Bontoyan, W. R.; Law, M. W.; Wright, D., Jr. *J. Agric. Food Chem.* **1979**, *27*, 631.
Hunt, D. F. *Prog. Anal. Chem.* **1973**, *6*, 359.
Hunt, D. F.; McEwen, C. N.; Upham, R. A. *Anal. Chem.* **1972**, *44*, 1292.
Lusby, W. R.; Oliver, J. E.; Kearney, P. C. *J. Agric. Food Chem.* **1980**, *28*, 641.
Oliver, J. E.; Kearney, P. C.; Kontson, A. *J. Agric. Food Chem.* **1979**, *27*, 887.
Smith, R. H.; Oliver, J. E.; Lusby, W. R. *Chemosphere* **1979**, *11/12*, 855.

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Oligomers and Quinones from 2,4-Dichlorophenol

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Incubation of 2,4-dichlorophenol with a phenol oxidase from the fungus *Rhizoctonia praticola* caused the formation of several phenolic and quinonoid oligomers. Some of these products were highly reactive and several could be isolated and identified. Mass spectrometric analysis indicated the formation of oligomers up to the pentamer. Two dimeric quinones were identified by mass and NMR spectroscopy as 2-(2,4-dichlorophenoxy)-6-chloro-1,4-benzoquinone and 2-(2,4-dichlorophenoxy)-1,4-benzoquinone.

Degradation of pesticides and other xenobiotics by biological or physicochemical factors often causes the formation of intermediary products which can react with themselves or with naturally occurring molecules. It is probable that in the soil environment the same agents which cause polymerization during the humification process oxidize or polymerize xenobiotics and consequently are responsible for their incorporation into soil organic matter.

Oxidation of phenols can yield quinones and radicals. Both molecular formulations are usually very reactive and form stable products by self-coupling or cross-coupling with available molecules. Although this reaction is well-known, it has received little attention as an alternate transformation reaction in determining the fate of xenobiotic products. The reason for this oversight can be explained by the difficulty in detecting and identifying compounds which are incorporated into complex, high molecular weight polymers.

In a previous investigation we selected 2,4-dichlorophenol as a representative phenolic intermediate from a pesticide (2,4-D, 2,4-dichlorophenoxyacetic acid), and we demonstrated the formation of oligomers and cross-coupling products with phenolic humus constituents in the presence of a laccase from the fungus *Rhizoctonia praticola* (Bollag et al., 1980). The purpose of this study was to investigate in more detail the lower molecular weight reactive intermediates formed initially by oxidation and by oxidative coupling of 2,4-dichlorophenol.

MATERIALS AND METHODS

An extracellular phenoloxidase was isolated from the growth medium of the fungus *R. praticola* and purified as previously described (Bollag et al., 1979). The enzyme (0.5 unit/mL) was incubated with 2,4-dichlorophenol at 100 μg/mL in 0.1 M phosphate buffer (pH 6.9) for 2 h at 30 °C. One unit of enzyme is defined as that amount which causes a change in optical density of 1.0 per minute at 468 nm in 3.5 mL of 0.1 M phosphate buffer solution (pH 6.9) containing 3.24 μmol of 2,6-dimethoxyphenol at 23 °C (Sjoblad and Bollag, 1977). The reaction mixture was extracted with an equal volume of methylene chloride, and the extract was evaporated to dryness after drying over anhydrous sodium sulfate. Enzyme boiled for 5 min was used as a control.

Analysis of the products was carried out by thin-layer chromatography (TLC) using silica gel F-254 plates with a layer thickness of 0.25 mm (Brinkman Instruments Inc., Westbury, NY) and ether–hexane (4:1 v/v) as a development solvent. For isolation of products, preparative silica gel F-254 plates (0.5-mm layer thickness) were used. Methylation was obtained by treating the products with diazomethane in ether.

