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Synthesis and Isolation of 1-Hydroxy-2-propyl 3-Chlorocarbanilate from Soybean Plants Treated with Isopropyl 3-Chlorocarbanilate

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The metabolism of isopropyl 3-chlorocarbanilate (CIPC) to form an alkyl hydroxylated metabolite in soybean shoots was indicated by alkaline hydrolysis–distillation of 3-chloroaniline from polar plant extracts. The presence of 1-hydroxy-2-propyl 3-chlorocarbanilate (III) in hydroponically grown plants was confirmed by autoradiograms of two-dimensional TLC plates, both of III and the acetate derivative of III, t_R of III on HPLC and t_R of the heptafluorobutyrate derivative of III on GLC. The presence of III in soil-grown plants was confirmed by the alkaline hydrolysis–distillation of 3-chloroaniline from polar extracts, t_R of the acetate of III on HPLC and GLC, and the mass spectral analysis of the acetate of III. Soil grown soybean shoots produce a higher percentage of III when compared to the aryl hydroxylated metabolites which are the predominant metabolites found in hydroponically grown plants. Synthetic III (mp 88.5–91.0 °C, 68%) was prepared by HCl cleavage of 1-triphenylmethoxy-2-propyl 3-chlorocarbanilate (V) (mp 138–139 °C, 82%) which was prepared from 1-triphenylmethoxy-2-propanol (IV) and 3-chlorophenyl isocyanate.

Hodgson (1967) reported two water-soluble metabolites of isopropyl 3-chlorocarbanilate in barley, cucumber, and soybean. James and Prendeville (1969) reported the isolation of suspected β-glycosidic conjugates and postulated that the isopropyl moiety of CIPC was oxidized by smartweed, tomato, and parsnip plants. Extensive studies of hydroponically grown soybeans by Still and Mansager (1971–1973) showed the presence of the aryl hydroxylated metabolites, isopropyl 5-chloro-2-hydroxycarbanilate (I) and isopropyl 3-chloro-4-hydroxycarbanilate (II), but provided no indication of alkyl hydroxylation. Unpublished work at PPG Industries (1973) on soybean plants confirmed the presence of I and II but also indicated the presence of a third metabolite. This paper reports the joint investigation resulting in the identification of this third metabolite, 1-hydroxy-2-propyl 3-chlorocarbanilate (III).

EXPERIMENTAL SECTION

Synthesis of Metabolites. Compounds I and II were prepared by the method of Grunow et al. (1970). Preparation of the compounds by a different route has been described by Bobik et al. (1972).
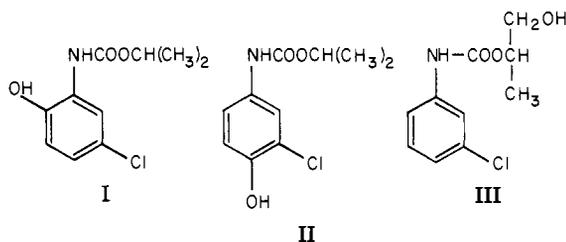
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In the following preparations, care was taken to use anhydrous solvents and reagents and to exclude atmospheric moisture from the reaction vessel.

Compound IV (Figure 1) was prepared from chlorotriphenylmethane and 1,2-propanediol in the presence of pyridine by the method of Havbrandt et al. (1969); 86% yield of IV; mp 99–100 °C (lit. mp 96–97 °C). The use of purified chlorotriphenylmethane (Renfrow and Hauser (1943)) was found essential for obtaining high purity product.

To a refluxing solution of 202.0 g (0.633 mol) of IV in 1000 ml of benzene was added 95.4 g (0.621 mol) of 3-chlorophenyl isocyanate over a 25-min period. After refluxing for 14 h, unreacted isocyanate was destroyed by adding 50 ml of isopropyl alcohol and refluxing for an additional 15 min. The product, 1-triphenylmethoxy-2-propyl 3-chlorocarbanilate, crystallized after the addition of 1500 ml of hexane. The product was removed by filtration, washed with 200 ml of hexane, and vacuum dried to yield 241.1 g (82%) of white crystals: mp 138–139 °C; ir (mull) 3310 and 1695 cm^{-1}; NMR (CDCl$_3$) δ 6.9–7.6 (m, 19, ArH), 6.72 (br s, 1, NH), 5.17 (m, 1, CH), 3.20 (d, J = 5 Hz, 2, CH$_2$), 1.24 (d, J = 6.5 Hz, 3, CH$_3$). Anal. Calcd for C$_{29}$H$_{26}$ClNO$_3$: C, 73.79; H, 5.55. Found: C, 73.52; H, 5.12.

