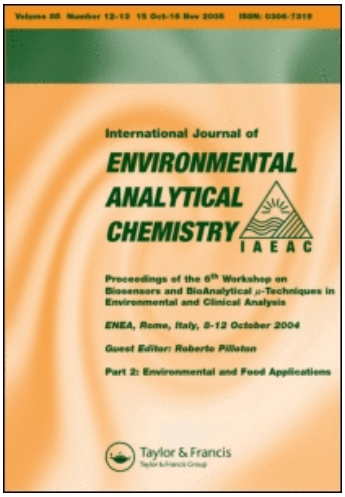

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Structural and Quantitative Analyses of 4-Chloroaniline-Derived Oligomers

K. E. Simmons[a]; R. D. Minard[a]; A. J. Freyer[a]; J. M. Bollag[a]
[a] Department of Agronomy and Department of Chemistry, The Pennsylvania State University, University Park, PA, U.S.A.

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Structural and Quantitative Analyses of 4-Chloroaniline-Derived Oligomers†

K. E. SIMMONS, R. D. MINARD, A. J. FREYER and J.-M. BOLLAG

Department of Agronomy and Department of Chemistry, The Pennsylvania State University, University Park, PA 16802, U.S.A.

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The oxidation of 4-chloroaniline by a peroxidase resulted in eight oligomeric products. A reverse-phase high performance liquid chromatography (HPLC) method was developed so that substrate disappearance and the corresponding product formations could be quantitatively monitored. The product mixture was isolated from the aqueous reaction solution with solid-phase extraction and the extract components were separated by thin-layer chromatography (TLC). The individual TLC bands were extracted for mass spectrometric and proton nuclear magnetic resonance analyses. The product mixture was found to contain dimers, trimers, and tetramers with benzoquinone monoimine, benzoquinone di-imine, diaminobenzene, and azobenzene structures. Analytical methodologies were specific for the study of the oxidative transformation of 4-chloroaniline, but they should be applicable to other aniline-derived oligomers.

KEY WORDS: 4-chloroaniline, aniline-derived oligomers, HPLC quantitation of substrate and products, peroxidase, oligomer structure determination.

INTRODUCTION

Substituted anilines are contaminants of agricultural soils. Oxidation of the anilines by enzymes, such as peroxidase[1,2] and laccase,[3]

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results in a variety of oligomeric compounds. A description of the oxidative oligomerization process would be helpful in understanding the transformation of substituted anilines in the soil. Complex soil matrices preclude *in vivo* study, so model reaction systems must be used. In order to define the reaction pathways which describe the oligomerization process, it is necessary to structurally characterize all products and to determine the relative amounts of substrate and products during the enzymatic reaction.

Gas–liquid chromatography[4–6] and high performance liquid chromatography (HPLC)[7–11] have been used in the analysis of substituted anilines. However, a method for separation and quantitation of the aniline-derived oligomers has not been previously reported.

The goals of this study were to structurally identify all oligomers resulting from the oxidoreductase transformation of 4-chloroaniline, and to develop a method for quantitation of substrate and products in the reaction solution. Results of the time course experiments are reported elsewhere, along with the proposed reaction mechanism scheme for the oxidative oligomerization of 4-chloroaniline.[12]

EXPERIMENTAL

Chemicals

4-Chloroaniline was purchased from Aldrich Chemical Company (Milwaukee, WI) and uniformly ring-labeled. ^{14}C-4-chloroaniline (10.24 mCi/mmol) was obtained from Pathfinders Laboratories, Inc. (St. Louis, MO). Both compounds were $^{+}$98% pure as confirmed by thin-layer chromatography (TLC) and HPLC.

Enzyme assay

Horseradish peroxidase with RZ (Reinheitszahl) of 0.43 and activity of 45 purpurogallin units/mg solid was purchased from Sigma Chemical Company (St. Louis, MO). A purpurogallin unit is defined (Sigma Chemical Company) as the amount of enzyme which forms 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. Purpurogallin is measured by absorption at 420 nm.

Enzyme assays were conducted in citrate–phosphate buffer (pH 4.2) with 0.023 units/ml peroxidase, 2.5 μmol/ml hydrogen peroxide, 1.0 μmol/ml 4-chloroaniline, and 0.32 μCi/ml ^{14}C-4-chloroaniline at 25°C for 30 minutes. Assay solutions containing boiled enzyme served as controls.