Subsequently some of the products were extracted from the thin-layer plates and further analyzed and purified by high-performance liquid chromatography (LC) using a Waters Associates (Milford, MA) Model 6000 equipped with an U6K injector and a 440 UV detector operating at a wavelength of 254 nm. The column used was a 30 cm × 3.9 mm (i.d.), packed with normal phase μ-Porasil, particle size 10 μm (Waters Associates, Milford, MA). Separation of the products was achieved with a mobile phase consisting of 95% hexane and 5% dioxane at a flow rate of 1.2 mL/min. The samples were passed through a Florisil precolumn (Supelcosil-ATF-120, 100–200 mesh, Supelco Inc., Bellefonte, PA) equilibrated with methylene chloride, and the column was eluted with the same solvent unless otherwise stated.

For isolation of quinone products, the sample in methylene chloride was placed on a Florisil column equilibrated with hexane. Elution of the column with hexane removed the various polymers, but the quinones were retained on the column. Subsequently they were removed from the Florisil column with methylene chloride and separated by high-performance LC.

Mass spectrometry was based on electron impact analysis at 70 eV with sample introduction by direct insertion probe on an AEI MS-902 mass spectrometer or by a directly coupled gas chromatograph on a Finnigan 3200 mass spectrometer (1.5 m × 1.8 mm i.d. glass column, packed with 3% OV-1 or 3% OV-17 on 100–120-mesh Chromosorb W). The products were injected in a meth-

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Table I. Mass Spectrometric Analysis of Oligomers Formed during the Incubation of 2,4-Dichlorophenol with an *R. praticola* Phenol Oxidase

	M^+	M^+ (loss of 1 Cl)	M^+ (loss of 2 Cl)	M^+ (loss of 3 Cl)
monomer	162			
dimer	322	302	268	
trimer	482	448	414	
tetramer	642	608	574	540
pentamer	802	768	734	700

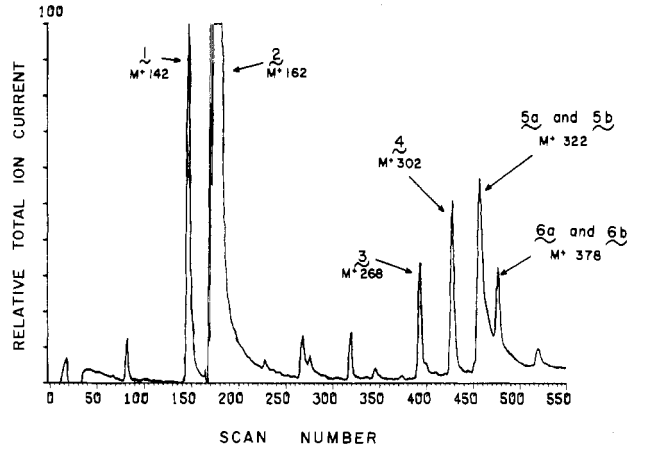


Figure 1. Gas chromatogram of a methylene chloride extract from a reaction mixture of 2,4-dichlorophenol and *R. praticola* phenol oxidase (see molecular structures of the identified products in Figure 2).

ylene chloride solution, and the column temperature was programmed from 100 to 250 °C at 8 °C/min. Molecular weights were verified by chemical ionization mass spectrometry using methane or isobutane. All m/z values reported herein are for the ^{35}Cl isotope. Ultraviolet absorption spectra were measured with a Bausch & Lomb Spectronic 505 spectrometer. Proton NMR spectra were taken on a Bruker 200-MHz instrument with a Fourier transform system.

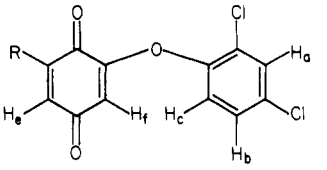
RESULTS

When 2,4-dichlorophenol was incubated with the phenol oxidase from *R. praticola*, the formation of various products could be determined by analysis with TLC, GC, and high-performance LC. Different chromatographic techniques were used depending on the compounds which had to be isolated.

