soil dissipation rates were the same for both immediate and delayed incorporation.

Application of pesticides under minimum-till practices may result in considerable pesticide loss (4–65% depending upon their vapor pressure) through volatilization unless the pesticide is protected by formulation or incorporation.

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Registry No. Butylate, 2008-41-5; heptachlor, 76-44-8; lindane, 58-89-9; dieldrin, 60-57-1.

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Diphenyl Ether Herbicides and Related Compounds: Structure–Activity Relationships as Bacterial Mutagens

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Several 4-nitrodiphenyl ether herbicides and related compounds or their nitroso, hydroxyamino, or amino derivatives are mutagens or promutagens in the *Salmonella typhimurium* (strain TA100)/microsome (S9) assay. Six of eleven 4-nitrodiphenyl ethers examined with no 3-substituent are direct-acting mutagens (4–9 revertants/nmol) and ten of eleven of the analogous 4-aminodiphenyl ethers (including those of the herbicides fluorodifen, nitrofen, and CNP) are mutagens (1–30 revertants/nmol) but only on metabolic activation (+S9). Herbicides with a 3-substituent (i.e., acifluorfen, acifluorfen-methyl, bifenox, bifenox free acid, chlomethoxynil, and oxyfluorfen) and their amino derivatives are generally not detected as mutagens (±S9). However, nitroso- and (hydroxyamino)nitrofen, nitroso-CNP, nitrosooxyfluorfen, and nitrosoacifluorfen are direct-acting mutagens (2–7 revertants/nmol). Rats reduce orally administered nitrofen, CNP, oxyfluorfen, acifluorfen, acifluorfen-methyl, and bifenox, presumably via nitroso and hydroxyamino intermediates, to the amino compounds that are excreted in the feces.

The herbicide nitrofen (Figure 1) is a teratogen (Gray et al., 1982) and carcinogen (Milman et al., 1978). It is also a promutagen in the *Salmonella typhimurium* assay, undergoing photochemical and metabolic activation to the nitroso and hydroxylamino derivatives (Draper and Casida, 1983). Aminonitrofen is both a promutagen and potent bactericide to *S. typhimurium* (Draper and Casida, 1983). This amino derivative also is an intermediary metabolite of nitrofen in rats (Costlow and Manson, 1983). These observations suggest that nitro reduction may play a role in some of the adverse toxicological properties of nitrofen. The relevance of these findings to related 4-nitrodiphenyl ethers, including other commercial herbicides, is not known.

This study examines the structure–activity relationships for bacterial mutagenesis and toxicity of nitrodiphenyl ethers and their nitroso, hydroxyamino, and amino derivatives tested in the *S. typhimurium* assay with and without metabolic activation. It also consideres the metabolism in rats of selected nitrodiphenyl ether herbicides to evaluate the possible presence of the nitroso, hydroxyamino, and amino derivatives as metabolites in mammals.

MATERIALS AND METHODS

Spectroscopy. Chemical ionization mass spectra (CI-MS) (70 eV, 0.8 torr of methane, solid probe) were recorded with a Finnigan Model 3200 instrument interfaced to a System Industries 150 data system. Proton nuclear

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Table I. Melting Points and ^{1}H NMR and CI-MS Data for 4-Nitrodiphenyl Ethers and Their Nitroso, Hydroxyamino, and Amino Derivatives

substituents	mp, °C	^{1}H NMR (acetone-d_6), chemical shifts, δ [a] $H_{3,5}$ (d)	$H_{2,6}$ (d)	CI-MS, m/e (%) [b] M + 1	M + 29	other
			4-Nitro			
none	55-57	8.26	7.12	100	7	
4′-F	71-73	8.25	7.12	100	8	
4′-Cl	74-75	8.27	7.17	100	10	
4′-Me	63-64	8.24	7.09	100	9	
4′-CN	142-145	8.33	7.32	100	11	
3′-Me	58-60	8.25	7.11	100	13	
3′,5′-Me_2	81-82	8.24	7.10	100	13	
2′-NO_2,4′-CF_3	86-88	8.37	7.43	100	17	309 (4),[c] 133 (65), 132 (87)
2′-NH_2,4′-CF_3		8.28	7.16	100	14	279 (32)[c]
2′,4′-Cl_2	68-70	8.22[d]	6.96[d]	100	11	
2′,4′,6′-Cl_3	107-108	8.20[d]	6.90[d]	100	11	
			4-Nitroso			
2′,4′-Cl_2		7.92[d]	7.03[d]	100	11	
2′,4′,6′-Cl_3		7.91[d]	6.97[d]	100	10	
			4-Hydroxyamino			
2′,4′-Cl_2		6.93[d]	7.01[d]	[e]		
			4-Amino			
none	81-82	6.69	6.78	100	16	108 (45),[f] 93 (78)[g]
4′-F	51-52	6.68	6.77	100	4	184 (36),[c] 108 (78),[f] 93 (55)[g]
4′-Cl	96-98	6.70	6.79	100	4	184 (41),[h] 108 (90),[f] 93 (55)[g]
4′-Me	123-125	6.67	6.75	91		108 (100)[f]
4′-CN	105-107	6.74	6.85	100	22	
3′-Me	71-73	6.68	6.76	100	18	108 (56)[f]
3′,5′-Me_2	42-43	6.67	6.75	100	9	
2′-NO_2,4′-CF_3		6.77	6.94	3		279 (8),[c] 108 (100)[f]
2′-NH_2,4′-CF_3		6.70	6.80	14		249 (22),[c] 108 (100)[f]
2′,4′-Cl_2	54-55	6.68[d]	6.85[d]	100	4	121 (36), 108 (50),[f] 93 (51)[g]
2′,4′,6′-Cl_3	67-70	6.61 (s)[d]		15		121 (39), 108 (16),[f] 93 (100)[g]