High performance liquid chromatography

At the specified incubation time, enzymatic activity was stopped by the addition of 2.5 ml of acetonitrile to an equal volume of the assay solution. The resulting 5.0 ml sample was passed through a 0.2 μm pore nylon-66 filter (Schleicher and Schuell, Keene, NH) to remove precipitated protein, and 175 μl were immediately analysed by HPLC. Approximately 300 injections of reaction samples were made with no noticeable loss of separation efficiency.

Analysis was performed on a Waters Associate (Milford, MA) high performance liquid chromatograph. The system consisted of a U6K injector, M45 and 6000A pumps run by a Model 720 Systems Controller, a Lambda Max 450 LC Spectrometer set at 280 nm (0.05 AUFS), and a Model 730 Data Module.

Reverse-phase separation was performed on a 15 cm × 4.6 mm Supelcosil LC-18 (bonded octadecylsilane) column of 5 μm particle size from Supelco, Inc. (Bellefonte, PA). The mobile phase at 1.5 ml/min was a mixture of aqueous component A (0.01 *M* KH_2PO_4, 0.05% triethylamine, pH 6.3) and organic component B (methanol, 0.1% triethylamine). For the first 3 minutes, the composition of the mobile phase was held at 40/60 (volume percent) A/B, then brought to 10/90 A/B (see Figure 1 for gradient curve shape) by 15 minutes, and this final composition was held for 5 minutes. The column was equilibrated for 10 minutes in 40/60 A/B before the next injection. HPLC retention times for individual products from the 4-chloroaniline reaction were verified from co-injections of a reaction sample solution and the pure product. The pure compounds were obtained from TLC preparatory-scale separations of the product mixture. Retention times were reproducible to within 0.05 minutes. The products were labeled as compounds B through I in order of increasing oligomer size.

HPLC was also used to isolate compound C for mass spectrometric analysis. The eluted peak of compound C was collected

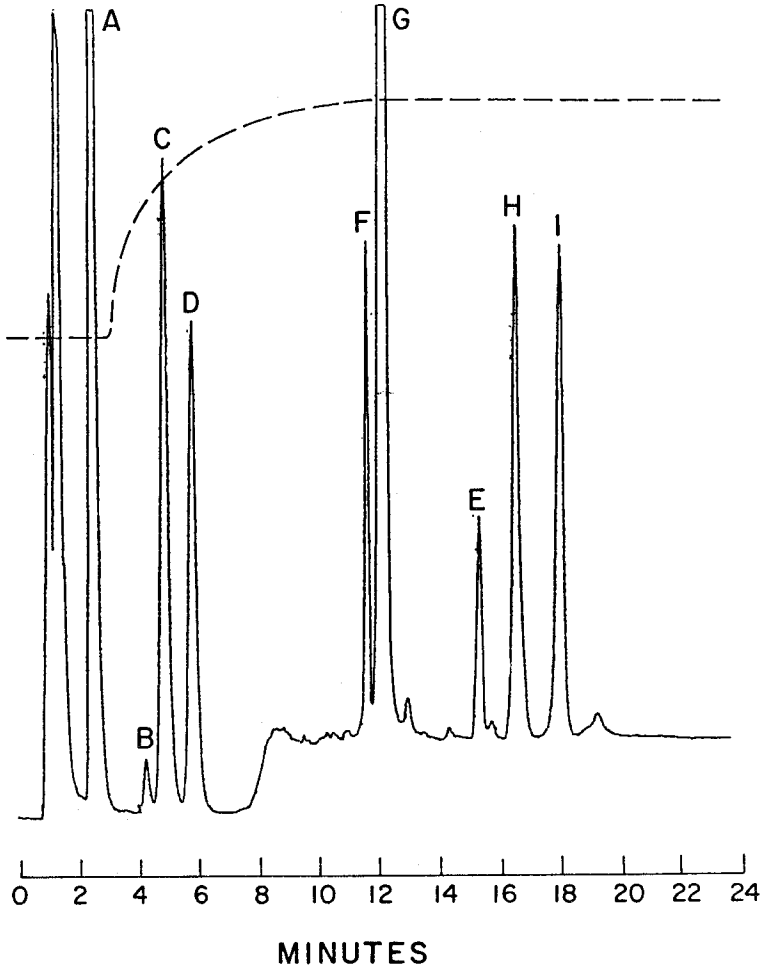


FIGURE 1 HPLC separation of 4-chloroaniline and the oligomeric products formed in the presence of an oxidoreductase. For compound identification, see Table I and Figure 2. The dashed line describes the gradient curve shape.

