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STRUCTURE ELUCIDATION OF INSECT PHEROMONES BY MICROANALYTICAL METHODS[1,2]

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Abstract—The isolation and identification of pheromones at the microgram level requires specialized techniques. The pheromones are obtained in the purity required for structural studies by high-speed, high-pressure liquid chromatography using silicic acid- and silver nitrate-treated columns and micropreparative GLC. Then, microchemical reactions, including hydrogenation, ozonolysis, and epoxidation are combined with infrared, nuclear magnetic resonance, and mass spectral studies to elucidate the structure of these compounds. In many cases these techniques are synergistic when combined. Thus, samples of 5–10 μg of most organic compounds under molecular weight 350 are sufficient for complete structural elucidation by using microchemical and spectroscopic techniques. The reduction in sample (2 μg) size necessary for good PMR spectra is significant because the information provided by this technique is often critical to the elucidation of a structure, and sensitivity of PMR has been one of the limiting factors in microspectroscopic analysis.

Key Words—insect pheromones, microspectroscopic techniques.

INTRODUCTION

Insect pheromones, which are highly active chemical messengers produced and used by the insects themselves, are now used as effective insect population

[1] Mention of a pesticide or a commercial or proprietary product in this paper does not constitute an endorsement of the product by the USDA.

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survey tools and will soon be used in insect control programs. Since most of these compounds are produced by the insects in only nanogram or picogram amounts their isolation and structure elucidation requires large numbers of insects or special techniques. For example, millions of boll weevils, *Anthonomus grandis*, were reared to obtain enough material to identify their four-component pheromone (Tumlinson et al., 1969). Fortunately, techniques and equipment have improved significantly in the last five years, so we now need only a few micrograms of a compound for identification. To a great extent this has eliminated the tremendous expense of rearing vast numbers of insects for pheromone identification. Recent studies have shown that pheromones are often multicomponent rather than single compounds, and trace amounts of synergizing or modifying compounds can be important. The isolation and identification of the complete pheromone complex, including the trace modifiers, is crucial to complete understanding of the effect of the phermone or insect behavior.

At the Insect Attractants, Behavior, and Basic Biology Research Laboratory we have adapted and improved existing techniques and devised new ones to enable us to isolate and identify compounds with minimal material. These procedures, to be described, are applicable to a wide range of problems in the life sciences.

PROCEDURES, RESULTS, AND DISCUSSION

Purification

The pheromones must be pure before they can be identified and usually purity is essential for biological activity. Trace amounts of isomers and other hard-to-separate compounds may have beneficial or deleterious effects on the activity of naturally derived and synthesized pheromones (Tumlinson et al., 1972, 1974*b*; Beroza et al., 1973).

High-speed, high-resolution liquid chromatography is one of our most important methods of pheromone purification. Recent developments, including high-pressure pumps and small particle, high-resolution columns, have placed this technique on a par with gas–liquid chromatography (GLC) in terms of speed, resolution, and convenience of operation. The major disadvantage of this type of liquid chromatography is the low sensitivity of the available detectors. Usually there is insufficient pheromone to be detected, but most natural extracts contain other compounds in sufficient quantities to serve as markers. We have used three types of columns with considerable success in isolating natural pheromones and purifying synthesized compounds.

Usually, our first step in the separation of the pheromone from the crude

extract is gel permeation chromatography. A glass column, 1.27 cm (ID) is packed to a height of about 90 cm with a hexane slurry of Poragel 60A, 37–75 μm (Waters Associates). The column is eluted with hexane at a flow rate of 300 ml/hr and a column inlet pressure of 40 psi. A 0.5-ml sample of concentrated crude extract is loaded on this column and the entire separation requires only about 1 hr. In this system, compounds with a molecular weight over 2000 will not permeate the pores of the packing and will be eluted virtually without holdup. Smaller molecules, which fall within the working range of the packing, are separated in reverse order by molecular size. With this column we have obtained a 20-ml fraction that contained all the active pheromone from the crude extract of 2500 *Heliothis virescens* females; it was pure enough to proceed directly to gas chromatographic separations.

