Isolation and Identification of Iturins as Antifungal Peptides in Biological Control of Peach Brown Rot with *Bacillus subtilis*

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The peach brown rot fungus, *Monilinia fructicola*, is inhibited by *Bacillus subtilis* and cell-free culture liquor of *B. subtilis*. Antifungal peptides of the Iturin family have been isolated from the culture liquor and are active against the mycelial growth of the spoilage organism. FABMS, MS/MS, and NMR were used for structure determinations.

Brown rot of stonefruit is the major cause of spoilage of postharvest peaches. Biological control of *Monilinia fructicola* (Wint.) Honey and other phytopathogenic fungi is of interest because some of the problem fungi are developing resistance to widely used fungicides such as benomyl and related benzimidazoles. *Bacillus subtilis* (Cohen) is being investigated as a biological control agent for efficacy against bean rust, *Uromyces phaeseoli* (Raben.) Wint. (Baker et al. 1983), and peach brown rot, *M. fructicola* (Wilson et al. 1985).

Since *B. subtilis* and related bacilli are known to produce a variety of antibacterial and antifungal peptide antibiotics (Korzybski et al. 1978), we suspected that similar substances might be responsible for the demonstrated activity against peach brown rot (Pusey et al., 1984). Therefore, we undertook the isolation and identification of the biologically active natural products from *B. subtilis* responsible for controlling *M. fructicola*, and we now report our results.

EXPERIMENTAL SECTION

Source of Organisms and Metabolites. A strain of *B. subtilis* (B-3), isolated from soil in Kearneysville, WV, was used in liquid fermentations to produce the antibiotic at the Southeastern Fruit and Tree Nut Research Laboratory, Byron, GA. *M. fructicola* was also supplied by the Byron location. The crude fermentation product was supplied as an acid precipitate after centrifugation as described by McKeen et al. (1986).

Isolation and Purification of Culture Liquor. The culture liquor (pH 7) was acidified to pH 3 with HCl. The resulting solution and precipitate was centrifuged to collect the active solid material. The solid was air-dried overnight and was then extracted with ether for 6 h in a Soxhlet extractor to remove nonpolar, biologically inactive impurities. The remaining solid was dissolved in 50:50 methanol-water, and the solution was chromatographed on a column of C-18 reversed-phase absorbent (Waters Prep Pak 500). Elution with a stepwise gradient of methanol and water (from water up to 80% methanol-water) eluted most of the active material as shown by bioassay. Further purification was achieved by droplet countercurrent chromatography (DCCC) (Buchi B-670) using chloroform-methanol-water (7:13:8) in the descending mode with the chloroform layer as the mobile phase. The main peaks (1 and 2) eluted in 300-500 mL of the chloroform layer.

Chemicals and Solvents. An authentic sample of Iturin A was obtained from Dr. Garold Gregory, U.S. Forest Service, who received the sample from Dr. F. Besson, University of Lyon, France. Bacitracin was purchased from Aldrich and Pfizer. Hydrochloric acid and ammonium hydroxide were reagent grade. Methanol and chloroform were HPLC grade from Fisher and Burdick and Jackson. Bonded-phase octadecylsilane on silica (ODS) was purchased from Waters Associates as a Prep-Pak 500 C-18 cartridge that was emptied for open-column use. TLC plates consisted of silica gel on aluminum support (Catalog No. 5539) (EM Science).

Analytical Methods. Open-column and DCCC fractions were analyzed by reversed-phase C-18 HPLC with UV detection at 210 nm. Separations, with gradient elutions starting at 50% MeOH in water, were performed on a Hewlett-Packard Model 1084B or a Waters Associates Model ALC202/401 with Model 680 gradient controller equipped with a Perkin-Elmer LC-55 variable-wavelength UV-vis detector. Preparative HPLC was performed with a Waters Associates Model 500A instrument, equipped with an ISCO V-4 variable-wavelength UV-vis detector.

HPLC columns used: Altex Ultrasphere C-18, 25 cm × 4.6 mm (i.d.), 5-μm particle size; Rainin Microsorb, C-18, 25 cm × 4.6 mm (i.d.), 5-μm; Waters, Nova-Pak C-18, 15 cm × 5 mm (i.d.), 5 μm, in a radial compression module, Model RCM-100.