Hydrogen chloride was passed through a sintered glass bubbler into a solution of 240.4 g (0.510 mol) of V in 1200 ml of chloroform. The temperature rose from 24 to 28 °C over a 24-min period and then dropped to 24 °C during the subsequent 115 min of the addition period. About 20 g of hydrogen chloride was added during the first 50 min and only an additional 4 g during the remaining 89 min. After sparging the solution with nitrogen for 70 min to remove excess hydrogen chloride, the solvent was removed under vacuum using a 25–35 °C water bath. The 262 g of solid residue was dissolved in 450 ml of carbon tetrachloride and 93 g of crude 1-hydroxy-2-propyl 3-chlorocarbanilate was obtained upon cooling. The product was
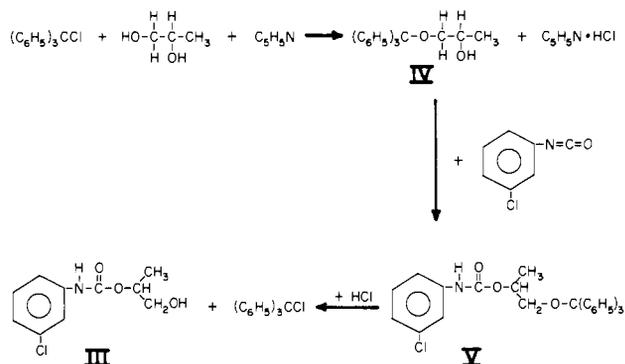
Figure 1. Synthesis of III.

dissolved in 1500 ml of chloroform and washed twice with 200-ml portions of 5% aqueous sodium bicarbonate solution and twice with 200-ml portions of water. Evaporation of the chloroform followed by recrystallization from a mixture of 500 ml of hexane and 525 ml of benzene gave 77.9 g (68%) of III as a white solid: mp 88.5–91.0 °C; ir (mull) 3340, 3260, 3190, and 1700 cm^{-1}; NMR (CDCl$_3$) δ 7.64 (br s, 1, NH), 7.47 (d of d, J = 1 and 1.5 Hz, 1, H'-2), 6.8–7.3 (m, 3, H'-4,5,6), 5.0 (m, 1, CH), 3.70–3.68 (d of d, J = 4 and 6.5 Hz, CH$_2$), 3.73 (s, OH), and 1.23 (d, J = 6.5 Hz, 3, CH$_3$). The 3.70–3.68 and the 3.73 signals total 3 H. Dilution by a factor of two moved the OH signal to 3.16 and revealed the splitting of the CH$_2$OH protons. Anal. Calcd for C$_{10}$H$_{12}$ClNO$_3$: C, 52.29; H, 5.27. Found: C, 51.96; H, 5.12.

Growth of Soybean Shoots. Soybean seeds (variety Hawkeye) were planted and grown for 2 weeks in a soil–sand mixture (2:1). The roots were then washed free of soil and the plants equilibrated for 2 days in aerated half-strength Hoagland and Aaron's nutrient solution. When the plants were in the 2–3 trifoliate leaf stage they were transferred to a treatment solution consisting of half-strength Hoagland and Aaron's solution to which had been added 1 ppm (4.7 × 10^{-6} M) ^{14}C-phenyl-labeled CIPC (New England Nuclear). The specific activity of the [^{14}C]CIPC was 20 μCi/mg which resulted in a specific activity of 4.4 × 10^4 dpm per ml of solution. Two plants were grown in each container with 300 ml of treatment solution. Additional nonradioactive nutrient solution was added as required. One plant from each container was harvested after 8 days and the other plant harvested after 16 days. The roots were severed from the shoots upon harvesting, and the shoots were stored in plastic bags in a freezer until analysis. The plants were grown indoors under artificial light at Barberton, Ohio, and analyzed there.