The second liquid chromatographic column that we have used extensively is a 0.64 (OD) × 50-cm stainless-steel column packed with 10 μm LiChrosorb (E. Merck) by the technique of Majors (1972). We normally use hexane and ether mixtures as solvent both in isocratic and gradient systems. The particular solvent system depends on the compounds to be separated. This column is useful in separating synthetic mixtures as well as natural extracts. Thus we found that we could separate (Z,E)-3,5-tetradecadien-1-ol acetate, a sex attractant for *Prionoxystus robiniae* (Doolittle et al., 1973), from its (E,E) isomer with 3% ether in hexane.

Our most useful liquid chromatographic column has been a 1.27 (OD) × 50-cm stainless-steel column dry-packed with Adsorbosil-2-ADN (Applied Science Laboratories), a 20% $AgNO_3$-coated silica gel used in thin-layer chromatography; benzene is used exclusively as the solvent. It is capable of eluting a wide range of organic compounds in a reasonable time, and the column can be reused indefinitely. The particle size of the Adsorbosil varies from about 2–11 μm, a wide range for high-performance liquid chromatography. Nonetheless, we have achieved complete separation of the (Z) and (E) isomers of at least two classes of compounds using this column. For example, the synthesized pheromone of *Synanthedon pictipes*, (E,Z)-3,13-octadecadien-1-ol acetate, contained about 3–5% of the (Z,Z)-isomer, rendering it almost totally unattractive to *S. pictipes* males (Tumlinson et al., 1974*b*). When this mixture was chromatographed on this column at a benzene flow of 6.5 ml/min and a column inlet pressure of 4700 psi, the (E,Z)-isomer eluted between 10 and 14 min after injection and the (Z,Z) isomer between 21 and 27 min. Similarly, the synthesized (Z) and (E) isomers of the aldehydic *H. virescens* pheromone were separated in 3 min at a flow of 4.0 ml/min.

Once a pheromone has been purified as much as possible by liquid chromatography we find micropreparative GLC our most reliable method of obtaining microgram quantities of naturally derived pheromones in a highly purified state. When 2–5% of the column effluent is directed to a flame

ionization detector and the remainder diverted to an efficient thermal gradient collector (Brownlee and Silverstein, 1968), we have achieved recoveries of the target compounds in excess of 90%. The components are collected and sealed in glass capillaries for subsequent purification or identification. Purity of the isolated pheromones is established by GLC on open tubular capillary columns coated by the method of Mon (1971). Using Carbowax 20M, Dexsil, and OV-101 phases in 0.76-mm × 60-m columns with over 60,000 theoretical plates, we are usually able to show that a compound is at least 99% pure.

Microchemical Techniques

Two microdegradative techniques that we find most useful in determining the number and position of olefinic bonds are hydrogenation over neutral palladium catalyst and ozonolysis. These techniques were developed on a microscale by Beroza and co-workers and the experimental techniques are adequately described elsewhere (Beroza and Sarmiento, 1966; Beroza and Bierl, 1966, 1967). The advantages of these procedures are that they can be performed with less than a microgram of material, and the products are available for analysis in a few minutes at most. The hydrogenation is carried out in the inlet of the gas chromatograph and the product is analysed without further handling as it is swept from the catalyst bed onto the GLC column by using hydrogen as a carrier gas. If the gas chromatograph is connected to a mass spectrometer the mass spectrum of the product is recorded as it is eluted from the GLC column. Under the proper conditions, this procedure only hydrogenates olefinic bonds; thus a vast amount of information is gained very quickly. For example, the mass spectrum of an unknown indicated that it was a straight-chain molecule with a molecular weight of 210. Thus, it could either be a 14-carbon alcohol with two olefinic bonds or 14-carbon aldehyde with one. Insufficient material was available for an infrared spectrum. Reduction of the compound in the inlet of the gas chromatograph and subsequent mass spectroscopy showed that the product had a molecular weight of 212. Thus, the unknown was a monounsaturated 14-carbon aldehyde. Similarly, the products of ozonolysis can be analyzed by GLC and mass spectroscopy with equal facility.

Another reaction that we have adapted for microanalysis is epoxidation of olefinic bonds. There are several instances when ozonolysis is not sufficient to locate with certainty all olefinic positions in a molecule. This is often the case when there are two or more olefinic bonds in a chain and the ozonolysis products are very small volatile molecules that are difficult to analyze.

