Unsuitability of Carbon-13 Nuclear Magnetic Resonance for Studying Pheromone Biosynthesis in the Cabbage Looper Moth (*Trichoplusia ni*)

Biosynthesis of the sex pheromone component (*Z*)-7-dodecenyl acetate was studied in female *Trichoplusia ni* (Hüber) (Lepidoptera:Noctuidae) by using ^{13}C labeling and ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy. Insufficient incorporation of ^{13}C was obtained to postualte a pathway of synthesis. Limitations of ^{13}C labeling and ^{13}C NMR spectroscopy in the study of insect pheromone biosynthesis are discussed.

Insect pheromones are among the most potent physiologically active substances known (Jacobson, 1965) and are produced and detected in extremely minute amounts by insects. Those interested in studying the mechanism of pheromone biosynthesis have had to resort to the most sensitive available techniques to carry out their investigations. Most authors (Berger, 1974; Mitlin and Hedin, 1974; Crew and Ross, 1975; Schmidt and Munroe, 1976; Jones and Berger, 1978; Dillwith et al., 1981) have relied on radiolabeling procedures. Although their studies have been adequate to establish that the pheromones are synthesized from certain precursors, our knowledge of pheromone biosynthesis is still quite limited.

Most, if not all, female lepidopteran sex pheromones that have been identified are aliphatic lipids (Tamaki, 1977). It has been suggested (Schmidt and Munroe 1976) that the route to their formation is similar to known pathways of synthesis for other animal lipids and involves a scheme similar to that catalyzed by the fatty acid synthetase complex (Lynen, 1967). Jones and Berger (1978) showed that sodium [1-^{14}C]acetate was incorporated into (*Z*)-7-dodecenyl acetate, a major component of *Trichoplusia ni* (Hüber) sex pheromone. Assay of the products from the partial chemical degradation of the labeled pheromone revealed the radioactivity was distributed on both sides of the double bond of the aliphatic alcohol part of the molecule as well as being present in the acetate group. Similar distribution of label was observed when [1-^{14}C]dodecanol was the precursor (Jones, 1980). The alcohol was apparently degraded before being incorporated into the pheromone. We then questioned whether dodecenol formation in vivo is accomplished by the assembly of two-carbon units as occurs in fatty acid synthesis.

^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy potentially offers an alternative to chemical degradation techniques (Sequin and Scott, 1974). Pheromone isolated from a synthesizing system that has been fortified with [1-^{13}C]- or [2-^{13}C]acetate should show ^{13}C enrichment of alternate and opposite carbons of those portions of the molecule that are produced by a fatty acid synthetase like system, depending on which of the two precursors was used. Findings of enrichment at other specific positions might be indicative of different pathways of synthesis. Reported here are the results of efforts to utilize ^{13}C NMR spectroscopy to determine the pattern of incorporation of [^{13}C]acetates into (*Z*)-7-dodecenyl acetate by female *T. ni*. Limitations and prerequisites in the application of this technique for the study of pheromone biosynthesis in insects are discussed.

MATERIALS AND METHODS

Assignment and Quantitation of the (*Z*)-7-Dodecenyl Acetate ^{13}C NMR Spectrum. Assignment of the spectrum of (*Z*)-7-dodecenyl acetate was performed by referring to published assignments and from observations on the progressive movement of the spectral lines of a solution of the compound as increments of shift reagent were added to it. Spectra were run on a 20-MHz computer-controlled FT-NMR (Varian CFT-20). One gram of (*Z*)-7-dodecenyl acetate was dissolved in an equal volume of $CDCl_3$ that contained tetramethylsilane (Me_4Si) as a reference. One hundred transients were accumulated, transformed, and plotted. Then 100 mg of the paramagnetic shift reagent europium [tris(1,1,1,2,2,3,3-heptafluoro-7,7,-dimethyl-4,6-octanedione)] [$Eu(fod)_3$] was added to the sample, and a second spectrum was acquired. This process was repeated 10 times with 100 mg of shift reagent being added each time and spectra being plotted following each addition. The series of spectra was compared to assess the effect of the shift reagent on the resonances of the various carbon atoms.

Quantitation of ^{13}C enrichment of a molecule depends on careful measurement of intensities of the resonance peaks of its CMR spectrum. Often the peak integrals are not proportional to ^{13}C enrichment for all positions in a molecule because of differences in nuclear Overhauser enrichments and relaxation times of the various carbon atoms (Levy, 1974). A technique for improving the accuracy of measuring peak intensities is to add a paramagnetic relaxation reagent such as tris(acetylacetonato)chromium(III), which results in the quenching of the nuclear Overhauser effects, shortens the relaxation times, and tends to equalize the intensities of peaks representing equal amounts of ^{13}C (Levy and Cargioli, 1973). A series of experiments was conducted in which solutions of 10–50% pheromone were made 0.1 M with this reagent and analyzed to determine if the variation in the intensities of peaks representing the different carbons of the pheromone could be eliminated by addition of the relaxaing reagent.