A mass spectrometric analysis of the methylene chloride extract of the reaction mixture revealed the formation of oligomeric products (Table I). The peaks obtained indicated that dimers to pentamers, formed under the selected conditions of the experiment, were volatile enough to be detected with the available mass spectrometer. The peaks with m/z 162, 322, 482, 642, and 802 showed isotopic patterns which correlate with 2, 4, 6, 8, and 10 chlorines, respectively. The masses and isotope patterns of the additional peaks correlated with oligomeric products in which one or more chlorines are lost as indicated in Table I.

When the products from the enzyme reaction of 2,4-dichlorophenol were extracted with methylene chloride and analyzed by GC–MS using a 3% OV-1 column, six major chlorine-containing components were observed (Figure 1). The first peak is 2-chloro-1,4-benzoquinone, **1** (M^+ 142, one chlorine). Attempts to isolate this compound by high-performance LC were unsuccessful since it appears to be a highly reactive compound. Indirect evidence for the identity of 2-chloro-*p*-benzoquinone was obtained by oxidizing chlorohydroquinone according to the method of Vliet (1922). The resulting product had the same high-performance LC retention time and mass spectrum as **1** (2-chloro-1,4-benzoquinone), thus confirming our structural assignment. The second (major) GC peak is the unreacted starting material, 2,4-dichlorophenol, **2** (M^+ 162, two chlorines).

Table II. Proton NMR Chemical Shifts and Coupling Constants for Compounds 3 and 4 (Assignments Have Been Confirmed by Off-Resonance Decoupling Experiments)



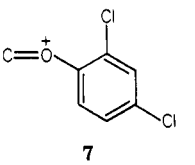
3, R = H_d
4, R = Cl

compd	proton		chemical shift, ppm	coupling constants, hertz
3	H_a	d[a]	7.715	J_{a-b} = 2.2
	H_b	dd	7.527	J_{b-c} = 8.6; J_{b-a} = 2.2
	H_c	d	7.419	J_{c-b} = 8.6
	H_d	d	6.915	J_{d-e} = 10.2
	H_e	dd	6.800	J_{e-d} = 10.2; J_{e-f} = 2.2
	H_f	d	5.736	J_{f-e} = 2.2
4	H_a	dd	7.744	J_{a-b} = 2.4; J_{a-c} = 4.4
	H_b	dd	7.555	J_{b-c} = 8.8; J_{b-a} = 2.4
	H_c	d	7.447	J_{c-b} = 8.8
	H_e	d	7.127	J_{e-f} = 2.4
	H_f	d	5.847	J_{f-e} = 2.4

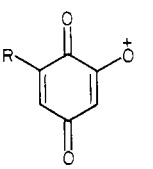
[a] d, doublet; dd, doublet of doublets.

The next two chlorine-containing components were the quinones, **3** (M^+ 268, two chlorines) and **4** (M^+ 302, three chlorines). For isolation of sufficient quantities of these phenoxyquinones for determination of their structures, the enzyme product mixture was purified by passing through a Florisil column (see Materials and Methods) and then separated by high-performance LC. The ultraviolet spectrometric analysis indicated a UV maximum at 276 nm for the quinone with M^+ 302 and at 255 nm for the quinone with M^+ 268.

High-resolution mass spectrometric analysis yielded an elemental composition of $C_{12}H_6O_3Cl_2$ for **3** (calculated 267.9693; found 267.9696) and $C_{12}H_5O_3Cl_3$ for **4** (calculated 301.9304; found 301.9321). Both spectra contain an ion at m/z 173 ($C_7H_3OCl_2$ by high resolution) corresponding to **7**, and **3** yielded a peak at 123 ($C_6H_3O_3$) and **4** a peak



7



8, R = H
9, R = Cl

at 157 ($C_6H_2O_3Cl$) which can be represented by the ions **8** and **9**, respectively. Therefore, the rings are linked through an oxygen.