Epoxidation is easily carried out by adding the olefin to chloroform that

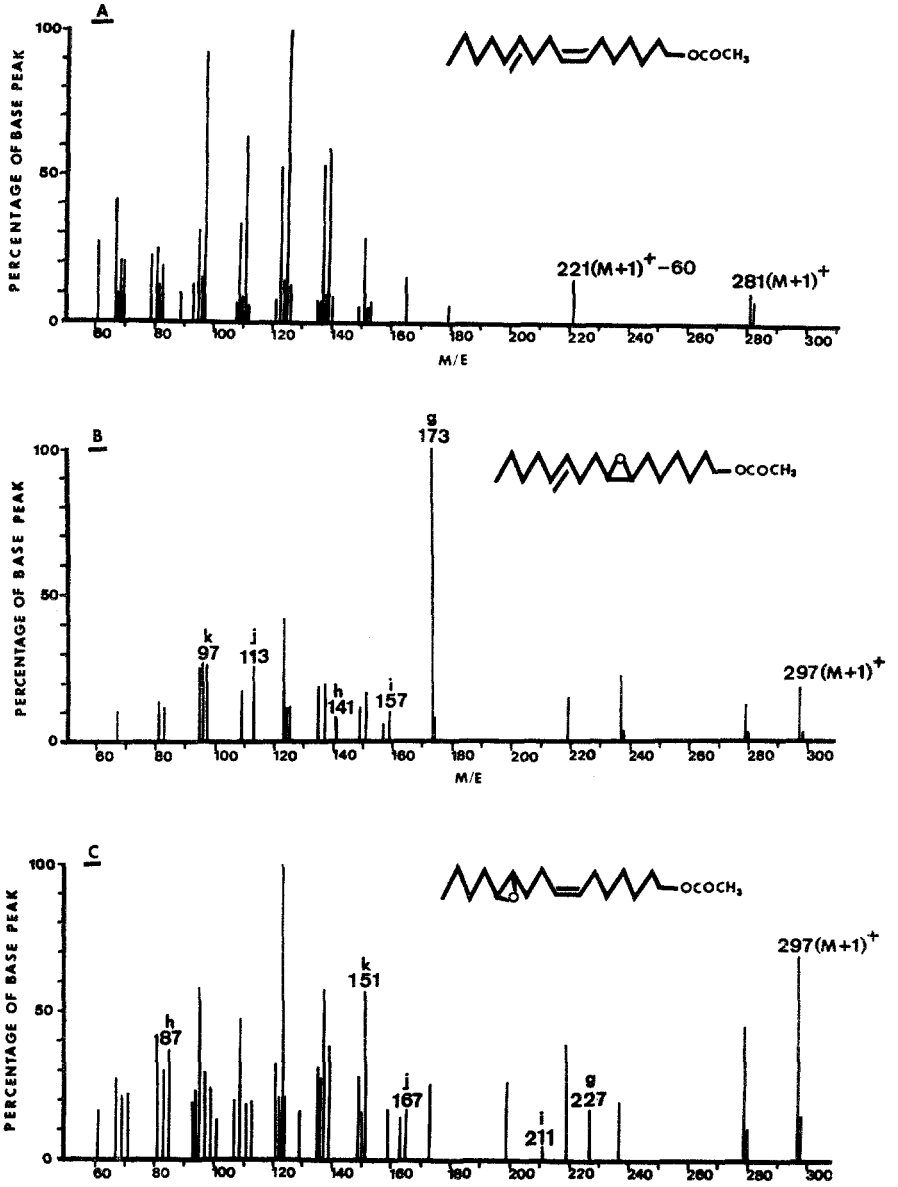

FIG. 1. Isobutane ionization mass spectra of: A, (Z,E)-7,11-hexadecadien-1-ol acetate; B, 7,8-epoxy-11-hexadecen-1-ol acetate; C, 11,12-epoxy-7-hexadecen-1-ol acetate. Spectra were obtained with 0.5 μg or less material. Direct comparison of the olefin and epoxide spectra facilitates location of the epoxide ring. A fragmentation scheme is shown in Figure 2.

