Isolation and Identification of Some Phytotoxic Compounds from Aqueous Extracts of Rye (*Secale Cereale* L.)

Donn G. Shilling,*[1] Louis A. Jones,* A. Douglas Worsham, Carol E. Parker, and Richard F. Wilson

Research has shown that certain weed species are suppressed by rye (*Secale cereale* L.) used as a mulch or cover crop in no-tillage cropping systems. To determine whether this suppression was caused by phytotoxic compounds, an aqueous extract of rye was partioned with diethyl ether–ethyl acetate, the organic phase was separated by preparative TLC, and biologically active bands were removed, extracted, and further separated by reversed-phase high-performance liquid chromatography. The collected fractions were evaporated and silyl and deuterated silyl derivatives prepared and analyzed by gas chromatography/mass spectrometry. Identified were two compounds not previously implicated in allelopathy, β-phenyllactic acid (β-PLA) and β-hydroxybutyric acid (β-HBA), noninhibitory succinic acid, and three other unidentified acids. Biological testing indicated that β-PLA was more inhibitory than β-HBA to hypocotyl and root growth of *Chenopodium album* and *Amaranthus retroflexus*. The isomeric α-PLA proved to be 17 times more active than β-PLA to hypocotyl growth to *A. retroflexus*.

Growth interference between plants can be caused by competitive factors (light, nutrients, water) and/or allelopathic chemicals, although differentiating between the two is difficult (Putnam and Duke, 1978). Allelopathic interactions from plant residues left on the soil in conjunction with no-till or minimum-till agriculture have been proposed to account for reduced crop yields (Guenzi and McCalla, 1966a, 1966b; Guenzi et al., 1967; Cochran et al., 1977). Leaving plant residues on the soil surface can result in the leaching of allelopathic compounds, thus exposing subsequent crops to potentially phytotoxic compounds in the soil. For example, various phenolic acids, extracted from stubble-mulch soils (Guenzi and McCalla, 1966a), were found to be phytotoxic to wheat seedlings and to crop growth (Patterson, 1981; Shettel and Balke, 1983). The extracts and residues of Johnson grass (*Sorghum halepense*) rhizomes have been shown to cause a decrease in soybean growth (Lolas and Coble, 1982), and Nicollier et al. (1983) isolated and identified two phytotoxic cyanogenic compounds from this source.

Putnam and Duke (1974) and Leather (1983) demonstrated that suppression of weed growth by crops could be enhanced by crop selection. Selected small-grain mulches have also been shown to suppress certain weed species while providing the growing crop with all the benefits of a no-till system (Overland, 1966; Barnes and Putnam, 1983; Liebl and Worsham, 1983; Shilling and Worsham, 1985). Liebl and Worsham (1983) reported the reduction of the biomass of pitted morning glory (*Ipomoea lacunosa* L.) in no-till corn (*Zea Mays* L.) by a cover crop of wheat (*Triticum aestivum* L.).

Several research groups have attempted the separation and identification of those compounds responsible for phytotoxic activity. Guenzi and McCalla (1966a) extracted plowed and subtilled soil with acetone and chromatographed the concentrate on a deactivated alumina column using various organic mobile phases. Identified by R_f and two-dimensional paper chromatography were vanillic, *p*-hydroxybenzoic, and protocatechuic acids. These acids, in addition to ferulic, *p*-coumaric, and syringic, were also found in the basic extract of the same soil. Chou and Patrick (1976), using paper chromatography, thin-layer chromatography (TLC), and gas chromatography (GC) identified 18 substituted aromatic acids from the aqueous extracts of decomposing corn residues and nine similar compounds from the aqueous extracts of decomposing rye residues. Benzoic, phenylacetic, 4-phenylbutyric, salicyclic, and *p*-hydroxybenzoic acids, common to both, were inhibitory to the growth of lettuce at concentrations of 25–50 ppm. Fay and Duke (1977) used paper chromatography and TLC to demonstrate that certain oat accessions exude the phytotoxic compound scopoletin (7-hydroxy-6-methoxycoumarin). Nicollier and Thompson (1982) chromatographed the aqueous extract of sweet clover and identified by TLC, GC, ^{1}H NMR, ^{13}C NMR, and mass spectrometry (MS) two new flavanoids as well as coumarin derivatives that inhibited tomato and radish seedling growth. Liebl and Worsham (1983) separated and identified by TLC the base-extracted phytotoxic substance 4-hydroxy-3-methoxycinnamic acid (ferulic acid) from wheat that inhibited seed germination and growth of pitted morning glory. The compound was found to decarboxylate in the presence of prickly sida seed carpels to 2-methoxy-4-ethenylphenol. Rye residues have been reported to reduce early-season biomass of common lambsquarters (*Chenopodium album* L.) by 80% and common ragweed (*Ambrosia artemisiifolia* L.) by 90% (Barnes and Putnam, 1983), confirming the results of Shilling and Worsham (1983, 1985).