from injections of samples which had been incubated for 2.5 min. The collected fractions were saturated with sodium bicarbonate and extracted with methylene chloride which had been treated with sodium sulfate and sodium carbonate. The methylene chloride ex-

tract was then dried with sodium sulfate and concentrated with a gentle stream of N_2.

The TLC isolate of compound H was further purified by HPLC for nuclear magnetic resonance (NMR) analysis. The isolate was dissolved in 50/50 (volume percent) H_2O/acetonitrile, and 200 μl was injected and eluted with a mobile phase composition of 10/90 (A/B). The peak (retention time 4.90 min) was collected from repeated injections. Combined fractions were diluted with an equal volume of water and extracted with two 50 ml portions of methylene chloride. The extract was passed through a sodium sulfate column and the solvent was evaporated under vacuum at 35°C.

Response factors

Substrate and products in the enzymatic reaction mixtures were quantitated on the HPLC system using the UV absorbance peak area and response factors (RF). The amount of each compound in an injection volume (Vinj) was determined using the equation:

$$\text{nmol} = \frac{\text{Peak area}}{\text{RF}}.$$

Response factors were obtained by correlating the UV absorbance peak area to the radioactivity of the same peak when ^{14}C-4-chloroaniline was included as substrate. HPLC fractions (0.75 ml) were collected by a Foxy Fraction Collector (ISCO, Lincoln, NE) and radioassays were performed with a Beta Trac 6895 scintillation counter (TM Analytic, Inc., Elk Grove Village, IL) after addition of 4 ml ScintiVerse II universal liquid scintillation cocktail (Fisher Scientific Co., Fairlawn, NJ). The radioactivity of the collected HPLC peak was used to obtain the amount of that compound in the sample injection according to the following equation:

$$\text{nmol} = \frac{\text{DPM of peak}}{\text{DPM of Vinj}} \times \frac{\text{nmol 4-CA}}{n} \text{ in Vinj}$$

where DPM is disintegrations per minute of ^{14}C, n is oligomer order ($n=2$ for a dimer, 3 for a trimer, 4 for a tetramer), and 4-CA is 4-chloroaniline. The response factor for each of the nine compounds

was then calculated by dividing the UV absorbance peak area by the amount of compound in the peak volume. To assure linearity of absorbance with varying compound concentrations, response factors were calculated from sample solutions with different substrate disappearances (20%, 38%, and 64%). The average values from duplicate injections of the three solutions were used for subsequent sample quantitations.

Thin-layer chromatography

TLC was used to isolate most of the products from the enzymatic oxidation of 4-chloroaniline. Assay solutions (50 ml) were extracted with a PrepSep C_{18} column from Fisher Scientific Company (Fairlawn, NJ). The vacuum-dried column was then eluted with 500 μl ethyl acetate and the eluant applied to a 20 cm × 20 cm × 0.25 mm Silica Gel 60 F-254 plate (EM Science, Darmstadt, W. Germany). The plate was developed with 20/80 (volume percent) ethyl acetate/hexane, and the separated compounds were extracted from the silica with ethyl acetate. All products isolated from TLC plates absorbed strongly at visible wavelengths.

Reduction and acetylation

The quinone monoimine (compound D) was reduced according to a modified method described by Vogel.[13] Approximately 5 mg of compound D was dissolved in 25 ml acetone. This solution was mixed with 5 g of ammonium chloride and 2 g of zinc dust in 25 ml of water. The stirred solution changed from yellow to colorless. The solution was then filtered into 100 ml of 0.1 *M* NaOH and the reduction product (an aminophenol) was recovered by methylene chloride extraction.