Capillary Gas Chromatography. The *N*-pentafluoropropionyl-D,L-amino acid *n*-propyl esters were separated on a 50 × 0.31 mm (i.d.) fused silica (FS) Chirasil-Val III capillary column using cold on-column injection (Arrendale and Chortyk, 1985). Cold on-column capillary GC conditions were as follows: injection volume, 1 μL; solvent, ethyl acetate; oven temperature, 65 °C, isothermal for 4 min and then programmed from 65 to 200 °C at 3 °C/min; probable linear velocity of He, 37 cm/s.

Capillary Gas Chromatography/Mass Spectrometry (GC/MS). The cold on-column injection GC/MS analyses of the *N*-pentafluoropropionyl-D,L-amino acid *n*-propyl esters were obtained with a Hewlett-Packard 5985B GC/MS system. The FS Chirasil-Val III capillary column was connected to the MS ion source with an open-split interface (Arrendale et al., 1984). The MS conditions were as follows: GC/MS interface zone temperature, 300 °C; ion source temperature, 200 °C; scan, 40-500 μm, scan rate, 400 μm/s; electron energy, 70 eV; electron multiplier voltage, 2000 V.

Fast atom bombardment (FAB) mass spectra were obtained with the MAT 731 double-focusing mass spectrometer with an Ion Tech gun (for positive-ion FABMS, including HRFABMS) and the JEOL HX110/HX110

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tandem double-focusing mass spectrometer with JEOL gun (for positive- and negative-ion FAB and collisionally induced decomposition (CID) MS/MS). The MAT instrument was operated at +8-kV accelerating voltage; the JEOL at ±10 kV with ∓18.5-kV postacceleration at the detector. The xenon neutral beam had 8-kV acceleration from the Ion Tech gun and 6 kV from the JEOL gun. Low-resolution FAB mass spectra were obtained at 1:2000 resolution; exact-mass measurements were made 1:10 000 resolution. CID MS/MS was performed with 1:1000 resolution in both MS-1 and MS-2. Helium was used as the collision gas at a pressure sufficient to reduce precursor ion abundance by 50%. Samples were dissolved in 1:1 methanol–glycerol for all FABMS analyses.

NMR. ^{1}H and ^{13}C NMR spectra were obtained on a Varian Associates XL-300 NMR spectrometer using solutions prepared in 100% dimethyl-d_6 sulfoxide (DMSO-d_6) and contained in 5-mm NMR tubes. ^{13}C NMR spectra, without the NOE enhancement, were obtained and peak areas integrated to determine the number of carbons giving rise to each peak. DEPT spectra (Doddrell et al., 1982) were obtained to determine the number of protons at each carbon.

Other Analyses. UV spectra were obtained with a Beckman DU-8 spectrophotometer, while infrared spectra were obtained with an Analect FX-6160 FTIR at 4-cm^{-1} resolution with a TGS detector. Optical rotation measurements were made with a Rudolph Autopol III polarimeter. Thin-layer chromatography of the peptides on silica gel were run in 80% ethanol–20% water. Peptide acid hydrolysates were chromatographed on silica gel plates with 70:30 ethanol–water for development. The plates were sprayed with ninhydrin and heated for color development of the amino acid derivatives.

Bioassays. *Fungal Bioassay.* The method and medium of McKeen et al. (1986) was used for the assay of spore germination of *M. fructicola*. Wells were formed in the agar surface with a cork borer (11 mm), and test solutions were then placed in the wells. When active compounds were present, circular zones of nearly total inhibition of fungal growth around the wells were evident in 3–4 days.

Wheat Coleoptile Bioassay. The etiolated wheat coleoptile assay was conducted according to Cutler (1984).

RESULTS AND DISCUSSION

The crude preparation containing the antibiotic peptides was found to be soluble in dilute ammonium hydroxide and could be precipitated by adjusting to pH 3 with hydrochloric acid. The process of dissolution and reprecipitation left a brown-black color in the supernatant, effecting a partial cleanup of the crude preparation. A further cleanup was accomplished by Soxhlet extraction of the crude solid with diethyl ether. The extraction removed a biologically inactive orange yellow oil representing approximately one-third of the weight of crude material. As an alternative the crude material was dissolved in 50:50 methanol–water and eluted from C-18 silica gel by stepwise gradients of methanol–water. The active peptides started to elute from the reversed-phase adsorbent at 65–70% ethanol–water and were completely eluted with 80% methanol–20% water. Bioassay of the C-18 column fractions with *M. fructicola* and with the wheat coleoptile bioassay showed activity in fractions that contained several peaks as analyzed by HPLC (210 nm). The wheat coleoptile bioassay showed 47–81% inhibition of etiolated wheat coleoptiles by the *B. subtilis* metabolites in impure and pure states. However, total inhibition was not observed, indicating a lag time necessary for inhibition. This effect was consistently observed in several tests. Five

