# Conversion of Pentahalogenated Phenols by Microperoxidase-8/$H_2O_2$ to Benzoquinone-Type Products

Ahmed M. Osman,*,† Maarten A. Posthumus,‡ Cees Veeger,† Peter J. van Bladeren,§ Colja Laane,† and Ivonne M. C. M. Rietjens†

*Department of Biomolecular Sciences, Laboratory of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands, Department of Biomolecular Sciences, Laboratory of Organic Chemistry, Agricultural University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands, and TNO Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ Zeist, The Netherlands*

*Received February 26, 1998*

This study reports the microperoxidase-8 (MP8)/$H_2O_2$-catalyzed dehalogenation of pentafluorophenol and pentachlorophenol, compounds whose toxic effects and persistence in the environment are well documented. The primary products of this dehalogenation reaction appear to be the corresponding tetrahalo-*p*-benzoquinones. Under the conditions used, the fluorinated phenol and its intermediate products are more susceptible to degradation than the corresponding chlorinated analogue and its products. The main degradation products of tetrachloro-*p*-benzoquinone and tetrafluoro-*p*-benzoquinone were identified as trichlorohydroxy-*p*-benzoquinone and trifluorohydroxy-*p*-benzoquinone, respectively. This secondary conversion of tetrafluoro-*p*-benzoquinone and tetrachloro-*p*-benzoquinone was not mediated by MP8, but was driven by $H_2O_2$. Evidence is presented for a mechanism where $H_2O_2$ molecules and not hydroxide anions are the reactive nucleophilic species attacking the tetrahalo-*p*-benzoquinones. In addition to the formation of the trihalohydroxy-*p*-benzoquinones, the formation of adducts of the tetrahalo-*p*-benzoquinone products with ethanol, present in the incubation medium, was observed. The adduct from the reaction of tetrachloro-*p*-benzoquinone with ethanol was isolated and identified as trichloroethoxyquinone. Thus, the present paper describes a system in which the formation of tetrahalo-*p*-benzoquinone-type products by an oxidative heme-based catalyst could be unequivocally demonstrated.

## Introduction

Microperoxidase-8 (MP8)[1] is a heme octapeptide derived from the enzymatic digestion of horse heart cytochrome *c*. Microperoxidases are often used as model systems for redox catalytic heme proteins such as peroxidases and cytochrome P450 enzymes (*1*). Recent investigations suggested that MP8 reacts with hydrogen peroxide to form intermediate compounds analogous to compounds I and II of horseradish peroxidase (*2*−*4*).

The elucidation of the degradation pathways of aromatic polyhalogenated compounds is of particular importance because of their persistence in the environment with potential deleterious effects to both plants and animals. Although peroxidase-catalyzed oxidation of chlorinated phenols to the corresponding benzoquinones was previously shown (*5*−*8*), neither the secondary degradation of the latter products nor the oxidation of pentafluorophenol has, to our knowledge, been reported. Previous work (*8*) showed that the MP8/$H_2O_2$ system is capable of catalyzing the dehalogenation of mono-*p*-halogenated phenols, resulting in the elimination of the halogen as anion and the formation of *p*-benzoquinone as the only dehalogenated product of the reaction. By using $H_2{}^{18}O_2$ and $H_2{}^{18}O$, it was demonstrated that the oxygen atom inserted into the product, *p*-benzoquinone, derived from the medium (water) and not from hydrogen peroxide.

The present study was carried out to investigate whether MP8 in the presence of $H_2O_2$ could also catalyze the dehalogenation of polyhalogenated phenols and give rise to reactive tetrahalo-*p*-benzoquinones. Direct proof of the formation of these reactive tetrahalo-*p*-benzoquinones would be of importance since their formation in oxidative (P450-catalyzed) dehalogenation reactions has always been derived from indirect evidence, namely, the detection of the reduced tetrahalohydroquinone analogues upon the addition of ascorbic acid or NADPH into the reaction mixtures (*9*, *10*). The results presented in this paper show that MP8 could indeed convert pentahalophenols to tetrahalo-*p*-benzoquinones as the primary metabolites. The absence of a large protein moiety in the case of MP8 as compared to cytochromes P450 apparently eliminates the swift protein binding, providing the possibility for direct detection of the reactive tetrahalo-*p*-benzoquinones.

## Materials and Methods

**Chemicals.** MP8 was prepared by limited proteolytic digestion of horse heart cytochrome *c* as described in the literature

* Address correspondence to this author at the Department of Biomolecular Sciences, Laboratory of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands. Phone: 31-317-482868. Fax: 31-317-484801.
† Laboratory of Biochemistry, Agricultural University.
‡ Laboratory of Organic Chemistry, Agricultural University.
§ TNO Nutrition and Food Research Institute.

[1] Abbreviations: MP8, microperoxidase-8; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography/mass spectrometry.

10.1021/tx980037l CCC: $15.00 
Published on Web 10/13/1998

(*11*), and its concentration was determined either by the chromogen method (*11*) or by measuring the absorbance at 397 nm ($\epsilon_{397\ nm} = 1.5 \times 10^5\ M^{-1}\ cm^{-1}$) in 0.1 M potassium phosphate (pH 7.0) (*3*). The purity of this preparation of MP8 was judged from HPLC analysis to be over 95%. Pentachlorophenol, tetrafluorophenol, tetrafluorohydroquinone, and protoporphyrin IX were obtained from Aldrich (Steinheim, Germany). Tetrachlorophenol and tetrachloro-*p*-benzoquinone (chloranil) were purchased from Janssen (Beerse, Belgium). Pentafluorophenol was from Sigma (St. Louis, MO), and tetrafluoro-*p*-benzoquinone was from Fluorochem (Derbyshire, U.K.). L-Ascorbic acid and hydrogen peroxide (v/v, 30%) were from Merck (Darmstadt, Germany). Hydrogen peroxide was diluted before use to a stock solution of 50 mM in demineralized water. The concentrations of the stock solutions of $H_2O_2$ were determined spectrophotometrically ($\epsilon_{240\ nm} = 39.4\ M^{-1}\ cm^{-1}$) (*12*); 91% enriched labeled $H_2{}^{18}O_2$ (v/v, 2%) was purchased from Icon (Sunnit, NJ).

