Transport and Metabolism of Pollen Suppressant SC-2053 in Wheat

John W. Cross,*,† Paul Herzmark,‡ William L. Guilford,§ Thomas G. Patterson,⊥ and Jeffrey N. Labovitz||

Sogetal, Inc., Hayward, California

SC-2053, 1-(4-chlorophenyl)-4-oxo-5-(methoxyethoxy)cinnoline-3-carboxylic acid, is a pollen suppressant (chemical hybridizing agent) in wheat. Metabolism of this compound was studied using [^{14}C]SC-2053 labeled uniformly in the chlorophenyl ring. Wheat plants were treated when the spike was 2–4 cm in length. The floral spike contained about 10% of the label taken up into the plant 4 days after treatment, 15% 14 days after treatment. Unchanged [^{14}C]SC-2053 plus two more polar metabolites were 90–95% of the total ^{14}C extracted from the plants 14 days after treatment. The more polar metabolite was a glycoside of the other. Treated wheat plants and wheat callus cultures metabolized [^{14}C]SC-2053 by a similar route. The aglycon purified from a callus culture filtrate was identified as 1-(4-chlorophenyl)-4-oxo-5-(2-hydroxyethoxy)cinnoline-3-carboxylic acid. This alcohol results from demethylation of the 5-methoxyethoxy group. In plants this metabolite is glycosylated, but in callus cultures only the initial demethylated compound accumulated in the medium. The major metabolite in mature straw was the glycoside.

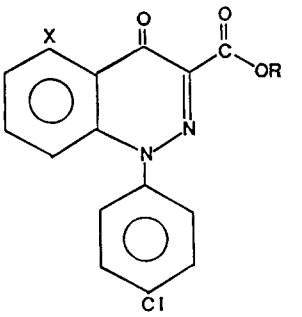
Keywords: *Chemical hybridization; CHA; O-demethylation; xenobiotic; male sterility; Triticum aestivum*

INTRODUCTION

SC-2053 is an active chemical pollen suppressant (chemical hybridizing agent or CHA) for wheat and other small grains (Guilford *et al.*, 1992; Wong *et al.*, 1992). The chemical structure of SC-2053 [1-(4-chlorophenyl)-4-oxo-5-(methoxyethoxy)cinnoline-3-carboxylate] is shown in Figure 1 (compound **I**).

The action of SC-2053 and several closely related compounds have been studied intensively in wheat (Schulz and Almeida, 1988; Cross *et al.*, 1989; Schulz *et al.*, 1993; Cross and Schulz, 1994; J. W. Cross, unpublished results). When applied to plants just prior to the initiation of meiosis, these compounds effectively inhibit development of early microspores at the uninucleate stage. This ultimately results in their death and cellular collapse. There is no effect on microspore meiosis. The sequence of events has been examined at the ultrastructural level with the closely related compounds, SC-1271 and SC-1058 [1-(4-chlorophenyl)-4-oxo-5-propoxycinnoline-3-carboxylic acid (CAS 130561-18-1) and 1-(4-chlorophenyl)-4-oxo-5-fluorocinnoline-3-carboxylic acid (CAS 143216-06-2), respectively] which are also potent chemical pollen suppressants (Schulz and Almeida, 1988; Cross *et al.*, 1989; Schulz *et al.*, 1993). With these two compounds the earliest identified effects are on the ultrastructure of the tapetal cells which surround the developing pollen grains in the anther. These effects include inhibition of the secretion of sporopollenin precursors. Similar effects have been observed with certain pollen suppressants belonging to other chemical series effective at this stage (Cross and Ladyman, 1991; Cross and Schulz, 1994). The molecular target of SC-2053 is not known.

We report here an investigation of the transport and metabolism of SC-2053 as a basis for understanding the mode of action of this compound.



	Compound	X	R
I	SC-2053	$CH_3OCH_2CH_2O$-	H-
II	SC-3095	$HOCH_2CH_2O$-	H-
III		sugar-OCH_2CH_2O-	H-
IV	SC-1231	HO-	H-
V	2053-ME	$CH_3OCH_2CH_2O$-	CH_3-
VI	3095-ME	$HOCH_2CH_2O$-	CH_3-
VII	3095-MEAc	$CH_3COOCH_2CH_2O$-	CH_3-

Figure 1. Structures of compounds described in the text.

MATERIALS AND METHODS

Analytic Methods. *Chemicals.* SC-2053 (**I**), SC-3095 (**II**), and SC-1231 (**IV**) were synthesized at Sogetal (Guilford *et al.*, 1992). [^{14}C]SC-2053 (40.5 mCi/mmol, uniformly labeled with ^{14}C in the chlorophenyl ring) was prepared by an unambiguous synthesis at Sogetal from

† Present address: National Science Foundation, 4201 Wilson Blvd., Suite 615, Arlington, VA 22230 (Internet 406-7188@mcimail.com).

‡ Present address: Department of Pharmacology, University of California, San Francisco, CA 94143.

§ Present address: 16530 Russell Ct., San Leandro, CA 94578.

⊥ Present address: 4202 Echo Ct., Pleasanton, CA 94588.

|| Present address: Lynx Therapeutics, 3832 Bay Center Place, Hayward, CA 94545.

0021-8561/95/1443-0548$09.00/0

Table 1. Chromatographic Properties of Compounds in the Text

compound	identity	HPLC t_R (min)	system[a]	TLC R_f	system[a]
I	SC-2053, free acid	17.6	1	0.24	3
II	SC-3095, free acid = aglycon	16.0	1	0.41	3
III	SC-3095-glycoside = glycoside	13.8	1		
IV	SC-1231 = phenol	18.3	1		

compound	identity	HPLC t_R (min)	system	GC t_R (min)	system
V	SC-2053-ME, methyl ester	7.9	2	6.3	4
VI	SC-3095-ME, methyl ester	5.6	2		
VII	acetyl-SC-3095-ME, acetylated methyl ester	8.6	2	5.9	4