[a] J for $H_{3,5}$ and $H_{2,6}$ = 9.2-9.3 for 4-nitro compounds, 8.8-9.1 for 4-nitroso derivatives, 9.1 for the 4-hydroxyamino compound, and 8.8-9.0 for the 4-amino derivatives. [b] Intensity relative to base peak. [c] $(M - F)^+$. [d] Chloroform-d. [e] Trimethylsilyl derivative gave M + 1 (14), M^+ (14), M − 15 (9), and M − 35 (5). [f] p-Quinonimine ion (C_6H_6NO). [g] Anilinium ion (C_6H_7N). [h] $(M - Cl)^+$.

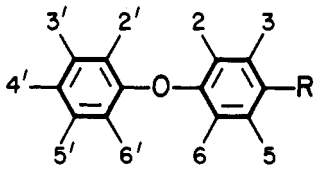
magnetic resonance (^{1}H NMR) spectra were determined with the UCB-250 (Chemistry Department, University of California, Berkeley) instrument at 250 MHz with samples dissolved in various deuterated solvents utilizing the proton-containing contaminant, e.g., acetone-d_5, as the internal reference (Tables I and II).

Chemicals. Structures and designations for the compounds are given in Figure 1. Herbicides were obtained from the following sources; acifluorfen, bifenox, and bifenox acid from Mobil Chemical Co. (Edison, NJ); fluorodifen from Chem Service (Westchester, PA); technical nitrofen (95.7%, TOK) [recrystallized from ethanol and then methanol–ethanol (9:1)] and oxyfluorfen (98.4%) from Rohm and Haas Co. (Philadelphia, PA); chlomethoxynil from Nihon Noyaku Co., Ltd., Osaka, Japan; CNP from Sankyo Co., Ltd., Tokyo, Japan. Acifluorfen-methyl was prepared by refluxing acifluorfen in methanol–H_2SO_4. Other chemicals were synthesized as outlined below to give melting points and NMR and MS data reported in Tables I and II.

Syntheses and Spectral Features. *Nitrodiphenyl Ethers.* A mixture of 4-chloronitrobenzene (50 mmol), a substituted phenol (120 mmol), KOH (120 mmol), powdered Cu (1.2 mmol), and water (33 mmol) was heated under reflux at 200 °C for 16–24 h (Sittig, 1977). Coupling proceeded slowly for monosubstituted phenols with ~50% isolated yields after 24 h, but this condition was not suitable for 2,4,6-trichlorophenol. The reaction mixture, usually a black tarry material, was dissolved in dichloromethane, washed with aqueous Na_2CO_3 (5% w/v) to remove unreacted phenols, and dried ($MgSO_4$). The nitrodiphenyl ethers were recovered by distillation off of unreacted chloronitrobenzene (bp ~65 °C/0.03 mmHg), crystallization, and, when necessary, treatment with activated charcoal for decolorization. Attempts were made to minimize colored byproducts by either eliminating the Cu catalyst or further washing the products with aqueous HCl, but neither procedure was successful. Preparative thin-layer chromatography (TLC) (silica gel, carbon tetrachloride) was used to obtain the pure 4′-CN and 4′-Me derivatives.

Aminodiphenyl Ethers. The nitro compound (1.0 g) in absolute ethanol (10 mL) was stirred vigorously with platinum(IV) oxide monohydrate catalyst (20 mg) under 1 atm of H_2 at room temperature for 18–24 h (Adams and Cohen, 1944). The amines were isolated in ~90% yield by TLC of the methylene chloride soluble products, using silica gel with ether for the amino derivatives of bifenox acid and acifluorfen and carbon tetrachloride–ether (3:1) in other cases. The amines chromatographed with mobilities of 0.2–0.8 relative to those of the nitro compounds except for the anthranilic acid analogues, aminobifenox acid and aminoacifluorfen, in which case the mobility of the amino compound exceeded that of the parent aryl nitro compound due to intramolecular hydrogen bonding. The amines autooxidized to colored derivatives when sorbed on silica gel for extended time periods. The amino derivatives of bifenox, bifenox acid, acifluorfen, and acifluorfen-methyl fluoresced blue under 360-nm light.

As an alternative, the nitro compounds were reduced with powdered Fe in refluxing ethanolic HCl (Draper and Casida, 1983) and the amines isolated in ~30% yield by



4-Nitrodiphenyl ethers (R = NO_2)

Unsubst. 4′-F 4′-Cl 4′-Me

4′-CN 3′-Me 3′,5′-Me_2

fluorodifen 2′-NO_2,4′-CF_3

nitrofen 2′,4′-Cl_2

CNP 2′,4′,6′-Cl_3

3-Substituted-4-nitrodiphenyl ethers (R = NO_2)

chlomethoxynil 3-MeO, 2′,4′-Cl_2

oxyfluorfen 3-EtO, 2′-Cl,4′-CF_3

bifenox acid 3-COOH, 2′,4′-Cl_2

acifluorfen 3-COOH, 2′-Cl,4′-CF_3

bifenox 3-COOMe, 2′,4′-Cl_2

acifluorfen-methyl 3-COOMe, 2′-Cl,4′-CF_3

Figure 1. 4-Nitrodiphenyl ethers and their nitroso, hydroxyamino, and amino derivatives. The names refer to 4-nitrodiphenyl ether herbicides.

crystallization or TLC. The low yields were due, in part, to competing reductive dehalogenation, i.e., nitrofen yielded significant amounts of dechlorinated amines. Reduction of fluorodifen yielded three amino derivatives: the 2′-amino analogue (R_f 0.52) and the 4-amino and 2′,4-diamino compounds (R_f ~0.14). The low-R_f fluorodifen amines were separated on three TLC developments with carbon tetrachloride–ether (3:1) with the diamine being slightly more polar.