The demonstrated phytotoxicity of rye residues prompted a study to identify possible allelopathic compounds present in aqueous extract of rye, and this report presents the preliminary results of that study.

MATERIALS AND METHODS

Extraction Procedure. Field-grown rye (Abruzzi) harvested at early-flowering stage on March 24, 1983, from the Central Crops Research Station (Clayton, NC) was harvested and air-dried for 7 days. The tissue (150 g) was cut into 7–8-cm sections, extracted with 3 L of distilled water for 10 h with agitation, filtered through cheesecloth, and then centrifuged at 28000*g* for 20 min. The supernatant was concentrated to 300 mL under reduced pressure at 50 °C.

An aliquot of the concentrate (240 mL) was adjusted to pH 2.5 with 2 N HCl and partitioned three times with 240 mL each of diethyl ether and ethyl acetate. The organic phases were combined and dried over $MgSO_4$. Following filtration, the filtrate was taken to dryness under reduced

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Table I. Tabulation of Important Ions Present in Mass Spectra, GC Retention Times (t_r), Molecular Weights (MW) of Unknown and Standard Compounds, and Number of Trimethylsilyl (Me_3Si) Groups per Molecule

			EI								
			M^+		$M^+ - 15$		CI (Me_3Si)				no. of Me_3Si
peak[a]	chemical	t_r[b]	Me_3Si	Me_3Si-*d*	Me_3Si	Me_3Si-*d*	M + 1	M + 29	M + 41	MW	gps
1	β-hydroxybutyric acid	16.1[c]	*d*	*d*	233	248	249	277	289	104	2
2	succinic acid	19.1[c]	262	280	247	262	263	291	303	118	2
3	unknown	22.1	216	225	201	207	217	245	257	144	1
4	β-phenyllactic acid	24.3[c]	*d*	*d*	295	310	311	339	351	166	2
5	unknown[e]	26.1	*d*	*d*	303	318	319	347	359	174	2
6	unknown[e]	27.3	327	345	312	327	328	356	368	183	2

[a] Refers to peaks shown in Figure 3. [b] Obtained on a Hewlett-Packard GC/MS 5985B using a 30 m × 0.25 mm i.d. fused silica column coated with DB-5, helium flow approximately 1 mL/min. See experimental procedures for details. [c] Compared to corresponding standards. [d] Not present in mass spectra. [e] See text for discussion.

pressure and the solid remaining reconstituted with 80 mL of diethyl ether–ethyl acetate (1:1).

The components in the organic phase were separated first by thin-layer chromatography (TLC). The organic phase (80 mL) was streaked onto 20 plates of 1000-μm silica GF (Analtech, Inc., Newark, DE) and developed with toluene–ethyl formate–formic acid (5:4:1) (Van Sumere, 1965). Compounds, located with ultraviolet light (UV) at 254 nm, appeared as dark bands against a fluorescent background. These were scraped and extracted from the silica gel with ethyl acetate–methanol (2:1). Following filtration, the filtrate was dried in vacuo and redisolved in 80 mL of methanol.

Preparatory to high-performance liquid chromatography (HPLC), 30 mL of the methanol solution, containing biologically active compounds (R_f 0.4–0.52), was taken to dryness in vacuo. The residue was then dissolved in 15 mL of 35% aqueous methanol, filtered (0.4-μm Millipore filter), and analyzed on a Waters Associates HPLC (Milford, MA; Model 6000A pumping system, Model U6K injector, Model 480 Lambda-max LC spectrophotometer) equipped with a μBondapak C_{18} column (7.8 mm × 30 cm). The solvent (CH_3OH–H_2O, 35:65) was delivered isocratically at a flow rate of 1.5 mL/min and the column effluent monitored at 350 nm (0.5 aufs for 6 min, 0.1 aufs for 6–30 min). Effluent was collected every 2 min with a total run time of 30 min. Each of the 15 fractions was taken to dryness and the residue dissolved in 15 mL of methanol and stored at 0 °C.

Ion-exchange chromatography of the biologically active TLC fraction using Dowex-1 (Sigma Chemical Co., St. Louis, MO) produced an effluent that was biologically inactive, indicating that phytotoxic compounds were acidic.

An extract blank (i.e., distilled water only) was similarly extracted to ensure that all identified compounds were of biological origin.