Trichlorohydroxy-*p*-benzoquinone was prepared as described in the literature (*13*) by dissolving solid chloranil (30 mg) in an ice-cold 2 M sodium hydroxide solution. This solution was acidified, keeping it in ice, with concentrated hydrochloric acid. The purity of this synthesized compound was judged from HPLC analysis which gave only one peak, and the compound was identified by mass spectrometry (MS) (data not shown). The recovery was quantitative. Trifluorohydroxy-*p*-benzoquinone was synthesized from tetrafluoro-*p*-benzoquinone following the same procedure described above for the synthesis of trichlorohydroxy-*p*-benzoquinone. The purity and the identity of this synthesized trifluorohydroxy-*p*-benzoquinone were judged from both HPLC and GC-MS analysis (see Results).

**Incubation Conditions.** A typical reaction mixture consisted of the halophenol (0.25−2 mM final concentration) and MP8 [7.5 $\mu$M final concentration in 0.1 M Tris-HCl (pH 7.0) containing 50% ethanol, or in some cases, when indicated, in 0.1 M potassium phosphate (pH 7.6)]. Sometimes, as indicated, ascorbic acid (0.5−3 mM final concentration) was added to the reaction mixture. Reaction mixtures were preincubated at 37 °C for 2 min. The reaction was started by the addition of hydrogen peroxide (2.5 mM final concentration). After 1 min of incubation, the reaction was stopped by freezing the samples in liquid nitrogen. Furthermore, since iron in the presence of $H_2O_2$ could lead to Fenton-type chemistry, the following incubations were carried out as control experiments: 2 mM pentachlorophenol was incubated either with 10 $\mu$M protoporphyrin IX or with 100 $\mu$M ferrous salts ($FeCl_2 \cdot 4H_2O$ or $FeSO_4 \cdot 7H_2O$) in a reaction mixture of 1 mL in 0.1 M Tris-HCl (pH 7.0) containing 50% ethanol. Following the addition of 2.5 mM hydrogen peroxide (final concentration) to the reaction mixture, the reaction was incubated for 1 min at 37 °C. The reaction was terminated by freezing the samples in liquid nitrogen.

**Effect of $H_2O_2$ on the Noncatalyzed Conversion of Tetrachloro-*p*-benzoquinone and Tetrafluoro-*p*-benzoquinone to the Corresponding Trihalohydroxy-*p*-benzoquinones.** To examine the effect of $H_2O_2$ on the noncatalyzed conversion of the tetrahalo-*p*-benzoquinones to the corresponding trihalohydroxy-*p*-benzoquinones, various concentrations of hydrogen peroxide (0−2.5 mM) were incubated with 1 mM either tetrachloro-*p*-benzoquinone or tetrafluoro-*p*-benzoquinone in 0.1 M Tris-HCl pH 7.0, containing 50% ethanol for 1 min at 37 °C. After 1 min of incubation, the samples were put in liquid nitrogen until analysis by HPLC.

**Isolation of Trichlorohydroxy-*p*-benzoquinone Obtained by Incubating Labeled $H_2{}^{18}O_2$ and Unlabeled $H_2O_2$ with Tetrachloro-*p*-benzoquinone.** To understand the source of the oxygen atom incorporated into the trichlorohydroxy-*p*-benzoquinone, formed from the reaction of $H_2O_2$ with the tetrachloro-*p*-benzoquinone, 2 mM tetrachloro-*p*-benzoquinone was incubated with 5 mM labeled $H_2{}^{18}O_2$ in a final volume of 1 mL of Tris-HCl (pH 7.0) containing 50% ethanol. A control experiment with normal $H_2O_2$ was also performed. In these incubations, the concentrations of both labeled and unlabeled hydrogen peroxide were increased 2-fold (relative to the standard incubation condition) to obtain more product formation. After 1 min of incubation, the samples were put into liquid nitrogen. The product, trichlorohydroxy-*p*-benzoquinone, formed in these incubations was isolated by HPLC and was identified by MS.

**Isolation of Trichloroethoxy-*p*-benzoquinone.** To isolate trichloroethoxy-*p*-benzoquinone, 6.2 mg of tetrachloro-*p*-benzoquinone was dissolved in 5 mL of absolute ethanol. Of this solution, 100 $\mu$L was taken and added to a mixture of 350 $\mu$L of 0.1 M Tris-HCl (pH 7.0) and 250 $\mu$L of absolute ethanol. The resulting solution was well mixed and incubated at 37 °C for 2 min. This sample was frozen in liquid nitrogen until analyzed by HPLC.

**High-Performance Liquid Chromatography (HPLC).** A volume of 10−50 $\mu$L of each of the reaction mixtures (see incubation conditions) was loaded onto a Lichrosphere RP8 column (150 × 4.6 mm). Elution was performed at 1 mL/min, starting with 100% solvent A (1% acetic acid in water) and then maintaining 100% of this solvent for 1 min, followed by a linear gradient to obtain 100% methanol in 25 min. For the isolation of trichloroethoxy-*p*-benzoquinone from tetrachloro-*p*-benzoquinone, the gradient was increased linearly to obtain 94% methanol in 19 min and was maintained at 94% methanol for 1 min, followed by a linear increase of the gradient to 100% methanol in 25 min. UV detection and measurement of UV absorption spectra of the eluted compounds were performed with a Waters 996 diode array detector.

**Analysis by Gas Chromatography−Mass Spectrometry (GS−MS).** For fluorinated derivatives, the gas chromatograph (Hewlett-Packard 5890) was equipped with a 30 m × 0.25 mm ID capillary DB 17 column (J and W Scientific, Folsom, CA). The carrier gas was helium at a flow rate of about 1 mL/min. A temperature gradient from 70 to 200 °C for 13 min was applied. The column was connected to a Hewlett-Packard 5970B mass spectrometer.

For chlorinated derivatives, GC was not successful, and the solutions in dichloromethane were introduced via a direct insertion probe into the ion source of a Finnigan MAT 95 mass spectrometer operated in the 70 eV, EI (electron impact) ionization mode at resolution RP = 1000 for the mass spectrum determination and RP = 7000 for the accurate mass measurements, respectively.

**Molecular Orbital Calculations.** Molecular orbital calculations were carried out on a Silicon Graphics Indigo$^2$ using Spartan version 5.0 (Wave Function Inc., Irvine, CA). The semiempirical molecular orbital method was used, applying the PM3 Hamiltonian. Geometries were optimized for all bond lengths, bond angles, and torsion angles.

