Diphenyl Ether Herbicides Remarkably Elevate the Content in *Spinacia oleracea* of (*E*)-3-(4-Hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxy-3-methoxyphenyl)ethyl]-2-propenamide

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Spinach (*Spinacia oleracea*) leaves treated with the diphenyl ether herbicide bifenox and held in the light for 48–72 h contain up to 23 ppm of a new phenolic amide identified by ^1H and ^{13}C NMR, MS, and synthesis as (*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxy-3-methoxyphenyl)ethyl]-2-propenamide. The level of this amide is elevated by 5- to >50-fold in the light compared to in the dark following treatment of spinach leaves with any one of six ortho-substituted diphenyl ether herbicides and two *N*-aryl-3,4,5,6-tetrahydrophthalimides considered to have the same mechanism of herbicidal action. Two meta-substituted diphenyl ether herbicides and thirteen herbicides of other types are less effective or ineffective in elevating the level of this amide in a light-dependent manner. The compound discovered in spinach was not detected in 20 other crops and weeds even when treated with the diphenyl ether acifluorfen and held in the light.

We observed that spinach (*Spinacia oleracea*) leaves treated with diphenyl ether herbicides contain high levels of a UV-absorbing amide present in no more than trace amounts in normal spinach. The finding that this compound was not a herbicide metabolite but instead was a natural constituent prompted the studies reported here on its identity (I, Figure 1) and possible relationship to the herbicidal action of diphenyl ethers.

MATERIALS AND METHODS

Chemicals. The sources and designations of herbicides are given in Table I. [*nitrophenyl*-^{14}C]Bifenox (7.3 mCi/mmol) was provided by Mobil Chemical Co. (Edison, NJ). Intermediates for synthesis of amide I were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Treatment of Plants. A fresh spinach leaf (1.5–3 g), preconditioned by holding its 5-cm stem in 5 mL of water for 4 h, was transferred to 20 mL of water to which the herbicide had been added in 200 μL of methanol with mixing. An alternative treatment method after preconditioning involved injection of the herbicide solution in acetone or water into the stem at 10 μL of solution/g of fresh leaf weight. Controls in each case were treated with the solvent containing no herbicide. Treated leaves were held in the dark or under fluorescent lamps for 24–72 h and then removed, frozen, and stored at −20 °C until analyzed.

Seedlings (2–15-cm height) of six broadleaf weeds, six grass weeds, and eight crops were sprayed with 1.2- or 2.4-ppm solutions of acifluorfen in water containing 0.5% (v/v) Tween-20 or with the Tween-20 solution without herbicide. The 1.2-ppm acifluorfen solution was sprayed on the broadleaf weeds and the 2.4-ppm solution on the grass weeds and crops to yield doses approximating 0.25 and 0.5 kg/ha, respectively. Treated plants were held in the light or dark for 30 h (broadleaf weeds) or 45 h (grass weeds and crops) prior to recording the symptoms and cutting at the soil surface and holding the stems and leaves at −20 °C until analyzed.

Isolation of Amide I for Structure Elucidation. One thousand spinach leaves (2.6 kg) were treated with bifenox (stem injection of 100 μg in 50 μL of acetone/leaf) or MC 7783 (potassium salt) (stem absorption from a 200-ppm aqueous solution) and held 48–72 h. The extraction and purification procedures were as described below for analysis with the following variations: methylene chloride replaced ethyl acetate as the extraction solvent; the second TLC purification used benzene–acetone (2:1) (R_f 0.17); acetone was used to extract the product from the silica gel. The yield was 18 mg of a pale green gum rendered colorless on HPLC purification [10 μ-Porasil; chloroform–acetonitrile–ethanol (80:20:1)].

