# Multiresidue Procedure for Halo- and Nitrophenols. Measurement of Exposure to Biodegradable Pesticides Yielding these Compounds as Metabolites

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The urinary level of phenolic compounds may be the key for establishing an index of exposure to pesticides containing this moiety as an easily hydrolyzed or metabolized portion of the molecule. A method has been developed for quantitating ten halo- and nitrophenols in rat urine which could result from exposure to and subsequent metabolism and excretion of a broad spectrum of pesticides. The procedure involves acid hydrolysis, extraction, derivatization, silica gel chromatography, and electron-capture gas chromatography. Male rats fed pesticidal compounds containing halo- and nitrophenol moieties at levels varying from factors of $10^{-5}$ to $10^{-2}$ of the $LD_{50}$ were used to establish the usefulness of this procedure for determining the extent of exposure to the biodegradable pesticides.

Determination of the urinary excretion of halo- and nitrophenol metabolites of biodegradable pesticides is of increasing interest to those involved in pesticide epidemiology. Metabolism studies (Menzie, 1969) of the biodegradable pesticides EPN, fenitrothion, dicapthon, methyl bromophos, C-9491, Dursban, DNOC, PCP, VC-13, and ronnel, which contain halo- or nitrophenol substituent groups in their molecular structures, indicate that these phenols are the major urinary metabolites. The purpose of this investigation was to develop a multiresidue method for the determination of low levels of halo- and nitrophenols in urine. The method is based on electron-capture gas chromatography of ethyl ether derivatives of the phenols (Bradway and Shafik, 1971; Shafik *et al.*, 1971a). Such a procedure may be of value in developing an exposure index to biodegradable pesticides based on levels of these urinary phenols.

## EXPERIMENTAL SECTION

**Apparatus and Equipment.** A Micro-Tek 220 gas chromatograph equipped with tritium foil electron-capture detector was used. A glass U-shaped column, 6 ft × ¼ in., was packed with 4% SE-30/6% QF-1 on 80/100 mesh Chromosorb W (HP). The gas chromatographic column was operated under the following parameters: nitrogen carrier gas flow rate, 60 ml/min; column temperature, 175°; inlet, 210°; detector, 210°; transfer line, 240°. Chromatographic columns were size 22, Kontes K-420100. Concentrator tubes were 25 ml, Kontes K-570050. Condensers were Kontes K-286810. Nitrogen evaporator was equipped with a water bath maintained at 40°. N-Evap was obtained from Organomation Associates, Worcester, Mass.

**Reagents.** The following were used: *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.); ethylating reagent (Stanley, 1966; Shafik *et al.*, 1971a); and silica gel, Woelm, activity grade I (Waters Associates, Inc., Framingham, Mass.). Dry adsorbent for 48 hr at 170° and store in a desiccator. Prepare daily 2 g of deactivated silica gel for each chromatographic column by adding 40 μl of benzene-extracted deionized or distilled water for each 2 g of dried silica gel in a tightly stoppered container. Rotate container until the water is evenly distributed throughout the adsorbent. Allow to equilibrate for 2 to 3 hr with periodic shaking. Prepare the chromatographic columns just before use. Deionized or distilled water used for deactivating the silica gel must be extracted twice with benzene. Prepare each day the anticipated amount of deactivated silica gel to be used. Larger amounts may be prepared by using the same ratio of water to dried silica gel.

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**Preparation of Standard Solutions.** The phenolic compounds were 95+% pure and a mixture of the ten compounds was prepared at the final concentrations as follows: 2,4-dichlorophenol (2,4-DCP), 0.4 μg/ml; 2,4,5-trichlorophenol (2,4,5-TCP), 0.1 μg/ml; and 3,5,6-trichloro-2-pyridinol (3,5,6-TCPyridinol), 0.1 μg/ml, Dow Chemical Co., Midland, Mich.; 3,6-dichloro-4-iodophenol (3,6-DCIP), 0.05 μg/ml, Ciba Agrochemicals Co., Summit, N. J.; 2,5-dichloro-4-bromophenol (2,5-DCBrP), 0.02 μg/ml, Cela, Ingelheim, West Germany; pentachlorophenol (PCP), 0.01 μg/ml, and *p*-nitrophenol (PNP), 0.2 μg/ml, Aldrich Chemical Co., Milwaukee, Wis.; *p*-nitro-*m*-cresol (PNC), 1.2 μg/ml, Chemagro Corp., Kansas City, Mo.; 2-chloro-4-nitrophenol (2-C-4NP), 0.08 μg/ml, American Cyanamid Co., Princeton, N. J.; 2,4-dinitro-6-methylphenol (DNOC), 0.4 μg/ml, Chemical Insecticide Corp., Edison, N. J. These compounds are the corresponding phenolic metabolites of pesticides listed in Column 1, Table II.

