Previously Unreported Homolog of Malathion Found as Residue on Crops

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An unknown compound, thought to be related to malathion, was found as a residue on malathion field-sprayed kale. The same compound was also found in the Malathion, 50% Emulsifiable Concentrate, which was used to prepare the aqueous spray, as shown by similar gas-liquid chromatographic retention times and thin-layer chromatographic R_f values. This observation indicated that the unknown compound was not an alteration product formed after the application of malathion. The compound was isolated and collected from the Malathion, 50% Emulsifiable Concentrate, by gas-liquid chromatographic fractionation. By the use of nuclear magnetic resonance and mass spectrometry, the compound was identified as the ethyl butyl mercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorodithioate, a homolog of malathion. The oxygen analog of this compound was five times as inhibitory as the oxygen analog of malathion toward bovine erythrocyte cholinesterase. The parent compound, as determined by gas-liquid and thin-layer chromatographic data, has since been found in one 1968 commercial formulation of malathion, and in samples of grains containing malathion residues.

In 1965, maturing field-grown kale was sprayed with an aqueous emulsion of malathion at the rate of 2.5 pounds of active malathion per acre. The purpose of the study was to look for other alteration products of malathion in addition to the known oxygen analog. Samples of kale were harvested at specified intervals after treatment, extracted, and cleaned up by the method of Storherr *et al.* (1964), and analyzed by gas-liquid chromatography (GLC) with a potassium chloride thermionic detector (KClTD) (Giuffrida, 1964). A prominent unidentified GLC peak appeared, as well as peaks showing residues of malathion and of the oxygen analog of malathion. The unidentified peak eluted from a column of 10% DC-200 on Gas Chrom Q and had a retention time of 2 compared with malathion.

A summary of the results of analysis of this malathion field-sprayed kale is shown in Table I. Quantitative data from GLC chromatograms indicated that residues of the unidentified compound were more persistent than those of malathion. Control kale plants from the same field were extracted and analyzed by GLC but no comparable peak was present. Analysis of the aqueous malathion spray solution by GLC showed the presence of this unknown material. Subsequent GLC analyses of individual samples of various malathion formulations affirmed the occurrence of the unidentified compound in Malathion, 50% Emulsifiable Concentrate, obtained in 1965, and in Malathion, 57% Emulsifiable Liquid, obtained in 1968. Measurement of GLC peak areas (assuming equivalent KClTD response) indicated a ratio of unidentified compound to malathion of about 1 to 5 in the formulations. The unidentified component was not present in several other formulations which were examined.

This paper reports the isolation procedure for the unidentified compound and its spectrometric characterization.

EXPERIMENTAL

Extraction and Cleanup. All kale samples were extracted with acetonitrile and cleaned up on a charcoal chromatographic column (Storherr *et al.*, 1964).

GLC Determination. A Barber-Colman Model 5360 gas chromatograph equipped with a potassium chloride thermionic detector (KClTD) was used for the pesticide determinations. The columns were 6-foot × 4-mm. I.D. coiled glass, packed with either 10% DC-200 on 80 to 100-mesh Gas Chrom Q or 2% diethylene glycol succinate (DEGS) on 80 to 100-mesh Gas Chrom Q. Nitrogen, at a flow rate of 120 ml. per minute, was the carrier gas for both columns. Column temperatures were maintained at 203° C. for the 2% DEGS column or 200° C. for the 10% DC-200 column.

Confirmation by Thin-Layer Chromatography. To confirm the presence of malathion and the unidentified compound in various spray and crop samples, unconditioned precoated TLC plates (Uniplate, Analtech, Inc., Wilmington, Del.) were spotted and developed as received. The solvent systems were benzene, chloroform, or 40% methyl ethyl ketone in *n*-heptane (v./v.); the plates were developed at room temperature (55 to 60% relative humidity) in the Chromaflex Sandwich Technique Apparatus (Kontes Glass Co., Vineland, N. J.). A modified enzymatic inhibition method (El Refai and Hopkins, 1965; Mendoza *et al.*, 1968) was used for detection of compounds showing cholinesterase inhibition.