[a] The systems are as follows: System 1: Gradient HPLC; for free acids and crude extracts; column, Dyanmax 60A C-18 (8 μm avg particle size, irregular silica-based, 4.6 × 250 mm i.d. plus guard cartridge); mobile phase, methanol 5% (constant) plus a linear gradient of 10–50% acetonitrile in 25 min, balance, solvent A (solvent A = 0.05 M triethylamine + 0.05 M acetic acid); elution, 1.0 mL per min at 40 °C. System 2: Isocratic HPLC; for methyl esters; column, Spherisorb C-18 (5 μm particle size silica-based, 4.6 × 250 mm); mobile phase, acetonitrile–water 1:1; elution, 1.0 mL per min at 40 °C. System 3: Thin-layer chromatography; for free acids; Analtech RPS-F (20 × 20 cm, 250 μm layer); mobile phase, buffer (buffer A diluted to 2 mM TEA–acetic acid with water) 75% and acetonitrile 25%. System 4: Gas chromatography; for acetylated methyl esters; direct injection at 320 °C; column, 0.53 mm × 15 m fused silica capillary bonded with 1 μm 95% dimethyl–5% diphenyl polysiloxane (R_{tx}-5, Restek Corp., Bellefonte, PA); carrier, high-purity helium, 12 mL/min; oven, 285 °C; detector, FID (H_2–air optimized) at 320 °C.

precursors prepared by Dr. Sung Rhee (SRI International, Menlo Park, CA). Solvents were glass distilled HPLC grade or equivalent. Sodium ascorbate was USP grade. All other chemicals were analytic reagent grade. Water for HPLC was purified with a Milli-Q system (Millipore, Bedford, MA).

Liquid Chromatography. Analysis and purification of CHA metabolites were accomplished with a Spectra-Physics (San Jose, CA) liquid chromatograph as described (Cross *et al.*, 1992). HPLC solvents were filtered through a 0.45 μm nylon filter (Nylaflo, Gelman Sciences, Ann Arbor, MI) before use and sparged with helium during chromatography. Samples were filtered through a 0.45 μm nylon filter, or centrifuged for 30 s in a microcentrifuge before injection. Radioactive metabolites were quantified by liquid scintillation counting of collected fractions or by on-line counting with a Flo-One Beta detector (Radiomatic Instruments, Tampa, FL). This instrument was used with a flow cell that mixes the chromatographic effluent with scintillation fluid. Chromatographic conditions are given in Table 1.

Gas Chromatography. Routine analyses were performed on a Perkin-Elmer Model 8500 gas chromatograph using the flame ionization detector (see Table 1). Electron impact ionization mass spectra were obtained by David Thomas (SRI International, Menlo Park, CA) on a Ribermag GC-MS Model R 10-10c, using a 30 m × 0.32 mm DB-5 capillary column eluted from 230 to 300 °C at 10 °C/min.

Metabolic Methods. *Growth of Plants.* Wheat (*Triticum aestivum* L. var. Yecora Rojo) was grown in a thermally regulated (65–75 °F) greenhouse in Hayward, CA (Cross *et al.*, 1992). Five plants per 15 cm square pot were seeded and then thinned to three plants per pot. In winter, supplemental illumination from high pressure sodium lamps provided a day length of at least 16 h and a daily mean quantum flux of at least 400 μE m^{-2} s^{-1}. Greenhouse conditions were relatively constant summer and winter because of the proximity to San Francisco Bay. Uptake and transport of SC-2053 were also studied in field-grown wheat (var. S83501). Male sterility in this report was scored by examination of dissected mature anthers under the stereomicroscope (greenhouse studies) or by finding open flowers and subsequent seed-set in bagged spikes (field studies).

Localization of SC-2053 Residues. Plants were labeled in the greenhouse with [^{14}C]SC-2053 for 3, 7, and 14 days or until senescence [see Cross *et al.* (1992) for methods]. After the treated leaves were rinsed, separate extracts were prepared from the leaf of application, the spike, and the remainder of the above-ground plant (remaining plant) as described in the next section. Aliquots of the surface rinse and of each extract were chromatographed. Unextracted ^{14}C was measured by combustion analysis. At each step an accounting was made of the ^{14}C label in each fraction.

Isolation of Metabolites from Plants. *Extractions.* Plant material was weighed, rinsed with water to remove surface radioactivity, blotted dry, and sealed in Zip-loc plastic bags. These were packed individually in dry ice until processing could continue. In selected experiments, the tissue was processed immediately and metabolites were identified which were chromatographically identical (system 1) to those stored on dry ice. The frozen tissue was powdered with excess dry ice in a coffee grinder sealed with Parafilm. To extract metabolites, hot water with 10 mM sodium ascorbate (dissolved immediately before the extraction) was poured over the frozen powder and stirred. The mixture was filtered while still hot. The filter cake was re-extracted and the filtrates were combined. This method had been shown to efficiently extract metabolites of this and related compounds without effect on the chromatographic profile of metabolites (Cross *et al.*, 1992). The recovery of radioactivity was monitored at each step.

Concentration and HPLC Analysis of Metabolites from Crude Plant Extracts. The extracts were adjusted to 0.1 M NaCl + 0.3 N H_3PO_4 (using a 20× stock solution of NaCl + H_3PO_4) and concentrated in portions on C-18 Sep-Pak cartridges (Waters Associates). The cartridges were rinsed with water, eluted with methanol/acetone (10:1 v/v), and evaporated to dryness under N_2. The concentrates (20–200 μL each) were analyzed using system 1 (Table 1).

Purification of the Glucoside. For preparative work, the concentrated extracts were divided into portions to avoid overloading the column (system 1). Fractions were collected and the metabolite peak fractions were pooled. The glycoside peak was 32% and the aglycon was 43% of the total radioactivity recovered at this step.

Purification of the Aglycon. The fractions containing the glycoside were evaporated to dryness under nitrogen, and digested with β-glucosidase (Sigma type II, almond) to release the aglycon. Reactions included 10 units of enzyme in 45 mM potassium acetate, adjusted to pH 5. Incubations were for 30 min at 37 °C and were terminated by heating the mixture for 1 min in a boiling

water bath. The precipitated protein was removed by centrifuging (1 min in the Microfuge) and the aglycon was purified by HPLC (system 1). The fractions containing the aglycon were pooled, evaporated under nitrogen, and analyzed by isocratic HPLC using the UV and on-line radioactivity detectors. The purified aglycon showed a radiochemical peak purity of 94% and a corresponding UV_{355} purity. The purified aglycon was subsequently methylated and acetylated as described above.