Reductive acetylation of compound D was also performed according to a modified method described by Vogel.[13] Approximately 5 mg of compound D was dissolved in 3 ml of acetic anhydride, then 0.25 g of zinc dust and 0.3 g of anhydrous sodium acetate were added. The mixture was warmed until colorless, then was refluxed for 2 min. Two ml of glacial acetic acid was added, and after heating to the boiling point, the solution was poured into 300 ml of cold H_2O. The acetylated product was recovered by extraction with methylene chloride.

Mass spectrometry

Molecular weights for the 4-chloroaniline products were determined by electron impact ionization using a Kratos MS 9/50 double-focusing mass spectrometer. In some cases, the molecular weight was confirmed by chemical ionization mass analysis on a Finnigan 3200. Sample introduction was by direct insertion probe.

Nuclear magnetic resonance

Product structures were confirmed with NMR. The data were obtained on a Bruker WM-360 instrument with acetone-D6 as solvent.

RESULTS AND DISCUSSION

The one-electron oxidation of many substituted anilines results in the formation of oligomeric compounds. In this study, the enzymatic oxidation of 4-chloroaniline produced eight oligomers ranging in size from dimers to tetramers.

Addition of peroxidase to a buffered aqueous solution of 4-chloroaniline initiated the reaction and dilution of the assay solution with an equal volume of acetonitrile served to halt enzyme activity. The diluted sample was injected for reverse-phase HPLC analysis, and substrate and products were separated with gradient elution (see Figure 1). Retention times for the nine compounds are listed in Table I.

The direct injection HPLC method described here is well suited for analysis of the oligomeric products of substituted anilines. Other analytical methods which involve extensive sample pretreatment procedures such as pH adjustment, extraction of the aqueous solution with an organic solvent, or concentration of the organic extract, can lead to low analyte recoveries and chemical transformation of reactive species. The direct injection method allows for quantitative analysis of substrate and products immediately following enzyme deactivation; therefore, transient species can be monitored and artifacts avoided. In this study, compound C, a very reactive di-imine dimer, was only detected when the direct injection method was applied. When an organic solvent or solid phase extrac-

TABLE I

Characterization of products formed by enzymatic oxidation of 4-chloroaniline

Compounds	HPLC retention time (min)	TLC Rf	TLC color	MW[b]	Formula
A — Monomer (4-chloroaniline)	2.45	0.30	colorless[a]	127	C_6H_6NCl
B — Dimer (*N*-(4-chlorophenyl)-*p*-phenylene diamine)	4.33	—	—	218	$C_{12}H_{11}N_2Cl$[c]
C — Dimer (*N*-(4-chlorophenyl)-*p*-phenylene di-imine)	4.93	—	—	216	$C_{12}H_9N_2Cl$
D — Dimer (*N*-(4-chlorophenyl)-benzoquinone monoimine)	5.86	0.49	yellow	217	$C_{12}H_8NOCl$[c]
E — Dimer (4,4-dichloroazobenzene)	15.40	0.86	yellow	250	$C_{12}H_8N_2Cl_2$
F — Trimer (2-(4-chloroanilino)-*N*-(4-chlorophenyl)-benzoquinone)	11.68	0.37	purple	342	$C_{18}H_{12}N_2Cl_2$[c]
G — Trimer (2-amino-5-chloro-benzoquinone)	12.16	0.63	red	377	$C_{18}H_{12}N_3Cl_3$[c]
H — Tetramer (2-(4-chloroanilino)-5-hydroxybenzoquinone-di-4-chloroanil)	16.61	0.59	green	467	$C_{24}H_{16}N_3OCl_3$[c]
I — Tetramer (2-amino-5-(4-chloroanilino)-benzoquinone-di-4-chloroanil)	18.03	0.69	brown	466	$C_{24}H_{17}N_4Cl_3$[c]

[a]Visualized using UV absorbance.
[b]Determined with mass spectrometry.
[c]Confirmed by high resolution analysis.

tion step preceded analysis, compound C was not detected with subsequent HPLC or TLC. The same HPLC and TLC analyses did, however, show the presence of a pentamer (m/z 578) which has been reported previously[1,3] as a product of 4-chloroaniline enzymatic oxidation. This oligomer was not found in the enzyme reaction solutions when the direct injection method was used. It appears that the pentamer is formed during extraction procedures and is not produced in the aqueous reaction solution.