4-Acetylamino-CNP was prepared by treatment of amino-CNP with acetylchloride and triethylamine in toluene: NMR (chloroform-*d*) δ 7.395 (d, 2 H), 7.39 (s, 1 H), 6.76 (d, 2 H), 2.14 (s, 3 H); (M + 1) 330 (24%).

(Hydroxyamino)diphenyl Ethers. Nitrofen and bifenox were converted to their respective hydroxyamines in ~80% yield by reduction with Zn and NH_4Cl in ethanol at room temperature under N_2 or Ar (Draper and Casida, 1983). The hydroxyamines were isolated by preparative TLC (silica gel, carbon tetrachloride–ether, 3:1) at ~5 °C. This procedure was not successful when applied to oxyfluorfen and chlomethoxynil.

Nitrosodiphenyl Ethers. Nitrosonitrofen, -CNP, -oxyfluorofen, and -acifluorfen-methyl were prepared by oxidation of the arylamines with *m*-chloroperoxybenzoic acid (MCPBA) in methylene chloride for 4 h at 0 °C (Okazaki et al., 1969). The products were isolated by washing with aqueous Na_2CO_3 and TLC of the organosoluble materials. The nitroso and nitro analogues were readily separated on aluminum oxide TLC with hexane–ether and chloroform–ether mixtures but not on silica gel chromatoplates. The nitroso derivatives were pale green or blue-green and reacted with pentacyanoammineferroate (PCAF) giving blue complexes (Draper and Casida, 1983).

Nitrosoacifluorfen was prepared by treatment of aminoacifluorfen (100 mg) with MCPBA (100 mg) in methylene chloride (6 mL) to 0 °C for 1 h. The mixture was stored at −65 °C and, as needed, the product was isolated by two TLC purifications at 5 °C (silica gel, ether–methanol, 4:1). R_f values for acifluorfen and its amino and

Table II. Melting Points and ^{1}H NMR and CI-MS Data for 3-Substituted 4-Nitrodiphenyl Ethers and Their Nitroso, Hydroxyamino, and Amino Derivatives

substituents	mp, °C	^{1}H NMR (acetone-d_6), chemical shifts, δ, and coupling constants, *J*, Hz			CI-MS, *m/e* (%)[a]		
		H_2 (d)	H_5 (d)	H_6 (dd)	M + 1	M + 29	other
4-Nitro							
3-MeO,2′,4′-Cl_2	111–113	6.96, *J* = 2.5	7.93, *J* = 9.0	6.55, *J* = 9.0, 2.5	100	12	
3-EtO,2-Cl,4′-CF_3	82–83	7.05, *J* = 2.5	7.95, *J* = 9.0	6.68, *J* = 9.0, 2.5	100	17	342 (16)[b]
3-COOH,2′,4′-Cl_2	165–167	7.32, *J* = 2.7	8.08, *J* = 8.9	7.25, *J* = 8.9, 2.8	1		310 (3),[c] 65 (100)
3-COOH,2′-Cl,4′-CF_3	163–165	7.46, *J* = 2.8	8.13, *J* = 8.9	7.38, *J* = 8.9, 2.8	31	5	344 (100)[c]
3-COOMe,2′,4′-Cl_2	86–88	7.30[d]	7.29, *J* = 9.6	7.29, *J* = 10, 2.8	11	6	310 (64),[c] 215 (100)
3-COOMe,2′-Cl,4′-CF_3	89–92	7.39[d]	8.17, *J* = 8.3, 0.9[e]	7.37, *J* = 2.7[d]	47	16	356 (26),[b] 344 (100)[c]
4-Nitroso							
3-EtO,2′-Cl,4′-CF_3		6.80, *J* = 2.2[f]	6.39, *J* = 9.1[f]	6.24, *J* = 9.0, 2.3[f]	100	12	326 (28)[b]
3-COOH,2′-Cl,4′-CF_3		6.81, *J* = 2.5[g]	6.50, *J* = 9.6[g]	6.81, *J* = 9.7, 2.7[g]			
3-COOMe,2′-Cl,4′-CF_3		7.44, *J* = 2.4	7.21, *J* = 8.8	7.30, *J* = 8.8, 2.5	100		340 (20),[b] 199 (48), 187 (40)
4-Hydroxyamino							
3-COOMe,2′,4′-Cl_2		7.49, *J* = 2.7	7.46, *J* = 9.3	7.28, *J* = 2.8[d]			
4-Amino							
3-MeO,2′,4′-Cl_2	oil	6.64, *J* = 2.5	6.71, *J* = 8.4	6.43, *J* = 8.4, 2.5	50	1	123 (100)[h]
3-EtO,2′-Cl,4′-CF_3	69–71	6.70, *J* = 2.5	6.78, *J* = 8.4	6.53, *J* = 8.4, 2.5	67	5	312 (69),[b] 137 (100)[h]
3-COOH,2′,4′-Cl_2	177–179	7.48, *J* = 2.9	6.89, *J* = 8.9	7.09, *J* = 9.0, 2.9	17		65 (100)
3-COOH,2′-Cl,4′-CF_3	158–161	7.59, *J* = 2.8	6.95, *J* = 8.9	7.17, *J* = 9.0, 2.9	100	8	312 (81),[b] 137 (25)[h]
3-COOMe,2′,4′-Cl_2	oil	7.45, *J* = 2.9	6.92, *J* = 8.9	7.11, *J* = 9.0, 2.9	100	7	280 (45),[c] 151 (58),[h] 166 (43)[i]
3-COOMe,2′-Cl,4′-CF_3	90–92	7.52, *J* = 2.9	6.95, *J* = 9.0	7.16, *J* = 9.0, 2.9	100	8	326 (95),[b] 151 (75)[h]