The structures were further defined by the NMR analysis (Table II). The proton assignments were con-

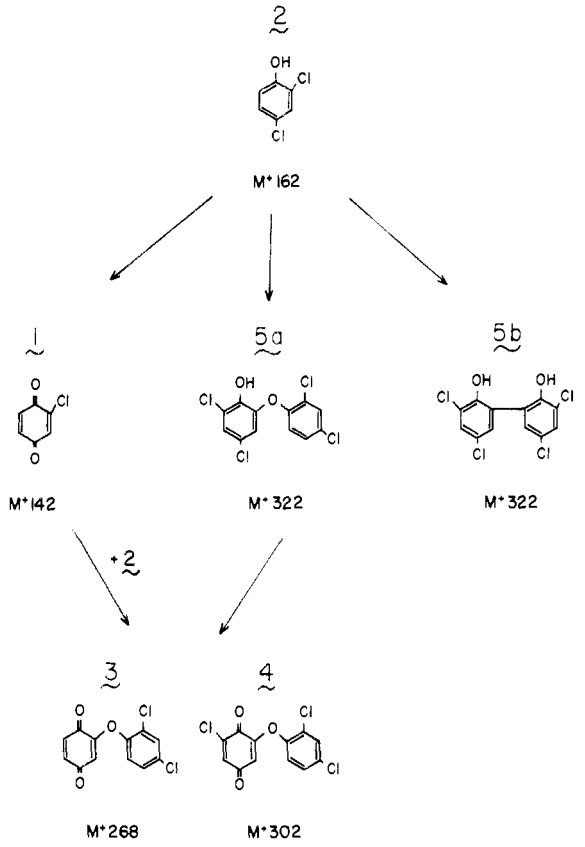
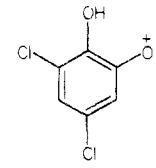


Figure 2. Probable pathway for the enzymatic products and their suggested molecular structure. 1, 2-chloro-1,4-benzoquinone; **2**, 2,4-dichlorophenol; **3**, 2-(2,4-dichlorophenoxy)-1,4-benzoquinone; **4**, 2-(2,4-dichlorophenoxy)-6-chloro-1,4-benzoquinone; **5a**, 2-(2,4-dichlorophenoxy)-4,6-dichlorophenol; **5b**, 3,3′,5,5′-tetrachloro-2,2′-diphenol.

firmed by off-resonance decoupling NMR studies. Carbon–carbon linkage would have produced a free phenolic hydroxyl. Neither **3** nor **4** contained an exchangeable phenolic OH proton, and the resonances at 5.74 + 5.85 ppm were too far downfield and were narrow and split, confirming the ring coupling as C–O and not C–C. The NMR spectrum also fixes the positional relation of the phenoxy and chlorine groups on the 1,4-benzoquinone as being 2,6 and not 3,6. These data allow us to assign **3** the structure 2-(2,4-dichlorophenoxy)-1,4-benzoquinone and **4** the structure 2-(2,4-dichlorophenoxy)-6-chloro-1,4-benzoquinone (Figure 2).

The next compound to appear in the gas chromatographic analysis (Figure 1) was compound **5** which has a molecular weight of 322 (four chlorines) and corresponds to the dimeric coupling product of 2,4-dichlorophenol reported earlier (Bollag et al., 1977). This peak actually consists of two different dimers which can be separated on a 3% OV-17 GC column.

By use of this phase, the first dimer to elute was determined to be the C–O coupled product **5a** by the presence of an ion at m/z 177 in its electron impact spectrum corresponding to the ion **10**. Dimer **5a** has been observed



10

by Plimmer and Klingebiel (1971) in the riboflavin photosensitized oxidation of 2,4-dichlorophenol.

The mass spectrum of the other dimer **5b** did not contain this ion and therefore must be C–C coupled. Methylation of the two dimers yielded products with molecular ions of 350 (**5b** dimethyl ether) and 336 (**5a** monomethyl ether) for the C–C and C–O coupled products, respectively.