Isolation by Gas-Liquid Chromatographic Fraction Collection. For fraction collection, 1 ml. of the Malathion, 50% Emulsifiable Concentrate, was diluted to 10 ml. with acetone and 50-μl. portions were slowly injected into a Packard gas chromatograph connected to a Packard GLC fraction collector. The gas chromatograph was equipped with a flame ionization detector and a straight 5-foot × 4-mm. (I.D.) glass column containing 20% DC-200 on 80 to 100-mesh Gas Chrom Q. The nitrogen flow rate through the column was 60 ml. per minute. The injector port, column, and de-

Table I. Residues Found by GLC[a] in Malathion Field-Treated Kale

Harvest Interval, Days after Spraying	Malathion, P.P.M.	Unknown Compound, Estimated, P.P.M.[b]	Malathion Oxygen Analog, P.P.M.
2	10.90	5.4	1.76
7	1.34	1.30	0.10
11	0.65	0.60	none
15	0.58	0.60	none

[a] The column used was 10% DC-200 on 80 to 100-mesh Gas Chrom Q. [b] The amount of unknown compound was estimated by comparing the peak area with that of malathion. The unknown compound was later identified as a butyl homolog of malathion.

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Table II. GLC Relative Retention Times and TLC Relative R_f Values of Malathion and the Malathion Homolog

Sample	Retention Time (Relative to Parathion)		R_f Value[a] (Relative to Malathion)		
	Column		Solvent System		
	I[b]	II[c]	I[d]	II[e]	III[e]
Malathion standard	0.90	0.76	1.00	1.00	1.00
Isolated malathion homolog[f]	1.75	1.10	1.46	1.29	1.22
Field-sprayed kale extract	0.91, 1.76	0.77, 1.12	1.00, 1.49	1.00, 1.32	1.00, 1.22
Malathion, 50% Emulsifiable Concentrate	0.91, 1.76	0.75, 1.11	1.02, 1.48	1.00, 1.29	1.00, 1.25
Malathion, 57% Emulsifiable Liquid	0.92, 1.76	0.77, 1.13	1.01, 1.47	N.A.[g]	N.A.[g]
Rice	0.92, 1.77	0.77, 1.13	1.00, 1.44	1.00, 1.27	1.00, 1.24

[a] Detection was by enzymatic inhibition (Mendoza *et al.*, 1968).
[b] Column I is 6-foot × 4-mm. (I.D.) coiled glass column packed with 10% DC-200 on 80 to 100-mesh Gas Chrom Q.
[c] Column II is 6-foot × 4-mm. (I.D.) coiled glass column packed with 2% DEGS on 80 to 100-mesh Gas Chrom Q.
[d] Solvent system I is 50 ml. of benzene. Values are averages of three determinations. The R_f of malathion was 0.20.
[e] Solvent systems II and III comprise a two-dimensional TLC system. The spotted sample was developed in solvent system II (50 ml. of chloroform), dried, exposed to 0.1 ml. of bromine in a 5-liter tank to obtain the P → O analogs of the compounds, and developed perpendicularly to the first direction with solvent system III (20 ml. of methyl ethyl ketone mixed with 30 ml. of *n*-heptane). R_f values for one determination are reported. The R_f of malathion in solvent system II was 0.38. The R_f of malathion oxygen analog was 0.44 in solvent system III.
[f] The GLC collected fraction contained about 5% malathion.
[g] Not analyzed.

tector temperatures were 220° C., 218° C., and 215° C., respectively. The center 60 to 70% portion of the unidentified peak eluting after malathion was collected on small glass beads (Giuffrida, 1965; Ives, 1968). It was necessary to fractionate several portions in order to collect 4 to 5 mg. of the unidentified material. The compound was removed from the glass beads with three 3-ml. acetone rinsings. The compound used in the characterization studies was obtained by evaporating the acetone at 40° C. under a stream of nitrogen.