## Results

**Incubation of Pentachlorophenol with MP8/$H_2O_2$.** Upon analyzing various conditions, it was found that the reaction products were relatively stable in 0.1 M Tris-HCl (pH 7.0) containing 50% ethanol which was consequently employed.

Figure 1A presents the results from HPLC analysis of the incubation of pentachlorophenol with MP8 in the presence of hydrogen peroxide. After 1 min of incubation, about 14% of the substrate was converted to products. No further degradation of the substrate was observed when the time of incubation was increased to 5 min, due to the inactivation of MP8 (*14*). No product formation was observed in control incubations in which either MP8 or $H_2O_2$ was omitted from the reaction mixtures. In the presence of MP8 and $H_2O_2$, three major products were found (Figure 1A). The peak with the retention time of 13.3 min, which is the dominant product, was identified as trichlorohydroxy-*p*-benzoquinone. This product was identified by comparing its retention time and the UV spectrum with those of an authentic trichlorohydroxy-*p*-

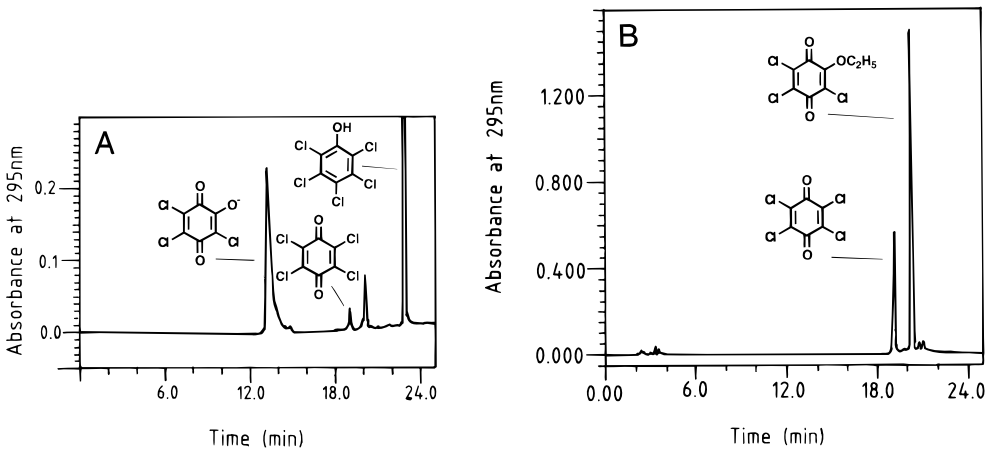


**Figure 1.** (A) HPLC elution profile of the incubation mixture of 7.5 $\mu$M MP8 and 2.5 mM $H_2O_2$ with 2 mM pentachlorophenol in 0.1 M Tris-HCl (pH 7.0) containing 50% ethanol. (B) HPLC profile of a sample of tetrachloro-*p*-benzoquinone dissolved in 0.1 M Tris-HCl (pH 7.0) containing 50% ethanol and incubated for 2 min at 37 °C.

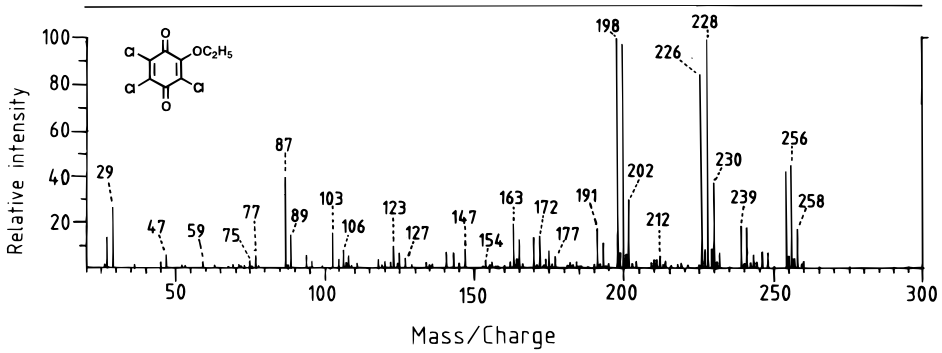


**Figure 2.** Mass spectrum (MS) of the product with a retention time of 20.1 min (Figure 1A), identified as trichloroethoxyquinone.

benzoquinone standard. This product gives the reaction mixture a characteristic purple color at neutral pH, that could be attributed to the anionic form of trichlorohydroxy-*p*-benzoquinone ($pK_a = 1.09$) (*15*). The protonated form of this product, obtained by dissolving it in 1 N HCl, as well as its reduced form obtained by incubating with ascorbic acid, resulted in the disappearance of the purple color. The peak with the retention time of 19 min was identified as tetrachloro-*p*-benzoquinone by comparing its retention time and UV spectrum with tetrachloro-*p*-benzoquinone. The peak at retention time 20.1 min is derived spontaneously from tetrachloro-*p*-benzoquinone. Dissolving tetrachloro-*p*-benzoquinone in the same buffer used for the enzymatic reaction and incubating for 2 min at 37 °C, omitting MP8 and $H_2O_2$, resulted in the formation of the metabolite with retention time 20.1 min (Figure 1B). This product was isolated by HPLC and analyzed by mass spectrometry (Figure 2). The mass spectrum shows a trichloro compound of molecular weight 254, losing masses 28 and 18 consecutively to form fragments at 226 and 198, respectively. Accurate mass measurement at a resolution of 7000 for the molecular ion peak *m*/*z* 254 was 253.9308 amu, in agreement with the elemental composition $C_8H_5Cl_3O_3$ (calculated mass = 253.9304). Therefore, the product can be identified as trichloroethoxy-*p*-benzoquinone. The slight deviation of the isotopic pattern of the peak clusters at *m*/*z* 226 and 254 from the theoretical pattern is due to the presence of a small amount of the reduced form of this compound (trichloroethoxyhydroquinone MW = 256). During freeze-drying, the reduction of this adduct by methanol occurred. We showed previously (*16*) that alcohols could act as reductants for activated benzoquinone-type products. No detectable amount of this adduct (trichloroethoxyquinone) was observed when a solution of tetrachloro-*p*-benzoquinone was dissolved in pure ethanol and incubated for 2 min at 37 °C. Moreover, the rate of formation of this adduct increased 9-fold when tetrachloro-*p*-benzoquinone was incubated in 0.1 M Tris-HCl (pH 8.0) containing 50% ethanol. This indicates that the aqueous buffered solution favored the interaction of ethanol with chloranil. Together, the results presented in Figures 1 and 2 demonstrate that tetrachloro-*p*-benzoquinone is the primary product resulting from the MP8-mediated dehalogenation of pentachlorophenol.