Soybean plants were grown in five 4 ft × 4 ft × 9 in. plastic lined flats. The flats were filled with 1 in. of gravel, 4 in. of untreated soil (silt loam with 2–3% organic matter), 1 in. of [^{14}C]CIPC treated soil, 1 lb of Amsoy variety soybean seed/box, and 1 in. more of treated soil on top. A commercial 12–12–12 grade fertilizer had been incorporated into the soil at a 50 lb/acre rate. The ^{14}C-labeled CIPC was dissolved in acetone and added to unlabeled commercial CIPC (Furloe 4EC). The resulting mixture was diluted with distilled water and incorporated into the 2-in. equivalent of soil by spraying and mixing the soil in a large plastic bag. The treatment rate was 2 lb/acre CIPC or 6.7 μCi of ^{14}C/ft^2. Plants were thinned at 24 days (first trifoliate stage) and the shoots harvested at 35 days or the second trifoliate leaf stage. The plants were grown in February at Boyce-Thompson Institute, Yonkers, N.Y., and were frozen upon harvest, shipped in dry ice, and

stored frozen until analyzed.

Extraction and Prehydrolytic Purification. All extractions of tissue used the modified Bligh-Dyer procedure of Still and Mansager (1973). This procedure involves extraction of the tissue with a one-phase 2:1:0.8 methanol–chloroform–water system followed by adjusting the solvent ratio to 2:2:1.8 which provides a polar–nonpolar phase separation.

Hydroponically grown shoots (1–2-g samples) were extracted using a Virtis homogenizer and a 50:1 solvent to tissue ratio. Small samples (50 g) of soil grown shoots were homogenized with a Tekmar homogenizer (Model MS-45N) using a 3:1 solvent to tissue ratio. The 4.6-kg sample of soil-grown shoots was macerated in a Hobart Food Chopper as described by Still and Mansager (1973).

The nonpolar and polar phases from the extraction of the hydroponically grown soybean shoots were concentrated with a rotary evaporator prior to spotting of the nonpolar phase on TLC or hydrolysis of the polar phase.

The polar extract from the 4.6 kg of shoot tissues from soil-grown plants was concentrated in a flash evaporator to 415 ml which removed the methanol. The solution was divided in two equal fractions and each half was passed through a 21 cm × 5 cm column of 20–50 mesh Amberlite XAD-2 resin. The column was washed with water and eluted with methanol following the procedure of Still and Mansager (1973). The methanol eluate was lyophilized to remove the methanol and redissolved in water for hydrolysis.

Identification of III from Hydroponically Grown Plants. The concentrated polar phase from the Bligh-Dyer plant extract was diluted 1:1 with concentrated hydrochloric acid and digested for 1 h at 65 °C. The solution was extracted with four 5-ml portions of chloroform, and the chloroform extract was then evaporated to 0.5 ml for thin-layer chromatography (TLC). Separation of metabolites in the hydrolyzed polar extract was effected by two-dimensional TLC using Brinkmann Laboratory 0.25 mm, silica gel F-254, 20 × 20 cm plates with fluorescent indicator. Nine plates were each spotted with 50 μl (50000 dpm) of the above chloroform extract. In the case of two of the plates, the material spotted on the plate was spiked with synthetic, nonradioactive I, II, and III. On these two plates nonradioactive standards were also spotted at the two corners of the plates adjacent to the radioactive spot to provide coordinates. A 60:40 hexane–acetone solution was used as the first developer, and a 90:9:1 chloroform–acetone–acetic acid solution was used as the second developer. The developed TLC plates were marked with dots of radioactive ink at three corners to permit subsequent orientation of the plates in relation to the developed autoradiographs. The TLC plates were packed with single-coated, blue-sensitive XB54 Eastman medical x-ray film and stored for 7 days before developing the films. All the films showed three major spots. The spots, visible by fluorescence quenching on the two TLC plates that had been spiked with nonradioactive standards I, II and III, were superimposable with the three autoradiograph spots. Compound III was isolated from the TLC plates by scraping the area on the plate which corresponded to the spot of III on the autoradiograph. The scrapings were slurried with a small amount of 95% ethanol, the solids were filtered out, and the filtrate was evaporated to dryness by blowing with a stream of nitrogen while heating in a 60 °C water bath. The residues were used in the following experiments.