Synthesis of Amide I. To a stirred solution of (4-hydroxy-3-methoxyphenyl)ethylamine hydrochloride (1.0 g, 5 mmol), (*E*)-4-hydroxy-3-methoxycinnamic acid (0.97 g, 5 mmol), and triethylamine (0.51 g, 5 mmol) in dry methylene chloride (30 mL) and tetrahydrofuran (5 mL) was added dicyclohexylcarbodiimide (1.0 g, 5 mmol) in methylene chloride (15 mL). The mixture was stirred at 25 °C overnight, filtered through a pad of Celite, and evaporated to dryness. The resulting gum was taken up in ethyl acetate and water (1:1 v/v) (75 mL), and the aqueous layer extracted with further portions of ethyl acetate (3 × 15 mL). The combined extracts were washed with $NaHCO_3$ solution (×3), saturated NaCl solution, and dried over $MgSO_4$. Filtration and concentration in vacuo yielded crude amide I as a white solid (1.1 g, 65%), further purified by recrystallization from ethyl acetate containing 5% ethanol to yield white crystals: mp 172–173 °C; UV_{max} (0.1 N NaOH) 360 nm (ε 26 000); UV_{max} (methanol) 323 nm (ε 21 000); diacetate (acetic anhydride/sodium acetate) mp 135.5–137 °C.

This reaction was satisfactorily applied on a 0.1-mmol scale in the synthesis of amide 1 and its analogue from (3-hydroxy-4-methoxyphenyl)ethylamine.

Analysis of Amide I. The plants were cut into small pieces which were soaked in acetone (15 mL/g of plant) overnight in the dark. After filtration with suction, the leaf pieces were homogenized with granular anhydrous Na_2SO_4 (4 g/g of plant) using a mortar and pestle and than additional acetone (10 mL/g of plant) was added and the mixture further homogenized and filtered. The residue was homogenized again with acetone and filtered as above. The combined filtrates were evaporated, followed by partitioning between hexane (20 mL), water (20 mL), and acetone (8 mL). The hexane phase was discarded, and the aqueous acetone layer further extracted with hexane (20 mL × 2) and ethyl acetate (20, 15, and 15 mL). The combined ethyl acetate extracts were evaporated and subjected to TLC as 2.5-cm bands on 0.5 mm thickness

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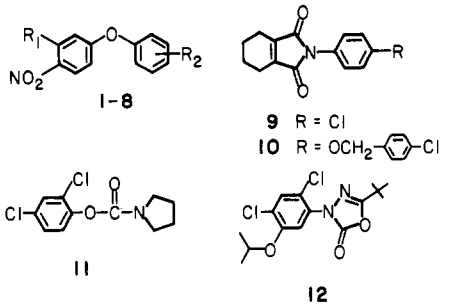
Table I. Content of Amide I in Spinach 48 h after Stem Injection with Various Herbicides at 50 ppm and Holding in the Light or in the Dark



no.[a]	compound name	substituents R_1	substituents R_2	amide, ppm dark	amide, ppm light
	Diphenyl Ethers				
	(A) Compounds with Meta Substituents Not Requiring Light for Activity				
1	TOPE	H	3-CH_3	<0.05	0.6
2	DMNP	H	3,5-$(CH_3)_2$	<0.05	<0.05
	(B) Compounds with Ortho Substituents Requiring Light for Activity				
3	nitrofen	H	2,4-Cl_2	0.3	1.6
4	fluorodifen	H	2-NO_2, 4-CF_3	<0.05	3.3
5	chlomethoxynil	CH_3O	2,4-Cl_2	<0.05	4.3
6	MC 7783	COOK	2,4-Cl_2	0.1	2.7
7	bifenox	$COOCH_3$	2,4-Cl_2	0.9	9.1
8	MC 10108	$COOCH_3$[b]	2-Cl, 4-CF_3	0.9	8.8
	Other Compounds				
	(A) Similar Action to Ortho-Substituted Diphenyl Ethers				
9	MK 616			0.1	1.9
10	MK 129			0.3	1.8
11	phenopylate				0.8
12	oxadiazon				0.1
	(B) Other Modes of Action				
13	alachlor			1.2	0.9
14	paraquat			0.8	1.2
15-23	others[c]				<0.05

