Synthesis and Herbicidal Activity of Cyperin

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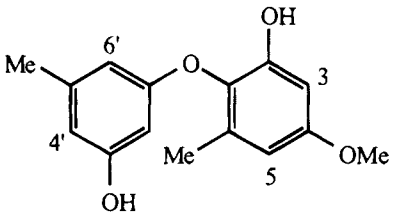
Cyperin is a phytotoxic diphenyl ether natural product. Total synthesis of cyperin has been achieved and its herbicidal activity evaluated. The synthesis is highlighted by an Ullmann coupling to construct the diphenyl ether moiety contained within cyperin. It was found that cyperin inhibited root growth of *Cyperus rotundus* grown on agar; however, root growth of *Cyperus* grown in soil was unaffected. Cyperin also inhibited growth of *Arabidopsis thaliana* and *Agrostis palustris* grown in agar. The mode of action of cyperin is different from that of commercial diphenyl ether herbicides that inhibit protoporphyrinogen oxidase.

Keywords: *Cyperin; herbicide; synthesis; phytotoxin; protoporphyrinogen oxidase; PROTOX*

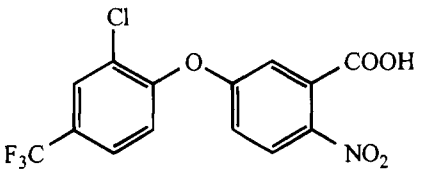
INTRODUCTION

Cyperin (Figure 1) is a natural product that has been isolated from fungal cultures of *Preussia fleischhakii* (Weber and Gloer, 1988), *Ascochyta cypericola* (Stierle et al., 1991), and *Phoma sorghina* (Venkaltsubbaiah et al., 1992). The latter two species are pathogens of purple nutsedge (*Cyperus rotundus*) and pokeweed (*Phytolacca americana*), respectively. The purified compound was shown to be phytotoxic to *C. rotundus* (Stierle et al., 1991) and several other plant species in a detached leaf assay (Venkaltsubbaiah et al., 1992).

Structure elucidation of cyperin revealed that it is a diphenyl ether (Stierle et al., 1991; Venkaltsubbaiah et al., 1992). Diphenyl ethers, a broad class of highly successful commercial herbicides exemplified by acifluorfen, oxyfluorfen, fomesafen, and lactofen, are known to elicit their herbicidal activity by inhibition of protoporphyrinogen oxidase (PROTOX), an enzyme in the porphyrin biosynthetic pathway [see review by Duke et al. (1991)]. The fact that cyperin was extremely phytotoxic to purple nutsedge, one of the world's most serious threats to crop plants, and its reported structure as a diphenyl ether peaked our interest in synthesizing and exploring the herbicidal utility of this compound. In this paper, we report the total synthesis of cyperin, its herbicidal activity, and results of the mode of action studies as a potential PROTOX inhibitor.



Cyperin



Acifluorfen

Figure 1. Structures of cyperin and acifluorfen.

MATERIALS AND METHODS

Materials. Commercial reagents were utilized without further purification. Anhydrous solvents were distilled before use.

General Experimental Procedures. Melting points are uncorrected and were determined in open capillary tubes using a Thomas-Hoover apparatus. ^{1}H NMR data were obtained with a Varian Unity 300 MHz instrument using residual solvent as an internal standard, and chemical shifts are reported in parts per million downfield from TMS. Data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and b, broad), coupling constant (hertz), integration, and assignment. ^{13}C NMR data were obtained with a Varian Unity 300 MHz instrument using residual solvent as an internal standard, and chemical shifts are reported in parts per million downfield from TMS. Data are reported as follows: chemical shift and assignment. Flash chromatography (Still et al., 1978) was performed on EM Reagents silica gel 60 (230–400 mesh). Mass spectral (MS) data were recorded on a Finnigan MAT PSQ 46 quadrapole spectrometer. Analytical thin layer chromatography (TLC) was performed with Merck 0.25 mm silica gel 60-F plates utilizing UV visualization.