Preparation of the Oxygen Analog of the Malathion Homolog. The oxygen analog of the malathion homolog was prepared by adding, with stirring, 250 ml. of a 0.01*M* benzene solution of *m*-chloroperbenzoic acid to 250 ml. of a benzene solution containing 0.3 ml. of the Malathion, 50% Emulsifiable Concentrate (Ruzicka *et al.*, 1967). After the mixture was allowed to react at room temperature for 30 minutes, a 250-ml. aliquot was passed through a dry 6-gram silica gel (13% $CaSO_4$) column under gravity and the eluate was discarded. Column elution with 20 ml. of acetone quantitatively removed the more polar reaction products, including the oxygen analog of malathion and the oxygen analog of the malathion homolog. Acetone was removed by air-jet evaporation and the resultant residue was dissolved in 25 ml. of methylene chloride. Acidic reaction products were partitioned from the methylene chloride phase into an aqueous phase by shaking with 25 ml. of a cold saturated $NaHCO_3$ solution in a 100-ml. separatory funnel. The lower methylene chloride phase was removed and reduced to 1 ml. by air-jet evaporation. This solution was spotted on three 0.5-mm. thick silica gel GF TLC plates and dried. The plate was developed in a sandwich chamber with 40% methyl ethyl ketone in heptane. The areas showing quenched fluorescence under ultraviolet light (254 mμ) at an R_f of 0.50 were scraped from the plates into a 10-mm. (I.D.) glass column having a fritted glass disk. The compound was then eluted from the adsorbent with 10 ml. of acetone. Aliquots of the acetone solution were evaporated and used to determine the amount of cholinesterase inhibition by the pH-stat titrimetric technique (Jacobsen *et al.*, 1957). Two- to six-microliter portions of this acetone solution were rechromatographed on the 0.25-mm. thick precoated Silica Gel GF plates with 40% methyl ethyl ketone in heptane in order to compare the R_f and the cholinesterase inhibition behavior of the prepared compound with that of the *in situ* bromine oxidation product (El Refai and Hopkins, 1965; Mendoza *et al.*, 1968) of the isolated homolog. A standard solution of the oxygen analog of malathion was also chromatographed for comparison purposes.

Instrumentation. The following instruments were used for the characterization studies: Beckman IR-10 infrared spectrophotometer; Bendix Model 14 Time-of-Flight mass spectrometer equipped with a vacuum lock direct inlet system (Damico, 1966a); and Varian HA-100 and A-60 NMR spectrometers equipped with a Varian C-1024 Time Averaging Computer. A Radiometer Copenhagen pH-stat titrator was employed in comparing inhibitory strengths of the oxygen analog of the malathion homolog with the malathion oxygen analog standard.

RESULTS

Chromatography. Gas-liquid and thin-layer chromatography confirmed the presence of the unidentified compound in both samples. Chromatographic characteristics of samples containing malathion, or the unidentified compound, or both are presented in Table II. The GLC relative retention times of malathion and the unidentified compound obtained from extracts of malathion field-sprayed kale were identical to those found in the ethyl acetate dilutions of the Malathion, 50% Emulsifiable Concentrate, of 1965. TLC confirmed the presence of the unidentified compound in both samples. The unidentified compound found as a residue in malathion field-sprayed kale was apparently the same as that found in the original Malathion, 50% Emulsifiable Concentrate.

Because the unidentified compound was present only in small amounts in the kale sample, it was isolated by GLC and collected as described above from the Malathion, 50% Emulsifiable Concentrate, for the characterization studies.