**Incubations of Pentafluorophenol with MP8/$H_2O_2$.** Figure 3A shows the HPLC chromatogram of the reaction mixture obtained from the incubation of pentafluorophenol with MP8 and hydrogen peroxide. The HPLC profile is analogous to that described above for pentachlorophenol. Pentafluorophenol was more readily converted than the chlorinated analogue. After 1 min of incubation, about 75% of the pentafluorophenol was converted as compared to 14% for pentachlorophenol. However, although readily observable, the product, tetrafluoro-*p*-benzoquinone (the minor peak with retention time 12 min in Figure 3A), was less stable as compared to tetrachloro-*p*-benzoquinone. One of the major products was identified as trifluorohydroxy-*p*-benzoquinone by comparing its retention time (8 min) and UV spectrum to an authentic reference compound synthesized from tetrafluoro-*p*-benzoquinone. As shown in Figure 3B, a minor product with a retention time of 2.3 min was also present in the preparation. This minor product increased

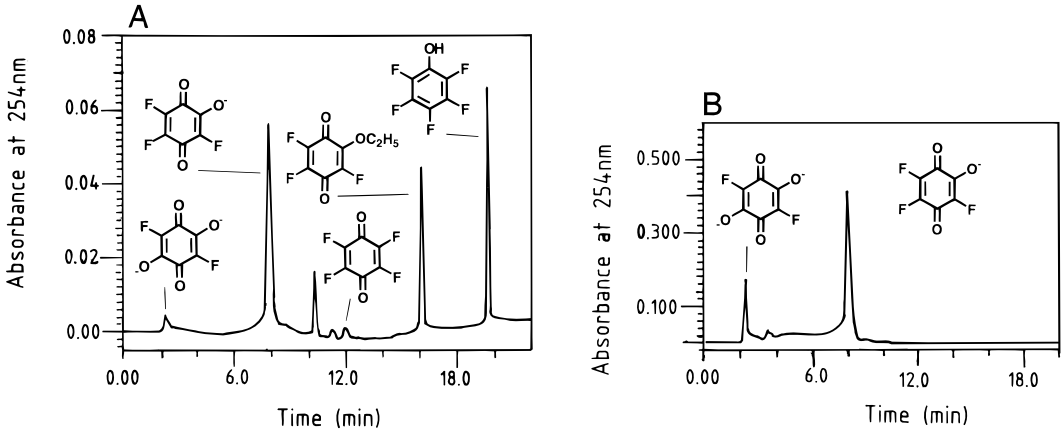


**Figure 3.** (A) HPLC elution profile of the incubation mixture of 7.5 $\mu$M MP8 and 2.5 mM $H_2O_2$ with 2 mM pentafluorophenol in 0.1 M Tris-HCl (pH 7.0) containing 50% ethanol. The p$K_a$ of trifluorohydroxy-*p*-benzoquinone is 1.73, and the two p$K_a$'s for 2,5-difluoro-3,6-dihydroxy-*p*-benzoquinone are p$K_{a1}$ = 1.4 and p$K_{a2}$ = 3.3 (*15*). The peak with retention time 10.4 min is a degradation product of trifluoroethoxy-*p*-benzoquinone. (B) HPLC chromatogram of the preparation of trifluorohydroxy-*p*-benzoquinone synthesized from tetrafluoro-*p*-benzoquinone.

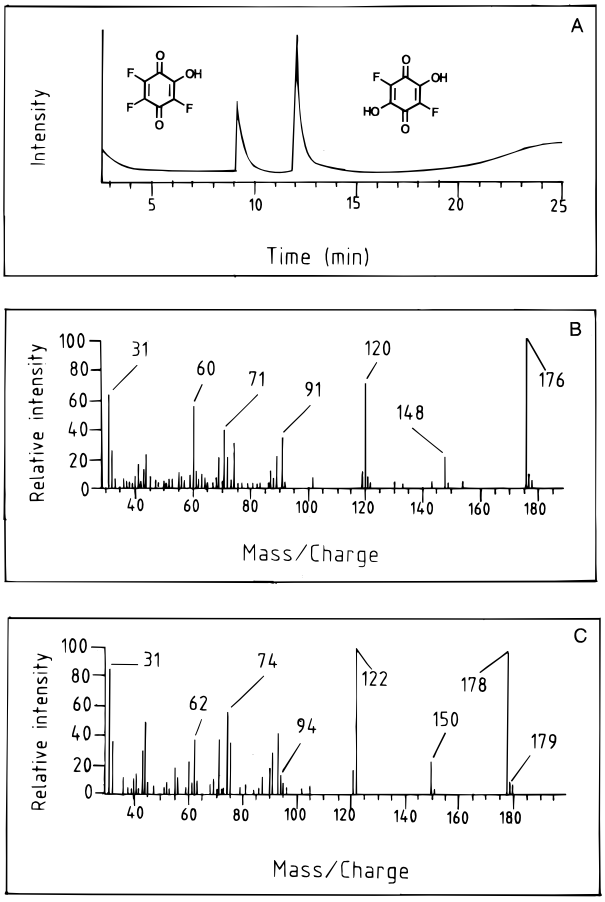


**Figure 4.** (A) Gas chromatographic analysis of the preparation of trifluorohydroxy-*p*-benzoquinone, freeze-dried overnight and dissolved in ethyl acetate. (B) Mass spectrum of the peak with retention time 12.1 min, identified as 2,5-difluoro-3,6-dihydroxy-*p*-benzoquinone. (C) Mass spectrum of the peak with retention time 9.2 min, identified as trifluorohyroxy-*p*-benzoquinone.