Qualitative Analysis. Extract samples (200 μL) that had been purified by TLC (R_f 0.4–0.52) and by HPLC (7–11 min) were dried at 40 °C under N_2 gas. Trimethylsilyl (Me_3Si) derivatives of these samples were formed by adding 25 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Pierce Chemical Co., Rockford, IL) and 25 μL of pyridine to a 1-mL vial and heating to 70 °C for 30 min (Pierce, 1983). Deuterated trimethylsilyl derivatives (Me_3Si-*d*) of these same samples were prepared by reaction with Deutero-regisil (Regis Chemical Co., Morton Grove, IL). Using the aforementioned derivatives, 1–4-μL samples were injected (splitless) into a 30-m (0.35-mm i.d.) DB-5 fused silica capillary column [95% dimethyl–5% diphenylpolysiloxane; J & W Scientific, Inc., Rancho Cordova, CA]. A Hewlett-Packard GC/MS 5985B was used to obtain retention times (t_r), and electron impact (EI) and methane positive chemical ionization (CI) mass spectra were taken of the derivatized samples and standard compounds. Helium was used as the carrier gas (ca. 1 mL/min) and methane as the makeup gas for CI runs. Chromatographic conditions were as follows: injection port temperature, 250 °C; detector temperature, 270 °C; column temperature, 50 °C hold for 2 min and then programmed to 280 °C at 8°/min. All mass spectra were obtained with an ionizing voltage of 70 eV. Gas chromatography using a Varian GC 3700 (Varian Associates, Sunnyvale, CA) was used to corroborate identifications by coinjection of known samples and their Me_3Si derivatives. The results are summarized in Table I, and a flow diagram summarizing the isolation procedures is shown in Figure 1.

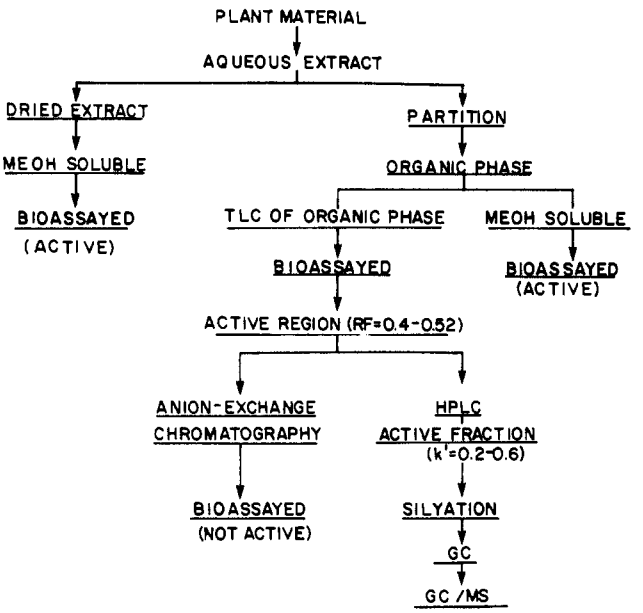


Figure 1. Flow diagram of isolation and identification techniques.

Bioassays. Biological activity of various isolated compounds was determined with *C. album* L. seed collected in North Carolina in 1981. Each sample (1.4 mL of methanolic sample) was placed into a 3-cm Petri dish and the solvent evaporated under a laminar flow hood at room temperature. Seventy seeds (0.035 g) were then placed in the Petri dishes to which was added 1.4 mL of filtered (0.2-μm Millipore filter) sterilized 15 mM MES buffer (2-morpholinoethanesulfonic acid; Sigma Chemical Co.) adjusted to pH 5.5. The dishes were kept in the dark at 25 °C for 84 h, exposed to 12-h fluorescent light (250 μeinsteins/m^2 per s), and then placed back in the dark for an additional 4 days (Karseen, 1970). Percent germination and root and hypocotyl lengths were then determined.

The following identified chemicals were obtained commercially and bioassayed with *C. album* and *Amaranthus retroflexus* L. (seeds collected in North Carolina in 1980): succinic acid (butanedioic acid, Sigma Chemical Co.),

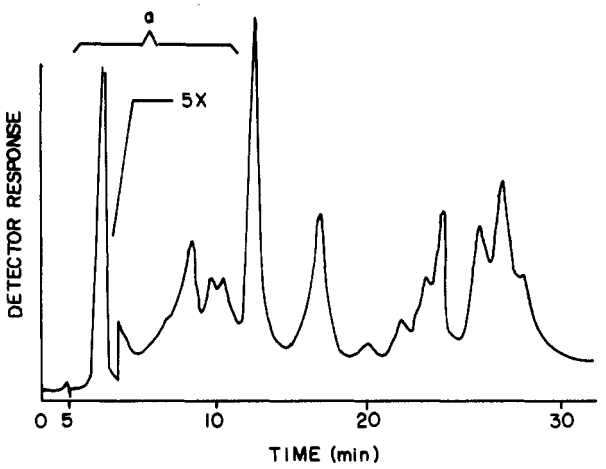


Figure 2. HPLC chromatogram of TLC region containing biologically active compounds indicated by region "a". See experimental procedures for details.

DL-β-hydroxybutyric acid (DL-3-hydroxybutyric acid, sodium salt; Sigma Chemical Co.; β-HBA), and L-β-phenyllactic acid (L-2-hydroxy-3-phenylpropanoic acid; Sigma Chemical Co.). *A. retroflexus* was also used to compare the relative activity of α- and β-phenyllactic acid (α-PLA, β-PLA), DL-2-hydroxy-2-phenylpropanoic acid (Pfaltz and Bauer, Inc., Stamford, CT), and tropic acid (DL-α-phenylhydracrylic acid; Sigma Chemical Co.). These bioassays were accomplished in a manner similar to that used for *C. album* except that 50 seeds (0.025 g) were germinated in the dark at 25 °C for only 60 h.