A DuPont Model 830 high performance liquid chromatograph (HPLC) equipped with ultraviolet photometer

Table I. CIPC Metabolite R_f Values on Silica Gel F-254

Compound	Hexane-acetone (60:40) developer	Chloroform-acetone-acetic acid (90:9:1) developer
I	0.48	0.42
II	0.41	0.32
III	0.35	0.14
Acetate of III	0.49	0.45

Table II. Hydrolysis and Steam Distillation of Metabolites Isolated by TLC

Tentative spot identity from R_f	Total ^{14}C in spot, dpm	Radioact. of fractions, % of total in spot			
		Residue	Steam distillate		
			Acidic extract	Basic extract	Unextractable
I	27381	94	1	1	5
II	3385	85	3	3	9
III	7046	39	4	51	6

detector operated at 254 nm was used. The column was 1 m × 2.2 mm i.d. stainless steel packed with ETH-Permaphase (DuPont) and was operated using a mobile phase of 1,4-dioxane (4%) in hexane at 1 ml/min, 400 psig, and 50 °C. The analysis of standard solutions showed III to be eluted in the fraction collected between 10.5 and 15 min. The residue from one TLC plate spot extract was injected into the chromatograph and fractions were collected for liquid scintillation counting (lsc). The residue from the extract of the scrapings of five unspiked TLC plates was dissolved in 200 μl of the mobile phase solvent and 100 μl injected into the chromatograph. The fraction with the retention time of III (10.5–15 min) was collected and used for gas chromatography. A Hewlett-Packard Model 7620A gas–liquid chromatograph (GLC) equipped with an electron-capture tritium radiation source detector was used for the determination of III. The 1.2 m × 4 mm i.d. glass column was packed with 5% OV-17 on 80–100 mesh Gas-Chrom Q packing and was operated at 170 °C. A standard solution containing 100 μg/l. of synthetic III in hexane containing 4% of 1,4-dioxane was prepared. To 2 ml of this solution was added 5 μl of heptafluorobutyryl chloride and 1 μl of triethylamine followed by a 5-min reaction time at room temperature. A 10-ml portion of pH 7 buffer solution was then added and the mixture shaken for 3 min. After phase separation, a 10-μl sample of the hexane phase was injected into the gas chromatograph giving a single peak with a retention time of 10 min (5% methane in argon, flow rate 60 ml/min). Similar derivatization of 2 ml of the above HPLC fraction gave a product having the same GLC retention time (10 min).

The spot corresponding to III was scraped from one of the TLC plates which had been prepared using extract spiked with nonradioactive standards. The residue from the extract of the scrapings was treated with 25 μl of acetic anhydride for 1 h at 135–140 °C, and the product was allowed to stand for 30 min with 0.5 ml of 5% sodium bicarbonate solution before extracting with chloroform. After the extract was evaporated to about 50-μl volume, it was spiked with nonradioactive III and the acetate of III (prepared from synthetic III by the same acetylation procedure). The solution was spotted on a TLC plate and two spots were found by fluorescence quenching on the developed plate. An autoradiograph of the TLC plate showed a single spot corresponding to the TLC spot of the acetate of III (Table I).

The spots of I, II, and III were separately scraped from a TLC plate. The scrapings were each refluxed with 35 ml of 25% aqueous sodium hydroxide for 3 h and then distilled until 20 ml of distillate had been collected in a receiver containing 10 ml of 1 N hydrochloric acid. The acidified distillate was extracted with three 5-ml portions of chloroform and then made basic by the addition of 15 ml of 1 N potassium hydroxide. The basic solution was then extracted with three 5-ml portions of methylene chloride. Liquid scintillation data on these extracts are shown in Table II.