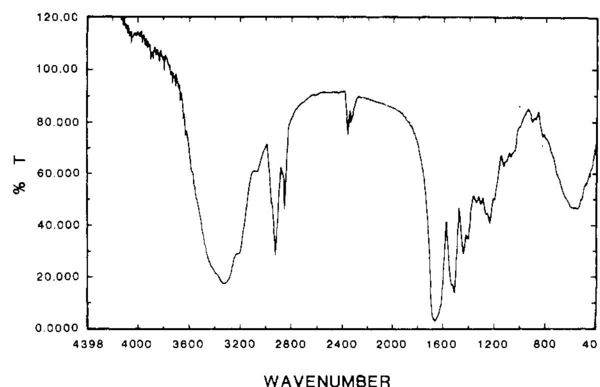

Figure 1. FTIR spectrum of PK1, shown to be Iturin A-2.

HPLC peaks having a profile very similar to those published by Isogai et al. (1982) for *B. subtilis* metabolites and collected from an analytical C-18 column were also active in the *M. fructicola* bioassay. Peak 1 (PK1), corresponding to Iturin A-2 according to Isogai's scheme, showed inhibition zones of 3.1, 13.8, and 19.2 mm for 1, 5, and 10 μg/treatment well. Fourier transform infrared spectra (FTIR) (Figure 1 shows PK1) of the collected peaks from HPLC were very similar to those of bacitracin, a *B. subtilis* peptide metabolite, as were the HPLC retention times. Despite these similarities, bacitracin was inactive against *M. fructicola*. The FTIR spectra had broad bands centered around 3310–3330 cm^{-1}, indicating associated NH, and amide I and II bands at 1654 and 1517 cm^{-1}.

Two major peaks were also separated by the open C-18 column chromatography step. Amino acid analyses by TLC and capillary GC of the acid hydrolysates of the two peaks showed the presence of serine, proline, tyrosine, aspartic acid, and glutamic acid. Another (nonprotein) amino acid appeared in the GC analysis but was not detected by the usual ninhydrin reagent on TLC [see Delcambe et al. (1976)]. Before hydrolysis the original peptides also were not detectable with ninhydrin on TLC. The EI GC/MS of the *N*-acetyl methyl ester of the nonprotein amino acid had ions in common with aspartic acid most notably at m/z 144 ($CH_3CON^+H{=}CHCH_2COOCH_3$; Delcambe et al., 1976). This result confirmed the β-amino acid structure and indicated the probable relationship to Iturin-type antibiotics.

After fractions from several open C-18 columns were accumulated and combined with those of similar composition, as indicated by HPLC, further purification of the major peaks was carried out with the DCCC. A mixture of the two major HPLC peaks, 1 and 2, was partially separated on the DCCC in 10-mL fractions from tubes 31–43, but the C-18 column accomplished most of the separation. The DCCC removed impurities that had coeluted from the C-18 columns and produced very pure samples of the two major peaks. It was subsequently found by HPLC that peak 2 was still a mixture of two compounds. This was confirmed by ^{13}C NMR, which showed an 80:20 ratio for the two components. The ^{13}C NMR data for peak 1 was nearly identical with data published earlier (Garbay-Jaureguiberry et al., 1978) and are summarized in Table I.

Comparison of the ^{1}H and ^{13}C NMR spectra of peaks 1 (Figures 2 and 3) and 2 with those reported for the cyclic peptides iturin A (Garbay-Jaureguiberry et al., 1978) and mycosubtilin (Delcambe et al., 1976) suggest that peaks 1 and 2 are similar cyclic peptides but differ from each other in the β-aminoiturinic acid. The ^{13}C data obtained for PK1 (Table I) suggested that it contains β-amino acids identical with those reported for iturin A (Garbay-Jaure-

Table I. ^{13}C Chemical Shifts and Probable Assignments for HPLC Peak 1[a]

residue	C_α	C_β	C_γ	C_δ	other
Tyr	56.2	34.9	127.8	129.7	114.9, 155.7
Ser	56.2	61.4			
Pro	60.7	29.0	25.3	47.1	
Gln	50.7	28.6	30.5		
Asn	50.7	34.6			
Asn (2)	49.6	36.3			
BNC_{14}	41.6	45.4			13.9, 22.0, 24.6, 26.4, 28.6 (6), 31.2, 35.3

[a] Carbonyl carbons at 170.2, 170.8 (3), 170.9, 171.2 (3), 171.8, 172.6, 173.1, and 174.1 ppm.