Weigh 10 mg of each of the ten analytical standards into separate 10-ml volumetric flasks. Add 5 ml of benzene to each flask and swirl the flask until the compound dissolves. In a well-ventilated hood, wearing disposable gloves, add diazoethane dropwise with a disposable pipet until a definite yellow color persists. Allow the solution to stand 15 min, and then bubble dry nitrogen through the solution until the yellow color disappears (5–10 min). Dilute to volume with benzene. From these ethylated stock standards, prepare a mixture of the ten compounds at the final concentrations listed above.

**Chromatographic Behavior of the Ten Ethyl Ethers on a Silica Gel Column.** Pipet an aliquot of the mixture of the ten ethylated compounds into a 15-ml graduated centrifuge tube. Evaporate the benzene to a volume of 0.3 ml using the nitrogen evaporator, and add 1.7 ml of hexane. To a chromatographic column lightly plugged with glass wool, add the 2.00 g of the partially deactivated silica gel and top with 1.5 g of anhydrous sulfate. Prewash the column with 10 ml of hexane and discard the hexane eluate. Transfer the standard mixture quantitatively to the column using 8 ml of the 20% benzene-in-hexane solution and collect this fraction in a 15-ml centrifuge tube (fraction I). Elute the column with 10 ml of 40% benzene-in-hexane (fraction II), followed by 10 ml of 60% benzene-in-hexane and 10 ml of 80% benzene-in-hexane, collecting the 20 ml in a 25-ml concentrator tube (fraction III). Elute the column with 10 ml of benzene and collect this fraction in a 15-ml centrifuge tube (fraction IV). Inject 5–10 μl from each fraction into the gas chromatograph to establish the elution pattern of the ten ethyl ethers from the silica gel column.

Under our laboratory conditions, fraction I (20% benzene-in-hexane) contained the ethyl ethers of 2,4-DCP, 2,4,5-TCP, 3,5,6-TCPyridinol, 3,6-DCIP, 2,5-DCBrP, and

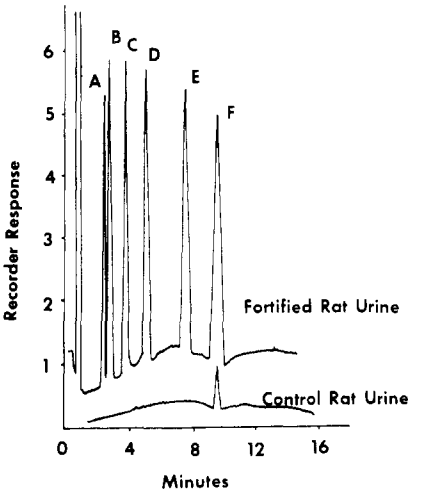


**Figure 1.** Chromatogram of the 20% benzene-in-hexane fraction of fortified and control rat urine. A. 2,4-DCP, 0.8 ppm; B. 3,5,6-TCPyridinol, 0.05 ppm; C. 2,4,5-TCP, 0.03 ppm; D. 2,5-DCBrP, 0.02 ppm; E. 3,6-DCIP, 0.03 ppm; F. PCP, 0.02 ppm.

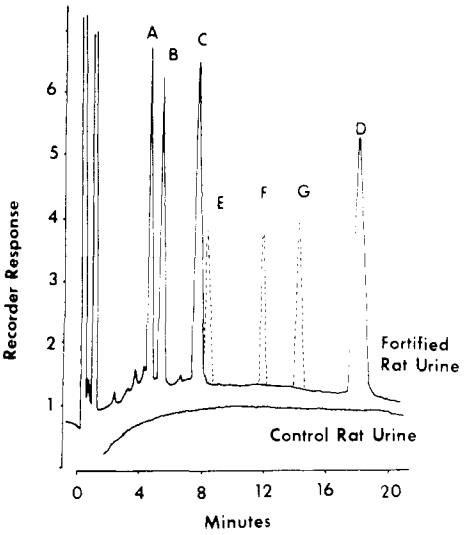


**Figure 2.** Chromatogram of the 60–80% benzene-in-hexane fraction of fortified and control rat urine: A. PNP, 0.1 ppm; B. PNC, 0.4 ppm; C. 2-C-4NP, 0.04 ppm, D. DNOC, 0.16 ppm; E. 2,4-D; F. 2,4,5-TP; G. 2,4,5-T.