Metabolism in Wheat Tissue Cultures. *Tissue Culture Methods.* Primary suspension cultures prepared from disorganized friable callus cultures of Yecora Rojo were obtained from Dr. Jim Wong (Sogetal). The cultures had been initiated from leaf bud explants cultured at 25 °C on Murashige and Skoog medium (without amino acids, plus 2 mg/L picloram, pH 5.8) and had been in culture 8–10 weeks at time of the metabolism study. The suspension was prepared by scraping 2–3 g of this friable callus into 35 or 40 mL of the same medium and incubating on an orbital shaker at 25 °C. As a prerequisite to metabolic experiments it was necessary to determine the tolerance of wheat callus to SC-2053. Inhibitory effects on the growth of wheat seedling tissue have been observed (Vesper and Cross, 1990). Wheat callus was exposed to 0, 0.2, 2, or 20 μM unlabeled SC-2053 for a period of 9 days and examined for viability. The solutions of SC-2053 were prepared by dilution of concentrated aqueous stocks and were filter-sterilized before addition to the growth medium. Under sterile conditions SC-2053 is quite stable in aqueous solution (unpublished data). Viability was evaluated microscopically on the basis of uptake and retention of fluorescein diacetate by cells (Widholm, 1972) and by their active cytoplasmic streaming across trans-vacuolar strands. Cultures with up to 20 μM SC-2053 had a high percentage of viable callus. To be conservative, for isolation of metabolites from culture medium we treated cultures with 10 μM [^{14}C]SC-2053.

Analysis of Metabolites from Callus Tissue. A friable wheat callus was suspended in medium plus [^{14}C]SC-2053. Each treatment was replicated with 0.28 and 1.28 uM [^{14}C]SC-2053. The cultures were terminated after 7 or 11 days and both the filtered media and the tissues were analyzed. Samples of media were also chromatographed at 2 and 3 days. The tissue was collected on Whatman No. 42 paper with suction, scraped off the filter, and extracted (3×) with ethanol using a glass–glass homogenizer. The combined extracts were evaporated to dryness under N_2, taken up in solvent A (Table 1) and chromatographed using system 1 (Table 1). The unextracted residue was counted (after drying under N_2 and heating at 50 °C for 30 min in Soluene).

Isolation of the Major Metabolite from Suspension Culture Medium. The filtered culture medium was adjusted to 0.1 M NaCl + 0.3 N H_3PO_4 and the metabolites were concentrated on C-18 cartridges (Spice, Rainin Instruments, Woburn, MA). Insignificant ^{14}C was found in the initial effluent. The cartridges were rinsed with water and then eluted with methanol/acetone 10:1 (v/v). This concentrate was evaporated to dryness under N_2 and taken up in solvent A. After clarification in a microcentrifuge, the metabolites were separated by HPLC using system 1 (Table 1). The unchanged SC-2053 plus the major metabolite accounted for 84% of the radioactivity and over 54% of the UV_{355}-absorbing material at this point. A single metabolite (labeled F-16) was 48% of the label and 29% of the UV_{355}-absorbing matter. The metabolite fractions were pooled and reanalyzed by isocratic HPLC (as in system 1, but a constant 67% solvent A, 28% acetonitrile, and 5% methanol) and TLC (system 3, Table 1, detection by autoradiography) showing a radioactive peak purity of >90%.

Purified metabolite F-16 was methylated with diazomethane and chromatographed by HPLC (system 2). The radiochemical peak purity of the methylated product (labeled as 16-ME) was 89%, and the UV_{355} peak purity was 90.6%. The balance cochromatographed with SC-2053. The methyl ester peak fractions were pooled, evaporated to dryness, and acetylated by warming (about 50 °C) in an excess of acetic anhydride in pyridine. The acetylated product (labeled as 16-MEAC) on HPLC had a peak purity at 355 nm of >91%. This derivative was submitted for GC-MS.

Table 2. Recovery of SC-2053 from Tissue Extracts

	percentage of tissue recovery[a]		
expt	1	2	3
length of exposure (days)	4	4	14
leaf of application[b]			
extract	47	65	49
unextracted[c]	6	11	16
total	53	77	65
spike			
extract	10	10	12
unextracted	<1	<1	3
total	11	10	15
remaining plant			
extract	32	10	14
unextracted	3	1	4
total	35	11	19

[a] Percentage of the radioactivity recovered in the pooled extracts plus the unextracted material determined by combustion analysis. [b] Not including compound still remaining on the leaf surface, which (as a percentage of the label applied) was 20% at 4 days, 24% at 14 days. [c] Burned.

RESULTS

1. Metabolism of SC-2053 in Whole Plants. These studies focused on the 2-week period after application when SC-2053 induces early effects on microspore development. Analyses of metabolites from mature, senescent plants were less complete, but suggested that the same metabolites are present (data not shown).

Localization of SC-2053 Residues. The results show that the bulk of the applied label (Table 2) remains in or on the leaf where it was applied. Excluding label rinsed off the surface of the leaf (20–24%, Table 2), the greatest part was found in the leaf of application (65% after 14 days), the spike (15% after 14 days), and the body (leaves and shoot) of the plant (19% at 14 days). Separate experiments (not detailed here) using combustion analysis of unextracted plant organs substantiated that little radioactivity can be recovered in the crown, tillers, or roots. For example, after 14 days the tillers (which did not receive direct application of labeled compound) contained only 1% of the quantity penetrating into the plant.

Extraction Efficiency. Combustion analysis also indicated that the hot water extraction was effective in removing most of the radioactivity from the tissue. Unextracted ^{14}C in all plant parts summed to 10%, 13%, and 23% of the total recovered in three separate experiments. In work with the related compound SC-1271 it was found that more exhaustive extractions and