[a] Sources: 1 and 2 synthesized for this study; 3 and 4 from Chem Service, Westchester, PA; 5 and 11 from Nihon Nohyaku Co., Ltd., Osaka, Japan; 6-8 from Mobil Chemical Co., Edison, NJ; 9 and 10 from Mitsubishi Chemical Industries, Ltd., Tokyo, Japan; 12 from Showa Rhodia Chemicals K.K., Tokyo, Japan; 13-23 from commercial sources. [b] The R_1 = -COONa analogue of 8 is acifluorfen. [c] Amitrole, atrazine, CDAA, chloroxuron, 2,4-D, dinoseb, diuron, EPTC, and pyrazon.

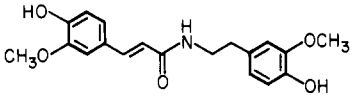


Figure 1. Structure of (*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxy-3-methoxyphenyl)ethyl)]-2-propenamide (amide I) from spinach leaves treated with diphenyl ether herbicides.

precoated TLC chromatoplates (silica gel 60 F-254, Merck) developed with chloroform–acetonitrile (4:1). Amide I was evident at R_f 0.14 as a UV-absorbing band when viewed at 254 nm and a blue fluorescent band at 366 nm. This UV-absorbing region of silica gel was extracted with 0.1 N NaOH (3, 2, and 2 mL) by using 1-min mixing and centrifugation to recover the supernatants. The combined alkaline extract was acidified with 1 N HCl (0.8 mL) and then extracted with ethyl acetate (4 mL × 3). After evaporation under N_2, a second TLC purification involved diisopropyl ether–acetone–methanol (13:6:1) (R_f 0.22). When necessary, a third TLC purification utilized benzene–acetone (3:2) (R_f 0.26).

Quantitative analysis of amide I purified as above was achieved in one of two ways. The preferred method, used in obtaining all tabulated data, involved adding the gel region following the two or three TLC purification steps to 0.1 N NaOH (3 mL), thorough mixing, centrifugation, and determination of the absorption spectrum of the soluble portion at 280–410 nm to check its purity in comparison with that of the synthesized material described above (Figure 2). Recoveries were essentially quantitative by this method. The absorbance at 360 nm, corrected for

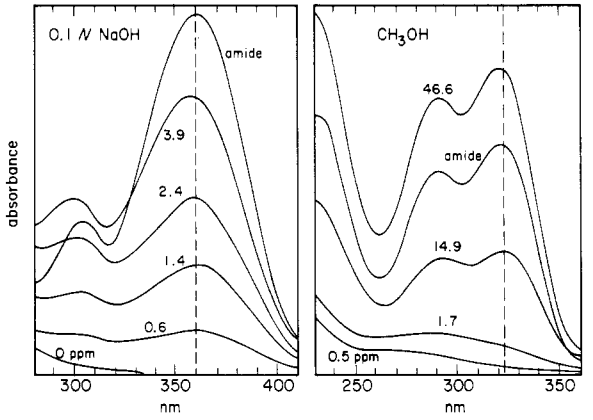


Figure 2. Absorption spectra in 0.1 N NaOH and methanol for amide I and purified extracts of treated spinach leaves. The spectra in 0.1 N NaOH are as follows: 0 ppm (TLC blank), 0.6 ppm (control plants), and 1.4, 2.4, and 3.9 ppm (treated plants 48 h after stem injection with phenopylate, MK-129, and fluorodifen, respectively). The spectra in methanol are for plants 0, 10, 20, and 30 h after placing stems in a 100-ppm solution of MC 7783 (free acid). The absorbances at 360 nm in 0.1 N NaOH and 323 nm in methanol are used in quantitating the amide concentration. Absorbances evident in these curves are not proportional to the amide ppm values since there were differences in the size of leaf samples analyzed.

a TLC silica gel blank, was compared directly to that of amide I. The alternative procedure utilized methanol at each stage of purification to extract the gel and finally methanol (4 mL) to determine product purity (230–360 nm) (Figure 2) and content of amide I (323 nm). This procedure required correction for recoveries during the two TLC steps which were 71, 95, 50, and 42% at 5, 10, 15, and 25 μg of amide, respectively. Amide I content is expressed as ppm relative to the plant fresh weight at the time of treatment.