PCP. The ethyl ethers of PNP, PNC, 2-C-4NP, and DNOC eluted in fraction III (60 and 80% benzene-in-hexane). Occasionally, traces of DNOC are found in fraction IV (benzene). Fraction II (40% benzene-in-hexane) did not contain any of the derivatives.

Elution patterns may vary from one laboratory to another, depending on the temperature and relative humidity. It is therefore necessary to establish an elution pattern under local conditions before attempting to analyze samples.

**Analysis of Urine.** Pipet 1 to 5 ml (the actual volume to be determined by the anticipated residue level) of urine into a 25-ml concentrator tube. Add dropwise a volume of concentrated HCl equal to one-fifth the amount of urine, and mix well. Fit a stoppered reflux condenser to the tube and heat in a boiling water bath for 1 hr while cooling the condenser with circulating ice water. Remove from the bath, cool, and rinse the sides and tip of the condenser with a total of 2 ml 0.1 *N* NaOH. Add 3 ml of anhydrous ethyl ether to the tube and mix contents vigorously on a Vortex mixer for 2 min; then centrifuge and transfer ethyl ether layer to a 15-ml centrifuge tube with a disposable pipet. Repeat the extraction with an additional 3-ml volume of ethyl ether and add the second ethyl ether extract to the centrifuge tube.

Add diazoethane dropwise with a disposable pipet until the yellow color persists. Let the solution stand 15 min; then bubble clean dry nitrogen through the solution to remove excess reagent. Concentrate the ethylated urine extract to approximately 0.3 ml, using the nitrogen evaporator. Add 2 ml of hexane and continue evaporating the ether-hexane solution to 0.3 ml.

Prepare a silica gel chromatographic column as previously described. Prewash the column with 10 ml of hexane and discard the washing. Transfer the concentrated urine extract quantitatively to the column using 2 ml of the 20% benzene-in-hexane. As soon as the solvent sinks in the sodium sulfate, add 8 ml of the 20% benzene-in-hexane to the column and collect the total volume of the 20% benzene-in-hexane (10 ml). This fraction contains the halogenated phenols. Continue eluting with 10 ml of 40% benzene-hexane and discard this fraction. Add 10 ml of 60% benzene-hexane, followed by 10 ml of 80% benzene-hexane and collect these fractions in a single tube. The combined 60–80% benzene-hexane fractions will contain the nitrophenols. Add 10 ml of benzene to column and collect eluate. Frequently a small amount of the DNOC is found in the benzene fraction. The urinary impurities eluted by the benzene solvent do not interfere with the gas chromatographic determination of DNOC, which has a relatively long retention time. The elution pattern of spiked control urine extracts must be established before the analysis of actual samples is undertaken.

**Table I. Electron-Capture Detector Sensitivities,[a] Limits of Detection and Recovery Data[b] for Ten Halo- and Nitrophenol Ethyl Ethers**

| Compound | Limits of | | |
|---|---|---|---|
| | Sensitivity, ng | Detection, ppm | Recovery, % |
| 2,4-DCP | 0.2 | 0.1 | 87–96 |
| 2,4,5-TCP | 0.02 | 0.01 | 85–95 |
| 3,5,6-TCPyridinol | 0.05 | 0.01 | 91–97 |
| 3,6-DCIP | 0.02 | 0.01 | 88–94 |
| 2,5-DCBrP | 0.02 | 0.01 | 88–96 |
| PCP | 0.01 | 0.01 | 92–96 |
| PNP | 0.1 | 0.02 | 85–98 |
| PNC | 0.3 | 0.05 | 88–98 |
| 2-C-4NP | 0.05 | 0.01 | 85–92 |
| DNOC | 0.1 | 0.05 | 86–96 |

[a] Based on 15% scale deflection. [b] Based on use of the described method.

## RESULTS AND DISCUSSION

Control rat urine samples were fortified with the sodium salts of the phenols. The samples were analyzed and aliquots of the 20% B-H and 60 + 80% fractions were injected separately into the gas chromatograph. Figure 1 illustrates chromatograms of the 20% B-H fraction (halogenated phenols) of spiked and control rat urine samples, and Figure 2 shows chromatograms of the 60 + 80% fraction (nitrophenols) of control and fortified rat urine samples. An average of 0.01 ppm of pentachlorophenol was routinely found in all control urine samples. The percent recovery, limits of detection in ppm, and detector sensitivity in nanograms (based on 15% scale deflection) are shown in Table I.

