration and washed with

methylene chloride. The organic solvent was removed *in vacuo*, leaving a very light yellow-colored oil, the hydrolyzed acetate, 18 mg. The compound crystallized within thirty minutes, and the spectral data of the hydrolyzed compound were found to be identical to that of the naturally occuring alcohol, **74**.

Reaction of Quinolizidine Alcohol, **74**, with 2-Chloro-4-nitrobenzoyl Chloride

To 2 eq. of 2-chloro-4-nitrobenzoic acid were added 1 ml thionyl chloride in 1 ml toluene in a 25 ml round bottom flask. The contents were refluxed under nitrogen for 7 hours with stirring. The solvent and the remaining thionyl chloride were removed *in vacuo*, leaving a light-yellow oil. The alcohol, 20 mg, was transferred in 1.5 ml pyridine-d_5 to the round bottom flask containing the acid chloride. To the contents 1.5 eq. DMAP were added, and the mixture was heated to 65 ^{0}C under nitrogen with stirring for five hours. The reaction mixture was allowed to cool to room temperature, and a ^{1}H NMR spectrum was taken. Only the derivative was observed; there appeared to be no starting material. The solvents were removed *in vacuo*, leaving a brown oil. The reaction mixture was permeated through Bio-Beads S-X8 gel with CH_2Cl_2-CH_3CN (3:2), to yield 21 mg (95%) of the

derivative, a light yellow oil. ^{1}H NMR analysis of the fraction showed the presence of a one-proton signal at 11.5 ppm, indicating the formation of the hydrochloride salt of the derivative. A portion of the derivative, 10 mg, spiked with 5 µl of freshly distilled TEA and chromatographed via CCC. One fraction eluted from the mobile phase, the deprotonated derivative, a clear oil. Attempts to crystallize the derivative were unsuccessful.

Reaction of Quinolizidine Alcohol, **74**, with 2-Phenylbutyryl Chloride

To 200 mg of 2-phenylbutyric acid were added 20 ml thionyl chloride in a 100 ml round bottom flask. The contents were refluxed under nitrogen with stirring for four hours. The excess thionyl chloride was removed *in vacuo*, leaving behind a slightly yellow oil, the acid chloride. To 21.5 mg (2 eq.) of the acid chloride were added 20 mg (1 eq.) of the quinolizidine alcohol, **74**, a catalytic amount of DMAP (0.1 eq.) in 1 ml dry benzene and 1 ml dry pyridine in a 15 ml pear-shaped round bottom flask as in the method described by Barnekow[53]. The reaction mixture was heated to 55 ^{0}C for 4.5 hours under nitrogen with stirring and then allowed to stand at room temperature for 12 hours. The solvent was removed *in vacuo*, leaving behind a yellow solid. The reaction

mixture was partitioned between 5% $NaHCO_3$ and methylene chloride, yielding the derivative in the organic layer. The aqueous layer was acidified to pH4 and partitioned with methylene chloride, yielding the unreacted acid in the organic layer. The solvent was removed *in vacuo*, giving 10 mg of the unreacted acid. An optical rotation performed on the acid ($CHCl_3$) gave no observable optical rotation.

Acetylation of Quinolizidine Diol, 77

To 0.9 mg of a fraction containing the diol, **77**, were added 0.3 ml of acetic anhydride and 0.5 mg of DMAP in 0.6 ml dry pyridine in a 1 ml microscale reaction vessel. The contents were heated to 55 °C for 1.5 hours under nitrogen with stirring. The solvent was reduced *in vacuo*, leaving a clear film. ^1H NMR revealed the presence of three acetate signals in the product. The compound had also been protonated. The reaction mixture was spiked with 5 µl of triethylamine and chromatographed via CCC, n-hexane-CH_2Cl_2-CH_3CN (50:15:35), reverse phase mode, yielding two fractions, A and B. Fraction B contained the triacetate, 0.7 mg, a clear film. EIMS gave m/z 463, corresponding to the triacetate.

Reaction of Quinolizidine Alcohol, 74, with 4-Bromophenyl Isocyanate

To 3.5 mg of the quinolizidine alcohol, **74**, were added 1.5 eq. 4-bromophenyl isocyanate (3.5 mg) and 0.5 eq. DMAP (0.75 mg) in 0.5 ml C_6H_6 in a 15 ml pear-shaped round bottom flask. The round bottom was sealed, and the contents were stirred occasionally at room temperature for 24 hours. EIMS revealed no reaction had taken place at that time. The reaction mixture was allowed to stand at room temperature for another 48 hours. EIMS gave m/z 504, the desired product, but revealed the presence of some as yet unreacted material. The reaction mixture was allowed to stand another 32 hours. When the contents were examined after this period, they had taken on the appearance of a brown sludge. EIMS no longer gave an M^+ at m/z 504.

Attempted Reaction of Quinolizidine Alcohol, 74, with Parabromobenzenesulfonyl Chloride

To 10 mg of the quinolizidine alcohol, **74**, were added 1.1 eq. of p-bromobenzenesulfonyl chloride in 1 ml pyridine-d_6 in a ^1H NMR tube. The contents turned a deep orange within minutes. A ^1H NMR spectrum was recorded immediately thereafter and at thirty minutes. The

spectra collected showed no reaction having taken place. To the tube was added 1.1 eq. of DMAP, and a ^1H NMR was taken after a period of 15 minutes. Again, no reaction products were observed. The reaction mixture was permeated through Bio-Beads S-X8 gel in CH_2-Cl_2-CH_3CN (3:2), yielding four fractions, the second of which was the unreacted compound. No derivative was observed in any of the fractions.

Second Attempted Reaction of the Quinolizidine Alcohol, **74**, with Parabromobenzenesulfonyl Chloride

To 14 mg of the quinolizidine alcohol, **74**, were added 1.1 eq. of p-bromobenzenesulfonyl chloride and 1.1 eq. of DMAP in 0.5 ml pyridine-d_5 in a 5 ml micro reaction vial. The reaction mixture was heated to 60 ^0C for 10 hours with stirring under nitrogen atmosphere. The contents were allowed to cool to room temperature, and a ^1H NMR was taken. The spectrum revealed that no reaction had taken place.

Attempted Cleavage of Indolizidine Monoenes, **86**

To 19.8 mg of a fraction containing a mixture of the indolizidine monoenes, **86**, were added 0.5 ml of a solution of dioxane-H_20 (12:4), in a 3 ml microscale reaction vessel. A catalytic amount of OsO_4 was added to

the contents, which turned a deep purple with stirring. The contents were stirred at room temperature for 30 minutes. Over a period of 25 minutes 2 eq. of $NaIO_4$ were added. The solution was partitioned between water and Et_2O immediately. The organic solvent was removed *in vacuo*, giving a dark solid, 2 mg. A ^1H NMR of the material yielded an unrecognizable profile. The remaining contents of the reaction vessel were partitioned once again, between CH_2Cl_2 and water. The organic solvent was removed *in vacuo*, leaving a dark green solid. ^1H NMR of the material again yielded a spectrum very similar to the previous one. A very weak signal at 9.5 ppm was observed for the aldehyde. Attempts to recover the presumed product via CCC were unsuccessful.

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