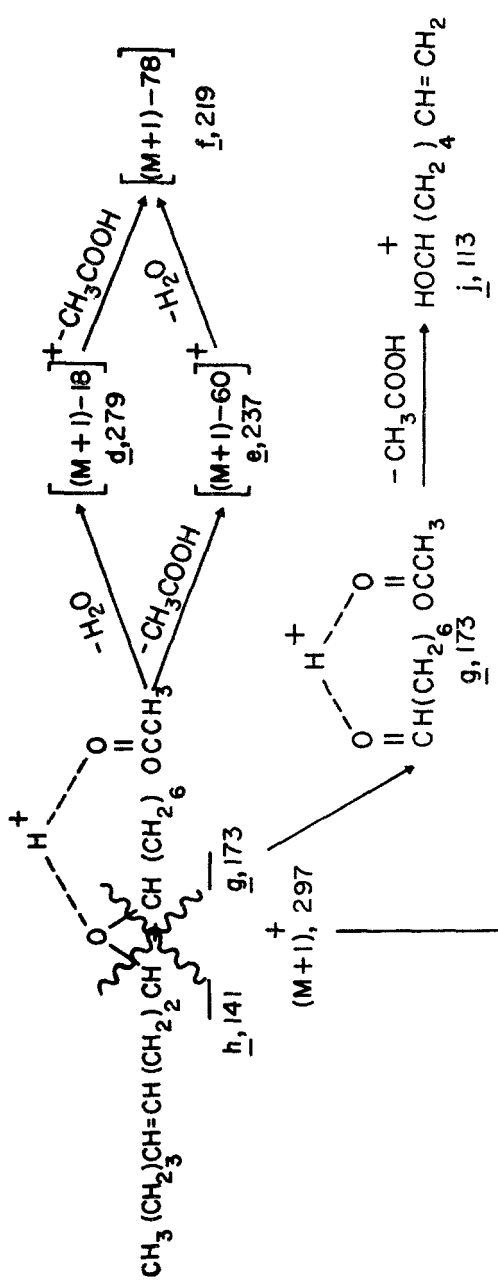

FIG. 2. Suggested isobutane ionization fragmentation scheme for epoxyhexadecen-1-ol acetates. The relative abundance of each ion is shown in Figure 1B.

contains a molar excess of 99+% *m*-chloroperbenzoic acid (Schwartz and Blumberg 1964) and allowing the mixture to stand at room temperature. When compounds containing two ethylenic bonds are epoxidized the reaction can be monitored by gas chromatography with a column containing 3% OV-210 on 100/120 mesh Varaport 30 and then stopped at the monoepoxide stage. The position of the olefinic bonds in a chain is then located by analysis of the monoepoxides by chemical ionization mass spectroscopy with isobutane as a reagent gas (Tumlinson et al. 1974*a*). Furthermore, the configuration of the olefinic bonds can be determined by infrared analysis of the monoolefins.

We used this technique in the analysis of a synthetic isomeric mixture of the Angoumois grain moth, *Sitotroga cerealella*, pheromone (Z,E)-7,11-hexadecadien-1-ol acetate (Vick et al., 1974). Although the mixture was separated into four isomers by chromatography, we did not know which component was the desired (Z,E)-7,11-hexadecadien-1-ol acetate. The most