[a] Intensity relative to base peak. [b] (M − F)$^+$. [c] Acylium ion. [d] Not resolved. [e] Para coupling. [f] Chloroform-*d*. [g] Dimethyl sulfoxide-d_6. [h] Substituted anilinium ion. [i] Substituted *p*-quinonimine ion.

nitroso derivatives were respectively 0.56, 0.98, and 0.08 (broad, tailing band reacting with PCAF). As an additional confirmation of structure, TLC-pure nitrosoacifluorfen (11 mg) was oxidized in ~80% yield to acifluorfen by treatment with $K_2Cr_2O_7$ (0.5 g) in water (1.0 mL) in an ice bath, addition of concentrated H_2SO_4 (1.8 mL), and holding at 65 °C for 1 h (Langley, 1955). The oxidation product was recovered by extraction into methylene chloride and identified by TLC and CI-MS.

4-Nitrodiphenyl Thioether. The thioether was prepared by a radical nucleophilic substitution reaction (Bunnett, 1978) on dropwise addition of thiophenol (0.5 mL in 5 mL of tetrahydrofuran) and then Na metal (~60 mg) to a solution of nitrofen (100 mg) in refluxing ammonia. After 15 min the ammonia was evaporated, and the crude product was dissolved in methylene chloride, washed with 5% NaOH and water, and dried (Na_2SO_4). Preparative TLC (silica gel, cyclohexane) gave nitrodiphenyl thioether (~20% yield based on nitrofen reacting): NMR (chloroform-*d*) δ 8.05 (d, 2 H), 7.52 (m, 2 H), 7.44 (m, 3 H), 7.16 (d, 2 H); (M + 1), 232 (100%); (M + 29), 260 (10%). Byproducts were diphenyl disulfide [*m/e* 219 (M + 1), 141 ($C_6H_6S_2$)] and aminodiphenyl ether (*m/e* 186, M + 1) but apparently not aminodiphenyl thioether.

Spectral Features (Tables I and II). NMR spectra of the 4-nitrodiphenyl ethers with 4′-Cl, 4′-Me and 4′-CN substituents revealed two sets of AX systems (eight protons). Spectra were more complex for the 3′-, 3′,5′- and 2′,4′-substituted nitrodiphenyl ethers, although the AX systems of the nitrophenoxy ring remained a common feature. Only in fluorodifen, with two electron-withdrawing substituents on the 2′,4′-disubstituted phenoxy ring, were protons downfield of the 3,5-doublet. The protons of the 4′-fluorophenoxy group were collapsed into a single complex resonance at δ 7.24–7.31 and those of the (trifluoromethyl)phenoxy group showed some signal broadening due to coupling with fluorine. The spectra of nitrodiphenyl ethers with 3,4,2′,4′-tetrasubstitution were consistent with two ring systems each consisting of three protons split by ortho, meta, and ortho/meta coupling.

In the 4-amino-4′-substituted diphenyl ethers, as in the nitro series, the AB and AX systems (both AX in the nitro series) were resolved, except for the 4′-F derivatives where coupling with fluorine resulted in double doublets for the 2′,6′- and 3′,5′-protons (ortho J_{H-F} = 8.8 Hz and meta J_{H-F} = 4.5 Hz). At high field, even the complex coupling patterns of 4-amino-3,2′,4′-substituted diphenyl ethers were completely resolved; the aromatic regions of aminochlomethoxynil and aminobifenox acid, for example, comprised a total of 16 signals within 0.6 ppm. Broad resonances were observed for the amine protons (δ 4.5–6.5), except in anthranilic acids where H bonding was significant.

The chemical shift of H-5 was useful in differentiating (hydroxyamino)bifenox (δ 7.46) from its nitro (δ 8.16) and amino (δ 6.92) analogues, and this derivative also exhibited signals at δ 8.81 and 9.25 possibly attributable to the amine and hydroxyamine hydrogens, respectively. For the nitro/nitroso/amino series of nitrofen, CNP, and acifluorfen-methyl, the 3,5- or 5-doublets were successively more shielded (upfield), but for nitroso derivatives of acifluorfen and oxyfluorfen the signals for the 5-protons were upfield of those for the corresponding protons of the amine analogues, possibly due to shielding in an intramolecularly H-bonded conformer.

The CI-MS quasimolecular ion (M + 1) was the base peak and prominent M + 29 ions were apparent for the nitrodiphenyl ethers except those with 3-COOH and 3-COOMe substituents, with particular instability for bifenox acid. Arylamines without 3-substituents usually cleaved, yielding *p*-quinonimine ion, often as the base fragment, and the anilinium ion in lesser abundance. The 3-substituted arylamines also fragmented at the ether linkage, but the anilinium ions predominated. A major M–F fragment was evident with most trifluoromethyl-containing compounds.