Identification of III from Soil-Grown Plants. CIPC

in chloroform extracts from the Bligh-Dyer extraction was separated from any unconjugated III with a silica gel column and then the CIPC was hydrolyzed and analyzed with a Bleidner apparatus using the procedure for unextracted plants (Ercegovich and Witkonton, 1972). For example, the nonpolar extract from 50 g of tissue was evaporated to about 2 ml. A 16 mm o.d. glass column was dry packed to a height of 15 cm with silica gel (Woelm, 60–150 mesh, 20% water added to dried material). A 1-cm layer of anhydrous sodium sulfate was added to the column top and 12 ml of 1:1 hexane–methylene chloride was added to the column and drained to the top of the packing. The chloroform extract was transferred to the column with 4 ml of 1:1 hexane–methylene chloride and the CIPC eluted with 75 ml of 1:1 hexane–methylene chloride. The eluent was evaporated in the presence of water in a three-necked 1-l. flask and treated as a crop sample from that point. With the CIPC present in the sample determined by this procedure and the total CIPC residue (CIPC + III) (Ercegovich and Witkonton, 1972), metabolite III was obtained by difference.

The polar extract from the Bligh-Dyer extraction contains the soluble conjugates of metabolites I, II, and III present. The presence of conjugates of III was checked by the Bleidner hydrolysis–distillation of the polar phase extract, concentration of the distillate, and analysis of the 3-chloroaniline present by GLC. The GLC procedure for the separation of 3-chloroaniline from other anilines was taken from Bombaugh (1965). The column used was 1.8 m × 0.5 cm o.d. packed with Siponate DS-10 on acid-washed Chromosorb W precoated with 2% NaOH. The carrier gas was nitrogen at 70 ml/min. The initial program temperature was 142 °C to a final temperature of 210 °C at 2 °C/min with an inlet temperature of 200 °C.

For the isolation of III, the XAD-2 purified polar extract was buffered at pH 4.8 with 0.1 M sodium acetate buffer, and 100 mg of cellulase (Calbiochem) was added. The sample was kept at 37 °C for 24 h and then extracted 3 times with equal volumes of diethyl ether. The ether phase was dried with sodium sulfate and concentrated by rotary evaporation. This solution was used to prepare the acetate derivatives of the metabolites by refluxing in acetic anhydride for 2–3 h, dilution with water, and neutralization with sodium bicarbonate. The resulting acetates were extracted with ether and concentrated by rotary evaporation. The ether phase containing the acetate derivatives was purified on a 4.5 mm × 183 cm Bondapak/C_{18}-Porasil HPLC column. Flow rate was 3.5 ml/min at 2200 psi. A uv detector was used with gradient elution from 1:1 H_2O–acetonitrile to 100% acetonitrile. This gradient provided maximum cleanup in minimum time as indicated by Still and Mansager (1975). The HPLC extracts containing the acetate of III were composited for further purification on GLC. The gas chromatograph used for metabolite purification and identification was equipped with a glass inlet and a 152 cm × 6.35 mm glass column

of 3% OV-1 on Gas-Chrom Q, 60–80 mesh. Nitrogen carrier gas was used with a flow rate of 60 ml/min. The column inlet temperature was 200 °C and the column oven temperature was programmed from 125 to 155 °C at a 1.0 °C/min rate, with no hold and 4 min to cool. The column effluent was split with a 10:1 stream splitter with the smallest portion passed to a flame detector. The components trapped from the GLC column were either assayed for radiocarbon by liquid scintillation counting or analyzed in the mass spectrometer with the use of a solid sample probe. The mass spectra were obtained on a Varian/MAT CH-5 DF.

RESULTS AND DISCUSSION

The first indication of the presence of a third CIPC metabolite occurred when 3-chloroaniline was found in the hexane extract from the Bleidner hydrolysis of the Bligh-Dyer polar phase and insoluble residue. CIPC itself was quantitatively extracted into the Bligh-Dyer nonpolar phase. Metabolites I and II were hydrolyzed to chloroaminophenols by the high alkalinity of the Bleidner hydrolysis and consequently were not distilled; thus, the presence of a new metabolite, hydroxylated and conjugated through the alkyl moiety of the molecule, was indicated. On the basis of this observation, the logical choice, compound III, was synthesized and efforts were made to confirm its presence in soybean plants.