RESULTS

Recognition and Identification of Amide I in Diphenyl Ether Treated Spinach. TLC analysis of a spinach leaf extract prepared 48 h after stem injection with [*nitrophenyl*-^{14}C]bifenox (5 ppm relative to leaf weight) revealed almost no metabolism of the bifenox (except for a small amount of hydrolysis) either in the light or in the dark. It was surprising, however, to observe on the F_{254} chromatoplates a band with strong UV quenching at 254 nm and fluorescence at 366 nm. This band was prominent for the leaf held in the light and absent or very faint for the leaf held in the dark. The UV-absorbing compound contained no ^{14}C and was therefore not a metabolite retaining the original diphenyl ether linkage or derived from the nitrophenyl moiety. It appeared possible that the UV-absorbing material was a spinach constituent enhanced in level by bifenox treatment and exposure to light.

Purification by preparative TLC and preliminary analysis of an impure sample indicated several aromatic protons and two aryl methoxy groups (^{1}H NMR) and at least one phenolic ring [blue-green $FeCl_3$ test and red shift (323 → 360 nm) in UV spectrum on basification]. In obtaining adequate material for high-resolution ^{1}H NMR and ^{13}C NMR, the extraction and chromatographic procedures involved the deliberate exclusion of methanol to avoid any possibility that phenolic methylation might occur during isolation. Eighteen milligrams of amide I was ultimately isolated from 2.6 kg of spinach treated with bifenox or MC 7783 (potassium salt), a content equivalent to 7 ppm.

Amide I from spinach showed strong IR bands at 3000 (wide skirt) and 1695 cm^{-1} (amide carbonyl) and UV_{max} 360 (0.1 N NaOH) and 323 (methanol). High-resolution MS revealed a strong molecular ion at *m/e* 343.1414 (calculated for $C_{19}H_{21}NO_5$: 343.1420). The gross skeletal structure of an *N*-phenylethyl cinnamide with each ring bearing a hydroxyl and a methoxyl substituent was obvious from the MS fragmentation pattern (Figure 3) in comparison with those of related compounds studied earlier (Rondest et al., 1968; Takemoto et al., 1975).

The high-resolution ^{1}H NMR spectrum revealed eight aromatic/vinylic protons at low field, two aryl methoxy groups, and four protons associated with two isolated methylene groups (Figure 3). The eight-proton resonance consisted of a pair of doublets (J = 15.6 Hz) flanking a six-proton multiplet. The multiplet was analyzed as two partially overlapping three-proton systems, each having one proton ortho coupled ($J \sim 8$ Hz), one proton meta coupled ($J \sim 1.8$ Hz), and one proton mutually ortho and meta coupled ($J \sim 8$ and ~ 1.8 Hz). ^{1}H NMR considerations alone were not adequate to assign the orientation of ring substituents of the individual cinnamoyl and (phenylethyl)amine residues. The lower field aromatic resonances of amide I had a marked similarity to those of 4-hydroxy-3-methoxycinnamic acid but not of 3-hydroxy-4-methoxycinnamic acid. Comparable similarities were not evident for the ^{1}H NMR spectra of (4-hydroxy-3-methoxyphenyl)ethylamine, (3-hydroxy-4-methoxyphenyl)-ethylamine, and amide I. Accordingly, the two amides