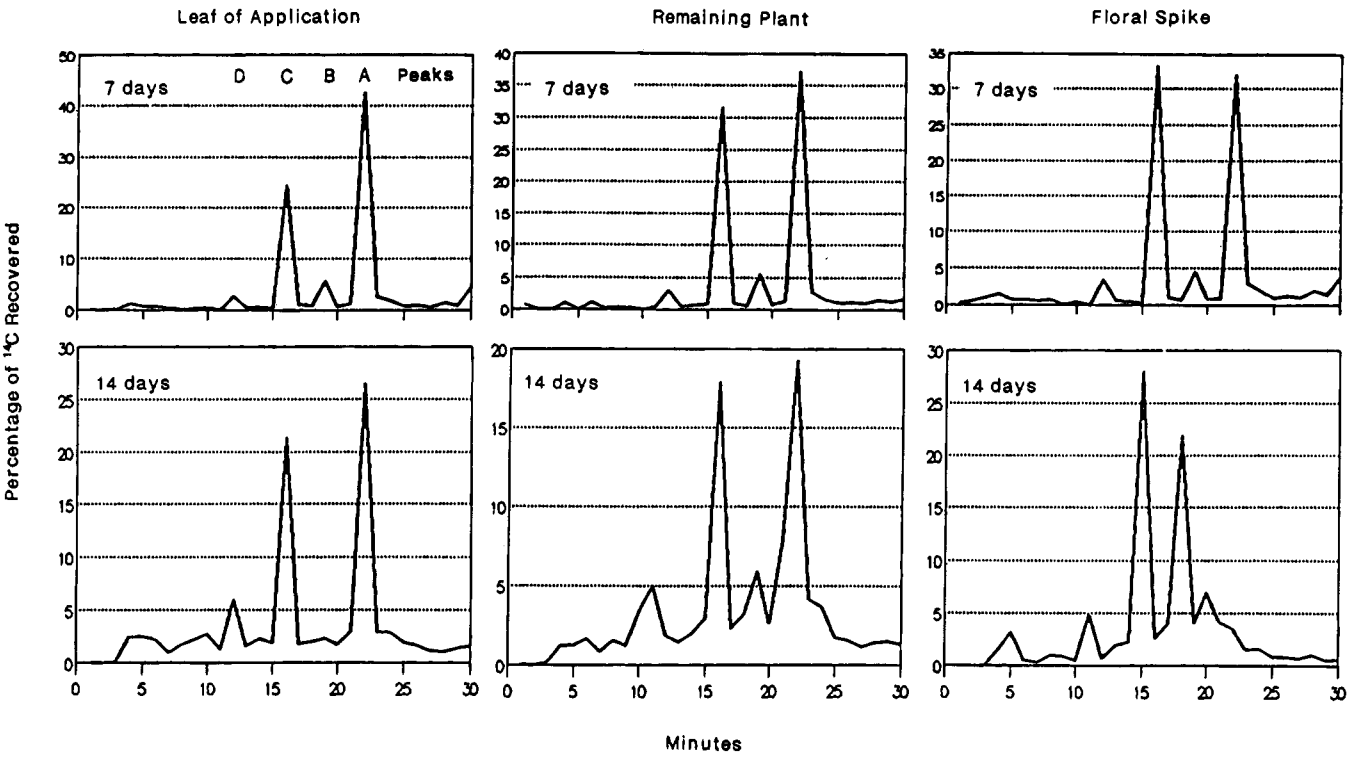


Figure 2. Metabolism of [^{14}C]SC-2053 by wheat plants. Chromatograms of concentrated crude extracts from plants treated in the greenhouse for 4 or 14 days. The extracts were concentrated on C-18 cartridges as described in the Materials and Methods Section and then chromatographed with system 1 (Table 1), but the column was eluted with a 30 min gradient resulting in somewhat longer retention times. Remaining plant is the aboveground plant minus the treated leaf (leaf of application) and the floral spike. The peaks identified **A**–**C** are discussed in the text: (**A**) is unchanged SC-2053, (**B**) is the metabolite aglycon, and (**C**) is the glycoside.

extractions with organic solvents did not extract additional metabolites not found in the initial aqueous extracts (Cross *et al.*, 1992).

Separation of Metabolites. Chromatograms of typical extracts from the first 2 weeks are shown in Figure 2. Four major peaks can be seen that are 5% or more of the total chromatogram. One peak (peak **A**) coelutes with unchanged SC-2053 (t_R 22 min under these conditions, system 1). In addition, the peaks of three other metabolites are discernible. These metabolites, labeled peaks **B**–**D**, eluted in advance of peak **A** at 19, 16, and 12 min, respectively (Figure 2). The same major metabolites (plus many minor peaks, less than 5% each) can also be observed in chromatograms of whole plants extracted at maturity (not shown). Peak **C** is the major metabolite in unhydrolyzed extracts at all time points. In some extracts there also may be a significant percentage of peak **B**, particularly in the spike extracts (Figure 2) and at maturity.

Minor Metabolites. Peak **D** appears in most chromatograms, but never accounts for more than 5–6% of the total. Other smaller peaks were observed, particularly after longer incubations (Figure 2). These minor metabolites have not been investigated further.

Enzymatic Digestion. Treatment of the crude, concentrated plant extract with β-glucosidase (Figure 3) quantitatively converted the major metabolite (peak **C**, eluting at 13.5 min) to a compound cochromatographing with peak **B** (17.5 min). The retention of peak **B** on the reversed-phase column indicates a substance intermediate in polarity between peak **C** and SC-2053. The radioactivity lost from peak **C** was matched by the increased radioactivity seen in peak **B**. No change in the area of peak **A** occurred, and no new peaks appeared.

Metabolic Sequence. These results suggested that SC-2053 is first metabolized to a hydroxylated product (peak **B**), which subsequently was glycosylated to form the β-glucoside (peak **C**). The glycoside (peak **C**) was the major metabolite to accumulate. This pathway for metabolism of SC-2053 was substantiated by the isolation and chemical identification of peak **B**. Further, the aglycon prepared enzymatically from peak **C** is identical to peak **B** (see below).

Kinetics of Metabolism in the Plant. Much of the metabolism in the plant appears to occur before the 3 day time point (Table 3). Experiments show considerable variation in the amount of SC-2053 remaining, particularly in the spike fraction.

2. Metabolism in Wheat Tissue Cultures. Wheat meristematic tissue cultures are free of photosynthetic pigments that complicate the purification of xenobiotic metabolites. Therefore, we initiated a study of SC-2053 metabolism in wheat callus culture.

Uptake of Compounds by the Callus. The wheat callus concentrated the ^{14}C label from the medium (Table 4). Assuming a fresh callus density of 1 g/cm^3, ^{14}C in the callus was roughly 7× that remaining in the medium (Table 4). Since the callus itself was a small percentage of the volume of the culture, the medium contained 85% of the label recovered at 11 days. This accumulation of label by the callus cells may be attributed to distribution of SC-2053 as a weak acid across the proton gradient of the plant cell membrane and the accumulation of fixed or membrane-impermeable metabolites. Only 4% of the applied label was associated with the unextracted residue (Table 3).

Metabolites in Callus Extracts. A major peak coeluting with SC-2053 and three major metabolites were separated by HPLC (Figure 4). At 11 days 50% of the