III from Hydroponically Grown Plants. After 16 days of root exposure to [^{14}C]CIPC, soybean shoots were processed using the modified Bligh-Dyer extraction procedure. The radiocarbon was partitioned into the Bligh-Dyer nonpolar fraction (10%), the polar fraction (79%), and the insoluble fraction (11%). Autoradiography of a thin-layer chromatographic plate of the nonpolar fraction contained only one significant ^{14}C spot which cochromatographed with CIPC.

After hydrolysis with HCl, the polar fraction was extracted with chloroform and 84% of the ^{14}C partitioned into the chloroform layer. Two-dimensional thin-layer chromatography and autoradiography of this chloroform extract yielded three radioactive spots which were superimposable with the nonradioactive standards I, II, and III. Relative amounts of each metabolite on the plate as determined by scraping and liquid scintillation counting were 72, 9, and 18%, respectively. Table I gives the R_f values for the metabolites.

The area on a replicate TLC plate corresponding to III but containing no synthetic III was scraped, eluted, and passed through the high performance liquid chromatograph. The HPLC fraction corresponding to the retention time of synthetic III had 99% of ^{14}C. The heptafluorobutyrate derivative of synthetic III also had the same GLC retention time as the derivatized HPLC fraction.

Another replicate TLC plate with a spot corresponding to III was scraped and eluted and then derivatized with acetic anhydride. The reaction mixture, along with synthetic III and the synthetic acetate of III, was spotted on a TLC plate for two-dimensional development. Fluorescence quenching showed two spots, one for III and one for the acetate of III. An autoradiogram showed radioactivity only in the spot corresponding to the acetate of III.

The areas corresponding to I, II, and III were scraped from TLC plates and used for alkaline hydrolysis and steam distillation. From radioassay it was found that the hydrolysis products of I and II were not steam distillable whereas the majority of the radioactivity of III steam distilled. This volatile ^{14}C could not be extracted from acid solution, but was extractable from basic solution as would

be expected for 3-chloroaniline (Table II).

III from Soil Grown Soybean Shoots. The soil grown plants treated with CIPC were produced in a sufficient quantity to isolate the new metabolite. Total CIPC residue (CIPC + III) in the plants was shown to be 0.54 ppm. CIPC was separated from III by Bligh-Dyer extraction, followed by silica gel chromatography of the nonpolar phase, and shown to be at a concentration of 0.03 ppm. Consequently 94% of the apparent CIPC in the shoots was metabolized to III.

Bligh-Dyer extraction of 4.6 kg of soil grown soybeans had a radiolabel distribution of 21% in the nonpolar phase, 50.5% in the polar phase, and 28.5% remaining as insoluble residue. An aliquot of this Bligh-Dyer polar extract was used for alkaline hydrolysis and steam distillation in a Bleidner apparatus. Of the radiolabel present, 73% distilled into the hexane phase. The hexane extract was concentrated by rotary evaporation for GLC. The GLC elution peak corresponding to 3-chloroaniline was trapped and found to contain 80% of the ^{14}C present in the extract.

The remainder of the polar phase was passed through XAD-2 resin. The average recovery of ^{14}C was 85.2% in the methanol eluent and about 6% was found in the water wash. A 4-h digestion of one-half of the polar extract with 40 mg of cellulase plus 50 mg of hesperidinase hydrolyzed only 9% of the ^{14}C-containing compounds. These conditions would be expected to hydrolyze most of the conjugates of I and II present. Continued hydrolysis for 20 h with an additional 40 mg of cellulase provided 52% more ether-soluble ^{14}C. The other half of the XAD-2 purified polar phase was hydrolyzed for 24 h with 100 mg of cellulase converting 85% of the ^{14}C to ether-soluble material. Thus, hydrolysis of conjugates of III was observed to be more difficult than the hydrolysis of the aryl-oxidized conjugates, I and II.

The acetylation of the hydrolyzed III gave very low yields when acetonitrile was used as solvent. When neat acetic anhydride was used, a yield of 70% was obtained. In general the acetylation of III appeared to be more difficult than the acetylation of I or II.