Ethylation of the phenols proceeds rapidly at room temperature, producing ethyl ether derivatives which are gas chromatographable and stable to silica gel column chromatography. Better gas chromatographic resolution of the more volatile phenols was achieved by preparing the ethyl

**Table II. The Relation between Total Dosage of Biodegradable Pesticides and Urinary Excretion of Halo- and Nitrophenols**

| Compound | Dose level $LD_{50}$ | nmol fed | Excretion of phenolic-type metabolites: Metabolite | nmol excreted | % of dose excreted | Days for complete excretion |
|---|---|---|---|---|---|---|
| VC-13 | $10^{-2}$ | 5140 | 2,4-DCP | 3470 | 67 | 3 |
| | $10^{-3}$ | 514 | | 359 | 70 | 1 |
| Ronnel | $10^{-4}$ | 234 | 2,4,5-TCP | 124 | 53 | 2 |
| | $10^{-5}$ | 23.4 | | 7.4 | 32 | 1 |
| Dursban | $10^{-3}$ | 232 | 3,5,6-TCPyridinol | 157 | 68 | 4 |
| | $10^{-4}$ | 23.2 | | 29.5 | 100+ | 4 |
| C-9491 | $10^{-4}$ | 314 | 3,6-DCIP | 7.6 | 2.4 | 2 |
| | $10^{-5}$ | 31.4 | | 0.834 | 2.7 | 1 |
| Bromophos | $10^{-4}$ | 617 | 2,5-DCBrP | 305 | 49 | 3 |
| | $10^{-5}$ | 61.7 | | 41.4 | 67 | 3 |
| PCP | $10^{-4}$ | 40.6 | PCP | 9.03 | 22 | 2 |
| | $10^{-5}$ | 4.06 | | 1.05 | 26 | 1 |
| EPN | $10^{-2}$ | 670 | PNP | 108 | 16 | 3 |
| | $10^{-3}$ | 67.0 | | 9.35 | 14 | 3 |
| Fenitrothion | $10^{-2}$ | 5430 | PNC | 3610 | 66 | 2 |
| | $10^{-3}$ | 543 | | 472 | 87 | 1 |
| Dicapthon | $10^{-2}$ | 8700 | 2-C-4NP | 3570 | 41 | 1 |
| | $10^{-3}$ | 810 | | 20 | 2.5 | 1 |
| DNOC | $10^{-2}$ | 910 | DNOC | 0 | 0 | |
| | $10^{-3}$ | 91.0 | | 0 | 0 | |

derivatives instead of the more commonly used methyl ethers.

Silica gel column chromatography serves two purposes: it provides a clean sample for gas chromatographic analysis and it conveniently separates the halogenated phenols, thus simplifying the gas chromatographic analysis.

A mixture of the ten phenols and three phenoxy acids, namely 2,4-D, 2,4,5-T, and silvex, can be determined in one sample. All of the halogenated phenols involved in this study are eluted with 20% benzene–hexane, while the nitrophenols and the phenoxy acids are eluted in the 60 and 80% benzene–hexane fractions. These herbicides are included in this report because they are detected as intact excreted residues in the urine using the present procedure. 2,4-DCP and 2,4,5-TCP, potential mammalian metabolites of 2,4-D and 2,4,5-T, are also determined in this procedure. The analysis of these herbicides and their metabolites has been described in detail in a previous report (Shafik *et al.*, 1971a). In the gas chromatographic step, the retention time of the ethyl ether derivative of 2-chloro-4-nitrophenol is almost identical to that of the ethyl ester of 2,4-D. In addition, these two derivatives are eluted in the 60 + 80% benzene–hexane fractions from silica gel. Confirmation of identity can be accomplished using the classical $NaHCO_3$ and acid extraction steps which separate carboxylic acids from phenols (Bakke and Scheline, 1969).

In order to determine if a correlation exists between exposure to intact pesticides and excretion of urinary phenolic metabolites, male Charles River rats weighing 190 to 220 g were dosed by gavage with peanut oil solutions of eight organophosphorus compounds, PCP, and DNOC in concentrations ranging from $10^{-2}$ through $10^{-5}$ of the $LD_{50}$ (Kenaga and Allison, 1969), as indicated in Table II. The doses were administered daily for 3 days to two rats at each dose level. The animals were maintained in stainless steel metabolism cages with the two rats administered the same dose regimen maintained in the same cage. Urine samples were collected at 24-hr intervals and stored in a freezer until analysis was performed.