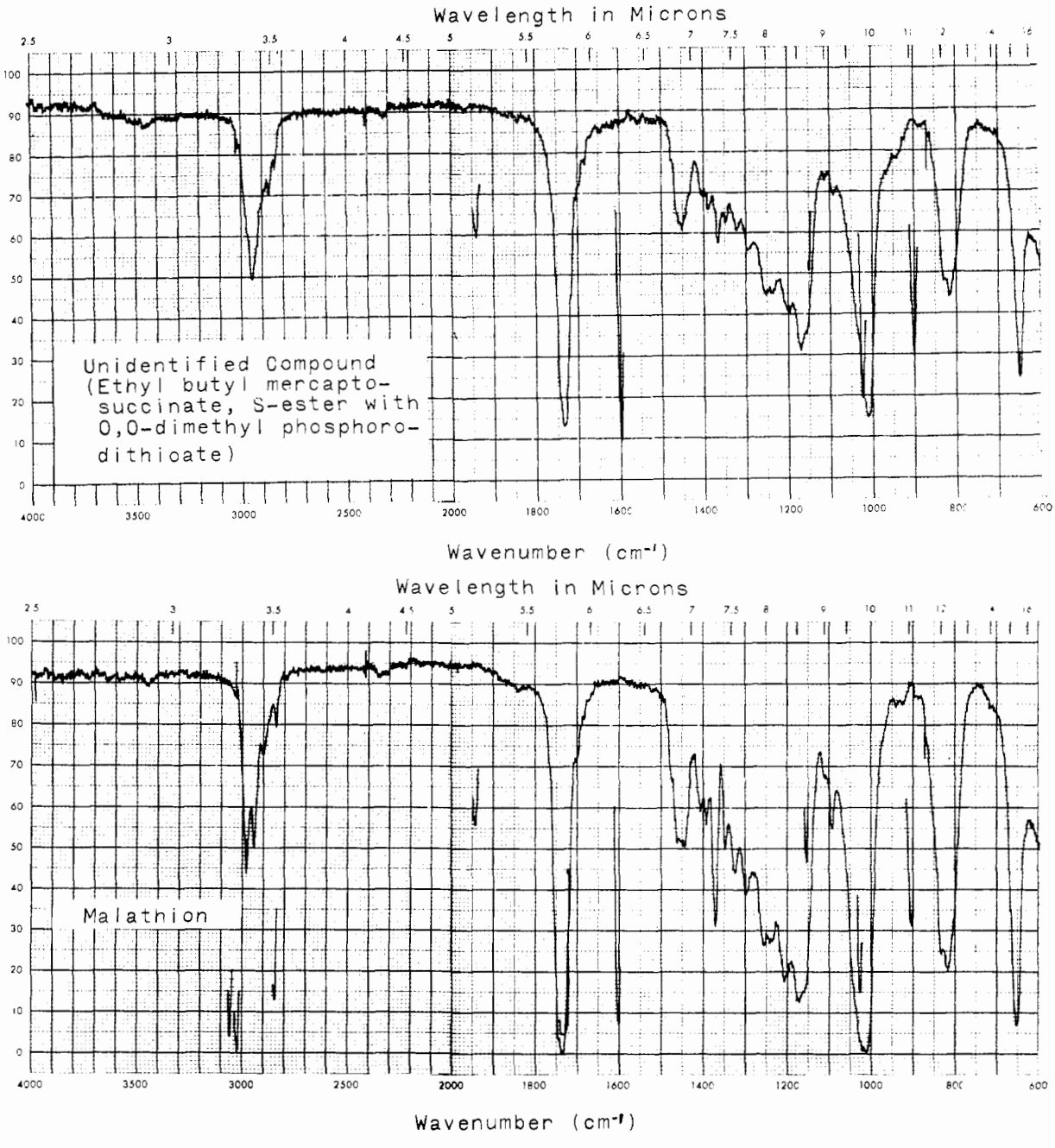
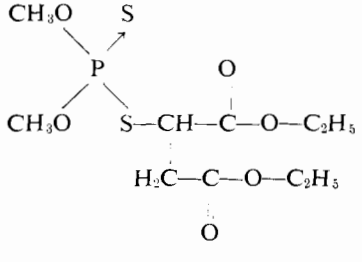


Figure 1. Top. IR spectrum of the malathion homolog (95%, by GLC). Bottom. IR spectrum of malathion (99.5%)

Spectra were obtained at a fast scan setting, with samples as neat thin films between NaCl prisms

Characterization Determinations. INFRARED SPECTROSCOPY. The IR spectra for both malathion and the unidentified compound are presented in Figure 1. The spectra show a remarkably close resemblance. The only conclusion possible from the spectra is that the unidentified compound is similar in structure to malathion. Malathion has the following structure:



MASS SPECTROMETRY. Pertinent mass spectral data for the isolated compound (Damico, 1966b) and for malathion are presented in Table III. The structure of the unkown compound was tentatively identified from the data in Table III and from the mass spectral correlations (Figure 2) of some organophosphorus esters (Damico, 1966a) as ethyl butyl mercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorodithioate.