Mutagenesis. The *S. typhimurium* mutagenesis assay (Ames et al., 1975) used the TA100 strain (sensitive to "base-pair" substitution mutagens) and the standard top agar-incorporation method for determination of mutagenic potency (revertant colonies per nanomole) and/or bactericidal activity. Microsomally activated assays involved the S9 fraction of rat liver from Aroclor-1254-treated animals (Ames et al., 1975). Dimethyl sulfoxide was the carrier solvent and the background reversion to prototrophy for histidine (120–150 colonies) was subtracted. Assays were scored as negative unless the net reversion rate exceeded the background rate (Brusick, 1980). 4-Nitroquinoline *N*-oxide (590 ± 94 revertants/nmol, –S9) and 2-aminofluorene (46 ± 26 revertants/nmol, +S9) were assayed as direct-acting and promutagen-positive controls, respectively, in each experiment. The mutagenic potencies are derived from the linear portions of the dose–response curves, and the standard deviations are calculated from 8 to 10 bioassay plates in this linear region. All critical comparisons (e.g., 4-nitrodiphenyl ethers, +S9) are based on simultaneous assays in the same experiment. The potency of each compound was then confirmed in at least one additional experiment.

Bactericidal activity is reported as the minimum dose (nanomoles per plate) destroying the "lawn" or decreasing the background reversion rate by more than 50%.

Photochemical activation utilized the procedure applied earlier to nitrofen (Draper and Casida, 1983). The nitrodiphenyl ether was irradiated for 2 and 6 h as a thin film (0.15 mg/cm^2) in a glass Petri dish fitted with a borosilicate glass cover by using lamps with maximal output at 360 nm in a Rayonet photoreactor (The Southern New England Ultraviolet Co., Middletown, CT). Photoproduct mixtures were dissolved in dimethyl sulfoxide and stored at –5 °C until bioassay.

Metabolism. Male Simonson albino rats (165–230 g) were dosed by stomach tube with various nitrodiphenyl ethers (100 mg/kg) by using methoxytriglycol as the carrier vehicle (40 mg/mL). Food was withheld for 18 h pretreatment and 6 h posttreatment, but water was always available. Metabolism cages were used to collect urine and feces for 48–120 h, with preservation by addition of toluene and storage at 0 °C, respectively. Daily urine samples were adjusted to 20 mL by addition of water and extracted with ether (20 mL × 3), the ether was dried (Na_2SO_4) and evaporated, and the residue was dissolved in ethyl acetate (10 mL). Bidaily feces collections were freeze-dried, homogenized in 50 mL of methanol in a Polytron, and centrifuged. A portion of the supernatant (5 mL) was combined with water (20 mL) and extracted with ether (4 × 20 mL), and the ether extracts were processed as above for analysis directly or following dilution.

The parent nitro compound, its amino derivative, and, in some cases, the nitroso compound were determined by electron capture gas–liquid chromatography (EC-GLC) with instrumentation, column, and operating parameters described earlier (Draper and Casida, 1983). A 5% OV-101 column was used in the EC-GLC analysis of nitrofen (230 °C), CNP (235 °C), oxyfluorfen (235 °C), and their metabolites. Retention times (min) were as follows: nitrofen, 11.5; nitrosonitrofen, 7.6; aminonitrofen, 8.9; CNP, 9.8;

Table III. Mutagenic and Bactericidal Potency of 4-Nitro- and 4-Aminodiphenyl Ethers to *S. typhimurium* TA100

	revertants/nmol			
	4-NO_2		4-NH_2	
substituents	−S9	+S9	−S9	+S9
	No 3-Substituent			
none	9 ± 1	5 ± 1	<0.1	13 ± 3
4′-F	5 ± 1	5 ± 1	<0.1	19 ± 4
4′-Cl	6 ± 1	*a*	<0.1	24 ± 9
4′-Me	7 ± 2	7 ± 1	<0.1	30 ± 8
4′-CN	4 ± 1	3 ± 1	<0.1	4 ± 3
3′-Me	4 ± 1	7 ± 2	<0.1	12 ± 3
3′,5′-Me_2	<0.1	2 ± 0.5	<0.1	6 ± 2
2′-NO_2,4′-CF_3	<0.1	0.5 ± 0.6	0.4 ± 0.3	1 ± 0.5
2′-NH_2,4′-CF_3	<0.1	<0.1	<0.1	<0.1
2′,4′-Cl_2	<0.1	<0.1	<0.1[b]	8 ± 2
2′,4′,6′-Cl_3	<0.1	*c*	<0.1[b]	6 ± 1
	3-Substituent Present			
3-MeO,2′,4′-Cl_2	<0.1	<0.1	<0.1	1 ± 1
3-EtO,2′-Cl,4′-CF_3	<0.1	<0.1	<0.1	<0.1
3-COOH,2′,4′-Cl_2	<0.1	<0.1	<0.1	<0.1
3-COOH,2′-Cl,4′-CF_3	<0.1	<0.1	<0.1	<0.1
3-COOMe,2′,4′-Cl_2	<0.1	<0.1	<0.1	<0.1[b]
3-COOMe,2′-Cl,4′-CF_3	<0.1	<0.1	<0.1	<0.1