Preparation of 3-(Benzyloxy)-5-hydroxytoluene (1). A 250 mL round-bottom flask was charged with 10.0 g (1.0 equiv) of anhydrous orcinol [prepared by refluxing orcinol monohydrate (Aldrich) in benzene with azeotropic removal of water for 3 h and removal of the benzene by concentration under reduced pressure] and 12.3 g (1.1 equiv) of potassium carbonate (K_2CO_3) in 100 mL of acetonitrile (MeCN), and 10.5 mL (1.1 equiv) of benzyl bromide (BnBr) was added. The reaction mixture was refluxed with stirring under nitrogen for 3 h, cooled, and vacuum filtered, and the filtrate was concentrated. Purification of the residue was carried out by flash column chromatography eluting with 10→20% ethyl acetate/hexanes gradient. Obtained was 4.3 g (25%) of **1** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.31–7.44 (m, 5H, Bn), 6.41 (s, 1H, Ar), 6.30 (s, 1H, Ar), 6.28 (s, 1H, Ar), 5.02 (s, 2H, CH_2), 4.91 (bs, 1H, OH), 2.27 (s, 3H, CH_3); TLC (20% EtOAc/hexanes) R_f = 0.25.

Preparation of 3-(Benzyloxy)-2-bromo-5-methoxytoluene (2). A 250 mL round-bottom flask was charged with 2.89 g (1.0 equiv) of 2-bromo-3-hydroxy-5-methoxytoluene (Cannon et al., 1971a,b) and 2.02 g (1.1 equiv) of K_2CO_3 in 50 mL of MeCN, and 1.74 mL (1.1 equiv) of BnBr was added. The reaction mixture was refluxed with stirring under nitrogen for 3 h, cooled, and vacuum filtered, and the filtrate was concentrated. Purification of the residue was carried out by flash column chromatography eluting with 5→10% ethyl acetate/hexanes gradient. Obtained was 3.72 g (91%) of **2** as a white solid: mp 45–48 °C; ^{1}H NMR ($CDCl_3$) δ 7.49 (d, 2H, J = 7.3 Hz, Bn), 7.31–7.42 (m, 3H, Bn), 6.44 (d, 1H, J = 2.7 Hz, Ar),

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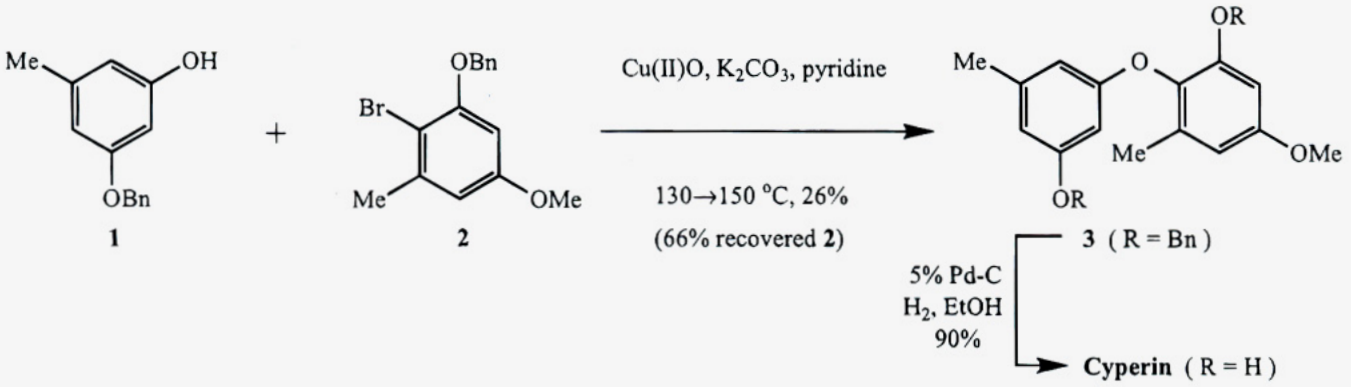

Figure 2. Synthesis of cyperin.

6.39 (d, 1H, J = 2.7 Hz, Ar), 5.12 (s, 2H, CH_2), 3.76 (s, 3H, OCH_3), 2.41 (s, 3H, CH_3); TLC (20% EtOAc/hexanes) R_f = 0.44.