Statistical Analysis. Data were examined by analysis of variance and regression analysis procedures using the general linear model procedure of Statistical Analysis System (SAS; Helwig and Council, 1979). Polynomial equations were best fitted to the data based on the significance level of the terms of the equations and R^2 value. The I_{50} predicted values [concentration (mM) required to cause 50% inhibition] were determined from the best fitted equations.

RESULTS AND DISCUSSION

The phytotoxic compounds of the aqueous rye extract were effectively partitioned into the ethyl ether–ethyl acetate phases as indicated by the level of inhibition of *C. album* hypocotyl length (96%), root length (93%), and germination (89%). Preliminary HPLC studies suggested that more than one compound was present in the TLC bands evaluated for biological activity. To assure that the biologically active compounds were collected, a relatively wide region was removed from the TLC plates (R_f 0.4–0.52).

Optimization of the HPLC separation of the bioassayed active fraction from TLC was realized with a C_{18} μBondapak column (Waters Associates, Milford, MA) and a mobile phase of CH_3OH–H_2O (35:65). Analysis of the active TLC fraction showed a minimum of 17 compounds, with k' values varying from approximately 0.20 to 5.0 (Figure 1). Bioassay of the collected peaks (collected every 2 min over 30 min) indicated that those compounds showing phytoxicity had k' values between 0.20 and 0.60 that included a minimum of five compounds (Figure 2). Repetitive injections and collections (15 fractions) gave sufficient sample for continued analysis.

Exploratory ion-exchange chromatography had previously indicated that the active components of the aqueous extracts were acidic since the eluent from the anionic exchange column proved to be noninhibitory when bioassayed.

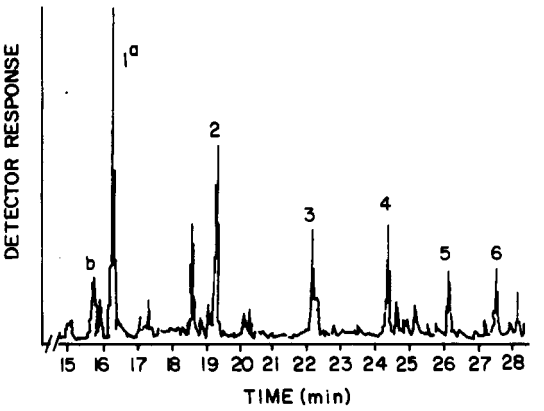


Figure 3. Reconstructed ion chromatogram obtained from the GC/MS of the Me_3Si derivatives of the active fraction separated and collected by HPLC. See experimental procedures for details. (a) Numbers refer to the compounds in Table I. (b) Peaks not numbered were present in the extract blank.

The collected HPLC and TLC (vide supra) fractions showing bioactivity were then analyzed by GC/MS after conversion to their trimethylsilyl (Me_3Si) derivatives using BSTFA (see experimental details). This technique permitted the GC separation of the mixture without the excessive tailing characteristics of underivatized acids (Pierce, 1983).

The GC/MS spectra data [electron impact (EI) and methane positive chemical ionization (CI)] of the Me_3Si and deuterated Me_3Si (Me_3Si-*d*) ether/ester derivatives, when compared with known compound derivative GC and GC/MS data, provided identification of three of the six peaks observed in the GC (Figure 3). They were β-hydroxybutyric acid (β-HBA; peak 1), succinic acid (peak 2), and β-phenyllactic acid (β-PLA; peak 4). The EI M^+ and $(M - CH_3)^+$ masses for the Me_3Si and Me_3Si-*d* derivatives and the CI masses of $(M + 1)^+$, $(M + 29)^+$, and $(M + 41)^+$ of these compounds are shown in Table I. The mass spectra of most silylated compounds yield weak or nonexistent molecular ions. However, silylated hydroxy esters have been reported to show masses of m/z 73 for $(CH_3)_3Si^+$, m/z 75 for $(CH_3)_2Si{=}OH^+$, and m/z 147 for $(CH_3)_2Si{=}O^+Si(CH_3)_3$ (Richter et al., 1967).