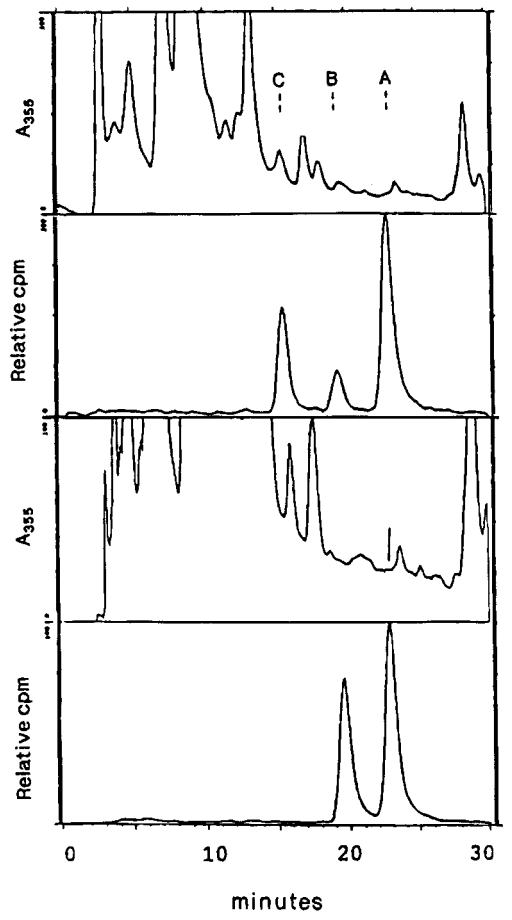


Figure 3. Treatment of SC-2053 metabolites with β-glucosidase. Crude whole-plant extracts were concentrated on C-18 cartridges, dried, dissolved in acetate buffer (pH 5.0), and digested with enzyme as described under Materials and Methods. Chromatography was as in system 1 (Table 1). Radioactivity is plotted relative to the highest peak: (above) before digestion (peak **A**) 59%, (peak **B**) 13%, and (peak **C**) 27% of label); (below) after digestion (**A**) 60%, (**B**) 40%, and (**C**) <1%.

Table 3. Percentage of Unchanged Compound Remaining in Plant Parts[a]

expt	days	percentage leaf	unchanged plant	[^{14}C]SC-2053 spike
1	3	28		50
2	4	49	50	44
3	4	67	72	82
4	14	52		5
5	14	36	33	21

[a] The percentage of unchanged SC-2053 (peak **A**) was determined from the integrated peak areas of each radiochromatogram. Experiments 2–4 are same as in Table 2. The heading designations are as follows: leaf, the leaf of application (not including material rinsed off the leaf surface); plant, all other aboveground plant parts; and spike, the inflorescence above the peduncle.

label cochromatographed with [^{14}C]SC-2053 (peak **A**, t_R = 17.6 min). The metabolites were designated as peaks **B**, **C**, and **D** (eluting at t_R = 16, 13.8, and 10.6 min, respectively). Peak **B** coelutes with the major metabolite peak seen in the culture filtrate. Peak **C** was converted by β-glucosidase into an aglycon co-chromatographing with peak **B** (not shown). Peak **D** (t_R = 10.6 min) was not changed by digestion with β-glucosidase. This metabolite (peak **D**) was not seen in the media (see below) and was not investigated further. Since the callus extracts totaled only 10% of the label recovered (Table 4), these metabolites together account for only about 5% of the total label.

Metabolites in Culture Media. Filtered culture media were chromatographed without prior concentration (Figure 4). At 11 days 21% of the label was associated with a single polar metabolite, from its retention (16 min) likely to be the same hydroxyl compound seen in cell and tissue extracts. Besides SC-2053 and the metabolite, the culture filtrate contained little radioactivity (Figure 4).

These results encouraged us to conclude that the major route for metabolism of SC-2053 in the wheat callus was qualitatively similar to that in the wheat plant. Therefore, we decided to first isolate the major metabolite of SC-2053 from these cultures.

3. Purification of the Metabolite from Culture Filtrates. The major metabolite (peak **B**) was prepared from a culture filtrate treated with 10 μM [^{14}C]SC-2053 for 26 days. HPLC revealed that this metabolite was 48% of the ^{14}C in the starting culture filtrate. The balance was unchanged [^{14}C]SC-2053. From the specific activity we calculated that the purification started with about 0.23 mg of metabolite. See Materials and Methods for details.

The peak fraction of the purified metabolite (fraction 16, F-16) was homogeneous by isocratic ion-pair chromatography (not shown). This fraction was compared chromatographically with metabolites previously isolated in crude extracts of treated plants. F-16 cochromatographed on HPLC and two systems of TLC with the aglycon released from the peak **C** of wheat plants by β-glucosidase. Therefore F-16 appears to be identical to the aglycon produced by metabolism of SC-2053 in wheat plants.

The purified tissue culture aglycon (F16) was then methylated. Radiochromatography confirmed a single product (F16-ME). Attempts to identify a peak corresponding to this product on the gas chromatograph (using the FID) were unsuccessful. Therefore, it was decided to derivatize the free hydroxyl group. An acetate ester (F16-ME-AC) was easily formed and gave a nice single peak on the gas chromatograph (see Table 1). This derivative was submitted for analysis by GC-MS (see below).

4. Partial Purification from Wheat Whole Plant Extracts. The metabolite from tissue culture medium cochromatographed on HPLC with the aglycon released by the principal metabolite from wheat plants. Further, the glucoside extracted from the tissue culture cochromatographed with the glucoside from plants and their aglycons also cochromatographed. This suggested, but did not prove, the identity of these compounds. Therefore the glucoside from whole plants was purified and analyzed.

The glucoside was extracted and the aglycon prepared from it by enzymatic hydrolysis. The aglycon was purified by reversed-phase HPLC, methylated, purified again by HPLC, and acetylated. The aglycon and the purified derivatives were compared with the compounds from tissue culture and with synthetic compounds.

5. Comparisons of the Isolated Metabolites and Synthetic Compounds. The aglycon, the methylated aglycon, and the methylated–acetylated aglycon from wheat plants cochromatographed on HPLC and on GC with F-16 (the tissue culture metabolite) and its corresponding derivatives, respectively (Table 1). The purified metabolite and its derivatives were also com-

Table 4. Uptake of SC-2053 by Wheat Callus Culture[a]

initial [^{14}C]SC-2053 in medium (uM)	radioactivity in fraction (dpm/gm f wt × 10^{-3})				total recovery (%)	concn in callus relative to medium (callus/medium)
	medium filtered (dpm/mL × 10^{-3})	extract	residue	extract + residue		
0.28	17	82	37	119	86	6.8
1.28	16	68	36	105	77	6.6

[a] The radioactivity in the medium, that extracted from the callus and the unextractable residue, were determined as described in the methods.