with time, accompanied by a parallel decrease of the concentration of trifluorohydroxy-*p*-benzoquinone. This indicates that this minor product is spontaneously generated from trifluorohydroxy-*p*-benzoquinone. Figure 4A shows the results obtained from the GC analysis of this mixture. The peak with the retention time of 12.1 min is the degradation product of trifluorohydroxy-*p*-benzoquinone. The mass spectrum of this degradation product (Figure 4B) shows a molecular ion peak at *m*/*z* 176, which is 2 amu lower than the molecular weight of trifluorohydroxy-*p*-benzoquinone, *m*/*z* 178 (Figure 4C). The mass spectrum of the degradation product (Figure 4B) exhibits spectral fragmentation similar to that of the parent compound, trifluorohydroxy-*p*-quinone (Figure 4C). However, the fragment *m*/*z* 62 ($C_2F_2$) is absent in the mass spectrum of the degradation product. This is consistent with the replacement of one of the neighboring fluorine atoms by a hydroxyl group. The product was, therefore, identified as 2,5-difluoro-3,6-dihydroxy-*p*-benzoquinone. Two other products (retention times of 16.2 and 10.4 min) were also found in the HPLC chromatogram of the incubation reaction mixture (Figure 3A). The peak with the retention time of 16.2 min appears to be the ethoxy adduct of tetrafluoro-*p*-benzoquinone, since from fluoranil, dissolved in 0.1 M Tris-HCl (pH 7.0) containing 50% ethanol, this peak was formed, as in the case of the chlorinated compound (data not shown). Thus, it is inferred that this product is the corresponding trifluoroethoxy-*p*-benzoquinone. Attempts to isolate this product failed. The product with the retention time of 10.4 min, shown in Figure 3A, is a degradation product from trifluoroethoxy-*p*-benzoquinone. Thus, it is concluded that tetrafluoro-*p*-benzoquinone is the primary product formed from the MP8/$H_2O_2$-mediated dehalogenation of pentafluorophenol.

**Effect of $H_2O_2$ on the Noncatalyzed Conversion of Tetrahalo-*p*-benzoquinones to the Corresponding Trihalohydroxy-*p*-benzoquinones.** As indicated in Figure 1B, tetrachloro-*p*-benzoquinone does not give rise to spontaneous formation of trichlorohydroxy-*p*-benzoquinone in the absence of MP8 and $H_2O_2$. Thus, the mechanism in which a hydroxide ion replaces the chlorine under alkaline conditions (*13*) does not occur significantly under our experimental conditions. When tetrachloro-*p*-benzoquinone was incubated with $H_2O_2$ in the presence and in the absence of MP8, in both cases the hydroxy compound was formed, showing that its formation depends only on $H_2O_2$. The data in Figure 5A show that the formation of trichlorohydroxy-*p*-benzoquinone from tetrachloro-*p*-benzoquinone increases with increasing concentration of $H_2O_2$ and is accompanied by a parallel decrease of the formation of the trichloroethoxy adduct. At low concentrations of hydrogen peroxide, the

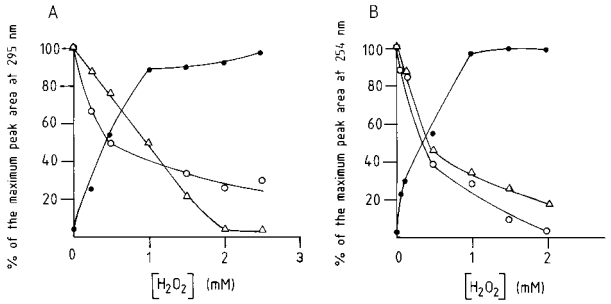


**Figure 5.** Effect of $H_2O_2$ on the spontaneous conversion of (A) tetrachloro-*p*-benzoquinone to trichlorohydroxy-*p*-benzoquinone and (B) tetrafluoro-*p*-benzoquinone to trifluorohydroxy-*p*-benzoquinone. Concentrations of hydrogen peroxide (0−2.5 mM) were incubated with 1 mM either tetrachloro-*p*-benzoquinone or tetrafluoro-*p*-benzoquinone in 0.1 M Tris-HCl (pH 7.0) containing 50% ethanol for 1 min at 37 °C. (○) Tetrahalo-*p*-benzoquinone; (△) trihaloethoxyquinone; (●) trihalohydroxy-*p*-benzoquinone. The maximum peak area of each product at the specified wavelength was taken as 100%.

formation of the trichloroethoxy adduct prevails over the formation of trichlorohydroxy-*p*-benzoquinone. In contrast, at concentrations of $H_2O_2$ above 1 mM, the formation of trichlorohydroxy-*p*-benzoquinone dominates over trichloroethoxy formation. Similar results were obtained in the case of the conversion of tetrafluoro-*p*-benzoquinone (Figure 5B). Hydrogen peroxide was shown also to stimulate the formation of trifluorohydroxy-*p*-benzoquinone from tetrafluoro-*p*-benzoquinone at the expense of the formation of trifluoroethoxy-p-quinone.

**Origin of the Oxygen in Trichlorohydroxy-*p*-benzoquinone Formed from Tetrachloro-*p*-benzoquinone with $H_2O_2$.** Tetrachloro-*p*-benzoquinone was incubated with labeled $H_2{}^{18}O_2$ or with unlabeled $H_2O_2$, and, as expected, the major product formed was trichlorohydroxy-*p*-benzoquinone. The mass spectra of the molecular ion region of trichlorohydroxy-*p*-benzoquinone, obtained with unlabeled and labeled $H_2{}^{18}O_2$ (Figure 6A,B), indicate the shift of the molecular ion isotope cluster peaks of the unlabeled compound (*m*/*z* 226, 228, 230, and 232) with 2 mass units, as can be expected for the incorporation of $^{18}O$. The small peak at *m*/*z* 226 in the labeled experiment originates from the unlabeled trichlorohydroxy-*p*-benzoquinone formed as a result of using not fully (91%) labeled $H_2{}^{18}O_2$.

The result of these experiments indicates that $H_2O_2$ is the source of the oxygen atom inserted into the trichlorohydroxy-*p*-benzoquinone.