Treatment of Insects. Insect rearing was performed as described in Jones and Berger (1978). A total of 7500 adult female moths (72 ± 12 h of age) were anesthetized with CO_2, and 5 μL of saline (9.0 g of NaCl, 0.2 g of KCl, 4.0 g of $CaCl_2$, and 4.0 g of glucose per L of 0.05 M Tris-HCl at pH 7.15) containing 90% 1-^{13}C-enriched sodium acetate (1×10^{-2} mol/mL) was injected into the abdomen of each moth. The moths were held for 1 h before being sacrificed for collection of pheromone.

Purification of Natural (*Z*)-7-Dodecenyl Acetate. Pheromone-producing glands were everted by squeezing the abdomens of the injected females and excised into hexane with iris scissors. After disruption of the tissue in a sonic homogenizer, the hexane extract was recovered by filtration and concentrated to a small volume. The oily residue was extracted 3 times with cold methanol (−15 °C), the methanol extracts were diluted with an equal volume of water, and the pheromone was recovered by partitioning with hexane. The crude pheromone was chromatographed by high-pressure liquid chromatography (HPLC) on a 9 mm × 50 cm preparative ODS column eluted with meth-

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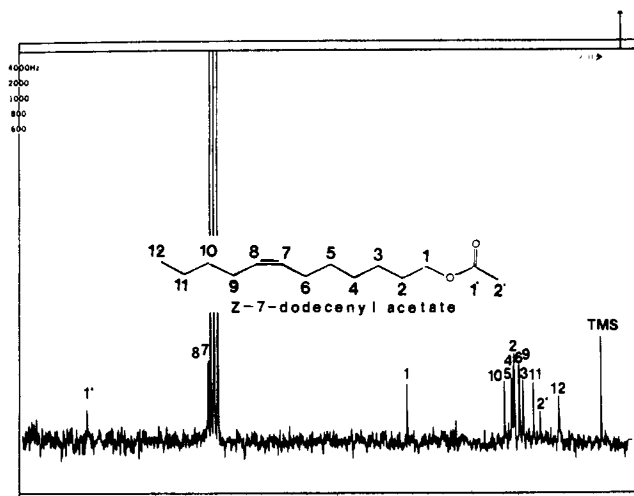

Figure 1. Proposed assignment of the ^{13}C NMR spectrum of (*Z*)-7-dodecenyl acetate.

Table I. Chemical Shifts for ^{13}C NMR Spectrum of (*Z*)-7-Dodecenyl Acetate and Lanthanide-Induced Shifts (Downfield) for Each Position after the Addition of Approximately One Gram of $Eu(fod)_3$

carbon position	chemical shift, ppm	lanthanide-induced shift, ppm
1'	169.78	2.01
8	130.18	0.10
7	129.94	0.10
1	64.30	3.07
10	32.37	0.10
5	30.03	0.23
4	29.26	0.44
2	29.12	1.14
6	27.47	0.17
9	27.31	0.11
3	26.24	0.73
11	22.70	0.06
2'	20.53	4.04
12	14.15	0.02

Table II. Comparison of ^{13}C NMR Spectra of Synthetic (*Z*)-7-Dodecenyl Acetate and Pheromone Isolated from [1-^{13}C]Acetate-Injected Moths

carbon position	synthetic		moth produced	
	shift, ppm	intensity	shift, ppm	intensity
1'	170.01	15	170.01	15
8	130.21	42	130.22	37
7	129.98	43	129.96	42
1	64.36	31	64.35	32
10	32.34	30	32.35	31
5	29.99	28	30.01	38
4	29.21	36	29.22	39
2	29.03	42	29.03	39
6	27.48	38	27.47	43
9	27.35	36	27.34	39
3	26.17	32	26.17	34
11	22.70	30	22.70	34
2'	20.53	17	20.53	19
12	14.18	25	14.19	27
		Me_4Si		
	0.00	51	0.00	50

anol–H_2O (83:17). Effluents containing the pheromone (monitored by a GLC 1.8 m × 2 mm i.d. glass column packed with 3% HiEff 8BP on Gas-Chrom Q, 100–120 mesh, at 170 °C) were combined, recovered in hexane, and rechromatographed on a 4.6 mm × 25 cm 10 μPorasil column eluted with hexane–chloroform (97:3).

Pheromone was analyzed by using a micro probe attachment on the spectrometer. This sample was dissolved in sufficient benzene-d_6 to make a total volume of 15 μL and analyzed in a 100 mm × 0.95 mm i.d. sample tube. A similar sample of synthetic pheromone was also prepared and analyzed for comparison. Approximately 100 000 transients were accumulated for computation of spectra for each sample.