Urine samples were analyzed for several days following the third dose until no detectable levels of the phenolic type metabolites were observed. This established the number of days required for total excretion of the metabolites. The percent of the total dose excreted as the phenolic metabolite was calculated from the sum of the amount of phenolic metabolite excreted each day and the total amount of pesticide fed in the 3-day period.

As indicated in Table II, the amount of urinary metabolites excreted is proportional to the dose of parent compound administered. The percent of the dose excreted in the urine as a phenolic metabolite of the pesticide fed indicates that low level animal exposure to VC-13, ronnel, Dursban, Bromophos, PCP, EPN, and fenitrothion can be detected. The low excretion rates of the phenols of C-9491 and dicapthon in urine indicate that the method cannot detect low-level exposure to these compounds. DNOC was not detected in the urine of rats fed $10^{-2}$ and $10^{-3}$ of the $LD_{50}$ of DNOC.

There are other pesticide chemicals not included in this investigation which may produce the same phenolic metabolites reported in this study. 2,4,5-TCP can be a urinary metabolite of ronnel, Gardona, lindane, 2,4,5-T, and Silvex. PNP in urine can result from exposure to EPN, ethyl parathion, and methyl parathion. The method is not capable of distinguishing between the sources of the urinary 2,4,5-TCP and PNP. When such a distinction is essential, the corresponding alkyl phosphate of the organophosphorous pesticide can be determined in the urine (Shafik *et al.*, 1971a). The free phenoxy acids of 2,4,5-T and Silvex can also be determined in the urine (Shafik *et al.*, 1971a). It must be emphasized that the rat-feeding experiment was designed only for the purpose of evaluating the method and not as a study of the metabolism of these compounds.

In conclusion, a multiresidue method has been developed for the determination of low levels of halo- and nitrophenols and phenoxy acids in rat urine which undoubtedly can be extended to environmental samples. Such a method should be useful, if properly evaluated on a monitoring and surveillance scale, in establishing human exposure to low levels of a large number of biodegradable pesticides.

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# Isolation, Identification, and Biogenesis of Bifunctional Compounds in Lavandin Oil

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Carbonyl compounds of lavandin were isolated by treating the oil with Girard T reagent in the presence of methanol and acidic ion exchange resin. The investigation of these materials resulted in the identification of 29 carbonyl compounds, of which 17 compounds are new in lavandin oil. They include ten aldehydes, four ketones, and three new bifunctional compounds. In another experiment, less volatile lavandin residue, obtained by molecular distillation of the oil, was also analyzed by both column chromatography and glc methods. This resulted in the identification of two alcohols, three lactones, and three more bifunctional compounds. Biogenesis of bifunctional compounds has been discussed in the light of photooxidation of linalyl acetate.

Lavandin oil is produced commercially in southern France from a hybrid plant (*Lavandula hybrida* Reverchon). Due to its importance as a fragrance material, various investigators have studied this oil and identified many of its components (Steltenkamp and Casazza, 1967). The major components of this oil are linalool and linalyl acetate. In addition, there are small amounts of carbonyl compounds which have also been investigated by two workers in two different laboratories. Igolen (1955) studied this oil and identified six carbonyl compounds. In 1960 Stadtler isolated the carbonyls of lavandin oil by Girard T reagent and identified 11 carbonyl compounds. The present paper reports the characterization of other carbonyl compounds of this oil, together with the constituents of less volatile residue.

## EXPERIMENTAL PROCEDURE

**Instrumental Methods.** Glc analyses were performed on F&M 810 instrument using 10% Carbowax 20M and 10% SE-30 coated on Anakrom ABS (80–100 mesh) packed in a stainless steel column (8 ft × 0.25 in. o.d.), unless specified. The following spectrometers were used: ir, Beckman IR-5A and IR-4; nmr, Varian 60 and HA 100 ($CCl_4$, TMS as internal standard); mass spectra, CEC Model 21-103 and AEI-MS9 for high-resolution spectra. Mass spectra major fragmentation peaks are recorded in decreasing order of intensity. Neutral alumina (Fisher Scientific, 80–200 mesh) was used for column chromatography.