**Effect of Ascorbic Acid and Fenton-Type Reaction Catalysts on the Degradation of the Pentahalogenated Phenols.** The formation of all products reported in Figures 1A and 3A was completely inhibited by the addition of 3 mM ascorbic acid to the reaction mixture (data not shown). This result is in agreement with previous findings (*8*), where also MP8-mediated oxidative dehalogenation of mono-*p*-halogenated phenols was completely suppressed by ascorbic acid. Finally, the possibility that Fenton-type chemistry might be involved in the MP8/$H_2O_2$-mediated oxidation of the pentahalogenated phenols was investigated. The incubation of 2 mM pentachlorophenol with 10 $\mu$M protoporphyrin IX or with 100 $\mu$M ferrous salts both in the presence and in the absence of $H_2O_2$ under experimental conditions similar to those used for the MP8-catalyzed reaction did not result in product formation. This result excludes the possibility that traces of iron cations, which might be liberated from the heme cofactor upon inactivation, could catalyze the oxidative dehalogenation of the pentahalogenated phenols. This implies that Fenton-type chemistry is not involved in the MP8-catalyzed oxidation of the pentahalogenated phenols. This conclusion is in agreement with a previous report where the superoxide anion and hydroxyl radical scavengers were shown to have no effect on the MP8/$H_2O_2$-mediated hydroxylation of aniline (*17*).

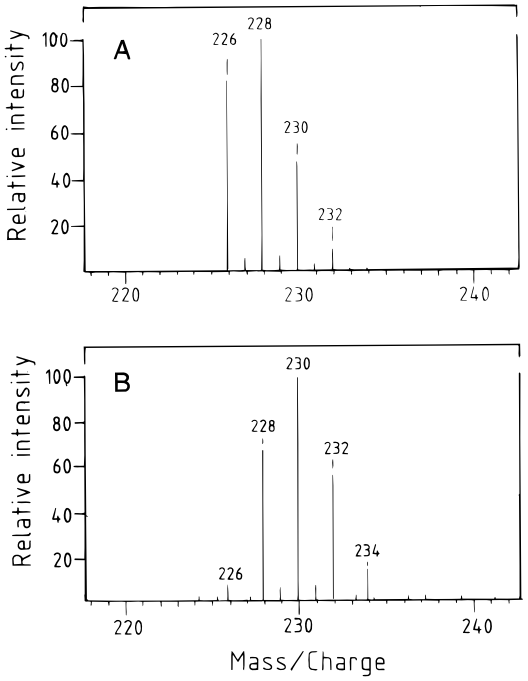


**Figure 6.** Mass spectra of the molecular ion region of trichlorohydroxy-*p*-benzoquinone obtained from the incubations of tetrachlorohydroxy-*p*-benzoquinone with (A) unlabeled $H_2O_2$ or with (B) labeled $H_2{}^{18}O_2$.

## Discussion

The results presented in this paper show that MP8, in the presence of hydrogen peroxide, can convert pentahalophenols to tetrahalo-*p*-benzoquinones as the primary products. The incubation of the pentahalophenols with MP8/$H_2O_2$ resulted in the formation of three principal products identified as trihalohydroxy-*p*-benzoquinone, tetrahalo-*p*-benzoquinone, and trihaloethoxy-*p*-benzoquinone. The active species of MP8 formed upon reaction with $H_2O_2$ could be analogous to compounds I and II of horseradish peroxidase (*2*−*4*). Previous studies (*8*) showed that ascorbic acid inhibited the MP8/$H_2O_2$-catalyzed dehalogenation of mono-*p*-halogenated phenols whereas ascorbic acid had no effect on the microsomal P450-mediated dehalogenation of the same substrates. Based on this observation as well as on studies of $^{18}O$-labeled $H_2{}^{18}O$ and $H_2{}^{18}O_2$, which showed that the oxygen atom inserted into the product, p-benzoquinone, originates from water, a peroxidase type of reaction rather than a P450 type of mechanism was suggested for this MP8/$H_2O_2$-mediated oxidative dehalogenation (*8*). It is important to stress that the results reported in the present study argue against the involvement of Fenton-type chemistry in this MP8/$H_2O_2$-mediated dehalogenation reaction.

A scheme outlining the mechanism of the MP8/$H_2O_2$-driven oxidative dehalogenation reaction is presented in