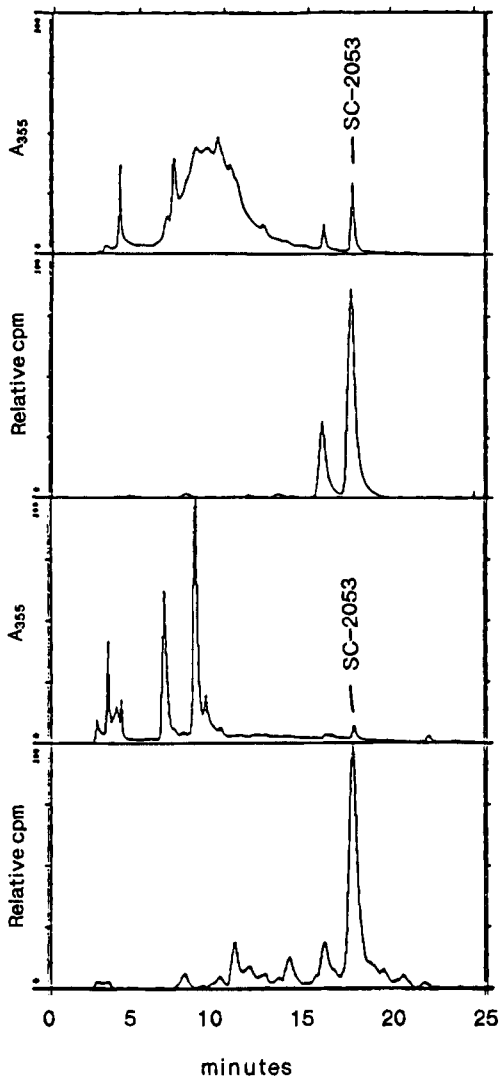


Figure 4. Metabolism of [^{14}C]SC-2053 by wheat callus suspension. Chromatography as in Table 1, system 1: (above) filtrate of culture medium; (below) extract of callus.

pared chromatographically (Table 1) with two potential structures prepared synthetically, SC-3095 (**II**) and SC-1231 (**IV**).

Comparison with SC-1231 (IV). This 5-OH compound would be the product of dealkylation at the base of the chain. Chromatography of SC-1231 shows it chromatographs at about t_R = 18.3 min, slightly *after* SC-2053. Comparisons of this peak with the profiles of the chromatograms of ^{14}C-labeled metabolites from tissue culture and wheat plants show less than 7% of the radioactivity eluted in this region in every instance.

Comparison with SC-3095 (II). This alcohol would be the product of demethylation within the methoxyethoxy chain of SC-2053. Purified F16 from tissue culture cochromatographs with SC-3095 by HPLC, and by TLC (Table 1). Furthermore, the methyl ester of this compound (SC-3095-ME, **VI**) cochromatographs with F16-ME on HPLC (Table 1). Since the methyl ester of SC-3095 (**VI**) did not give a peak in the GC (as for F16-ME, see above), F16-ME and **VI** were then acetylated. These derivatives, **VII**, and F16-ME-AC cochromatograph on HPLC and GC. By chromatography of the compounds and their derivatives, therefore, the aglycon from wheat (peak **B**), the metabolite from tissue culture medium filtrate (F-16), and SC-3095 are identical.

6. Chemical Identification of Metabolites. The acetylated methyl ester of the aglycon metabolite (16-ME-AC) and synthetic **VII** were analyzed by GC-MS. Their total ion chromatograms and GC mass spectra of these compounds were essentially identical. The data for 16-ME-AC are shown (Figure 5). The structure of the aglycon metabolite was also assigned as **II** on the basis of a comparison of the mass spectra obtained on **V** (Figure 5) and **VII**. The key fragments from the mass spectral fragmentation pattern of **VII** are shown in Figure 6. The parent ion of *m/e* = 416 indicated the loss of a carbon atom in the metabolism of **I**. The fragmentation pattern of **V** and **VII** were identical below *m/e* = 311, with the exception of a peak at *m/e* = 43 in the spectrum from **VII**, which was obtained by the partial loss of the acetyl group.

DISCUSSION

Transport. A foliar application of SC-2053 is sufficient to cause complete male sterility in wheat (Wong *et al.*, 1992). Under favorable conditions, a high percentage of the applied compound penetrates into the leaf where it is applied, but half to three-fourths of the compound taken up remains at the point of application. The remaining compound is transported upward in the plant. As much as 10–15% of the amount taken up through the leaf reaches the floral spike. It has been proposed that phloem mobility is an essential attribute for a CHA (Hsu and Kleier, 1990), but we did not investigate the mechanism of this transport.

Identification of Metabolites. Tissue cultures have proven very useful for research in xenobiotic metabolism (Mumma and Hamilton, 1979; Lewer and Owen, 1989; Winkler and Sandermann, 1989). Ease of purification makes the culture filtrate an ideal source for isolation and identification of xenobiotic metabolites. In our case the medium of a treated wheat suspension culture contained a substantial concentration of the initial major metabolite of SC-2053, identified by GC-MS as desmethyl-SC-2053 (**II**). Extracts of wheat plants and wheat callus treated with SC-2053 contained the same metabolite. In both callus and plant the glucoside **III** was a major metabolite of SC-2053.

II had been suggested as a possible metabolite before initiation of this research. The related compound, SC-1271, is metabolized by oxidation of its 5-propyloxy group (Cross *et al.*, 1992). Several other pollen suppressants in the phenylcinnoline carboxylate series with 5-alkoxy substituents were also metabolized by oxidation and formed β-glycoside-sensitive products, but phenylcinnoline carboxylates lacking 5-alkoxy side chains