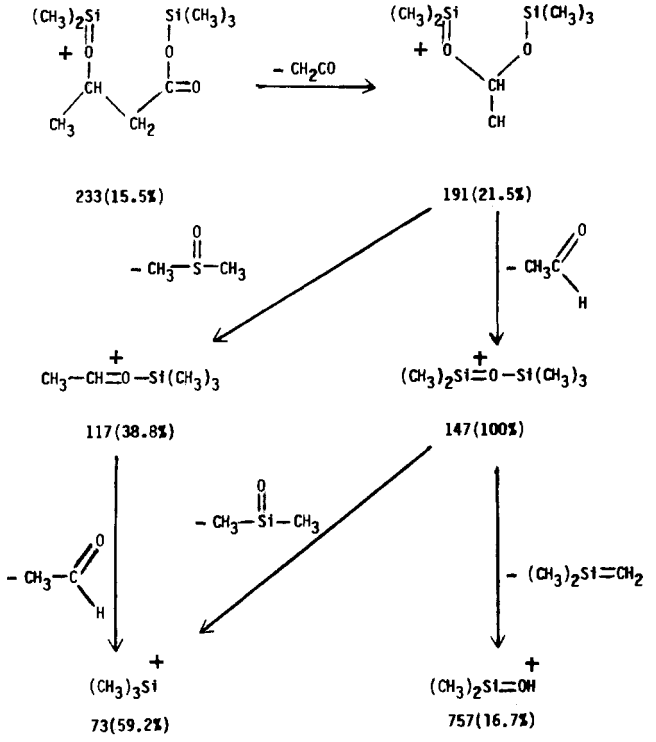
For the Me_3Si ether/ester of β-HBA, no molecular ion was observed but a moderate m/z 233 (15.5%), representing $(M - CH_3)^+$, was present (Table I). Suggesting that at least two $OSi(CH_3)_3$ moieties were in the derivative was the base peak m/z 147 (100%), $(CH_3)_2Si{=}O^+Si(CH_3)_3$. Since the EI MS of the Me_3Si-*d* ether/ester derivative gave an $(M - CH_3)^+$ of m/z 248, 15 mass units higher than the nondeuterated Me_3Si derivative, and since the CI spectrum showed the characteristic $(M + 1)^+$, $(M + 29)^+$, and $(M + 41)^+$ peaks (Budzikiewicz et al., 1967), the molecular weight of 248 was confirmed.

Of particular diagnostic value was the m/z 117 (38.8%) fragment that was assigned the structure $CH_3CH{=}^+OSi(CH_3)_3$ on the basis of the proposed fragmentation pathway in Scheme I. The general carbon skeleton $CH_3CH(OH)CH_2R$ where R was the carboxyl Me_3Si group is consistent with the proposed fragments.

The GC retention time and MS data of an authentic Me_3Si derivative of β-HBA were identical to those of the suspect compound, thus confirming the identification.

The other hydroxy acid identified was β-phenyllactic acid (β-PLA; peak 4, Table I) whose EI MS produced m/z 295 (7.5%), presumed to be the $(M - CH_3)^+$ ion. The Me_3Si-*d* derivative gave an m/z 310 peak for a difference of 15. As before, two $OSi(CH_3)_3$ groups were suggested by the fragment m/z 147 (60.8%), $(CH_3)_3SiO^+{=}Si(CH_3)_2$.

Scheme I. Fragmentation Pattern of β-Hydroxybutyric Acid, Bis(trimethylsilyl) Derivative



This was subsequently confirmed by CI MS. The $(M - CH_3)^+$ fragment, followed by successive losses of CO and $(CH_3)_2Si{=}O$, resulted in the intense m/z 193 (85.1%). This was assigned the structure $C_6H_5CH_2CH{=}O^+Si(CH_3)_3$ and is completely analogous to the β-HBA fragment of $CH_3CH{=}O^+Si(CH_3)_3$, m/z 117. This suggested a carbon skeleton of $C_6H_5CH_2CH(OH)R$ where R was the carboxyl Me_3Si group for β-PLA. The pathway in Scheme II accounts for the major fragment ions observed.