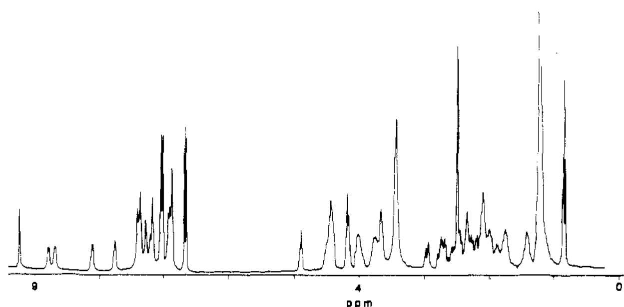

Figure 2. ^{1}H NMR spectrum of PK1, shown to be Iturin A-2.

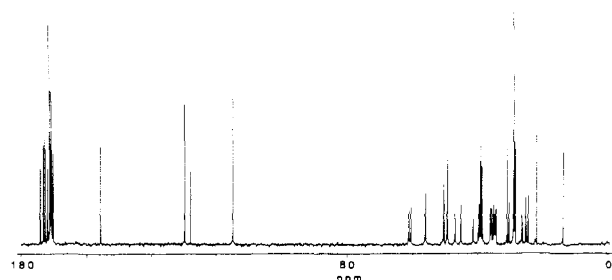

Figure 3. ^{13}C NMR spectrum of PK1, shown to be Iturin A-2.

guiberry et al., 1978). Integration of the ^{13}C spectrum of peak 1 further suggested that the β-amino acid portion of the molecule contain 15 carbons; however, the EI-MS data are more reliable, and PK 1 is shown to have 14 carbons in the β-amino acid. The carbon chemical shift of the methyl carbon further suggests that the β-amino acid portion of the compound is a straight-chained β-amino acid. While it is not possible to determine whether the amino acids are D or L and the sequence of the amino acids from the NMR spectra, it is clear from the ^{13}C and ^{1}H NMR spectra and EI-MS that PK 1 contains tyrosine, serine, proline, three aspargine, glutamine, and 3-aminotetradecanoic acid residues.

The NMR data obtained for PK 2 suggests that it is a mixture of at least two compounds in the ratio of 80:20, with the major component containing an iso β-amino acid. Because PK 2 is a mixture of at least two compounds, it is not possible from the NMR data to determine the structures of the β-amino acid portions of the compounds other than the above conclusion that the two major components contain iso and normal β-amino acids in the ratio of approximately 80:20.

Further confirmation of the structures of peak 1 and the two components of peak 2 comes from positive- and negative-mode FABMS, which indicate the molecular weights are 1042, 1056, and 1056 (Tables II and III). The FABMS and CID MS/MS spectra of these compounds are consistent with our MS spectra of authentic Iturin A-2 (Isogai), an antifungal peptidolipid (Besson et al., 1984) of the structure *Ser-Asn-Pro-Gln-Asn-Tyr-Asn-X* where X

Table II. Assignments of Peaks (m/z) in the Positive-Ion FAB Mass Spectra

PK1	PK2A/2B	assignment
1043	1057	$(M + H)^+$
818	832	Asn-Ser-X-Asn-Tyr-Asn[c]
801[a]	815[a]	Pro-Asn-Ser-X-Asn-Tyr
773	787	(Pro-Asn-Ser-X-Asn-Tyr − CO)
755	769	(Pro-Asn-Ser-X-Asn-Tyr − CO, H_2O)
638[a]	652[a]	Pro-Asn-Ser-X-Asn
610	624	(Pro-Asn-Ser-X-Asn − CO)
541	555	Asn-Ser-X-Asn
(524)[a]	538[a]	Pro-Asn-Ser-X
520	520	(Asn-Tyr-Asn-Gln + H)
427	441	Asn-Ser-X
409	423	(Asn-Ser-X − H_2O)
392	392	(Asn-Tyr-Asn + H)
323	323	(X-Asn − NH_3)
299[a]	299[a,b]	(Pro-Asn-Ser + H)
212[a]	212[a]	(Pro-Asn + H)
184[a,b]	198[a,b]	$C_{12}H_{26}N/C_{13}H_{28}N$

[a] Major ion in CID MS/MS spectrum of (M + H). [b] Assignment verified by exact-mass measurement. [c] X = $NHCHC_{11}H_{23}CH_2CO$ (PK1) or $NHCHC_{12}H_{25}CH_2CO$ (PK2A/2B).