Preparation of 2,3′-Bis(benzyloxy)-5′,6-dimethyl-4-methoxydiphenyl Ether (3). A 100 mL round-bottom flask was charged with 3.12 g (1.0 equiv) of **1**, 4.47 g (1.0 equiv) of **2**, and 4.23 g (2.1 equiv) of K_2CO_3 in 15 mL of pyridine. The mixture was incubated at 130 °C for 3 h, and 0.58 g (0.5 equiv) of copper(II) oxide (CuO) was added. The reaction mixture was heated at 150 °C with stirring under nitrogen for 72 h, cooled, diluted with diethyl ether, and vacuum filtered through Celite. The filtrate was concentrated under reduced pressure and the residue purified by flash column chromatography eluting with 5% ethyl acetate/hexanes. Obtained was 1.66 g (26%) of **3** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.12−7.39 (m, 10H, Bn), 6.47 (s, 1H, Ar′), 6.42 (d, 1H, J = 2.4 Hz, Ar), 6.38 (d, 1H, J = 2.4 Hz, Ar), 6.31 (m, 2H, Ar′), 5.12 (s, 2H, CH_2), 4.97 (s, 2H, CH_2), 3.77 (s, 3H, OCH_3), 2.28 (s, 3H, CH_3′), 2.17 (s, 3H, CH_3); MS m/z 458 (M^+); TLC (20% EtOAc/hexanes) R_f = 0.38.

Preparation of 2,3′-Dihydroxy-5′,6-dimethyl-4-methoxydiphenyl Ether (Cyperin). A Parr flask was charged with 0.49 g (1 equiv) of **3** in 25 mL of ethyl alcohol (EtOH), and 0.49 g (1 × wt equiv) of 5% palladium on carbon (Pd-C) was added. The reaction mixture was subjected to hydrogen at 50 psi for 4 h and the catalyst removed by vacuum filtration through Celite. The filtrate was concentrated and the residue purified by crystallization from petroleum ether/diethyl ether mixtures. Obtained was 0.26 g (90%) of cyperin as yellow needles: mp 116−117 °C [lit. (McGahren et al., 1970) mp 121.5−122.5 °C]; ^{1}H NMR ($CDCl_3$) δ 6.45 (d, 1H, J = 2.9 Hz, Ar), 6.26−6.35 (m, 3H, Ar, Ar′), 6.12 (s, 1H, Ar′), 5.51 (bs, 1H, OH), 5.32 (bs, 1H, OH), 3.77 (s, 3H, OCH_3), 2.22 (s, 3H, CH_3′), 2.06 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 158.1 (C-1′), 156.5 (C-4), 156.1 (C-3′), 148.6 (C-2), 140.6 (C-5′), 132.9 (C-1), 131.9 (C-6), 109.7 (C-4′), 107.6 (C-6′), 107.5 (C-5), 98.8 (C-2′), 98.7 (C-3), 54.9 (4-OCH_3), 20.9 (5′-CH_3), 15.7 (6-CH_3); MS m/z 261 (M^+); TLC (20% EtOAc/hexanes) R_f = 0.06.

Herbicidal Effects of Cyperin. Three *Cyperus* species, *rotundus*, *esculentus*, and *iria*, were evaluated under greenhouse conditions for the preemergence (PRE) and postemergence (POST) herbicidal effects of cyperin. PRE applications were made to a sandy loam soil containing 0.5% organic matter, and POST applications were made to the foliage of the emerged *Cyperus* growing in a commercial potting mixture. Cyperin was dissolved in 50/50 acetone/water with the addition of 0.25% v/v X77 nonionic surfactant. The cyperin solution was applied using a laboratory belt sprayer delivering 400 L/ha spray volume. Cyperin applications were made at two concentrations, 5 and 10 kg/ha. Visual observations were made several times during the study, and, at 42 days after application, the *Cyperus* was unearthed and root growth evaluated.

In an agar-based assay, *Arabidopsis thaliana*, *Agrostis palustris* (bent grass), and *C. rotundus* (purple nutsedge) were grown in 0.7% agar in continuous light at room temperature. Herbicidal effects of cyperin were rated on a scale of 0−9, where 0 means no effect and 9 is complete kill of the plant.

Cucumber Leaf Disk Leakage Assay. The assay was performed according to previously described procedures (Kenyon et al., 1985). Cucumber leaf disks (5 mm diameter) were incubated with various inhibitors in buffer containing 1 mM MES (pH 6.5) and 1% sucrose for 16 h in darkness. The disks were further incubated for 4 h in light. Conductivity measurements were done at various times during the experiment as indicated in Figure 6.

Figure 3. Herbicidal activity of cyperin on *C. rotundus*.

Table 1. Herbicidal Activity of Cyperin[a]

	concentration (μM)							
	1000	500	250	125	62.5	31.3	15.6	7.8
Arabidopsis	8	8	7	6	5	5	1	0
Agrostis	9	9	7	5	2	0	0	0

[a] Herbicidal effects were rated on a scale of 0−9, where 0 means no effect and 9 is complete kill of the plant.