The nine compounds in an injection volume were quantitated using response factors. The response factors, as listed in Table II, are the slopes of calibration curves of UV absorbance peak area vs. amount for each compound. The minimum detectable amounts (nmol/injection volume) for the compounds in Table II were 1.0 of 4-chloroaniline, 0.2 of compound E, 0.1 of compunds B, C, and D, and 0.05 of compounds F, G, H, and I.

When ^{14}C-4-chloroaniline is included as substrate, radioassay of HPLC eluate fractions can be used for repetitive quantitations of substrate and products. The accuracy and precision of radio-

TABLE II

Comparison of the use of response factors vs. radioassay of HPLC fractions in the quantitation of 4-chloroaniline and its products

Compound	Response factor $\times 10^3$ ($\pm$s.d.)	Replicates[a]	Calculated amounts (nmol/injection volume)	
			Using response factors	Using radioactive peak count
A — 4-Chloroaniline	165 $\pm$ 17	8	64	63
B — Dimer	1646 $\pm$ 411	4	—[b]	—[b]
C — Dimer	2312 $\pm$ 92	4	—[b]	—[b]
D — Dimer	2455 $\pm$ 196	6	0.3	0.3
E — Dimer	761 $\pm$ 68	6	1.0	0.9
F — Trimer	3140 $\pm$ 440	6	0.56	0.43
G — Trimer	3853 $\pm$ 262	6	3.72	3.57
H — Tetramer	3860 $\pm$ 425	6	0.70	0.62
I — Tetramer	3145 $\pm$ 252	6	0.96	0.83

[a]Number of replicate samples used in response factor calculations.

[b]Compounds B and C were not present in the 60-minute reaction sample.

chromatography methods are well documented.[14] However, when large numbers of column eluate fractions are collected, the method can be laborious and time-consuming. The use of UV absorbance response factors affords a more convenient method. Quantitations of substrate and products by the radioassay method and by the UV absorbance response factor method were performed on the same 60-minute reaction sample. Table II shows that values calculated by the two methods are in agreement, even for amounts that approach the detection limits.

Radiochromatography was used to ascertain that all reaction products were represented in the chromatogram of Figure 1. When ^{14}C-4-chloroaniline was included as substrate, recovery of radioactivity from HPLC column eluate fractions was 88% ± 4 s.d. for six control samples and 88% ± 2 s.d. for six reaction samples. Because the radioactive recoveries for control and reaction solution injections were the same, the nine peaks of Figure 1 are representative of the total initial substrate radioactivity. Apparently no other products were formed.

Preparation of samples for structure identifications involved isolation of the oligomeric mixture from aqueous solution using solid-phase extraction, and subsequent separation of the reaction products with TLC. The individual compounds were extracted from the TLC plate for mass spectrometric and proton NMR analyses. HPLC was used to isolate compound C for mass spectrometric analysis and to purify compound H for NMR analysis.

Structural elucidation studies were performed on the eight oligomers formed by enzymatic oxidation of 4-chloroaniline. The results are given below, and the proposed product structures are shown in Figure 2.

Compounds B and C

Compounds B and C were not found on the TLC plates and compound B was not produced in sufficient amounts for isolation by HPLC. Compound C, however, was isolated by HPLC and repeated injections of the collected peak showed a continuous conversion of compound C to compounds B and D. When the compound C fraction was saturated with sodium bicarbonate to prevent chemical hydrolysis of the analyte, only compounds C and B were found upon repeated injections.