Table III. Assignments of Peaks (m/z) in the Negative-Ion FAB Mass Spectra

PK1	PK2A/2B	assignment
1041	1055	(M − H)
1011	1025	(M − 31) (Ser)
998	1012	(M − 44) ($CONH_2$)
983	997	(M − 59) (Asn)
969	983	(M − 73) (Gln)
934	948	(M − 108) (Tyr)
766	780	Gln-Pro-Asn-Ser-X-Asn[a]
702	716	(Ser-X-Asn-Tyr-Asn − 2 H)
651-3	665/6	Gln-Pro-Asn-Ser-X
615	615	(Asn-Tyr-Asn-Gln-Pro − H)
539		(Asn-Ser-X-Asn − 2 H)
509	523	(Asn-Tyr-Asn-Gln-Pro − BzOH)
501	515	(X-Asn-Tyr − 2 H)
425	439	(Asn-Ser-X − 2 H)
395	409	(X-Asn-Tyr − BzOH)
390	390	(Asn-Tyr-Asn − H)
338	338	(Gln-Pro-Asn − H)
276	276	(Asn-Tyr − H)
224	224	(Gln-Poro − H)
196	196	(Gln-Pro − H, CO)
170	170	(Asn-Tyr − BzOH)
96	96	(Asn − NH_4)

[a] X = $NHCHC_{11}H_{23}CH_2CO$ (PK1) or $NHCHC_{12}H_{25}CH_2CO$ (PK2A/2B).

[normal C_{14} β-amino acid (X = $NHCH[C_{11}H_{23}]CH_2CO$) or iso or anteriso C_{15} β-amino acid (X = $NHCH[C_{12}H_{25}]CH_2CO$)] is part of the cyclic peptide structure of eight amino acid residues. This corresponds to the structural representation *-S-N-P-Q-N-Y-N-X-*, as assembled from the MS fragments shown in Figure 4A,B. The structure of Iturin A had been determined by electron ionization mass spectrometric analysis of its permethylated derivative (Peypoux et al., 1978). FABMS spectra for Iturin A have not been reported previously.

FABMS is often useful in amino acid sequencing of a single peptide or peptides present in mixtures (Biemann, 1986). In the positive mode the major fragmentations occur by cleavage on either side of the amide nitrogen with or without concomitant proton transfer. The usual result is a series of ions spaced by increments corresponding to amino acid residues the identity of which may be deduced by calculating the mass differences between the ions. In the present case, the positive mode FABMS for PK 1 includes the protonated molecular ion of the intact peptide (m/z 1043, M + H) and fragment ions whose nominal

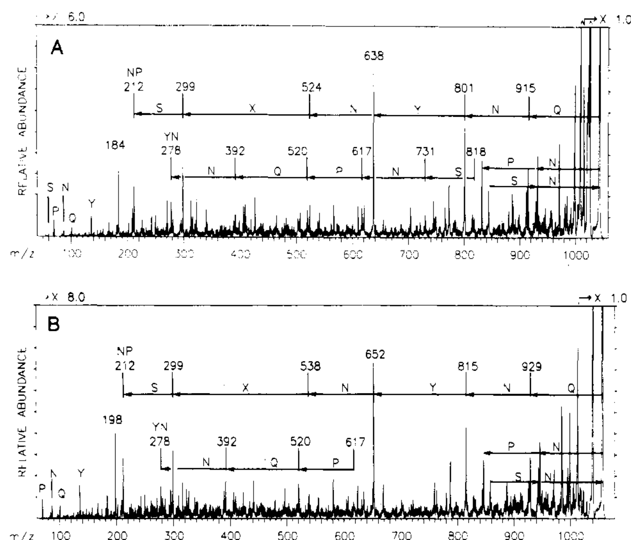

Figure 4. FAB CID MS/MS spectrum for $(M + H)^+$: (A) PK1, shown to be Iturin A-2; (b) PK2A, shown to be Iturin A-3 (Isogai).