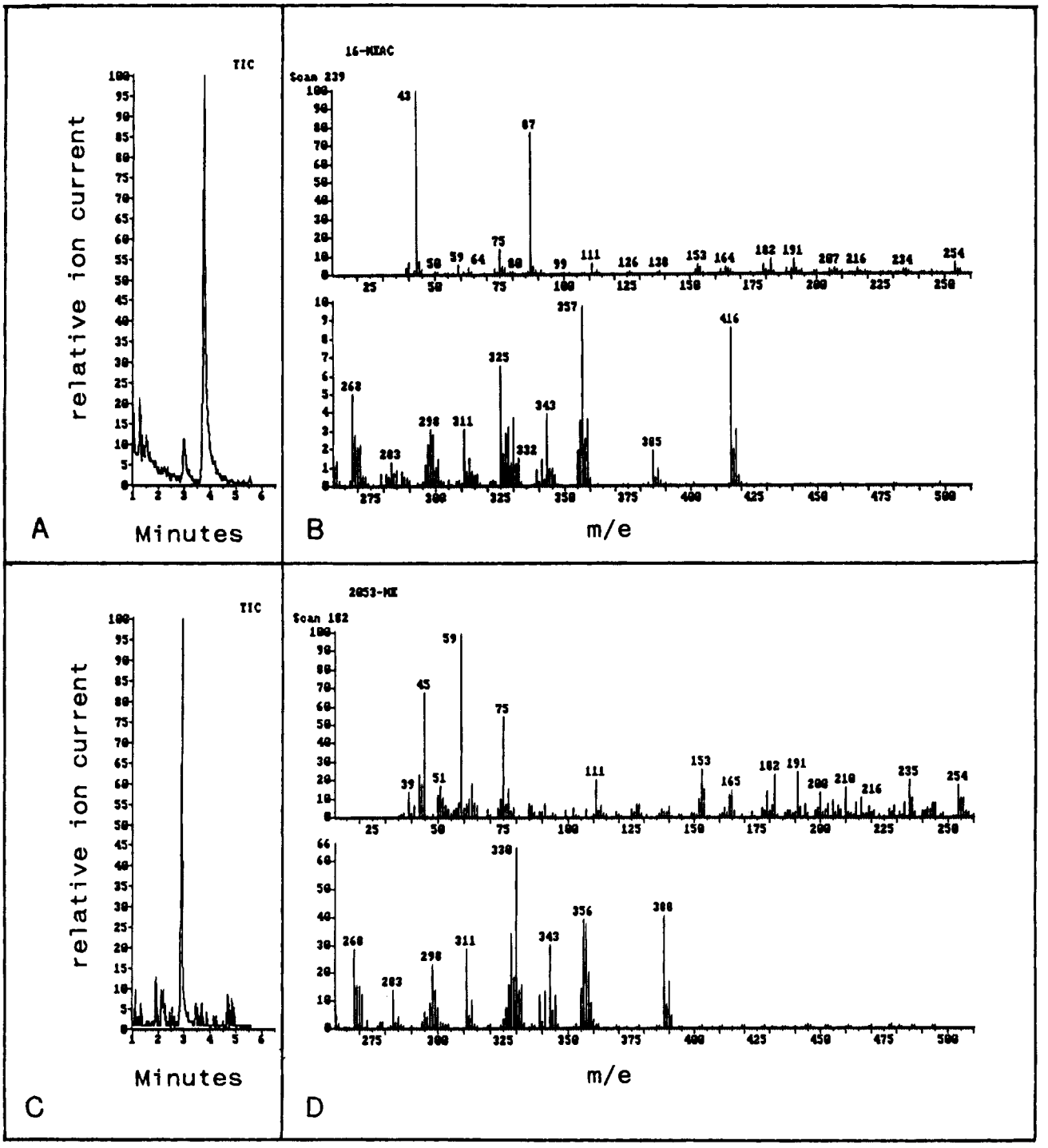


Figure 5. GC-MS of purified F16-ME-AC (A and B) and SC-2053 methyl ester (C and D): (A and C) chromatograms of the total ion current; (B and D) mass spectra of the F16-ME-AC peak eluting at 3.8 min (B), and of the SC-2053 methyl ester peak eluting at 2.9 min (C). The total ion chromatogram of synthetic **VII** and its mass spectrum are almost identical to that of F16-ME-AC.

were not rapidly metabolized (Cross and Herzmark, unpublished results). The most likely side-chain metabolite of SC-2053 would be a desmethyl (**II**), or a desalkyl (**IV**) derivative. Therefore, **II** and **IV** had been synthesized and were available for comparisons when the metabolites were isolated.

Digestion of peak **C** with β-glucosidase produced an aglycon (peak **B**) that eluted between **III** and SC-2053 on reversed-phase chromatography, so we presumed it to be intermediate in polarity. However, the 5-OH structure, **IV**, was more strongly retained on C-18 columns than SC-2053. This strengthened the view that the free hydroxyl would be on the side chain, rather than a ring. This was confirmed when **II** was shown by cochromatography and GC-MS to be identical to the hydroxyl compound from wheat callus culture filtrates.

Identity of the Glycoside. β-Glucosidase is not specific for β-glucosides and may hydrolyze other β-glycosides. Acyl glucosides are often partially degraded by both α- and β-glucosidases (Frear *et al.*, 1978). Our investigation does not rule out the presence of acyl glucosides.

Possible Mechanisms of Metabolism. Formation of O-glycosylated derivatives is a common fate for xenobiotic compounds in plants. These conjugates are formed in two distinct enzyme-catalyzed steps, (1)

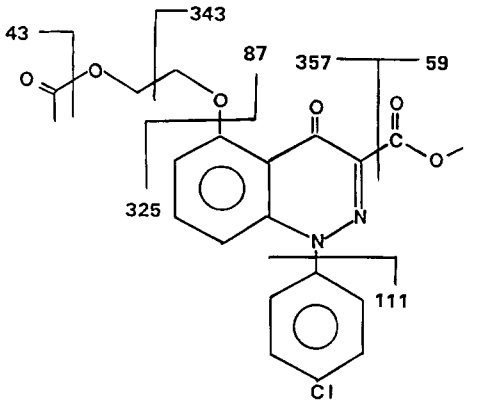


Figure 6. Mass spectral fragmentation pattern of **VII**.

oxidation, followed by (2) glycosylation. The oxidative step generally results from the action of a mixed-function oxidase, cytochrome P450, using NADPH as a substrate (Salaun *et al.*, 1981). Glycosylation is accomplished by a separate enzyme, usually a UDP-glucose glucosyltransferase, located in the cytoplasm (Lamoureux and Rusness, 1986).

Cytochrome P-450 in plants accounts for aliphatic and aromatic hydroxylation of many native and xenobiotic compounds (Salaun *et al.*, 1981; Borlakoglu and John, 1990; McFadden *et al.*, 1989). Like their mammalian counterparts, these reactions are catalyzed by specific isozymes. Enzymatic demethylation reactions have been shown in plant tissue cultures (Christinaki *et al.*, 1987). However, most examples deal with oxidation of methylated phenolics, rather than the oxidation of a methyl ether, as described here. Cytochrome P-450 from animals does catalyze O-demethylation of aliphatic ethers (Shoemaker *et al.*, 1982). Therefore, the mechanism of the initial oxidation of SC-2053 is uncertain.

Glycosylation of xenobiotics can occur rapidly after the hydroxylation of the substrate, with little free hydroxy compound present in the cell (Lamoureux and Rusness, 1986). In the callus culture, large amounts of **II** were found in the medium. Very little of the glucoside was formed by the callus, and this is found exclusively in the tissue extract. This suggests that the cell membrane is permeable to **II**, allowing it to leak out. In a suspension culture where the extracellular free space is large, the cellular concentration of **II** would remain low, effectively reducing its glycosylation. In the whole plant the extracellular volume is small. The fact that **III** does accumulate to high levels suggests that the portion of **II** subject to transport may be small. Direct measurement of the transport of **II** would be required to resolve this issue.