The molecular ion of the unknown was established as *m/e* 358. The intense peak at *m/e* 125 and the absence of a peak at *m/e* 97 suggested a phosphorodithioate methoxy ester. Previous data (Damico, 1966a) showed that the phosphorodithioate methoxy esters yield a peak at *m/e* 125 $[(CH_3O)_2PS]+$. The phosphorodithioate ethoxy esters have a peak at *m/e* 97, [(HS) (HO) PO]+, which is usually absent in the phosphorodithioate methoxy esters (Damico, 1966a).

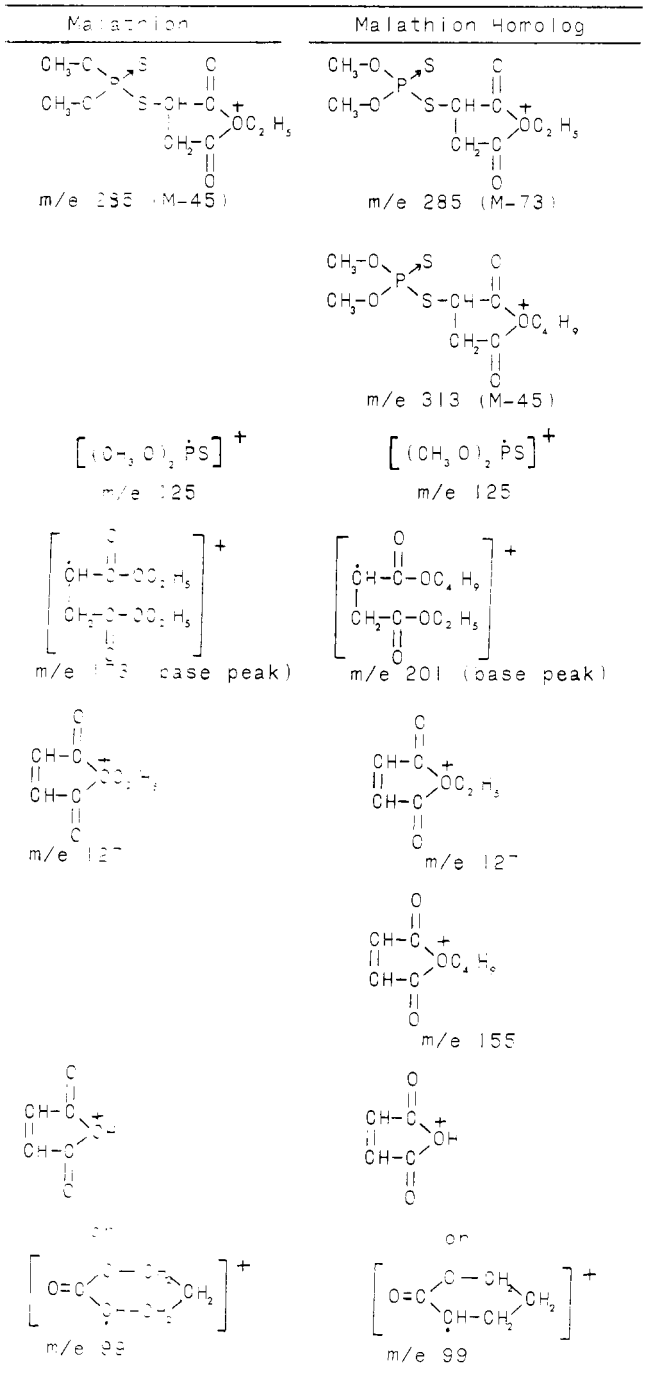


Figure 2. Comparison of postulated mass spectrum fragmentation structures of malathion and the malathion homolog

See Table III for peak intensities and Damico (1966a) for postulated malathion fragment ions

Subtraction of the methoxy phosphorodithioate group from the molecular ion left a residual moiety of 201 mass units. The phosphorodithioates yield an intense peak for this type of cleavage (Damico, 1966a); inspection of Table III showed that the *m/e* 201 ion of the unknown was in fact the base peak. The high ion currents observed at *m/e* 99 (Figure 2) and *m/e* 127 indicated that the *m/e* 201 moiety was very similar to the moiety of malathion, *m/e* 173, which is attached to the sulfur atom that is covalently bonded to the phosphorus atom. The *m/e* 155 ion suggested the presence of a butoxy group in the *m/e* 201 moiety. The M-45 and M-73 peaks were a further indication that both an ethoxy and a butoxy group were present in the molecule. Accordingly, the mass spectral

Table III. Selected Mass Spectral Data for Isolated Malathion Homolog and Malathion

	Relative Intensities,[a]	
m/e	Isolated compound	Malathion
97	0	0
99	50	22
125	81	51
127	54	78
155	10	...
157	...	9
173	...	100
201	100	...
285	8 ($M-C_4H_9O$)	11 ($M-C_2H_5O$)
313	5 ($M-C_2H_5O$)	...
330	...	7 (Molecular weight)
358	11 (Molecular weight)	...