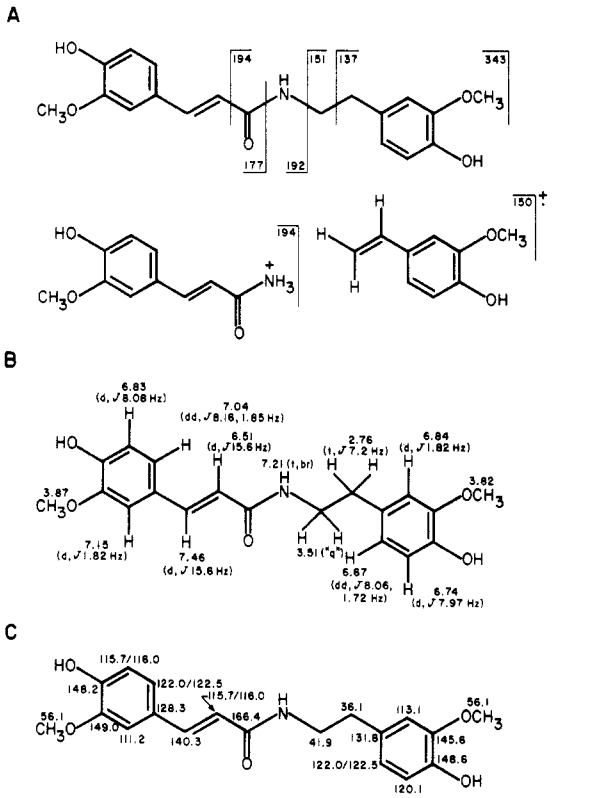


Figure 3. MS fragmentation and ^{1}H NMR and ^{13}C NMR assignments for amide I. (A) EI-MS (20 eV). (B) ^{1}H NMR (250 MHz, acetone-d_6, δ, tetramethylsilane). (C) ^{13}C NMR (62.8 MHz, acetone-d_6, δ, tetramethylsilane).

were synthesized by condensation of 4-hydroxy-3-methoxycinnamic acid with the appropriate (hydroxymethoxyphenyl)ethylamines in the presence of dicyclohexylcarbodiimide. The ^{1}H NMR spectrum of the synthetic amide derived from (4-hydroxy-3-methoxyphenyl)ethylamine was totally superimposable on that of the plant-derived material ($\Delta\delta < 0.005$), while the spectrum of the compound derived from (3-hydroxy-4-methoxyphenyl)-ethylamine was essentially superimposable except for the resonances due to the phenylethylamine aromatic protons. Synthetic amide I had identical ^{13}C NMR ($\Delta\delta < 0.1$) with the plant product (Figure 3). Tentative ^{13}C NMR assignments for the amide are based upon comparisons of its ^{13}C NMR spectrum with those of the diacetate and ferulic acid and consideration of the assigned spectrum of vanillin (Levy et al., 1980) and the calculated shifts for the amide (Ewing, 1979).

Effect of Herbicides on Content of Amide I in Spinach and Other Plants. The relationships of the amide content in spinach to diphenyl ether concentration and treatment time are shown in Figure 4. The amide content is increased at levels as low as 5–10 ppm of MC 7783 (free acid) with a treatment time of 26 h. The magnitude of increase is more than 20-fold in the light with a much smaller increase in the dark. Formation of amide I is almost linear with time, reaching 22–32 ppm over a 72-h period of exposure to 10 ppm of MC 7783 (free acid) or bifenox.

Spinach 48 h after stem injection with 50 ppm of six ortho-substituted diphenyl ether herbicides (**3–8**, Table I) produced 5- to >50-fold more amide in the light than in the dark. Diphenyl ethers **1** and **2** with meta substituents were less effective. *N*-Aryl-3,4,5,6-tetrahydrophthalimides **9** and **10** were as effective as diphenyl ether **3** in inducing amide formation. Other herbicides had lower activity (**11**, **12**, and **15–23**) or, as with alachlor (**13**) and