The final chlorine-containing compound **6** from the 3% OV-1 column (Figure 1) had a molecular weight of 378 (four chlorines) and showed ions at M – 15 and M – 43 in its mass spectrum. This implies that an acetyl group is present. The only reasonable source for this two-carbon substituent appeared to be the ethanol that was used to dissolve the 2,4-dichlorophenol initially. This could have been oxidized during the reaction to acetaldehyde or acetic acid which was subsequently incorporated into this product. This was confirmed when the enzymatic reaction was run using 2,4-dichlorophenol dissolved in methanol. In this case, no material with the retention time or mass spectra of **6** was formed. This product can also be separated into two isomers, **6a** and **6b**, on 3% OV-17 which have very similar mass spectra. Unfortunately, we have not been able to isolate enough of this material to allow structural assignment.

DISCUSSION

Musso has carried out fundamental studies on the chemical oxidation of phenols and has reviewed the literature dealing with phenol coupling (Musso, 1963). In many cases, phenols are oxidized to *o*- or *p*-quinones which can be isolated or which react with the starting phenol, oxidation intermediates, or added nucleophilic reagents to produce more complex products (Musso et al., 1964).

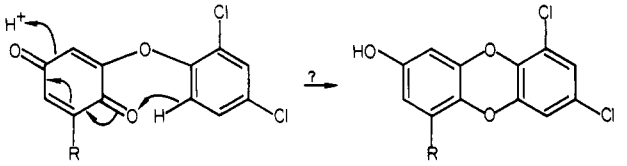
Oxidation of phenols to quinones is also catalyzed by various phenol oxidases such as laccases, tyrosinases, or peroxidases. Evidence obtained by Happold and Raper (1925) indicated that phenols were first oxidized to *o*-quinones by a "tyrosinase" and Pugh and Raper (1927) obtained indirect evidence of the formation of *o*-benzoquinone from catechol by isolating its dianilino adduct. In the biological oxidation of phenol it was also observed that, concurrent with quinone production, a dimerization reaction takes place in which a phenolic compound undergoes addition to the quinone. For example, Forsyth et al. (1960) isolated a dimerized quinone, diphenylene dioxide 2,3-quinone by oxidation of catechol with a "polyphenol oxidase". Küster and Little (1963) showed the formation of quinonoid products, fumigatin and spinulosin, by the fungi *Aspergillus fumigatus* and *Penicillium spinulosum*.

Whether the incorporated oxygen in the compounds reported in this paper is from water or molecular oxygen remains to be determined. Another question is whether the quinone dimers **3** and **4** come from the phenol dimer **5a** or are formed by addition of the phenol **2** to the monomeric quinone **1** followed by oxidation (with subsequent loss of chlorine in the case of **3**). Addition of phenols to quinones is a well-known reaction (Strauss et al., 1925; Teuber and Glosauer, 1965), and the extensive production of **5a** and the conversion of **2** to **1** makes the formation of **3** and **4** from **5a** a reasonable possibility.

Although from chemical oxidation we might expect the formation of *o*-quinones and products derived therefrom (Teuber and Glosauer, 1965), in the present study the identified quinones are all *p*-quinones even though this involves the elimination of chlorine. If 3,5-dichloro-1,2-benzoquinone is formed in this reaction, it must be reacting to form products such as polymers that are undetectable. Nucleophilic displacement of chlorine has been noted in

the photodecomposition of *p*-chlorophenoxyacetic acid by Crosby and Wong (1973), but the mechanism of this process for the reactions in Figure 2 is not clear.

The phenoxy quinones 3 and 4 are interesting structures because of the possibility of their conversion to dibenzodioxins 11 by an acid-catalyzed cyclization mechanism such as



3, R = H
4, R = Cl

11, R = H, Cl

Although hydroxy chlorinated dibenzodioxins have not been characterized in terms of their toxicological properties, the existence of a possible mechanism for their formation from 2,4-dichlorophenoxyacetic acid (2,4-D) in soil may be of consequence for the environmental impact of the use of 2,4-D. Conditions for this transformation are being explored in our laboratories.