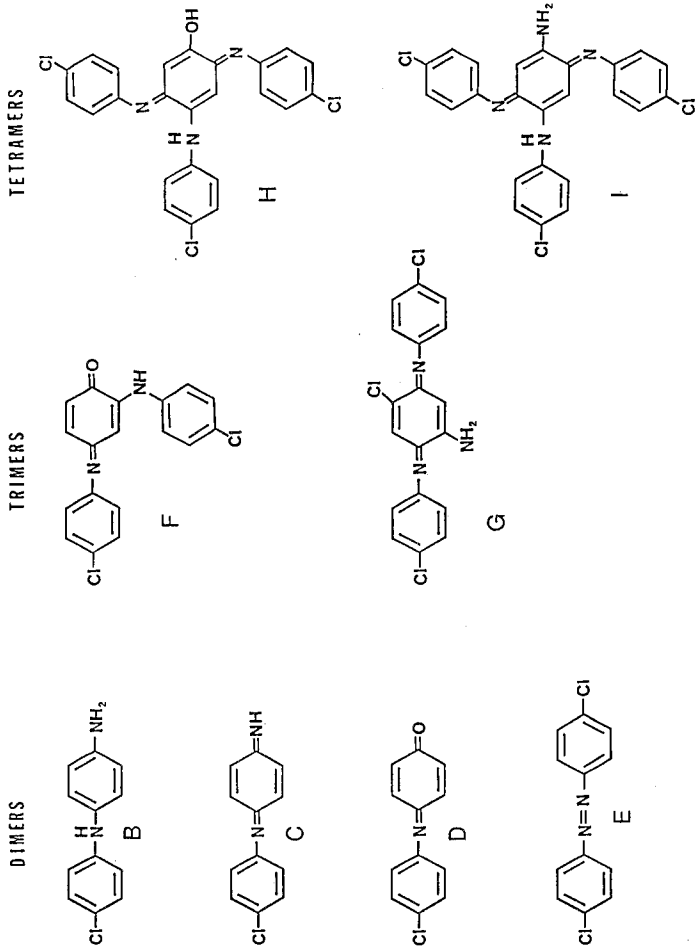


FIGURE 2 Proposed structures of the oligomers produced by oxidoreductase transformation of 4-chloroaniline.

Because compound C appeared to be rapidly hydrolyzed to compound D, a benzoquinone monoimine dimer, compound C was proposed to be a benzoquinone di-imine dimer.[15] However, high resolution mass spectrometric analysis of compound C gave a molecular weight of 218.059 amu for elemental composition $C_{12}H_{11}N_2Cl$, which describes a diamine dimer. We postulated that compound C was reduced (nominally, addition of 2 hydrogen atoms) to compound B in the electron impact ionization chamber. Quinone-type compounds, such as compound C, are known to have high potentials as oxidizing agents since reduction produces a stable aromatic ring.[16] This facile reduction of the di-imine to a diamine could also explain the presence of compound B in the reaction medium. Thus, we analyzed the di-imine dimer (C) as a diamine dimer (B).

The HPLC retention time of compound B (4.33 min) relative to compound C (4.93 min) also supports the reduced-oxidized structure relationship for B and C. The oxidized di-imine form (C) would be expected to have less hydrogen bonding interaction with the methanolic mobile phase, and hence be retained longer than the reduced diamine form (B). The same type of reverse phase retention behavior was observed for compound D (5.86 min) and its aminophenol reduced form (5.23 min).

Based on the above evidence, we propose the structures of *N*-(4-chlorophenyl)-*p*-phenylene diamine (with a calculated molecular weight of 218.016 amu for elemental composition $C_{12}H_{11}N_2Cl$) for compound B and *N*-(4-chlorophenyl)-*p*-phenylene di-imine (with a calculated molecular weight of 216.045 amu for elemental composition $C_{12}H_9N_2Cl$) for compound C.

Compound D

Compound D was believed to have been formed by nonenzymatic hydrolysis of the terminal imine moiety of compound C (see the preceding descriptions of compounds C and B). High resolution mass spectrometric analysis of compound D yielded a molecular weight of 217.031 amu for the elemental composition $C_{12}H_8NOCl$ (calculated molecular weight of 217.030 amu). The presence of the quinone carbonyl moiety was confirmed by reduction and reductive acetylation studies. Mass spectra of the reduced and acetylated

compound D yielded m/z 219 and m/z 261, respectively, which correspond to the molecular ions for each of these compounds.

Proton NMR analysis data for compound D is listed in Table III. Proton assignments for Hb, Hc, He, and Hd were made with reference to work on similar compounds by Iwan *et al.*[17] and Bollag *et al.*[18].

Based on the mass spectrometry and proton NMR data, compound D was identified as *N*-(4-chlorophenyl)-benzoquinone monoimine.