[a] Intensities were normalized to the most intense peak which was assigned a value of 100.

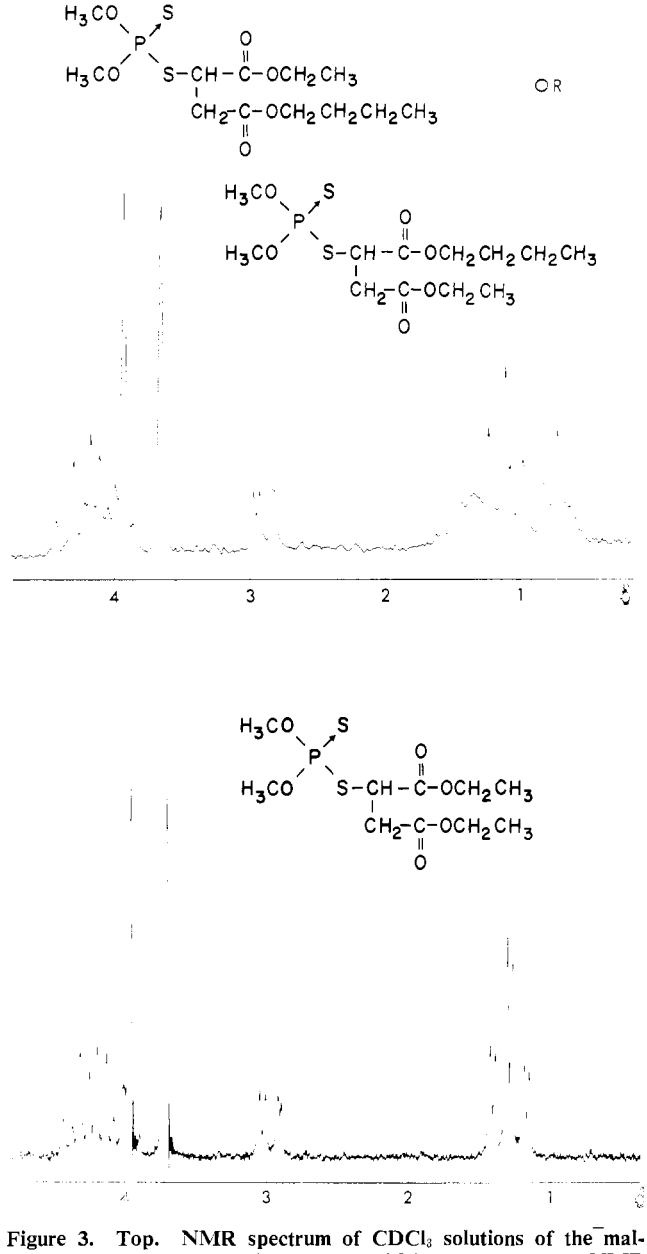
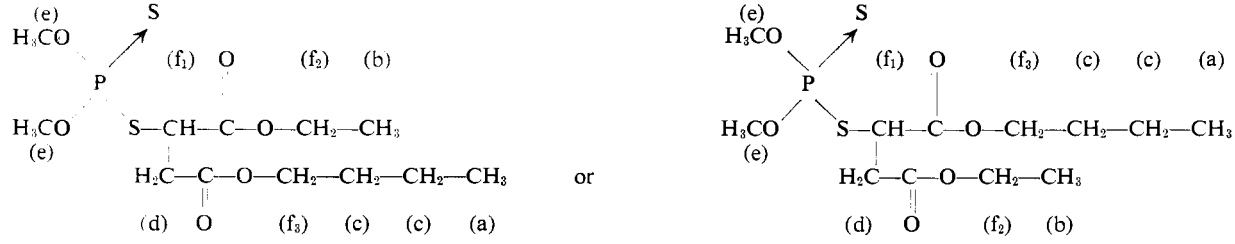