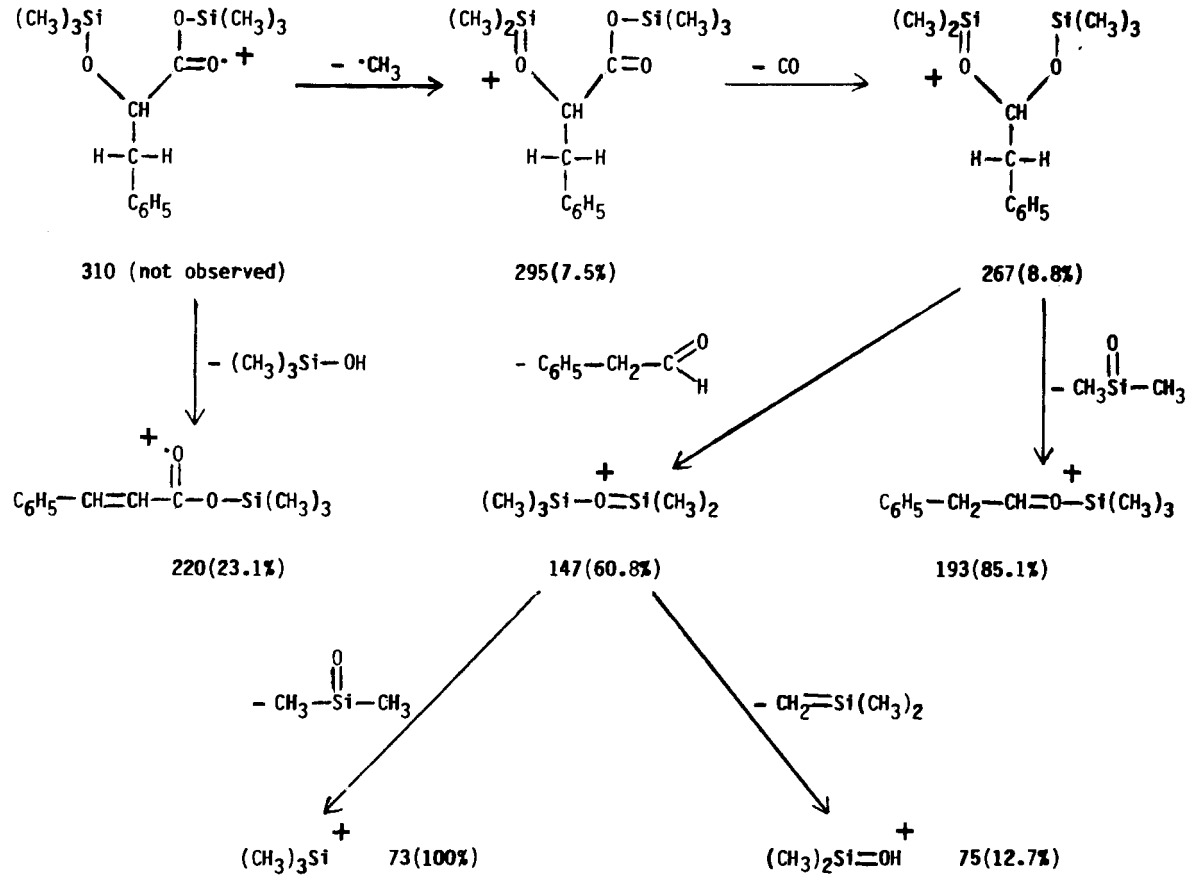
The $(Me_3Si)_2$ derivative of succinic acid was identified and confirmed similarly. Interestingly, only a very small molecular ion, m/z 262 (0.9%), was observed, the $(M - CH_3)^+$ fragment of m/z 247 (14.7%) being the more intense. The base peak of m/z 147 (100%) was assigned the structure $(CH_3)_2Si{=}O^+Si(CH_3)_2$ and again indicated the presence of two $OSi(CH_3)_3$ groups. The molecular weight of 262 was confirmed by CI mass spectrometry and the Me_3Si-*d* derivative.

The fragmentation pathway for succinic acid, bis(trimethylsilyl) derivative, is envisioned in Scheme III.

In all the spectra examined, those fragments identified as containing one or two silicon atom(s) exhibited the "A + 2" isotope cluster. For one silicon atom, the ratio is 100:5.1:3.4, and for two atoms, the ratio becomes 100:10:7.1:0.4 (McLafferty, 1980). Thus, for the m/z 145 fragment from β-PLA-Me_3Si, $(CH_3)_3SiO^+{=}Si(CH_3)_2$, the ratio was m/z 147 (60.8%), m/z 149 (9.8%), m/z 149 (5.1%), and m/z 150 (0.3%). For that fragment from β-HBA-Me_3Si, the ratio was m/z 147 (100%), m/z 148 (15.6%), m/z 149 (8.2%), and m/z 150 (1.0%). Indeed the $(M - CH_3)^+$ ion for this derivative showed the same ratio progression: m/z 233 (15.5%), m/z 234 (2.9%), m/z 235 (1.4%), and m/z 236(0.3%). Of interest was the fact that there was no fragment observed between m/z 147 and m/z 235 for β-HBA-$(Me_3)_2$.

As shown in Table I, three peaks were not identified. Peak 3 was the only one of the six identified as having a single $RC(=O)OSi(CH_3)_3$ function. Peak 5 showed two Me_3Si groups. Peak 6, however, gave two suspected M^+ of m/z 327 (11.6%), m/z 328 (2.3%), and m/z 329 (0.9%).

Scheme II. Fragmentation Pattern of β-Phenyllactic Acid, Bis(trimethylsilyl) Derivative



Scheme III. Fragmentation Pattern of Succinic Acid, Bis(trimethylsilyl) Derivative