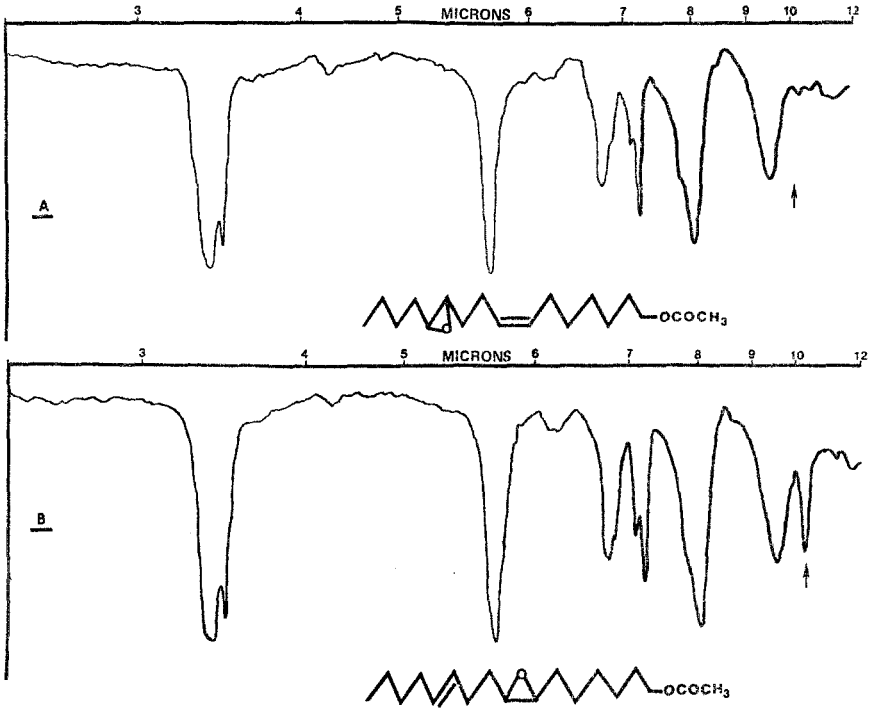

FIG. 3. Infrared spectra of: A, 11,12-epoxy-(Z)-7-hexadecen-1-ol acetate, and B, 7,8-epoxy-(E)-11-hexadecen-1-ol acetate. The arrow in each spectrum indicates the characteristic frequency of the trans olefinic absorption (10.2 μm, 980 cm^{-1}). The absence (3A) or presence (3B) of this band reveals whether the nonepoxidized olefinic band is cis or trans, respectively.

likely component was epoxidized, and the monoepoxides were analyzed by isobutane ionization mass spectroscopy. Figure 1 shows the mass spectra of the original component and the two monoepoxides. Figure 2 shows the suggested fragmentation pattern for 7,8-epoxy-11-hexadecen-1-ol acetate, which is similar to that observed for 11,12-epoxy-7-hexadecen-1-ol acetate (Figure 1) and is typical for acetates of straight chain epoxy alcohols. Thus, when the spectra of the original olefin and the two monoepoxides are compared, the location of the olefinic bonds is easily established. Spectra of this type can be easily obtained with 0.5 μg of sample.

Figure 3 shows the infrared spectra of these two olefinic epoxides. The trans band at about 970 cm^{-1} that appears in one spectrum and is absent in the other (see arrows) establishes the configuration of the double bond in each compound. Thus, we have conclusive proof that the component we selected was (Z,E)-7,11-hexadecadien-1-ol acetate.

Mass Spectroscopy

Recently developed, highly sensitive spectroscopic equipment has greatly facilitated analysis of microsamples. Only five years ago at least 100 μg of sample was required for complete spectroscopic analysis by infrared, proton magnetic resonance, and mass spectroscopy. With the most sophisticated equipment and techniques available today, it is possible to obtain all three spectra with less than 5 μg of a compound, and with 10 μg of sample the analyses are routine. In fact, the most difficult task involves handling and transferring these small samples without considerable loss.

Usually the first spectrum obtained of a sample is the mass spectrum because so little material is required and the information derived is very helpful in maximizing the effectiveness of the other spectral analyses. The advent of chemical ionization mass spectroscopy has increased the analytical capability of this technique even more. Since the reagent gas reacts with the sample molecule in the ion source of the mass spectrometer, the reagent gas may be varied considerably (Hunt and Ryan, 1971, 1972*a*,*b*; Hunt et al., 1971) so a particularly suitable reactant for a class of compounds may be chosen. Alternatively, a compound may be subjected to various ionizing gases to yield more structural information. As an example, Hunt et al. (1972) found that when D_2O was used as the reagent gas, the number of active hydrogens in a sample could be determined because they were all replaced with deuterium.

An additional advantage of chemical ionization mass spectroscopy is that the reagent gas can often be used as the carrier gas for the interfaced gas chromatograph. Thus, the total effluent from the GLC column is directed into the ion source, and no sample is lost.