*Isolation of Monocarbonyls and Bifunctional Compounds from Lavandin Oil.* A solution of lavandin oil (500 g) in methanol (5 l.) was treated with Girard T reagent (30 g) and acidic ion exchange resin (5 g) as described by Stanley *et al.* (1961), with regeneration of the carbonyls with purified formaldehyde (Hunter and Struck, 1962). The crude carbonyl compounds (4 g) thus isolated were first fractionated into 25 fractions (Figure 1) on a SE-30 column. Each fraction was collected from the end of the glc column in a Dry Ice-cooled glass tube and rechromatographed on a Carbowax 20M column. The pure compound thus isolated was analyzed by ir, nmr, and mass spectrometry.

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*Isolation of Bifunction Compounds and Lactones from Lavandin Residue.* Lavandin oil (5 kg) was distilled (25°, 0.1 mm) to separate the volatile material (4950 g) from a dark-colored gummy residue (50 g). This residue, which possessed strong lavandin odor, was chromatographed on neutral alumina (Activity I, 500 g). Benzene (2 l.) eluted 15 g of colored liquid possessing a strong odor. This liquid was again chromatographed on neutral alumina (Act. 1, 200 g) to obtain the fractions given in Table I. Fractions 1B, 1C, and 1D, which possessed lavandin odor, were further fractionated on a glc SE-30 column (0.25 in. × 4 ft) and the major peaks of each fractions were collected and analyzed by ir, nmr, and mass spectrometry.

## SYNTHESIS

**Compound 1.** Selenium dioxide oxidation of linalyl acetate **13** in ethyl alcohol (Sathe *et al.*, 1966) yielded **1**: ir (neat) 3.25, 3.38, 3.41, 3.5, 3.55, 3.7, 5.74 (-$OCOCH_3$), 5.9 (C=CCHO), 6.09, 6.9, 7.1, 7.3, 8.0, 8.52, 9.05, 9.25, 9.6, 9.7, 10.05, 10.65, 10.8, 12.2 $\mu$; nmr ($CCl_4$) $\delta$ 1.51 (s, 3 H, $CH_3CO$), 1.68 (s, 3 H, $CH_3C$=C), 1.93 (s, 3 H, $OCOCH_3$), 2.24 (m, 2 H, $CH_2$), 5.0–6.0 (m, 3 H, $CH_2$=CH), 6.28 (m, 1 H, -CH=CC=O), 9.22 (s, 1 H, CHO); mass spectrum showed a peak at $m/e$ 150 (M − 60) and major peaks at $m/e$ 43, 41, 45, 93, 91, 79, 55, 67, 53.

**Compound 2.** Selenium dioxide oxidation of lavandulyl acetate in the same way yielded **2**: ir (neat) 3.25, 3.4, 3.5, 3.7, 5.72 (-$OCOCH_3$), 5.9 (C=CCHO), 6.05, 6.9, 7.1, 7.25, 7.32, 8.1, 8.55, 9.6, 10.0, 10.3, 10.65, 11.15, 12.4 $\mu$; nmr ($CCl_4$) $\delta$ 1.7 (br s, 6 H, $CH_3C$=C), 1.99 (s, 3 H, -$OCOCH_3$), 2.5 (m, 3 H, $CH_2C$=C), 4.05 (br d, 2 H, $CH_2OAC$), 4.83 (br d, 2 H, C=$CH_2$), 6.3 (br, 1 H, CH=CCHO), 9.2 (s, 1 H, CHO); mass spectrum showed a peak at $m/e$ 150 (M − 60) and major peaks at $m/e$ 43, 44, 41, 93, 91, 55, 53, 107, 109.

**Compound 3.** Photooxidation of linalyl acetate **13**, as described below, yielded **3** as a minor component: ir (neat) 3.25, 3.36, 3.42, 5.75 (-$OCOCH_3$), 5.89, 5.96 (C=CC=O), 6.08, 6.13, 6.98, 7.05, 7.3, 7.99, 8.05, 8.5, 9.05, 9.32, 9.55, 9.8, 10.18 (*trans*-CH=CH), 10.45, 10.75, 11.75, 12.6 $\mu$; nmr ($CCl_4$) $\delta$ 1.53 (s, 3 H, $CH_3CO$), 2.0 (s, 3 H, -$OCOCH_3$), 2.2 (s, 3 H, $CH_3COC$=C), 2.75 (d, 2 H, $CH_2C$=C), 5.0-6.5 (m, 5 H, CH=CHC=O and $CH_2$=CH); mass spectrum showed a peak at $m/e$ 136 (M − 60) and major peaks at $m/e$ 43, 91, 93, 119, 77, 121, 65.