masses are appropriate for the sequence previously assigned to Iturin A (Table II). The low-resolution FABMS assignments are not completely unambiguous, however; the peaks observed could also accommodate the assembled structures (from Figure 4A,B) of P-*S*-*Y*-*N*-*N*-*N*-*Q*-*X*- and P-*T*-*Y*-*N*-*N*-*N*-*Q*-*X*- (T = threonine), X = $C_{13}H_{25}NCO$, for peaks 1 and 2, respectively. For either Iturin A or the alternative structures, fragments would be predicted at *m/z* 818 (loss of Ser(Thr)Pro or the unknown amino acid) and at *m/z* 184 (198), and *m/z* 299 and sequence-related ions could be assigned. Selection between the two possibilities was made on the basis of the exact-mass measurements of the ions at *m/z* 184.2076 (calcd for $C_{12}H_{26}N$, *m/z* 184.2065) and *m/z* 299.1361 (calcd for $C_{12}H_{19}N_4O_5$, *m/z* 299.1361) in the spectrum of peak 1 and *m/z* 198.2225 (calcd for $C_{13}H_{28}N$, *m/z* 198.2222) in the spectrum of peak 2. Further support for the Iturin A structure was provided by the positive-mode FAB CID MS/MS spectra (Figure 4A,B). In each MS/MS spectrum, sequence ions consistent with the reported Iturin A structure were observed. Each also included a product ion at *m/z* 887 that corresponds to loss of the long-chain alkyl group. The predominant sequence ion series are indicated in Figure 3. In these CID MS/MS spectra, as is usual for peptides (Biemann, 1986), the sequence ions stand out more clearly than in normal scans.

The negative-mode FABMS spectra (Table III) also contain fragments that are sequence specific as well as high-mass fragments that arise by loss of the amino acid side chains. Most cleavages in the negative-ion spectra occur adjacent to glutamine (Gln) or aspargine (Asn).

Capillary gas chromatography (Figures 5 and 6) of the *N*-pentafluoropropionyl *n*-propyl ester derivatives of the hydrolyzed amino acids show the presence of D-tyrosine and D,L-aspartic (asparagine) acid in a 2:1 ratio, respectively, consistent with the structure of Iturin A reported by Peypoux et al. (1978).

The β-amino (iturinic) acid portion of peaks 1 and 2 was shown by EI GC/MS to be C-14 and C-15 β-amino acids by molecular ions at *m/z* 431 and 445. Nagai et al. (1979) found the absolute configuration of an iturinic acid from Iturin A to be *R* at the C_3 center. The molecular rotation $[\phi]$ of peak 1 was determined to be −16.7 at 22.7 °C and 589 nm. Considering the above evidence and that the peptides are products of a *B. subtilis* strain, we conclude that our major and minor peptides, PK1, PK2a, and PK2b, and later eluting peaks (by HPLC) are the same as re-

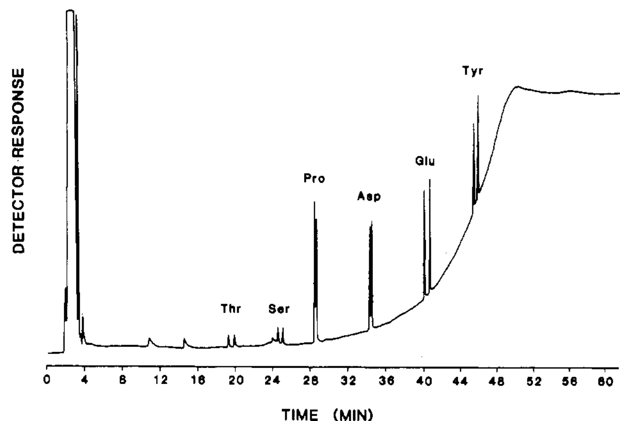

Figure 5. Capillary gas chromatogram of standard D,L-amino acids on chiral column, polysiloxane stationary-phase containing *N*-propioyl-L-valine *tert*-butylamide groups. Column was 50 m × 0.31 m, programmed from 65–200 °C at 3 °C/min.