RESULTS AND DISCUSSION

Synthesis. Although monobenzylated orcinol (**1**) has been prepared previously (Cannon et al., 1972), we found it more expedient to prepare this compound in one synthetic step. Water-free orcinol was treated with a slight excess of benzyl bromide (BnBr, 1.1 equiv) and potassium carbonate (K_2CO_3, 1.1 equiv) in refluxing acetonitrile (MeCN) for 3 h. The desired monobenzylated product **1** was prepared in multigram quantities and readily isolated by flash column chromatography in near statistical yield (25%) accompanied by minor amounts of starting orcinol as well as bisbenzylated product. The known 2-bromo-3-hydroxy-5-methoxytoluene was prepared according to the literature procedure (Cannon et al., 1971a,b) and subsequently benzylated under standard conditions [BnBr (1.1 equiv), K_2CO_3 (1.1

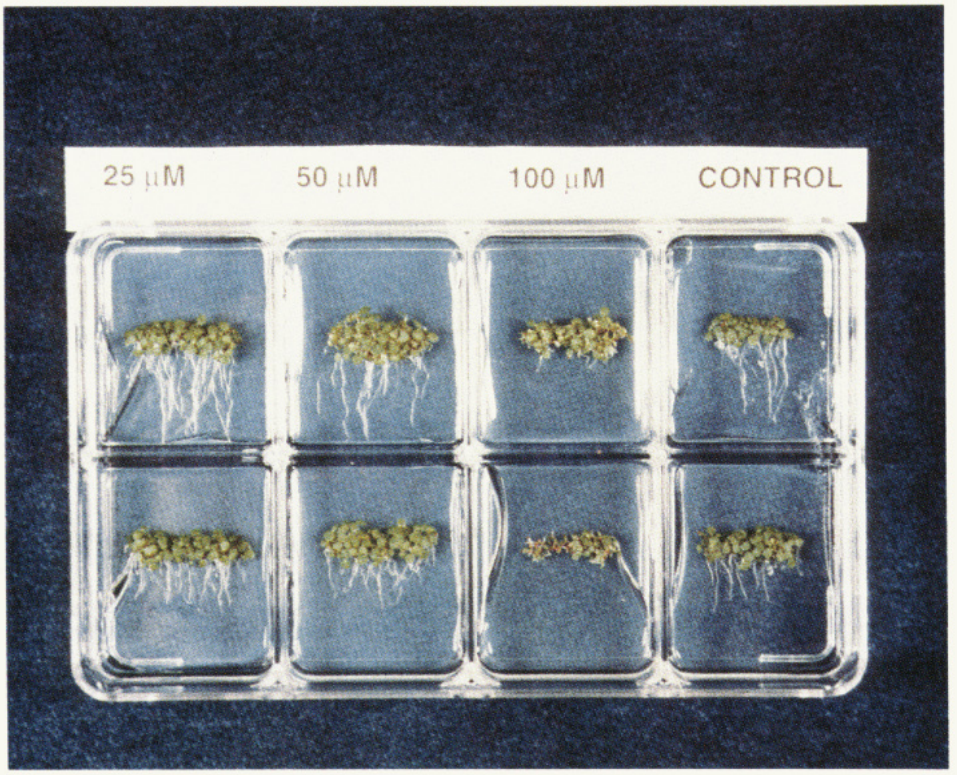

Figure 4. Herbicidal activity of cyperin on *A. thaliana*.