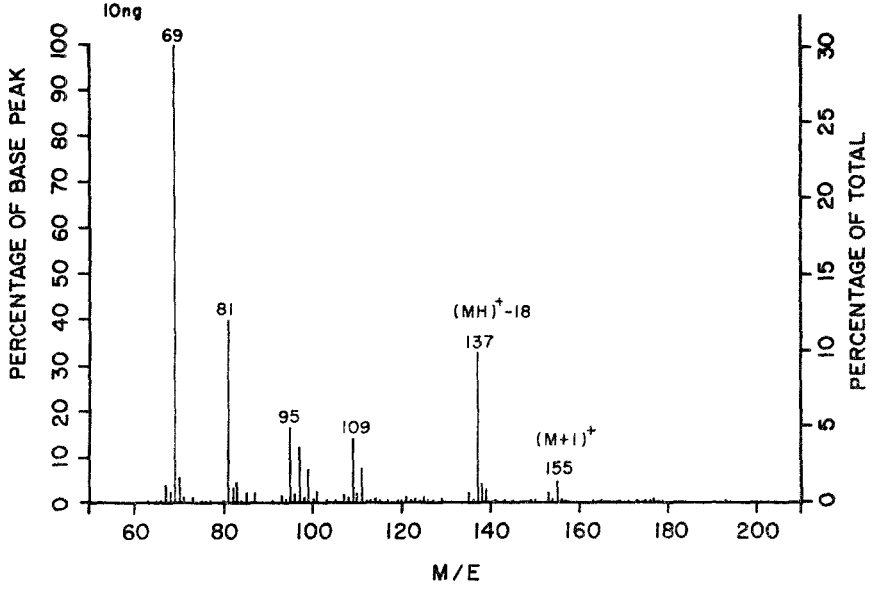

FIG. 4. Methane ionization mass spectrum of 10 ng of *cis*-2-isopropenyl-1-methyl-cyclobutaneethanol. Background peaks were subtracted by the computer.

The convenience and sensitivity of this technique is illustrated by Figure 4. A 10 ng sample of *cis*-2-isopropenyl-1-methyl-cyclobutaneethanol, a component of the boll weevil pheromone (Tumlinson et al., 1969), dissolved in about 0.2 μl of hexane was injected onto a 3% OV-1 on 100/120 mesh Varaport 30 column in a Varian Model 1400 gas chromatograph. The total effluent of the column was admitted to the source of a Finnigan Model 1015C chemical ionization mass spectrometer. Methane, the carrier gas, also served as the reagent gas at a source pressure of about 1 torr. Data acquisition and reduction were accomplished with a Systems Industries Disc System 150 computer interfaced to the mass spectrometer.

Infrared Spectroscopy

Infrared spectra are valuable in organic structure determinations, particularly in functional group analysis. Since others have published micro-infrared techniques recently, we will not discuss this at length. For example, Price et. al. (1967) reported good infrared solution spectra with 5 μg of benzyl acetate by using a specially designed cell. More recently, King (1973) published good solution spectra of 2,6-dimethoxyphenol with only 0.3 μg of sample in a microcavity cell by using a beam condenser and a Fourier transform mid-infrared spectrometer.

Micro-NMR Spectroscopy

High-resolution proton magnetic resonance (PMR) is probably the least sensitive of the three spectrometric techniques. Although Lundin et al. (1967) predicted that the minimum sample required for a compound of molecular weight 200 should be about 1 μg with Fourier transform high-resolution PMR, there have been no reports of usable spectra obtained with less than 20 μg thus far.

Our PMR spectra are obtained with a Bruker HX-90 spectrometer interfaced to a Nicolet 1080 Fourier transform data system and equipped with a high-sensitivity proton probe insert. Of several commercial PMR cells, we found a microtube prepared to our specifications by Wilmad Glass Co. (Figure 5) most compatible with our spectrometer. The optimum solvent volume in this tube was about 75 μl. Commercially available spectroquality carbon disulfide and carbon tetrachloride were purified by filtration through columns of silica gel Woelm (activity grade 1) and aluminum oxide Woelm

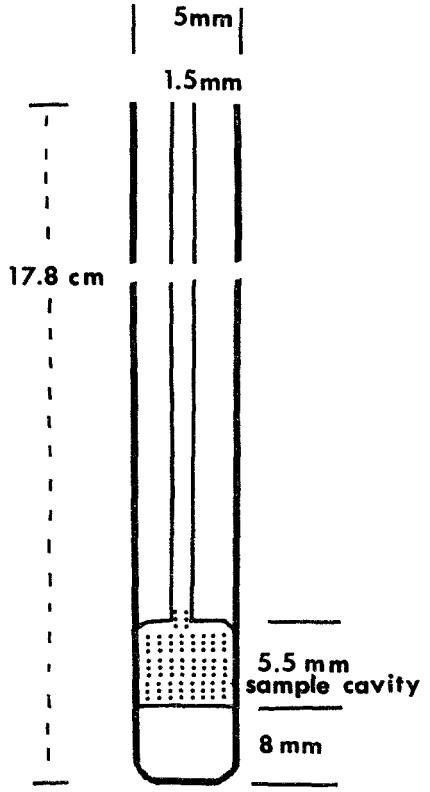

FIG. 5. Micro-NMR tube used to obtain spectra of 2-μg samples. Volume of the sample cavity is 75 μl.