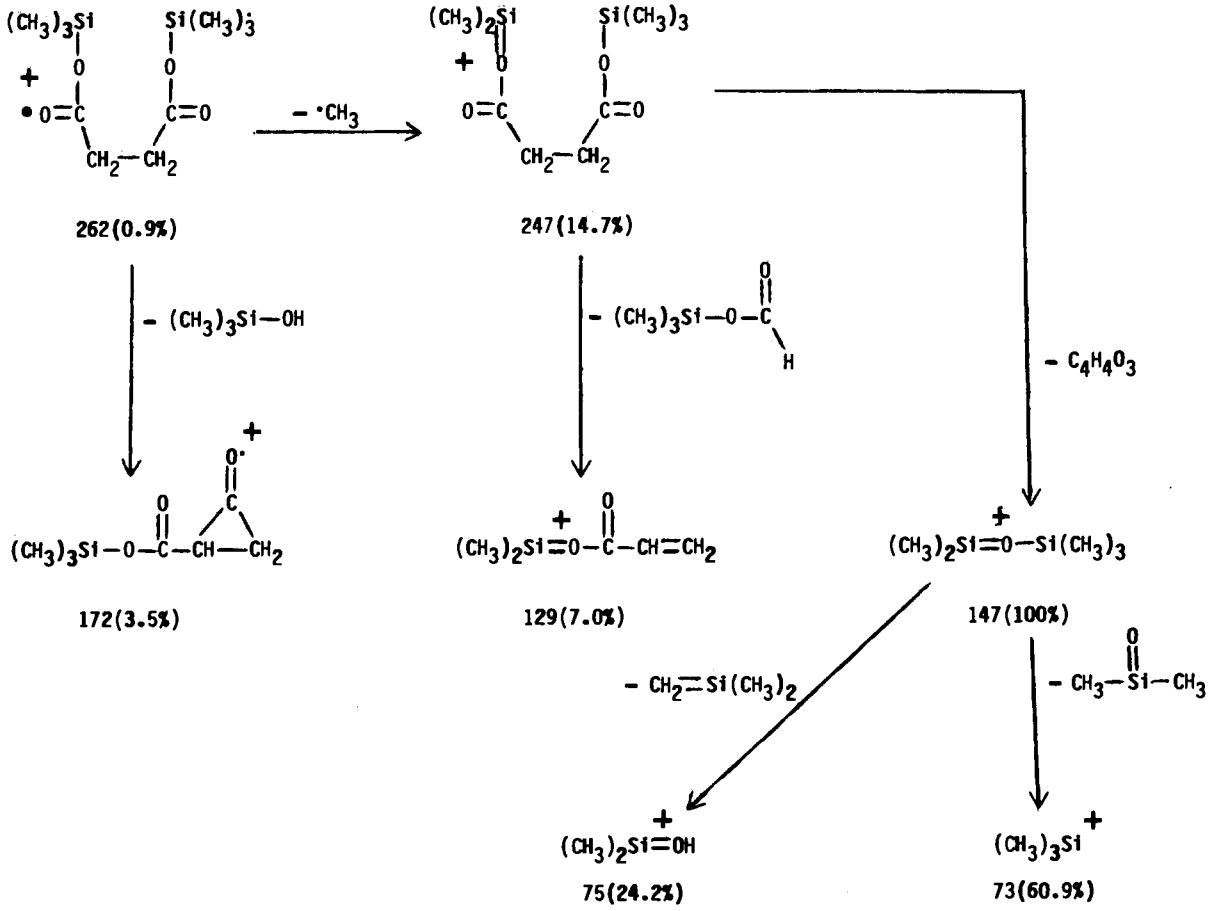


Table II. Concentration of DL-β-Hydroxybutyric Acid (β-HBA), L-β-Phenyllactic Acid (β-PLA), DL-α-Phenyllactic Acid (α-PLA), and DL-α-Phenylhydracrylic Acid (Tropic Acid) Required To Cause 50% Inhibition of Growth (I_{50})

	C. album		*A. retroflexus*			
growth parameter	β-HBA	β-PLA	β-HBA	β-PLA	α-PLA	tropic acid
hypocotyl length (HL)	12.1[a]	6.09[b]	10.72[e]	2.71[f]	0.16[i]	5.44[j]
root length (RL)	6.73[c]	4.79[d]	7.41[g]	1.54[h]	2.82[k]	5.59[l]

[a] HL = 3.15–0.13 (β-HBA), R^2 = 0.84. [b] HL = 2.80–0.23 (β-PLA), R^2 = 0.85. [c] RL = 2.02–0.15 (β-HBA), R^2 = 0.51. [d] RL = 2.30–0.24 (β-PLA), R^2 = 0.95. [e] HL = 1.93–0.09 (β-HBA), R^2 = 0.65. [f] HL = 1.82–0.39 (β-PLA) + (β-PLA)², R^2 = 0.92. [g] RL = 1.63–0.11 (β-HBA), R^2 = 0.67. [h] RL = 2.10–0.85 (β-PLA) + 0.11 (β-PLA)², R^2 = 0.90. [i] HL = 1.97–8.28 (α-PLA) + 13.12 (β-PLA)², R^2 = 0.98. [j] RL = 1.84–0.41 (α-PLA) + 0.03 (α-PLA)², R^2 = 0.88. [k] HL = 1.85–0.17 (tropic acid), R^2 = 0.86. [l] RL = 1.90–0.17 (tropic acid), R^2 = 0.72.