[a] Bactericidal at 25 nmol/plate and no evidence for mutagenesis at 10 nmol/plate. [b] Bactericidal at 200–500 nmol/plate. Other nitro and amino compounds not specifically designated were not bactericidal at 2500 nmol/plate. [c] Bactericidal at 35 nmol/plate.

nitroso-CNP, 6.8; amino-CNP, 7.8; oxyfluorfen, 8.2; aminooxyfluorfen, 6.4. 3-COOMe-substituted diphenyl ethers were not resolved from their corresponding amino derivatives with the nonpolar liquid phase but were separated by using a 1.5 m × 4 mm (i.d.) 3% OV-101/5% OV-210 packed column. Retention times (min) for bifenox (220 °C) and acifluorofen-methyl (210 °C) and their metabolites were as follows: bifenox, 12.1; aminobifenox, 7.8; acifluorofen-methyl, 9.8; aminoacifluorofen-methyl, 5.9.

A modified procedure was used to analyze the feces from acifluorofen-treated rats. The methanol extract (10 mL, extraction procedure as above) was combined with water (25 mL) and extracted with ether (4 × 35 mL) and the organic phase was dried ($MgSO_4$), concentrated, and separated by TLC (carbon tetrachloride–ether, 1:1). Aminoacifluorfen was detected by its blue fluorescence under long-wavelength UV light (R_f 0.29), eluted from the adsorbent with methanol, and determined spectrophotometrically in acetonitrile solution (λ_{max} = 350 nm, ϵ_{350} = 4500 L M^{-1} cm^{-1}).

TLC and CI-MS were used to confirm the identities of the nitro and amino derivatives in feces extracts. The ether-soluble products were separated by TLC (silica gel, carbon tetrachloride–ether, 3:1 for most compounds but 1:1 for acifluorfen and aminoacifluorfen). Metabolites were compared to standards, and the corresponding bands, detected by quenching at 254 nm or fluorescence at 360 nm, were eluted with methanol for CI-MS analysis.

RESULTS

Mutagenicity. *Nitrodiphenyl Ethers (Table III).* 4-Nitrodiphenyl ether and its 3′- and 4′-monosubstituted analogues were mutagens without activation (−S9) with potencies ranging from 4 to 9 revertants/nmol. The S9 fraction reduced the mutagenic potency of the unsubstituted and 4′-CN compounds. Metabolic activation as mutagens occurred only with the 3′-Me and 3′,5′-Me_2 compounds. Diphenyl ethers with a 2′-substituent were marginally active (fluorodifen) or inactive with or without S9. The increased bactericidal activity on S9 activation for the 4′-Cl and 2′,4′,6′-Cl_3 derivatives limited considerably the sensitivity of the mutagenesis assays. The 2′,4′-Cl_2 compound was negative or only marginally active under the present assay conditions and a number of variations thereof (Draper and Casida, 1983). The 3-substituted analogues were not detected as mutagens, even on S9 fortification.

Aminodiphenyl Ethers (Table III). Only aminofluorodifen was mutagenic without S9 and its activity was marginal. All monoamino compounds without a 3-substituent underwent S9 activation, exhibiting a potency greater than that for the corresponding nitro compound (except the 4′-CN derivative). None of the 3-substituted analogues were detected as mutagens (±S9), with the exception of chlomethoxynil.

Nitroso- and (Hydroxyamino)diphenyl Ethers (Table IV). Nitroso- and (hydroxyamino)nitrofen and nitrosoacifluorfen were mutagens without S9 but underwent S9 activation. Nitroso-CNP and nitrosooxyfluorfen also were active without S9, and the activity of nitrosooxyfluorfen was diminished in the presence of the S9 fraction. (Hydroxyamino)bifenox and nitrosoacifluorfen-methyl could not be adequately tested for mutagenesis because they were potent bactericides.

Other Diphenyl Ethers. 4-Nitrodiphenyl thioether was not bactericidal (>430 nmol/plate) but was a potent mutagen (90 and 30 revertants/nmol, −S9 and +S9, respectively). In contrast, 4-acetylamino-CNP was neither bactericidal (>300 nmol/plate, ±S9) nor mutagenic (<0.4 revertant/nmol, ±S9).

Bactericidal Activity (Tables III and IV). The nitrodiphenyl ethers were not highly bactericidal with or without activation (minimum bactericidal dose, 1250 to >2500 nmol/plate, ±S9) with three exceptions: the unsubstituted compound (500 nmol, −S9 only) and the 4′-Cl and 2′,4′,6′-Cl_3 compounds (25 and <35 nmol, respectively, +S9 only). The bactericidal aminodiphenyl ethers were restricted to the 2′,4′-Cl_2 and 2′,4′,6′-Cl_3 compounds (200–500 nmol, +S9) and aminobifenox (500 nmol, +S9 only). Bactericidal nitroso and hydroxyamino compounds were nitrosonitrofen (400 nmol, +S9 only), (hydroxyamino)nitrofen (300 nmol, ±S9), and (hydroxyamino)-bifenox and nitrosoacifluorfen-methyl (100–150 nmol, −S9 only).