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LITERATURE CITED

Bollag, J.-M.; Liu, S.-Y.; Minard, R. D. *Soil Sci. Soc. Am. J.* **1980**, *44*, 52.
Bollag, J.-M.; Sjoblad, R. D.; Liu, S.-Y. *Can. J. Microbiol.* **1979**, *25*, 229.
Bollag, J.-M.; Sjoblad, R. D.; Minard, R. D. *Experientia* **1977**, *33*, 1564.
Crosby, D. G.; Wong, A. S. *J. Agric. Food Chem.* **1973**, *21*, 1049.
Forsyth, W. G. C.; Quesnel, V. C.; Roberts, J. B. *Biochim. Biophys. Acta* **1960**, *37*, 322.
Happold, F. C.; Raper, H. S. *Biochem. J.* **1925**, *19*, 92.
Küster, E.; Little, B. T. *Biochim. Biophys. Acta* **1963**, *67*, 288.
Musso, H. *Angew Chem.* **1963**, *75*, 965.
Musso, H.; von Gizycki, V.; Zahorsky, V. I.; Bormann, D. *Justus Liebigs Ann. Chem.* **1964**, *676*, 10.
Plimmer, J. R.; Klingebiel, U. I. *Science* **1971**, *174*, 407.
Pugh, C. E. M.; Raper, H. S. *Biochem. J.* **1927**, *21*, 1370.
Sjoblad, R. D.; Bollag, J.-M. *Appl. Environ. Microbiol.* **1977**, *33*, 906.
Strauss, F.; Bernoully, O.; Mautner, P. *Justus Liebigs Ann. Chem.* **1925**, *444*, 165.
Teuber, H. J.; Glosauer, O. *Chem. Ber.* **1965**, *98*, 2643.
Vliet, E. B. *Org. Synth.* **1922**, *2*, 85.

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Coupling Reactions of 2,4-Dichlorophenol with Various Anilines

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Cross-coupling between various aromatic compounds in the presence of an enzyme can lead to hybrid products. When a fungal phenol oxidase was incubated with 2,4-dichlorophenol and various halogenated anilines, the formation of hybrid oligomers was determined. Mass spectrometric analysis indicated two types of trimers which consisted of a phenoxyquinone dimer coupled with an aniline molecule or a quinone coupled with two aniline molecules. Since the incubation of anilines alone with the fungal phenol oxidase did not cause the formation of oligomeric products, it was assumed that the coupling of aniline to enzymatic products of 2,4-dichlorophenol has to be of chemical origin. Quinone products from the enzyme reaction of 2,4-dichlorophenol were found to react with the anilines in the absence of phenol oxidase. This indicated clearly that both enzymatic and nonenzymatic causes were responsible for the formation of cross-coupling products.

Oxidation and coupling of phenols is known to occur either through enzymatic or nonenzymatic systems. Enzymatic polymerization of phenolic compounds is an important reaction for the biosynthesis of a wide range of natural products (Taylor and Battersby, 1967) and is important as a key reaction in the humification process (Flaig et al., 1975).

In soil, phenolic and other aromatic degradation products of lignin, together with microbially synthesized phenolic compounds, can form complexes with amino compounds and undergo enzymatic or chemical polymerization to form humic acid type polymers (Haider et al., 1975). If humic acids are formed by the polymerization of phenols and other products, it seems reasonable to assume that opportunities will exist for the incorporation of xenobiotic substances into humus substances. The cross-coupling reactions between 2,4-dichlorophenol and phenolic humus constituents in the presence of a fungal laccase serve as a model for this hypothesis (Bollag et al., 1980).

Haider et al. (1965) studied the linkage of amino acids and peptides with phenols during oxidation with phenol oxidases. The binding to nitrogen of amino acids was found mostly in the oxidation products of dihydroxyphenols like catechol, protocatechuic acid, and hydroquinone. Ladd and Butler (1966), synthesizing model phenolic polymers from catechol and *p*-benzoquinone, also concluded that amino compounds could be incorporated into the polymers, and they compared the resulting products with humic acids.

Many of the microbially synthesized phenols can be oxidized to semiquinonoid or quinonoid intermediates which strongly react with other phenolic or nitrogenous substances. The same reaction can take place with either naturally occurring or xenobiotic compounds.

Our interest focused on anilinic compounds which are often intermediary degradation products of various pes-

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