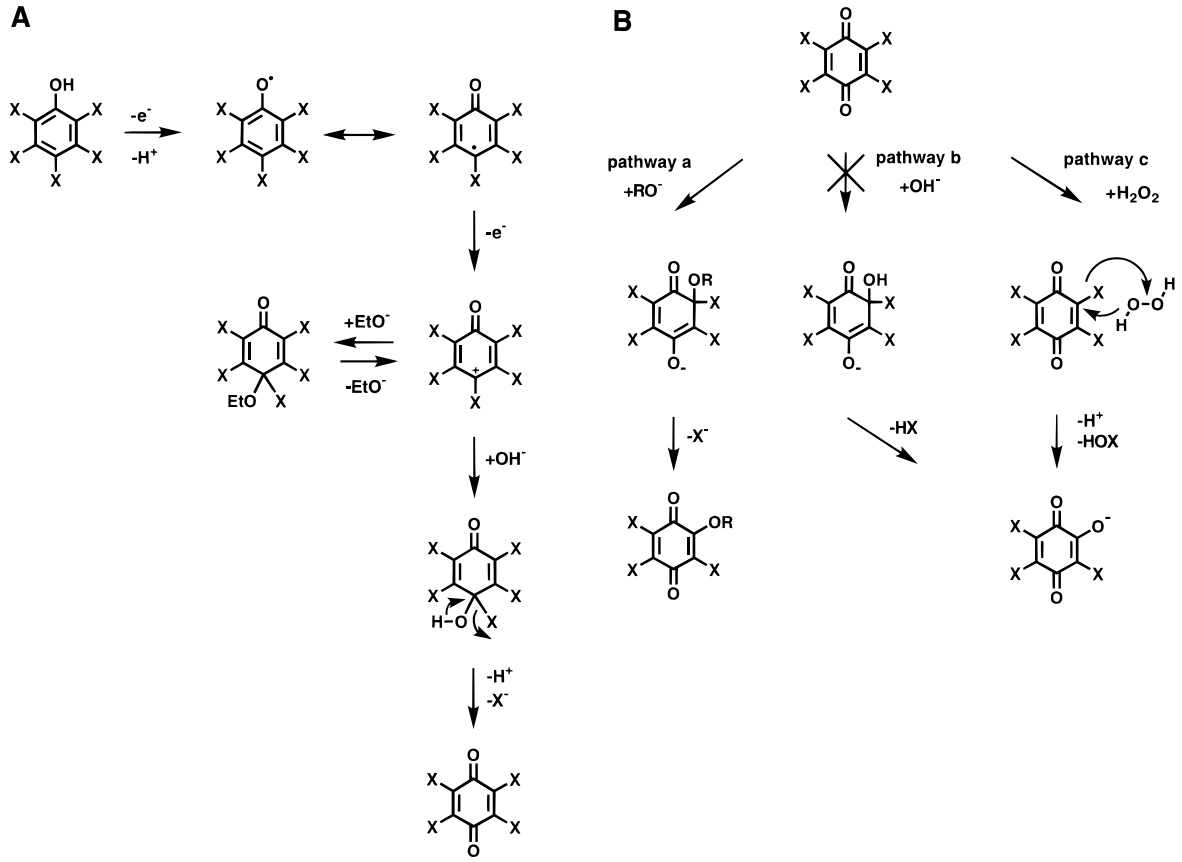


**Figure 7.** (A) Mechanism of the MP8-catalyzed dehalogenation of pentafluorophenol and pentachlorophenol to the corresponding tetrahalo-*p*-benzoquinones (*8*). The active species of MP8 formed upon addition of $H_2O_2$ could catalyze two consecutive one-electron oxidations of the pentahalogenated phenols, leading to the formation of carbocation derivatives which are attacked by $OH^-$ to give the tetrahalo-*p*-benzoquinones (the active species of MP8 formed upon reaction with hydrogen peroxide are supposed to be high-valent iron−oxo intermediates that could be analogous to compounds I and II of horseradish peroxidase; see refs *2*−*4*). (B) Mechanism of the spontaneous conversions of tetrachloro-*p*-benzoquinone and tetrafluoro-*p*-benzoquinone to trichlorohydroxy-*p*-benzoquinone and trifluorohydroxy-*p*-benzoquinone, respectively. The tetrahalo-*p*-benzoquinones could undergo the following nucleophilic reactions: nucleophilic attack by the solvent molecules, i.e., $EtO^-$ (pathway a) or $OH^-$ (pathway b). The reaction with water is insignificant under our experimental conditions. Nucleophilic attack by $H_2O_2$ molecules (pathway c). All these reactions lead to the displacement of the halogen (X = $F^-$ or $Cl^-$) as an anion and the formation of the corresponding trihalohydroxy-*p*-benzoquinones and ethoxy adducts as indicated.

Figure 7A. The active high-valent iron−oxo intermediates of MP8 could catalyze two consecutive one-electron oxidations of the pentahalogenated phenols. This would lead to the formation of carbocation intermediates, which are then subjected to nucleophilic attack by a hydroxide ion, giving rise to the formation of the tetrahalo-*p*-benzoquinones and the elimination of the halogen at the para position as an anion. The solvent consists of water and ethanol; however, the reaction of an ethoxide anion does not lead to a product, since an adduct is formed which has no acidic proton as in the case of the hydroxylated adduct and, as a result, cannot give rise to elimination of the halogen anion and the formation of the corresponding quinone (Figure 7A). This mechanism is consistent with the higher rate of conversion observed in the case of pentafluorophenol when compared with the rate of conversion of pentachlorophenol, since pentafluorophenol is oxidized more easily (*18*). This conclusion is also consistent with the data obtained from molecular orbital calculations, where the energy of the highest occupied molecular orbital, *E*(HOMO), of pentafluorophenol, computed to be −3.87 eV, is higher than the *E*(HOMO) of pentachlorophenol (−4.02 eV). This proposed mechanism is also in agreement with the previously suggested sequential one-electron oxidations of pentachlorophenol and 2,4,6-trichlorophenol by horseradish peroxidase (*6*, *7*) and by lignin peroxidase (*5*). It was even demonstrated that HRP-catalyzed oxidation of pentachlorophenol results in the formation of ESR-detectable pentachlorophenoxyl intermediates (*6*). Although steric restrictions and electrostatic repulsions beween the halogen substituents do not favor interactions between the phenoxyl radicals, we cannot yet exclude disproportionation between the radical intermediates as an alternative pathway to regenerate the parent phenol and form the cation (Figure 7A).

Once formed, the tetrahalo-*p*-benzoquinones react with solvent molecules and with hydrogen peroxide present in the incubation mixture, giving rise to the corresponding trihaloethoxy-*p*-benzoquinones and the corresponding trihalohydroxy-*p*-benzoquinones (Figure 7B). The fact that adducts are formed from tetrachloro-*p*-benzoquinone with ethanol in aqueous buffered solutions could explain the observations reported in the literature on the instability of tetrachloro-*p*-benzoquinone in solutions of 50% aqueous ethanol buffered at different pH values (*19*, *20*). Because the nucleophilic reactivity of the ethoxide ion is greater than that of the hydroxide ion (*21*, *22*), the ethoxide and not the hydroxide adduct is formed. The lack of detection of trichlorohydroxy-*p*-benzoquinone reported for the peroxidase-mediated oxidation of pentachlorophenol in previous studies (*5*, *6*) could be at-

tributed to the relatively lower concentrations of the pentahalogenated phenol and $H_2O_2$ used in these studies.

While ethoxide ions derived from ethanol molecules react with the tetrahalo-*p*-benzoquinones, leading to the formation of the trihaloethoxy-*p*-benzoquinones (pathway a, Figure 7B), the results presented in this study clearly show that the formation of the trihalohydroxy-*p*-benzoquinones did not result from the expected reaction of hydroxide ions (pathway b, Figure 7B) as was suggested to be the case under alkaline conditions (*13*). Instead, hydrogen peroxide acts as the nucleophile in the conversion of tetrahalo-*p*-benzoquinones to the corresponding trihalohydroxy-*p*-benzoquinones and provides the oxygen atom incorporated into the trichlorohydroxy-*p*-benzoquinone (pathway c, Figure 7B). The neutral $H_2O_2$ molecule is known to be a good nucleophile that has a higher reactivity than water (*23*).

The results presented here imply that plants and animals could degrade polyhalophenols to reactive halogenated benzoquinones through a peroxidase pathway. The likelihood that mammalian peroxidases might convert the polyhalophenols to reactive intermediates has important implications with respect to the cytotoxic effects of these products in vivo, since it provides an additional bioactivation pathway comparable to the cytochrome P-450 catalyzed bioactivation of polyhalophenols.

**Acknowledgment.** This study was supported by TMR large-scale NMR facility Grant ERB-CHGE-CT940061, by a grant for the Research School Environmental Chemistry and Toxicology (M and T) and TNO Nutrition and Food Research Institution, Zeist, The Netherlands, and by EU Copernicus Grant ERBIC 15 CT961004. We thank Mr. Martin Bouwmans for having drawn some of the figures.