Table IV. Mutagenic and Bactericidal Potency of 4-Nitroso- and 4-(Hydroxyamino)diphenyl Ethers to *S. typhimurium* TA100

substituents	revertants/nmol		minimum bactericidal dose, nmol/plate	
	−S9	+S9	−S9	+S9
No 3-Substituent				
4-NO,2′,4′-Cl_2[a]	4 ± 1	17 ± 4	1900	400
4-NHOH,2′,4′-Cl_2[a]	4 ± 1	9 ± 2	300	300
4-NO,2′,4′,6′-Cl_3	3 ± 1	3 ± 1	>3300	>3300
3-Substituent Present				
3-COOMe,4-NHOH,2′,4′-Cl_2	[b]	<0.2	150	1500
3-COOH,4-NO,2′-Cl,4′-CF_3	2 ± 1	4 ± 1	>1040	>1040
3-COOMe,4-NO,2′-Cl,4′-CF_3	[b]	<0.4	110	>280
3-EtO,4-NO,2′-Cl,4′-CF_3	7 ± 3	1 ± 0.5	>1200	>730

[a] Draper and Casida (1983). [b] No evidence of mutagenicity at nonbactericidal doses of 30–60 nmol/plate.

Mutagenicity and Bactericidal Activity on Photochemical Activation of Nitrodiphenyl Ethers. Chlomethoxynil underwent distinct photochemical activation after 2 h of irradiation, yielding a photoproduct mixture with a mutagenic activity of 4 revertants/μg but no toxicity; the mutagenic component(s) was (were) deactivated by S9. After 6 h of irradiation the photoproduct mixture from chlomethoxynil was bactericidal (250 μg/plate) but not mutagenic. Weakly mutagenic photoproduct mixtures (0.4–1 revertant/μg) also were formed at 2 and/or 6 h from the 3′,5′-Me_2 derivative (−S9), fluorodifen (−S9), and oxyfluorfen (+S9). Acifluorfen, however, did not yield active photoproduct mixtures on irradiation of the free acid in thin film (2 or 6 h, ±S9). Photoproduct mixtures from CNP and nitrofen were bactericidal at 250–500 μg/plate, but only the photolysate from nitrofen was mutagenic. The S9 fraction detoxified bactericidal constituent(s) in bifenox photolysates and increased the potency of those produced by CNP.

Metabolism (Table V). The six herbicides gave essentially none of the parent compounds or amino derivatives in the urine, except for 0.4% amino-CNP and 0.03–0.05% aminonitrofen and aminooxyfluorfen relative to the administered dose; aminoacifluorfen also was a minor urinary metabolite. The nitro and amino compounds appeared in the feces, with particularly large amounts (5–12%) of the amino derivatives from oxyfluorfen, acifluorfen, and acifluorfen-methyl. The nitroso derivatives were not detected in the feces of nitrofen- and CNP-treated animals. In these metabolism studies, the total mass balances were low (2–24% of the dose), indicating that the compounds analyzed here may not be the major excreted metabolites.

Table V. 4-Nitrodiphenyl Ether Herbicides and Their Metabolites in the Feces and Urine of Orally Treated Rats

compound	% of dose in feces[a]		
	4-NO_2	4-NH_2	total
No 3-Substituent			
nitrofen	0.8[b]	1.2	2
CNP	11.0[b]	2.0	13
3-Substituent Present			
bifenox	7.5[c]	3.7[b,d]	11
acifluorfen		11.5[b-e]	11.5[f]
acifluorfen-methyl	0.7[c]	9.3[b,d]	10
oxyfluorfen	18.7[b]	5.3[b,c]	24

[a] Data for 0–48 h (CNP), 0–72 h (nitrofen), 0–96 h (bifenox, acifluorfen, and acifluorfen-methyl), and 0–120 h (oxyfluorfen). Urinary products in the same period were as follows: 0.003% nitrofen and 0.05% amino-nitrofen; 0.08% CNP and 0.4% amino-CNP; <0.01% bifenox, aminobifenox, acifluorfen-methyl, and amino-acifluorfen-methyl; present but not quantitated for acifluorfen and aminoacifluorfen; 0.06% oxyfluorfen and 0.03% aminooxyfluorfen. Nitroso derivatives in the urine and feces were <0.01% with CNP and nitrofen; they were not analyzed in the other cases. [b] Identified by CI-MS quasimolecular ion. [c] Identified by isotope cluster for diagnostic fragment at *m/e* 310 (bifenox, acylium) and *m/e* 215 (bifenox fragment), *m/e* 312 (aminoacifluorfen, M − F) and *m/e* 137 (aminoacifluorfen, $C_7H_7NO_2$), *m/e* 344 (acifluorfen-methyl, acylium), and *m/e* 312 (amino-oxyfluorfen, M − F). [d] Blue fluorescence under 360-nm light. [e] 10% in 0–48 h and 1.5% in 48–96 h. [f] Amino-acifluorfen only since acifluorfen was not analyzed.

DISCUSSION

Some 4-nitrodiphenyl ethers with no 3-substituent are detected as mutagens without S9 while others require microsomal activation. 4-Nitrodiphenyl thioether is the most potent mutagen in this study, undergoing deactivation by the S9 system, possibly attributable to sulfur oxidation. An earlier investigation also noted the mutagenic activity of 4-nitro- and 4,4′-dinitrodiphenyl ether (Shimizu and Takemura, 1976). The only two compounds undergoing S9 activation (3′-Me and 3′,5′-Me_2) also differ from the other nitrodiphenyl ethers in their mechanism of herbicidal activity, acting in the dark as well as in the light (Matsunaka, 1969). The nitrodiphenyl ethers that are mutagens without S9 fortification probably undergo activation by bacterial nitro reduction [cf. Blumer et al. (1980)]. Addition of a 2′-substituent to 4-nitrodiphenyl ethers or a 5-Me substituent to the 3-Me analogue reduces or destroys the direct mutagenic activity. The structure–activity relationships probably represent a balance of substituent effects on lipophilicity, steric parameters, and electron density, for both activation and expression of genotoxicity.