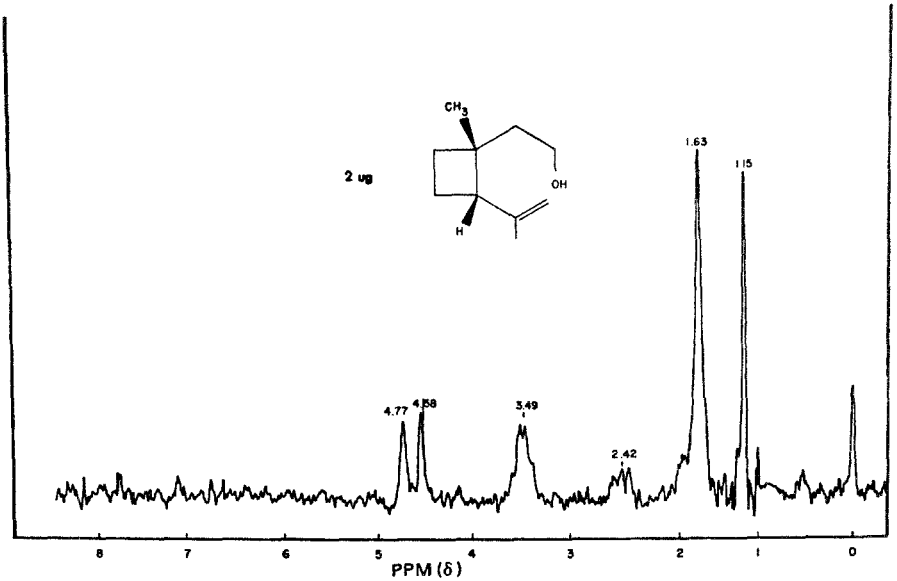

FIG. 6. 90-MHz proton NMR spectrum of 2 μg of *cis*-2-isopropenyl-1-methylcyclobutaneethanol. Concentration was 0.026% in 75 μl of CS_2. Tetramethylsilane was the internal standard. 60,000 transients of the sample were obtained and 60,000 background transients were subtracted by the computer. The free induction decay was stored in 4K memory. The sweep width was 1000 Hz. A high-sensitivity proton-receiver coil was used.

basic (activity grade 1[3]), followed by distillation in an all-glass system to remove particles of silica or alumina. Hexaflurobenzene (Thompson-Packard, NMR grade) at a concentration of about 5% (v/v) was used for the internal lock signal, and tetramethylsilane (Thompson-Packard, NMR grade) was used as an internal reference; in the small amounts used both produced no significant interference and needed no purification.

The PMR spectrum in Figure 6 illustrates the sensitivity of the technique. The design of the sample cell is critical because best results are achieved with the minimum amount of solvent that will fill the receiver coil area of the probe. If solvent impurities did not interfere, usable spectra could be obtained with less than 2 μg of sample. The major impurity in both carbon tetrachloride and carbon disulfide produces a singlet at 1.04 ppm downfield from TMS and interferes with the methyl signals of most organic compounds. This signal was decreased significantly by filtration of the solvent through silica gel, alumina, and distillation; the peak was smaller with carbon disulfide than

[3] "Purification of solvents by Woelm active aluminas," Waters Associates, Inc., Milford, Massachusetts.

with carbon tetrachloride. Although the addition of shift reagents [$Eu(fod)_3$, Penninsular ChemResearch] and trichloroacetyl isocyanate (TCAIC) shifted the impurity signal downfield, the impurity could not be removed by distillation of the solvent from TCAIC or 3-nitrophthalic anhydride. Also, addition of small amounts of water to the solvent produced a second peak, and the impurity peak remained at 1.04. We concluded that carbon disulfide is the best solvent for micro-PMR studies. Additionally, CS_2 facilitates sample recovery by gas chromatography since it produces a small signal and is very volatile.

Undoubtedly, techniques, procedures, and equipment will continue to improve and the amount of material necessary for identification of pheromones and other natural products will decrease even further. One of the promising new areas of investigation is ^{13}C nuclear magnetic resonance spectroscopy. This technique is presently limited by its sensitivity since mg samples are required. However, the vast amount of information that a ^{13}C spectrum yields, particularly with complex molecules, makes it a valuable method of structure elucidation (Nakanishi et al., 1973). In the near future, the problem of lack of sensitivity will be overcome and ^{13}C NMR will be a primary method of microstructure elucidation.

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