Separation of the acetylated derivative of III by HPLC is shown in Figure 2. Fraction 1 was the elution of the acetates of I and II (11.6% of the ^{14}C), fraction 2 the acetate of III (72.3%), and fraction 3 (16%) would contain some tailing of the acetate of III. Free metabolites are eluted prior to fraction 1 with this system. Figure 3 shows the GLC tracing of fraction 2. The peak corresponding to the acetate of III was trapped for mass spectral analysis.

Mass spectral evaluation of the acetate of III provided fragment ions at m/e 271, 229, 153, 127, and 101. The molecular ion for the acetate of III has a calculated m/e of 271, loss of the acetate gives m/e of 229, and splitting of the molecule into aromatic, aliphatic, and carbon dioxide portions yields a 3-chloroaniline fragment m/e 127 and an ester $CH(CH_3)CH_2OC(=O)CH_3$ of m/e 101. The m/e 153 peak corresponds to:

$$\left[\text{Cl} \overset{}{\underset{\text{O}}{\bigcirc}} N=C=O \right]^{-}$$
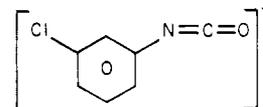
The data presented in Table II for hydroponically grown plants indicate that only 19% of the identified metabolites were alkyl oxidized while 81% were aryl oxidized. This may be compared to the soil-grown plants where 73–88% of the identified metabolites was the alkyl oxidized metabolite III. Metabolite III has been found in both hydroponically and soil-grown soybean shoots. However, it clearly is present as a much larger percentage of the total
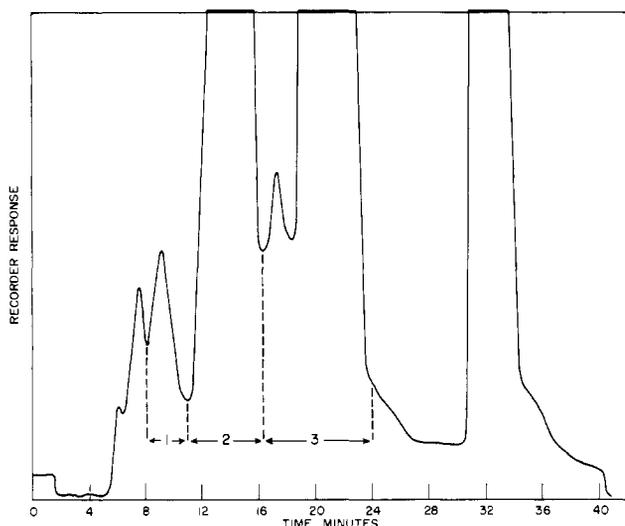
Figure 2. High-performance liquid chromatographic separation and purification of the acetate of metabolite III. Fraction 2 was collected for further purification by GLC.
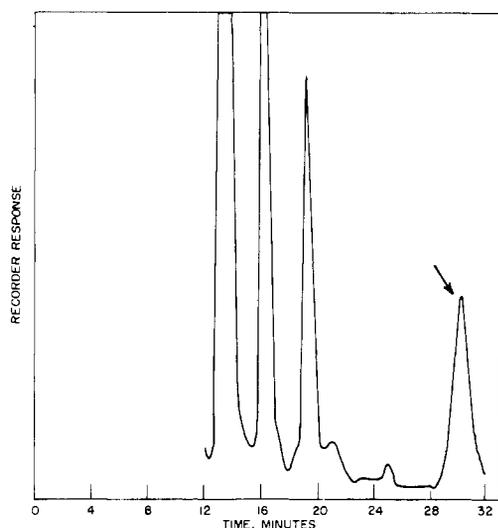
Figure 3. Gas–liquid chromatograph of fraction 2 from HPLC. Arrow indicates the peak corresponding to the acetate of metabolite III. This peak was trapped for mass spectral analysis.

metabolites in soil grown plants. In several hydroponic studies, Still and Mansager (1971, 1972, 1973) have found less than 5% of III. However, PPG Industries personnel (unpublished) have found III to be the predominant metabolite in field-grown soybean shoots. The reason for the preference of aryl hydroxylation in the hydroponic studies and alkyl hydroxylation in the soil studies is not known.

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