The 4-aminodiphenyl ethers without 3-substituents, except diaminofluorodifen, undergo S9 activation to mutagenic products, and bioassays of the amino analogues +S9 provide the most sensitive test condition. Mutagenicity in this series appears to be enhanced by 4′-position π- or σ-donors and high lipophilicity. A 2′-substituent may reduce the potency, but the 3-substituent is most important. Thus, nitrodiphenyl ethers with a 3-alkoxy, -carboxy, or -carbomethoxy substituent differ from the other compounds in showing little or no mutagenic activity for the amino derivatives with S9. Their nitroso and hydroxyamino derivatives, however, are mutagens or bactericides. It therefore appears that *S. typhimurium* strain TA100 and the S9 system are not efficient in reducing the nitro group or in oxidizing the amino function of diphenyl ethers with 3-substituents.

Mammals readily reduce the nitro group of the 4-nitrodiphenyl ethers via nitroso and hydroxyamino intermediates. Nitrofen and nitrosonitrofen, for example,

are reduced to the amino derivatives by rat liver preparations with NADPH under anaerobic conditions (Draper and Casida, 1983). Reduction also is a major metabolic pathway for nitrofen in rats (Costlow and Manson, 1983) and in ruminants or rumen fluid (Gutenmann and Lisk, 1967; Hunt et al., 1977) and for CNP in fish (Kanazawa and Tomizawa, 1978). Oxyfluorfen undergoes metabolic O-deethylation and nitro reduction in rats (Adler et al., 1977). The present study establishes in vivo reduction in rats not only of nitrofen and oxyfluorfen but also of CNP, bifenox, acifluorfen, and acifluorfen-methyl to their amino derivatives. It is not known whether the extensive nitro reduction is due to metabolism in mammalian tissues or by gut microflora.

This investigation establishes that several 4-nitrodiphenyl ethers including important herbicides undergo nitro reduction in mammals via putative nitroso or hydroxyamino intermediates that are bacterial mutagens. A simultaneous but independent study considering mutagenicity and nitro reduction in *Salmonella* has led to the same conclusion (Miyauchi et al., 1983).

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Registry No. Fluorodifen, 15457-05-3; nitrofen, 1836-75-5; CNP, 1836-77-7; acifluorfen, 50594-66-6; acifluorfen-methyl, 50594-67-7; bifenox, 42576-02-3; bifenox free acid, 53774-07-5; chlomethoxynil, 32861-85-1; oxyfluorfen, 42874-03-3; nitrosonitrofen, 73143-91-6; (hydroxyamino)nitrofen, 76532-45-1; nitroso-CNP, 73143-92-7; nitrosooxyfluorfen, 86823-15-6; nitrosoacifluorfen, 86823-16-7; 4-$FC_6H_4OC_6H_4NO_2$-4, 2561-25-3; 4-$ClC_6H_4OC_6H_4NO_2$-4, 1836-74-4; 4-$MeC_6H_4OC_6H_4NO_2$-4, 3402-74-2; 4-$CNC_6H_4OC_6H_4NO_2$-4, 17076-68-5; 3-$MeC_6H_4OC_6H_4NO_2$-4, 2303-25-5; 3,5-$Me_2C_6H_3OC_6H_4NO_2$-4, 1630-17-7; 2-NH_2-4-$CF_3C_6H_3OC_6H_4NO_2$-4, 24276-91-3; 4-$FC_6H_4OC_6H_4NH_2$-4, 36160-82-4; 4-$ClC_6H_4OC_6H_4NH_2$-4, 101-79-1; 4-$MeC_6H_4OC_6H_4NH_2$-4, 41295-20-9; 4-$CNC_6H_4OC_6H_4NH_2$-4, 17076-69-6; 3-$MeC_6H_4OC_6H_4NH_2$-4, 56705-84-1; 3,5-$Me_2C_6H_3OC_6H_4NH_2$-4, 86823-17-8; 2-NO_2-4-$CF_3C_6H_3OC_6H_4NH_2$-4, 24219-87-2; 2-NH_2-4-$CF_3C_6H_3OC_6H_4NH_2$-4, 24219-88-3; 2,4-$Cl_2C_6H_3OC_6H_4NH_2$-4, 14861-17-7; 2,4,6-$Cl_3C_6H_2OC_6H_4NH_2$-4, 26306-61-6; 3-COOMe-4-$NOC_6H_3OC_6H_3Cl$-2-CF_3-4, 86823-18-9; 3-COOMe-4-$HONHC_6H_3OC_6H_3Cl_2$-2,4, 76532-49-5; 3-MeO-4-$NH_2C_6H_3OC_6H_3Cl_2$-2,4, 59683-60-2; 3-EtO-4-$NH_2C_6H_3OC_6H_3Cl$-2-CF_3-4, 64378-95-6; 3-COOH-4-$NH_2C_6H_3OC_6H_3Cl_2$-2,4, 59216-76-1; 3-COOH-4-$NH_2C_6H_3OC_6H_3Cl$-2-CF_3-4, 74274-36-5; 3-COOMe-4-$NH_2C_6H_3OC_6H_3Cl_2$-2,4, 59216-75-0; 3-COOMe-4-$NH_2C_6H_3OC_6H_3Cl$-2-CF_3-4, 58105-66-1.

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