Figure 3. Top. NMR spectrum of $CDCl_3$ solutions of the malathion homolog (95%). Time-average of 36 scans. Bottom. NMR spectrum of $CDCl_3$ solutions of malathion

Table IV. NMR[a] Correlations of Ethyl Butyl Mercaptosuccinate, *S*-Ester with *O,O*-Dimethyl Phosphorodithioate (Isolated Compound)

Absorption	Chemical Shift, δ	Description	No. Protons
a	0.66	triplet (J_{HH} = 7 Hz)	
b	1.11	triplet (J_{HH} = 7 Hz)	10
c	~1.00–1.67	complex splitting pattern	
d	~2.87–2.97	AB portion of ABX spectrum	2
e	3.81	doublet due to H-P coupling (J_{HP} = 16 Hz)	11
f	~4.04–4.41	overlapping multiplets	

Proposed structures:



[a] Data for $CDCl_3$ solution collected with Varian A-60 and HA-100 spectrometers in combination with C-1024 Time Averaging Computer.

data are consistent with the structures postulated for the unknown compound in Table IV.

NUCLEAR MAGNETIC RESONANCE SPECTROMETRY. The NMR spectra of the $CDCl_3$ solutions of the unknown compound and malathion were obtained for confirmation of the mass spectral data and are presented in Figure 3.

The interpretation of the NMR spectrum of the unknown compound was based on its resemblance to the malathion spectrum, where bands can be quite readily assigned to the various types of protons. In particular, the appearance of bands and their positions in the regions of δ 3.8 and δ 4.2 are almost the same as in the malathion spectrum. Therefore, the areas under these bands, as determined by integration, were used to establish the number of protons in the unknown compound. The unknown was thus found to contain 23 protons, that is, four more than malathion.

From the similarity of the bands at ~δ 4.2 and ~3.8, it was concluded that the CH_3O— and CH—C(O)— groups occurring in malathion exist also in the unknown compound. Furthermore, the pattern at δ 2.9 is quite analogous to that of the central CH—CH_2— protons in malathion; in both cases, their relative intensity is 2, and the center of the corresponding band in malathion is at δ 3.0. Thus the four additional protons must be located on the alkoxy chain, probably in —CH_2— groups.

The possibility of the —$OCH_2CH_2CH_3$ groups, in lieu of the two —OCH_2CH_3 groups in malathion, can be excluded; in such a case the bands for the two CH_3 protons (perturbed triplets) would be expected to almost coincide, but the two triplets observed here are separated by about 0.45 p.p.m. Therefore only structures with one ethyl and one *n*-butyl group (Table IV) appear to be compatible with the spectrum. A further indication for an *n*-butyl group is that one of the CH_3— triplets appears at rather high field (δ 0.66), which is typical of CH_3— groups on a rather long aliphatic chain.

STUDIES WITH THE OXYGEN ANALOG OF THE HOMOLOG. The prepared oxygen analog of the malathion homolog had the same TLC R_f value as the bromine oxidation product (El Refai and Hopkins, 1965; Mendoza *et al.*, 1968) of the isolated malathion homolog (see Table II, footnote *e*). Two other slightly inhibitory spots were also present; one corresponded to the oxygen analog of malathion. Visual comparison of these areas indicated that approximately 90% of the inhibition was due to the desired oxygen analog. It was also estimated that 90% of the total peak area by GLC equipped with KCl thermionic and electron capture detectors was that of the oxygen analog of the malathion homolog.

The prepared oxygen analog homolog was five times as active as the oxygen analog of malathion as an inhibitor of bovine erythrocyte cholinesterase as measured by the pH-stat method.

Following isolation and identification of ethyl butyl mercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorodithioate, in these laboratories, several FDA District laboratories which had reported finding unidentified GLC peaks in samples of wheat and rice containing malathion were asked to send portions of these samples. In several of these samples, residues were found giving GLC peaks with retention times identical to those of the butyl homolog of malathion (Table II).

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