Relationship of Metabolism to CHA Activity. An ideal plant growth regulator would be metabolized rapidly after the temporal window for activity has expired. In the case of the phenylcinnolinecarboxylates this would correspond to the period of pollen exine wall development (Schulz *et al.*, 1993; Cross and Schulz, 1994). Compounds which are metabolized only in the leaves would be risky if they persist or accumulate in the growing zones of the plant. This would explain the phytotoxicity associated with application of pollen suppressants at inappropriate stages in the development of wheat plants (Cross and Ladyman, 1991). Our results show that SC-2053 does not accumulate in growing points other than the spike, and the metabolic pathway of this compound is identical for different parts of the plant.

LITERATURE CITED

Borlakoglu, J. T.; John, P. Cytochrome P-450-dependent metabolism of xenobiotics. A comparative study of rat hepatic and plant microsomal metabolism. *Comp. Biochem. Physiol.* **1989**, *94C*, 613–617.

Christinaki, H.; Bister-Miel, F.; Hammoumi A.; Bury, M.; Guignard, J. L.; Viel, C. Biotransformation of isopapaverine by *Silene alba* cell suspensions. *Phytochemistry* **1987**, *26*, 2991–2994.

Cross J. W.; Ladyman J. A. R. Chemical agents that inhibit pollen development: tools for research. *Sex. Plant Reprod.* **1991**, *4*, 235–243.

Cross, J. W.; Schulz, P. Chemical induction of male sterility. In *Pollen Biotechnology for Crop Production and Improvement*; Sawhney, V. K., Shivanna, K. R.; Cambridge University Press: New York, 1995; in press.

Cross, J. W.; Patterson, T.; Labovitz, J.; Almeida, E.; Schulz, P. J. Effects of chemical hybridizing agents SC-1058 and SC-1271 on the ultrastructure of developing wheat anthers (*Triticum aestivum* L. var. Yecora Rojo). In *Current Topics in Plant Physiology*; Lord, E., Bernier, G., Eds.; American Society of Plant Physiologists: Rockville, MD, 1989; Vol. 1, pp 187–188.

Cross, J. W.; Herzmark, P.; Guilford, W. L.; Patterson, T. G.; Labovitz, J. N. Transport and metabolism of pollen suppressant SC-1271 in wheat. *Pestic. Biochem. Physiol.* **1992**, *44*, 28–39.

Frear, D. S.; Swanson, H. R.; Mansager, E. R.; Wien, R. G. Chlorambem metabolism in plants: Isolation and identification of glucose ester. *J. Agric. Food Chem.* **1978**, *26*, 1347–1351.

Guilford, W. J.; Patterson, T. G.; Vega, R. O.; Fang, L. F.; Liang, Y.; Lewis, H. A.; Labovitz, J. N. Synthesis and pollen suppressant activity of phenylcinnoline-3-carboxylic acids. *J. Agric. Food Chem.* **1992**, *40*, 2026–2032.

Herzmark P. Optimizing Carbosorb and Permafluor V use by the Packard Sample Oxidizer Model 306. *Packard Perform. Note* **1989**, *20*, 1–2.

Hsu, F. C.; Kleier, D. A. Phloem mobility of xenobiotics. III. Sensitivity of unified model to plant parameters and application to patented chemical hybridizing agents. *Weed Sci.* **1990**, *38*, 315–323.

Lamoureux, G. L.; Rusness, D. G. Xenobiotic Conjugation in Higher Plants. *ACS Symp. Ser.* **1986**, *No. 299*, 62–105.

Lewer, P.; Owen, W. J. Amino acid conjugation of triclopyr by soybean cell suspension cultures. *Pestic. Biochem. Physiol.* **1989**, *33*, 249–256.

McFadden, J. J.; Frear, D. S.; Mansager, E. R. Aryl hydroxylation of diclofop by a cytochrome P450 dependent monooxygenase from wheat. *Pestic. Biochem. Physiol.* **1989**, *34*, 92–100.

Mumma, R. O.; Hamilton, R. H. Xenobiotic metabolism in higher plants: *In vitro* tissue and cell culture techniques. In *Xenobiotic metabolism: In vitro methods* Paulson, G. D., Frear, D. S., Marks, E. P., Eds. ACS Symposium Series 97; American Chemical Society: Washington, DC, 1979; pp 35–76.

Salaun, P.; Benveniste, I.; Reichart, D.; Durst, F. Induction and specificity of a cytochrome P-450 dependent laurate n-chain hydroxylase from higher plant microsomes. *J. Biochem.* **1981**, *119*, 651–655.

Sanders, D.; Slayman, C. L. Transport at the plasma membrane of plant cells: A review. In *Plant membrane transport*; Dainty, J., De Michelis, M. I., Marre, E., Rasi-Caldogno, F., Eds.; Elsevier: Amsterdam, 1989, pp 3–11.

Schulz, P. J.; Almeida, E. The effects of the chemical hybridizing agents (Sogetal, Inc., Hayward, CA) on the ultrastructure of developing wheat anthers (*Triticum aestivum* L. var. Yecora Rojo). *Am. J. Bot.* **1988**, *75*, part 2, 46 (abstr).

Schulz, P. J.; Cross, J. W.; Almeida, E. Chemical agents that inhibit pollen development: effects of the phenylcinnoline carboxylates SC-1058 and SC-1271 on the ultrastructure of developing wheat anthers (*Triticum aestivum* L. var. Yecora Rojo). *Sex. Plant Reprod.* **1993**, *6*, 108–212.

Shoemaker, D. D.; McManus, M. E.; Hoerauf, R.; Strong, J. M. Studies on the O-demethylation of misonidazole by rat liver microsomes. *Cancer Treat. Rep.* **1982**, *66*, 1343–1347.

Vesper, M. J.; Cross, J. W. A chemical pollen suppressant inhibits auxin-induced growth in maize coleoptile sections. *Plant Physiol.* **1990**, *92*, S-70.

Widholm, J. M. The use of fluorescein diacetate and phenosafranine for determining viability of cultures plant cells. *Stain Technol.* **1972**, *47*, 189–194.

Winkler, R.; Sandermann, H. Plant metabolism of chlorinated anilines: isolation and identification of N-glucosyl and N-malonyl conjugates. *Pestic. Biochem. Physiol.* **1989**, *33*, 239–248.

Wong, M.; Blouet, A.; Guckert, A. Effect of a chemical hybridizing agent on winter wheat. In *Proc. Congr. Eur. Soc. Agron.* **1992**, *2*, 152–153 (Scaife, A., Ed.).

Received for review June 1, 1993. Revised manuscript received November 15, 1994. Accepted November 30, 1994.[⊗] Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

JF930224J

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1995.