Two typical silicon "A + 2" clusters at M − 15⁺ [*m/z* 312 (100%); *m/z* 313 (21.7%); *m/z* 314 (11.6%), *m/z* 315 (2.6%)] were observed. With a suspected loss of *m/z* 74, $(CH_3)_2Si{=}O$, *another* two-silicon "A + 2" cluster was observed [*m/z* 237 (56.2%); *m/z* 238 (14.2%); *m/z* 239 (3.5%); *m/z* 240 (2.2%)]. The odd molecular weight suggests that peak 6 is either an amino acid with three Me_3Si moieties or is a mixture of two compounds, one of which is an amino acid.

Having identified β-PLA, β-HBA, and succinic acid, their biological activity was examined using *C. album* and *A. retroflexus*. The predicted I_{50} values (the concentration (mM) required to cause 50% inhibition) were derived from the best fitted polynomial equations (Table II). Included are the results obtained with the isomeric α-PLA acid and DL-α-phenylhydracrylic acid or tropic acid.

As shown in Table II, both β-PLA and β-HBA inhibited hypocotyl growth of *C. album*, the inhibitory action of β-PLA being twice that of β-HBA. For root growth, the I_{50} was lower for both compounds and little difference was observed between the two. The effect of β-PLA upon hypocotyl growth inhibition of *A. retroflexus* was 4 times that of β-HBA and approximately 5 times more inhibitory to root length. The isomer α-phenyllactic acid (α-PLA) and the isomeric tropic acid (3-hydroxy-2-phenylpropanoic acid) were also evaluated for phytotoxicity on *A. retroflexus*. Hypocotyl growth was 17 times more sensitive to α-PLA than the β-isomer. However, root growth was more sensitive to β-PLA than to α-PLA, and tropic acid was less active than both. Succinic acid was shown to be nonphytotoxic (data not shown).

It should be noted that these differences in species sensitivity could be attributed to the duration of the experiments (8 days for *C. album*, 60 h for *A. retroflexus*), the longer time allowing microbial degradation to produce lower concentrations and/or metabolism by the plant. No definitive conclusions can be drawn as to the applicability of these data to explaining possible allelopathic associations under field conditions since phytotoxicity was determined only under laboratory conditions. However, both β-PLA and β-HBA are easily extracted with water and could thus be leached from rye mulch under field conditions to inhibit weed growth. The data also suggest that subtle differences in structure result in differences in activity as well as species and tissue specificity. The increase in activity caused by structural changes also (α vs. β) indicates that evaluating natural products as new herbicidal chemistry has potential.

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Registry No. β-HBA, 300-85-6; β-PLA, 156-05-8; succinic acid, 110-15-6.

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Qualitative and Quantitative Changes in the Protein Composition of Peanut (*Arachis hypogaea* L.) Seed following Infestation with *Aspergillus spp.* Differing in Aflatoxin Production

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Peanut (*Arachis hypogaea* L.) seed CV. Early Bunch was inoculated with four different *Aspergillus* lines (NRRL 2999, NRRL 502, NRRL 3357, NRRL 3239) differing in their aflatoxin production capacities. Analyses of the seeds showed that *Aspergillus spp.* infestation caused a decrease in oil, iodine value, soluble carbohydrates, and protein content. Gel filtration studies indicated changes in the seed protein composition while one-dimensional gel electrophoresis revealed that following fungal infestation arachin became more acidic accompanied by the appearance of a basic protein. Two-dimensional gel electrophoresis showed gradual disappearance of a high molecular weight (70 000) polypeptide with an apparent p*I* between 5.8 and 6.4. In addition, several polypeptides with molecular weights between 16 000 and 34 000 also appeared after 9 days of infestation.

Under favorable conditions of temperature and moisture, *Aspergillus spp.* infestation causes rapid changes in seed composition and quality. It has been demonstrated that the seed components such as proteins and carbohydrates not only serve as nutrient source for fungi during their invasion (Krupa and Branstrom, 1974; Zscheile, 1974) but are also involved in aflatoxin biosynthesis (Buchanan and Lewis, 1984; Shih and Marth, 1974). The seed components that are affected by *Aspergillus spp.* infestation include dry matter, protein, oil, fatty acids, carbohydrates, and amino acids (Ward and Diener, 1961; Deshpande and Pancholy, 1979; Cherry et al., 1975; Cherry and Beuchat, 1976). Previous studies have indicated that major storage proteins are converted to numerous low molecular weight components following fungal invasion (Cherry et al., 1976; Cherry et al., 1978). In addition to proteins, enzymes such as esterase, leucine aminopeptidase, catalase, alcohol dehydrogenase, alkaline phosphatase, glucose 6-phosphate dehydrogenase, mannitol dehydrogenase, and malate dehydrogenase are also affected by fungal infestation (Cherry et al. 1978; Buchanan and Lewis, 1984; Cherry et al., 1972). Earlier studies were mainly aimed at determining the overall changes in the protein and enzyme patterns and thus made no special attempt to determine the changes in specific proteins and to identify the seed proteins/polypeptides that may be preferentially utilized by the fungi. Hence, this study was initiated to identify the

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