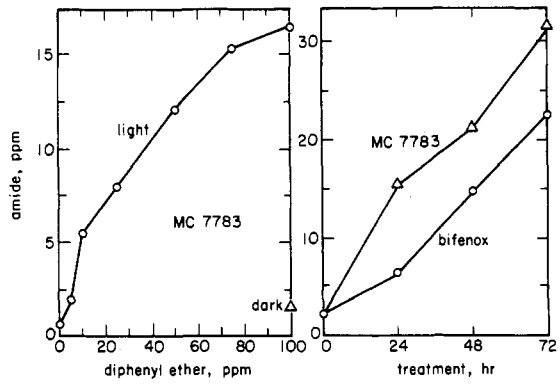


Figure 4. Relationship of amide I content in spinach to MC 7783 (free acid) concentration with treatment time of 26 h and to treatment time with MC 7783 (free acid) and bifenox concentration of 10 ppm.

paraquat (14), produced small amounts of the amide in both the light and the dark. The TLC characteristics and absorption spectrum of the amide were identical as induced by all compounds examined, e.g., fluorodifen, MK-129, and phenopylate (Figure 2).

The presence of this amide or diphenyl ether enhancement of its level appears to be specific for spinach. Thus, the amide content was less than 0.5 ppm for the following plants [with the indicated acifluorfen dose levels, time of exposure, and magnitude of symptoms in the light (none showed symptoms in the dark)]: broadleaf weeds (0.25 kg/ha; 30 h) *Abutilon theophrasti* (velvetleaf) (+), *Amaranthus retroflexus* (redroot pigweed) (+), *Brassica kaber* (wild mustard) (±), *Datura stramonium* (jimsonweed) (+), *Ipomoea purpurea* (tall morning glory) (+), and *Solanum nigrum* (black nightshade) (+); grass weeds (0.5 kg/ha; 45 h) *Avena fatua* (wild oats) (−), *Bromus tectorium* (downy brome) (+), *Cyperus esculentus* (yellow nut sedge) (+), *Echinochloa crus-galli* (water grass) (+), *Lolium multiflorum* (annual ryegrass) (±), and *Setaria lutesens* (yellow foxtail) (±); crops (0.5 kg/ha; 45 h) *Beta vulgaris* (sugar beets) (+), *Glycine max* (soybean) (+), *Gossypium hirsutum* (cotton) (+), *Oryza sativa* (rice) (±), *Sorghum bicolor* (sorghum and milo) (+), *Triticum vulgare* (wheat) (−), and *Zea mays* (corn) (+).

DISCUSSION

This study appears to be the first report of amide I in spinach or other plants. The (4-hydroxy-3-methoxyphenyl)ethylamine moiety is known to be present in higher plants but not until now condensed with the commonly occurring ferulic acid. The positions of the hydroxy and methoxy substituents in amide I are those most commonly encountered in naturally occurring phenylethylamine and cinnamic acid derivatives. Related botanicals are known with the *N*-phenylethyl cinnamide skeleton and other combinations of hydroxy, methoxy, and methylenedioxy substituents (Rondest et al., 1968; Smith, 1977; Takemoto et al., 1975). There are also natural amides derived from condensation of cinnamic acids with other amines, e.g., putrescine, spermidine, and spermine (Samborski and Rohringer, 1970; Martin-Tanguy et al., 1978, 1979).

Several lines of evidence associate amide I with spinach and the diphenyl ether type of action. Diphenyl ether herbicides increase the level of this amide in spinach by 5- to >50-fold. Twenty other plants examined probably do not contain this amide even with diphenyl ether treatment. The most effective compounds are ortho-substituted diphenyl ethers that require light for their herbicidal action. Two *N*-aryltetrahydrophthalimides that also require light to give herbicidal effects apparently identical with those of the diphenyl ethers (Matsunaka, 1976, 1979) also result in enhanced amide levels. Meta-substituted diphenyl ethers that do not require light for their herbicidal action are less effective in elevating the amide levels. The amide per se is probably not involved directly or indirectly in the herbicidal action of diphenyl ethers since the amide content is significantly elevated by diphenyl ether concentrations that have little or no visible effect on the spinach. However, knowledge of the mechanism by which diphenyl ethers elevate the content of this amide may be relevant to understanding the biochemical basis for their inhibition of weed growth.

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