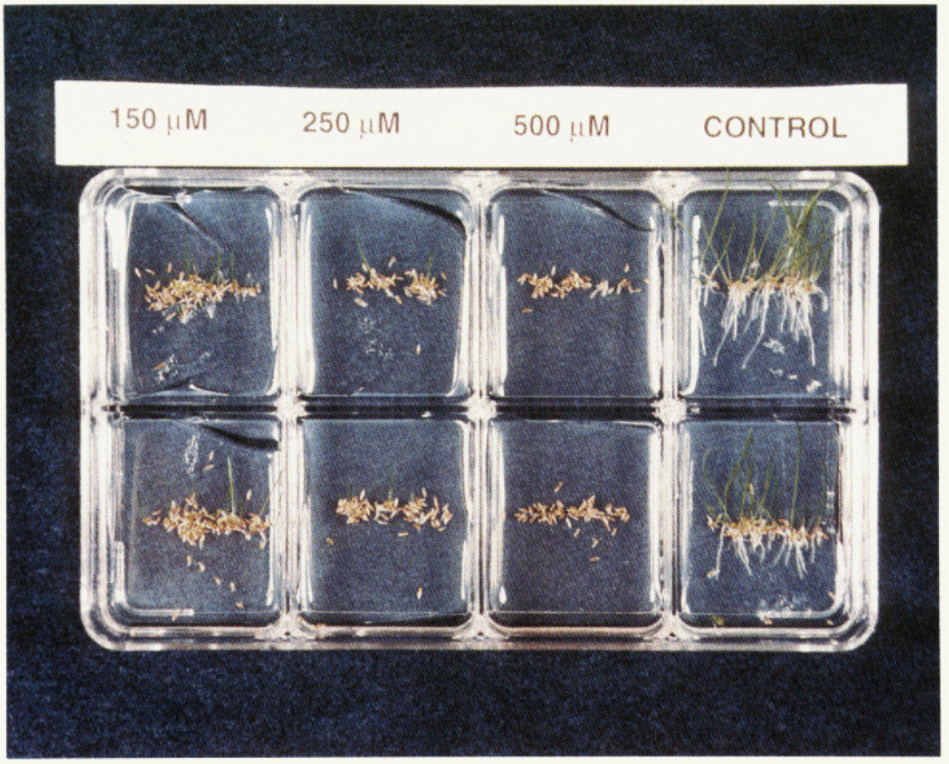

Figure 5. Herbicidal activity of cyperin on *A. palustris*.

equiv), MeCN, reflux] to afford the aryl bromide **2** (91%). Ullmann coupling of **1** and **2** has been investigated by other workers (Cannon et al., 1972) with the aid of copper bronze as the catalyst and potassium methoxide as the base under melt conditions to give the diphenyl ether **3**. We found it more convenient to employ the conventional conditions of copper(II) oxide (CuO, 0.5 equiv) as the catalyst and K_2CO_3 (2.1 equiv) as the base with pyridine as the solvent. The reaction mixture was heated at elevated temperature (130→150 °C) for 72 h and the product isolated by flash column chromatography. In this way, a slightly improved yield of **3** was readily obtained (26%) and, advantageously, the starting materials **1** and **2** (66%) could also be recovered from the reaction mixture [intermediates **1**–**3** have been further characterized than reported by Cannon et al. (1971a,b, 1972)].

Removal of the benzyl protecting groups of **3** was effected by hydrogenation over 5% palladium on carbon (Pd-C) in ethyl alcohol (EtOH) (see Figure 2). Synthetic