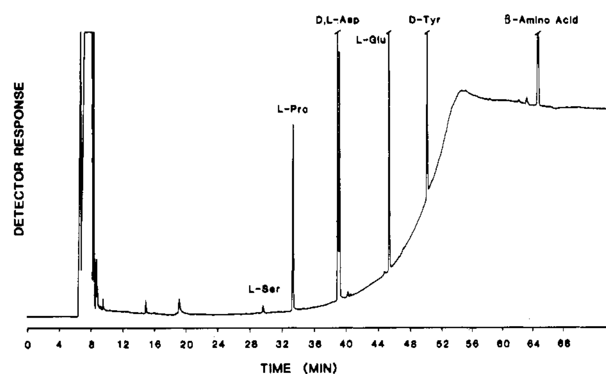

Figure 6. Capillary gas chromatograms of PK1 amino acids on chiral column as in Figure 5.

ported by Delcambe et al. (1976) and Isogai et al. (1982). As reported earlier (Delcambe et al., 1976), these peptides have medicinal value, and now we have evidence of the potential for use in agriculture, particularly in view of their low toxicity and low allergenic properties in topical applications. However, systemic effects must be evaluated since these surface active peptides may be associated with hemolysis (Quentin et al., 1982).

ACKNOWLEDGMENT

The MIT Mass Spectrometry Facility is supported by NIH Division of Research Resources Grant No. RR00317 (K. Biemann).

Registry No. PK1, 83785-07-3; PK2 (isomer 1), 83777-00-8; PK2 (isomer 2), 83777-01-9.

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Received for review June 15, 1987. Accepted October 23, 1987. Mention of firm names does not imply endorsement by the U.S. Department of Agriculture of the products. No discrimination or preference is intended. The use of firm names and products is for identification only.

Metabolism of Pentachlorophenol in Cell Suspension Cultures of Wheat (*Triticum aestivum* L.). Tetrachlorocatechol as a Primary Metabolite

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Wheat cell suspension cultures were incubated with [U-^{14}C]pentachlorophenol (PCP; 1 ppm, 48 h, 27 °C). Soluble metabolites were formed in ~50% yield, another ~31% of the applied radioactivity being incorporated into the "insoluble" residue. The soluble metabolite fraction, and its β-D-glucoside conjugate components, the total "insoluble" residue, and its lignin components, were all found to contain a novel polar PCP derivative besides smaller amounts of tetrachlorohydroquinone and PCP. The novel derivative also predominated in intact wheat plants and was identified as tetrachlorocatechol by TLC, HPLC, GC, and EI as well as CI mass spectroscopy. Tetrachlorocatechol is a potential mutagen, so that the soluble and "insoluble" conjugates formed in wheat from PCP may present a toxicological hazard.

Pesticide metabolism in plants often leads to significant incorporation of the radioactive label into the insoluble residue fraction. The lignin component of "insoluble" plant cell wall fractions has been recognized to be a major site of covalent binding, as demonstrated for 2,4-D (Scheel and Sandermann, 1981), benzo[*a*]pyrene quinones (Trenck and Sandermann, 1981), chlorinated anilines (Trenck et al., 1981; Arjmand and Sandermann, 1985a, 1986), and pentachlorophenol (PCP; Scheel et al., 1984). A number of additional reports on the lignin incorporation of pesticide chemicals exist (Baldwin, 1977; Roberts, 1984). However, these studies failed to establish the covalent nature of binding and/or to exclude labeling by refixation of radioactive carbon dioxide [see Sandermann et al. (1983)].

A stepwise approach has been suggested for the toxicological evaluation of plant insoluble residues (Huber and Otto, 1983; Kovacz, 1986). The nature of the bound radioactive material should be elucidated for residues corresponding to more than 10% of the applied radioactive pesticide (Huber and Otto, 1983; Kovacz, 1986). Pentachlorophenol has previously been shown to be incorporated to 37% into the insoluble residue of cultured wheat cells, the lignin cell wall component representing a major binding site (Sandermann et al., 1984; Scheel et al., 1984). High rates of incorporation into the insoluble residue have also been observed in parallel studies with wheat cell cultures (Langebartels and Harms, 1984; Harms and Langebartels, 1986; Schuphan et al. 1984) and in whole plant studies employing [U-^{14}C]pentachlorophenol, e.g. in rice (Haque et al., 1978; Weiss et al., 1982), corn (Cole and Metcalf, 1977), alfalfa and rye (Gile and Gillett, 1979), and wheat

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0021-8561/88/1436-0370$01.50/0 © 1988 American Chemical Society