Compound E

Compound E migrated on the TLC plate as a bright yellow band (Rf 0.86) and had an HPLC retention time of 15.40 minutes. Mass spectrometric analysis gave an m/z of 250 for the molecular ion. Identification of compound E as 4,4-dichloroazobenzene was based on correlating these data with previous reports.[1,2]

Compound F

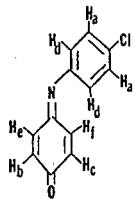
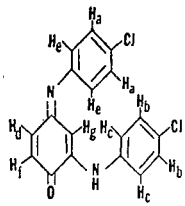
Compound D was dissolved in a solution of 4-chloroaniline, and subsequent TLC and HPLC analyses of the mixture showed the presence of 4-chloroaniline and compounds D, F and H. If pure compound F was dissolved in a solution of 4-chloroaniline, compounds F and H, and 4-chloroaniline were detected with TLC and HPLC. The results of these experiments implied that compounds F and H were formed nonenzymatically by sequential condensations of compound D and 4-chloroaniline.

Mass analysis of compound F gave a molecular weight of 342.031 amu corresponding to the elemental composition $C_{18}H_{12}N_2OCl_2$ (calculated molecular weight of 342.033 amu). Proton NMR data for compound F are listed in Table III. Ha and Hb assignments were made with reference to work on similar compounds by Bollag *et al.*[18] Homonuclear decoupling experiments showed *ortho* coupling between Ha and He, Hb and Hc, and Hd and Hf. *Meta* coupling between Hd and Hg was also found. Nuclear Overhauser effect difference experiments showed Hg to be in the 3-quinone position.

Signals for protons attached to the nitrogen (or oxygen) atoms were not observed in NMR analyses of compounds F, G, H, or I.

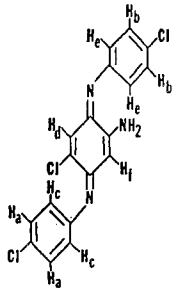
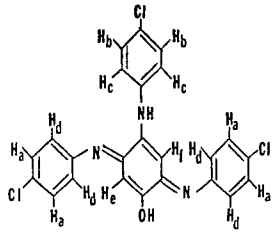
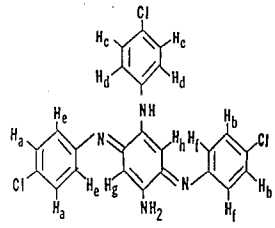
TABLE III

Proton nuclear magnetic resonance data for oligomers of 4-chloroaniline

Compound	Chemical shift ppm	Splitting[a] (integral)	Proton assignments	Coupling constant Hz
 Compound D	7.47	dd (2H)	H_a	6.8 2.0
	7.32	dd (1H)	H_b	10.0 2.6
	7.14	dd (1H)	H_c	10.4 2.6
	6.97	dd (2H)	H_d	6.8 2.0
	6.68	dd (1H)	H_e	10.0 2.0
	6.56	dd (1H)	H_f	10.4 2.2
 Compound F	7.42	m (2H)	H_a	
	7.35	m (2H)	H_b	
	7.30	m (2H)	H_c	
	7.25	dd (1H)	H_d	9.0 2.5
	6.96	m (2H)	H_e	
	6.76	d (1H)	H_f	9.0
	6.34	t (1H)	H_g	2.5

[a] *s* = singlet; d = doublet; dd = doublet of doublets; t = triplet; m = multiplet.

TABLE III (continued)

Compound	Chemical shift ppm	Splitting[a] (integral)	Proton assignments
 Compound G	7.48	m (2H)	H_a
	7.40	m (2H)	H_b
	7.00	m (2H)	H_c
	6.95	s (1H)	H_d
	6.84	m (2H)	H_e
	5.81	s (1H)	H_f
 Compound H	7.44	m (4H)	H_a
	7.35	m (2H)	H_b
	7.32	m (2H)	H_c
	7.02	m (4H)	H_d
	6.10	m (1H)	H_e
	6.00	m (1H)	H_f
 Compound I	7.40	m (2H)	H_a
	7.39	m (2H)	H_b
	7.33	m (2H)	H_c
	7.28	m (2H)	H_d
	6.95	m (2H)	H_e
	6.89	m (2H)	H_f
	6.05	s (1H)	H_g
	5.63	s (1H)	H_h

[a] s = singlet; d = doublet; dd = doublet of doublets; t = triplet; m = multiplet.

Such a signal is usually a broad multiplet which scarcely rises above baseline. The compounds mentioned were not present in sufficient quantities to produce a discernible signal for the amino or phenolic protons.