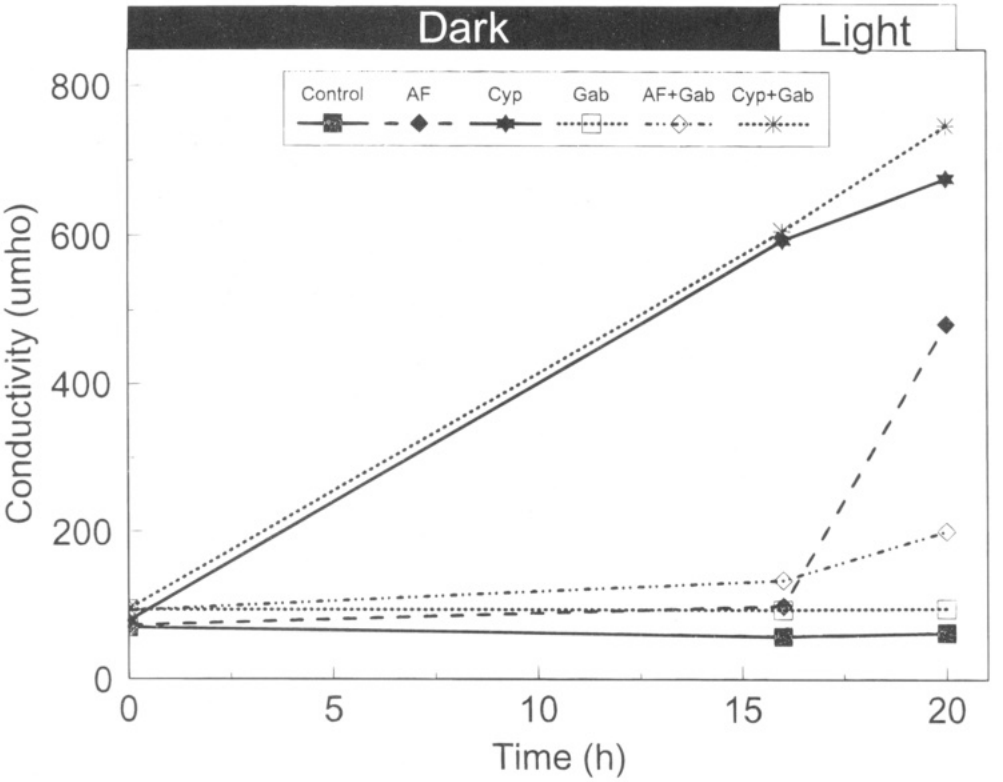

Figure 6. Comparison of the effect of 50 mM acifluorfen (AF), 1 mM cyperin (Cyp), or 330 μM gabaculine (Gab) on electrolyte leakage from cucumber disks. Disks were incubated in darkness for 16 h before exposure to light.

cyperin, identical in all respects to natural cyperin, was obtained (90%) as yellow needles upon crystallization from petroleum ether/diethyl ether mixtures and was used as technical grade material in herbicidal evaluation studies.

Herbicidal Effects of Cyperin. In greenhouse tests, both PRE and POST applications of cyperin at 5 and 10 kg/ha failed to cause any observable effects on either the foliage or the roots of any of the *Cyperus* species tested. The cyperin rates are believed to be sufficiently high to enable a compound of reasonable potency to demonstrate its biological activity. Lack of activity in the greenhouse prompted us to evaluate cyperin in an agar-based test. Interestingly, cyperin-treated plants were taller than the control (Figure 3) which, at first, suggested that cyperin was not herbicidal. However, examination of the roots revealed a significant inhibition of root growth at high rates of cyperin (greater than 500 mM). *Arabidopsis* and *Agrostis* were more sensitive to cyperin than *Cyperus* (Table 1; Figures 3, 4, and 5). The difference between root inhibition of *C. rotundus* when grown in agar versus natural soil may be a factor of concentration and/or availability and uptake.

Mode of Action of Cyperin. Since cyperin is a diphenyl ether, it is possible that cyperin exerts its herbicidal activity in a similar fashion as the diphenyl ethers that inhibit PROTOX. Therefore, we compared cyperin with acifluorfen to determine whether cyperin has a similar mode of action. Cucumber disk assay is a simple and quick way to evaluate PROTOX inhibitors (Kenyon et al., 1985). PROTOX inhibitors cause accumulation of high levels of protoporphyrin IX in the cytosol. Exposure of the treated disk to the light causes photoperoxidation of the membranes facilitated by protoporphyrin IX, resulting in leakage of electrolytes into the buffer. This process can be reversed by the addition of gabaculine to the medium. Gabaculine is a compound that inhibits an enzyme earlier in the pathway, thus preventing accumulation of protoporphyrin IX, and no significant leakage from the disks occurs.

As expected, acifluorfen caused electrolyte leakage in the light, which could be significantly reduced by including gabaculine in the medium. In contrast, cyperin caused cell leakage in the dark. Furthermore, addition of gabaculine did not stop the cell leakage (Figure 6). A visual examination of the disks revealed that cyperin-treated leaf disks were shrunken and did not resemble acifluorfen-treated disks. As stated earlier, PROTOX-inhibiting diphenyl ethers need light to be herbicidal. In a separate test, cyperin was herbicidal even in the absence of light (results not shown). Additionally, a cyperin sample was provided for an independent direct enzyme assay for PROTOX activity and an I_{50} = 60 μM was determined (S. O. Duke, U.S. Department of Agriculture, personal communication, 1994). This inhibition constant is very high and would not be sufficient for any herbicidal activity. These results suggest that the herbicidal activity of cyperin is not due to PROTOX inhibition *in vivo*.

ACKNOWLEDGMENT

Generous assistance from the Analytical, Physical, and Biochemical Research Section of the Agricultural Research Division of American Cyanamid is gratefully appreciated. Performance of the direct enzyme correlation study by S. O. Duke is also appreciated.

Supplementary Material Available: Reproductions of high-field ^{1}H NMR and ^{13}C NMR spectra for cyperin, a tabulation and comparison of the ^{13}C NMR spectrum of synthetic cyperin, and comparison with natural cyperin (3 pages). Ordering information is given on any current masthead page.

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Received for review August 1, 1994. Accepted December 14, 1994.[⊗]

JF940432R

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