RESULTS AND DISCUSSION

Our proposed assignment of the ^{13}C NMR spectrum for (*Z*)-7-dodecenyl acetate is presented in Figure 1 and Table I. The assignments of the spectral lines were based on correlations with published spectra for dodecanyl acetate (Sadtler Research Laboratories, 1976) and 2-ethylhexyl acetate, 1-decanol, and oleic acid (Johnson and Jankowski, 1971) and the magnitude of the lanthanide-induced shifts that were observed after the addition of the paramagnetic shift reagent, $Eu(fod)_3$, as they are related to the proximity of saturated carbons to the carbonyl oxygen (Batchelor et al., 1974). Assignments of the resonances of positions 7 and 8 adjacent to the double bond were based on data presented by Batchelor et al. (1974). This ^{13}C NMR spectrum differs from that published by Barabas et al. (1978) in that our assignment of the spectral lines for C_4 and C_5 are reversed as are those for C_6 and C_9. Our principal reason for making these assignments was our observations on the magnitude of the shifts in resonances induced by the $Eu(fod)_3$. Barabas et al. (1978) presented no shift data.

Very strong quenching of the intensities of the spectral lines was observed with the samples of pheromone to which the relaxing reagent was added. Only a very weak spectrum was obtained with a 50% solution of pheromone. It was concluded that this technique would be of little value in our studies of (*Z*)-7-dodecenyl acetate synthesis since the quantity of pheromone available from moths would be, at best, a few milligrams.

The extract of the pheromone glands from the 7500 moths resulted in the recovery of 2.6 mg of pheromone free of detectable impurities. Results of the ^{13}C NMR spectroscopic analysis of this sample of moth-produced pheromone are tabulated in Table II. Data for a similar amount of synthetic pheromone containing only natural abundance of ^{13}C are also presented for comparison. It is apparent that the amount of enrichment of ^{13}C was quite low. A statistical comparison of odd- and even-carbon intensities between moth-produced and synthetic (*Z*)-7-dodecenyl acetate showed no significant difference at $P = 0.05$ (paired *t* test). It is noteworthy that odd-carbon intensities in moth-produced pheromone, where enrichment would be expected, were significantly higher at $P = 0.3$ than at the same carbon poistions in synthetic pheromone. A similar comparison of even-carbon intensities showed no significant difference even at $P = 0.5$. It is not possible to postulate a pattern of synthesis of the pheromone. We were disappointed to find little or no evidence of enrichment in $C_{1'}$. Earlier work with [1-^{14}C]acetate (Jones and Berger, 1978) indicated that approximately 0.5% incorporation in the $C_{1'}$ position had been realized 1 h after treatment.

Obviously our attempts to use ^{13}C labeling and ^{13}C NMR spectroscopy to elucidate the pattern of incorporation of acetate into (*Z*)-7-dodecenyl acetate were not successful. The utility of the technique is limited by the minimum size of sample that can be analyzed and by the minimum percentage of enrichment of ^{13}C that can be detected over and above the 1.1% already present in natural compounds. Both factors were serious obstacles in this study.

We chose for our studies an insect that is a very abundant producer of pheromone in comparison with most other insects that have been studied. Many more insects would be required to produce sufficient pheromone for study with a less prolific species. Improvement in the percentage of incorporation of a precursor into the pheromone is considered essential. The 1-h incubation time was chosen because previous work using [^{14}C]acetate (Jones and Berger, 1978) indicated that the maximum level of incorporation was obtained at this time. A much higher percentage of incorporation of ^{14}C was obtained with isolated glands incubated in vitro, but the yield of pheromone was much less (Jones, 1979). Regardless of the techniques employed it is our estimate that approximately 2–3 mg of pure (*Z*)-7-dodecenyl acetate containing 10–12% ^{13}C would be required to readily detect a pathway of synthesis such as that catalyzed by the fatty acid synthetase complex.

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Determination of Carbofuran's Phenolic Metabolites in Hops

The phenolic transformation products of carbofuran were isolated from hop cone extracts by high-pressure liquid chromatography (HPLC) using a reverse-phase, octadecylsilane column. HPLC column fractions were treated with fluorodinitrobenzene, and the dinitrophenyl ether derivatives were determined quantitatively by electron capture gas chromatography. Detection limits were 0.30 mg/kg for 2,3-dihydro-2,2-dimethyl-7-benzofuranol and 2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol and 1.0 mg/kg for 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol in fortified hop samples. At the limit of detection the average through-the-method recovery for these phenolic residues was 81%.

Soil application of the *N*-methylcarbamate pesticide carbofuran results in systemic activity in plants against insects and nematodes. Oxidative metabolism of carbofuran introduces hydroxyl and carbonyl substituents in the 3 position, and these primary metabolites may be hydrolyzed and/or conjugated. In alfalfa, for example, residues are primarily in the form of glucosides of 2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol (II), 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol (III), and 3-hydroxycarbofuran, and small amounts of the free phenols (Figure 1) are present as well (Knaak et al., 1970). This diversity of chemical forms complicates the analysis of carbofuran residues in crops.

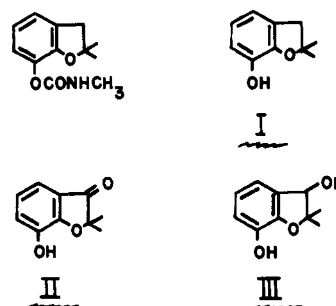

Figure 1. Carbofuran and its phenolic metabolites.

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