Evidence gathered from the mass spectrometric and NMR analyses, and from the nonenzymatic reaction experiments, led to the identification of compound F as 2-(4-chloroanilino)-*N*-(4-chlorophenyl)-benzoquinone.

Compound G

High resolution mass spectral analysis of compound G gave a molecular weight of 377.009 amu for the elemental composition $C_{18}H_{12}H_3Cl_3$ (calculated molecular weight of 377.007 amu).

The structure in Table III was proposed for compound G in a previous report[19] but proton NMR data were not published. In our studies, decoupling experiments showed *ortho* coupling between Ha and Hc, and between Hb and He. Equivalent protons on the two *N*-substituted aromatic rings showed different chemical shifts which would be predicted from their proximity to the di-imine ring chlorine. Thus, because the Ha and He protons are one atom closer to and more influenced by the chlorine than the Hb and He protons, signals for the Ha and He are shifted downfield relative to Hb and He. Hd is *ortho* to the di-imine ring chlorine, so its signal is shifted downfield from Hf. The NMR data for compound G confirmed the structure of 2-amino-5-chlorobenzoquinone-di-4-chloroanil.

Compound H

HPLC and TLC analyses of a solution containing compound F and 4-chloroaniline also detected the presence of compound H. Figure 2 shows that compound H is a tetramer in the oligomeric series containing compound D (dimer), compound F (trimer), and compound H. The addition of 4-chloroaniline at the 2-quinone position of the dimer produces the trimer, and addition at the 5-quinone position of the trimer produces compound H, a tetramer.

Mass analysis of compound H yielded a molecular weight of 467.033 amu for the elemental composition $C_{24}H_{16}N_3OCl_3$ (calculated molecular weight of 467.036 amu).

Data from proton NMR analysis established that compound H has a substituted benzoquinone di-imine structure, rather than the benzoquinone monoimine structure found for compounds D and F. Structure assignments for compounds D and F showed that the 4-chloroaniline ring is either conjugated to the benzoquinone ring through an imine bond or is substituted on the benzoquinone ring through amine bonds. Comparison of chemical shifts for corresponding protons on the *p*-chloroimine and the *p*-chloroaniline ring of compounds D and F showed that downfield chemical shifts for protons *ortho* to chlorine and upfield chemical shifts for protons *meta* to chlorine are obtained when the aromatic ring is conjugated to the benzoquinone ring. The chemical shift data for compound H indicate that two *p*-chloroimine rings and one *p*-chloroamine ring are substituted on the benzoquinone ring. This evidence, along with mass spectrometric data, led to the assignment of the structure 2-(4-chloroanilino)-5-hydroxybenzoquinone-di-4-chloroanil to compound H.

Compound I

When a solution of 4-chloroaniline was added to an isolate of compound C (a dimer), compound I was formed as confirmed by TLC and HPLC. High resolution mass spectrometry gave a molecular weight of 466.050 amu for the elemental composition $C_{24}H_{27}N_4Cl_3$ (calculated molecular weight of 466.052 amu) which suggested that compound I is the condensation product of two molecules of 4-chloroaniline and one molecule of compound C.

Proton assignments for compound I in Table III were made with reference to those for compound H. As with compound H, compound I contained two *p*-chloroimine aromatic rings, although chemical shift values for corresponding protons of the respective compounds were slightly different. Decoupling experiments confirmed the *ortho* positional relationship for proton pairs Ha and He, Hb and Hf, and Hc and Hd.

The evidence described above led to assigning the structure of 2-amino-5-(4-chloroanilino)-benzoquinone-di-4-chloroanil to compound I.

CONCLUSION

This report describes chemical structures for the oligomeric products obtained from the enzymatic oxidation of 4-chloroaniline. The product mixture contained eight oligomers ranging in size from dimer to tetramer with benzoquinone monoimine, benzoquinone diimine, diaminobenzene, and azobenzene structures.

A direct injection reverse-phase HPLC method for the determination of substrate and product amounts in the reaction solution is also presented. The HPLC method is ideal for the analysis of substituted anilines and their oligomeric oxidation products in that transient species are detected and artifacts are avoided. Both the product structure descriptions and the reaction component quantitation method can be applied to the elucidation of the oxidative transformation of 4-chloroaniline in the soil.

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