## References

(1) Adams, P. A., and Louw, J. (1995) Dioxygen bond scission and heme degradation in hemoproteins: a kinetic study of chemical model systems using ferrimyoglobin and hemepeptide: non-hemepeptide complexes as catalysts for peroxidasic reduction of hydrogen peroxide. *J. Chem. Soc., Perkin Trans. 2*, 1683−1690.

(2) Wang, J. S., and Van Wart, H. E. (1989) Resonance Raman characterization of the heme *c* group in *N*-acetyl-microperoxidase-8, a thermal intermediate spin-high spin state mixture. *J. Phys. Chem.* **93**, 7925−7931.

(3) Wang, J. S., Baek, H. K., and Van Wart, H. E. (1991) High-valent intermediates in the reaction of $N^{\alpha}$-microperoxidase-8 with hydrogen peroxide: models for compounds 0, I and II of horseradish peroxidase. *Biochem. Biophys. Res. Commun.* **179**, 1320−1324.

(4) Low, D. W., Winkler, J. R., and Gray, H. B. (1996) Photoinduced oxidation of microperoxidase-8: Generation of ferryl and cation-radical porphyrins. *J. Am. Chem. Soc.* **118**, 117−120.

(5) Hammel, K. E., and Tardone, P. J. (1988) The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular fungal lignin peroxidase. *Biochemistry* **27**, 6563−6568.

(6) Samokyszyn, V. M., Freeman, J. P., Maddipati, K. R., and Lloyd, R. V. (1995) Peroxidase-catalyzed oxidation of pentachlorophenol. *Chem. Res. Toxicol.* **8**, 349−355.

(7) Wiese, F. W., Chang, H. C., Lloyd, R. V., Freeman, J. P., and Samokyszyn, V. M. (1998) Peroxidase-catalyzed oxidation of 2,4,6-trichlorophenol. *Arch. Environ. Contam. Toxicol.* **34**, 217−222.

(8) Osman, A. M., Boeren, S., Veeger, C., and Rietjens, I. M. C. M. (1997) MP8- dependent oxidative dehalogenation: evidence for the direct formation of 1,4-benzoquinone from 4-fluorophenol by a peroxidase-type of reaction pathway. *Chem.-Biol. Interact.* **104**, 147−164.

(9) Den Besten, C., Van Bladeren, P. J., Duizer, E., Vervoort, J., and Rietjens, I. M. C. M. (1993) Cytochrome P450-mediated oxidation of pentafluorophenol to tetrafluorobenzoquinone as the primary reaction product. *Chem. Res. Toxicol.* **6**, 674−680.

(10) Van Ommen, B., Adang, A. E. P., Brader, L., Posthumus, M. A., Müller, F., and Van Bladeren, P. J. (1986) The microsomal metabolism of hexachlorobenzene: origin of the covalent binding to protein. *Biochem. Pharmacol.* **35**, 3233−3238.

(11) Aron, J., Baldwin, D. A., Marques, M. M., Pratt, J. M., and Adams, P. A. (1986) Heme and hemoproteins. 1. preparation and analysis of the heme-containing octapeptide (microperoxidase-8) and identification of the monomeric form in aqueous solution. *J. Inorg. Biochem.* **27**, 227−243.

(12) Nelson, D. P., and Kiesow, L. A. (1972) Enthalpy of decomposition of hydrogen peroxide by catalase at 25 °C (with molar extinction coefficients of $H_2O_2$ solutions in the UV). *Anal. Biochem.* **49**, 474−478.

(13) Hancock, J. W., Morrell, C. E., and Rhum, D. (1962) Trichlorohydroxyquinone. *Tetrahedron Lett.* **22**, 987−988.

(14) Spee, J. H., Boersma, M. G., Veeger, C., Samyn, B., Van Beeumen, J., Warmerdam, G., Canters, G. W., Van Dongen, W. M. A., and Rietjens, I. M. C. M. (1996) The influence of the peptide chain on the kinetics and stability of microperoxidases. *Eur. J. Biochem.* **241**, 215−220.

(15) Wallenfels, K., and Friedrich, K. (1960) Über Fluorchinone, II. *Chem. Ber.* **93**, 3070−3082.

(16) Osman, A. M., Boeren, S., Boersma, M. G., Veeger, C., and Rietjens, I. M. C. M. (1997) Microperoxidase/$H_2O_2$-mediated alkoxylating dehalogenation of halophenol derivatives in alcoholic media. *Proc. Natl. Acad. Sci., U.S.A.* **94**, 4295−4299.

(17) Osman, A. M., Koerts, J., Boersma, M. G., Boeren, S., Veeger, C., and Rietjens, I. M. C. M. (1996) Microperoxidase/$H_2O_2$-catalyzed aromatic hydroxylation of aniline proceeds by a cytochrome P450-type of oxygen-transfer reaction mechanism. *Eur. J. Biochem.* **240**, 232−238.

(18) Forbes, E. J., Richardson, R. D., Stacey, M., and Tatlow, J. C. (1959) Aromatic polyfluoro-compounds. Part II. Penta-fluorophenol. *J. Chem. Soc.*, 2019−2021.

(19) Birks, J. B., and Slifkin, M. A. (1963) Interaction of Amino acids, Proteins, and Amines with Chloranil. *Nature* **197**, 42−45.

(20) Slifkin, M. A., Sumner, R. A., and Heathcote, J. G. (1967) The interactions of chloranil in aqueous solvents. *Spectrochim. Acta* **28A**, 1751−1756.

(21) Miller, J. (1968) Medium, catalytic and steric effects. In *Aromatic nucleophilic substitution* (Eaborn, C., and Chapman, N. B., Eds.) pp 331−332, Elsevier Publishing Co., Amsterdam.

(22) Murto, J. (1964) Kinetics of the reactions of 2,4-dinitrofluorobenzene with hydroxide and alkoxide ions in alkanol−water mixtures. *Acta Chem. Scand.* **18**, 1029−1042.

(23) Curci, R., and Edwards, J. O. (1992) Activation of hydrogen peroxide by organic compounds. In *Catalytic Oxidations with Hydrogen Peroxide as Oxidant* (Strukul, G., Ed.) pp 57−60, Kluwer Academic Publishers, Dordrecht.

TX980037L