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# Removal of Estrogenic Activity and Formation of Oxidation Products during Ozonation of 17α-Ethinylestradiol

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This study investigated the oxidation of the oral contraceptive 17α-ethinylestradiol (EE2) during ozonation. First, the effect of ozone ($O_3$) on the estrogenic activity of aqueous solutions of EE2 was studied using a yeast estrogen screen (YES). It could be shown that $O_3$ doses typically applied for the disinfection of drinking waters were sufficient to reduce estrogenicity by a factor of more than 200. However, it proved impossible to completely remove estrogenic activity due to the slow reappearance of 0.1−0.2% of the initial EE2 concentration after ozonation. Second, oxidation products formed during ozonation of EE2 were identified with LC-MS/MS and GC/MS and the help of the model compounds 5,6,7,8-tetrahydro-2-naphthol (THN) and 1-ethinyl-1-cyclohexanol (ECH), which represent the reactive phenolic moiety and the ethinyl group of EE2. Additionally, oxidation products of the natural steroid hormones 17β-estradiol (E2) and estrone (E1) were identified. The chemical structures of the oxidation products were significantly altered as compared to the parent compounds, explaining the diminished estrogenic activity after ozonation. Overall, the results demonstrate that ozonation is a promising tool for the control of EE2, E2, and E1 in drinking water and wastewater.

## Introduction

In recent years, several studies have reported the occurrence of a large number of pharmaceuticals in the aquatic environment (*1*−*3*). The presence of pharmaceutical compounds in water resources makes it necessary to assess water-treatment processes with respect to their removal efficiency for such compounds. Several studies have recently shown that ozonation is a very promising technology for the oxidation of pharmaceuticals during water treatment (*4*−*6*). The results of these studies are based on the disappearance of the parent compounds. At $O_3$ doses typically used in water treatment, full mineralization of pharmaceuticals is not achievable, and consequently ozonation results in the formation of oxidation products. Depending on the functional group that is attacked by $O_3$ and its location in the molecule, changes in the molecular structure might be significant enough to destroy the pharmacological effects of the parent compound. However, it cannot be excluded that in some cases oxidation products still produce the original pharmacological effects. To confirm that ozonation is suitable for the removal of pharmaceuticals, it should be at least shown that there is high evidence that pharmacological effects are significantly reduced. So far, the formation of ozonation products has been investigated for very few pharmaceutical compounds (*7*, *8*), and the decrease in pharmacological effects never has been checked in a comprehensive manner.

In the present study, the oral contraceptive 17α-ethinylestradiol (EE2) was selected on the basis of its LOEC (lowest observed effect concentration) of 0.1 ng/L for vitellogenin induction in rainbow trout (*9*). In effluents of sewage treatment plants (STPs), its concentration typically ranges from <0.5 ng/L to 10 ng/L (*10*). In vivo tests showed that EE2 is approximately 11−27 times more potent than the female sex hormone 17β-estradiol (E2) (*11*). On the basis of in vivo estrogenic potency, EE2 might be the most important endocrine disruptor in STP effluents together with the octyl- and nonylphenols (*12*).

The estrogenic activity can be considered the primary pharmacological effect of EE2. With the yeast estrogen screen (YES) described by Routledge and Sumpter (*13*), a robust and easy to handle test was available to quantify the effect of ozonation on the estrogenic activity of pure aqueous solutions of EE2. In detail, the effects of substoichiometric $O_3$ doses, resulting in a partial transformation of EE2, as well as the effects of higher $O_3$ doses typical for drinking water treatment have been investigated in bench-scale experiments. In the second part of this study, a number of oxidation products formed during the ozonation of EE2, E2, and E1 were identified.

During ozonation, micropollutants such as EE2 can be oxidized either by ozone ($O_3$) directly or by hydroxyl radicals ($^{\bullet}OH$), which are formed as a consequence of $O_3$ decay. The two oxidants vary strongly in their reactivity. $O_3$ attacks selectively certain functional groups, whereas $^{\bullet}OH$ is a nonselective oxidant that reacts fast with a large number of moieties. Consequently, most $^{\bullet}OH$ are scavenged by the water matrix in natural waters. EE2 reacts very fast with $O_3$ and $^{\bullet}OH$ (*5*). However, due to the higher selectivity of $O_3$, oxidation by $O_3$ is normally the predominant process. Therefore, the present study focused on direct $O_3$ reactions and reactions with $^{\bullet}OH$ were suppressed by the use of scavenger compounds such as *tert*-butyl alcohol (TBA). The performance of ozonation processes with respect to the oxidation of micropollutants can be assessed with $O_3$ and $^{\bullet}OH$ exposure (i.e., concentration of oxidant integrated over the reaction time) (*14*). When $^{\bullet}OH$ is scavenged, the oxidation of micropollutants is only a function of $O_3$ exposure. This parameter is also used to assess the disinfection efficiency of ozonation processes. Therefore, $O_3$ exposure allows us to compare the removal of estrogenic activity to the inactivation of microorganisms.

## Experimental Section

**Standards and Reagents.** Estrone (E1), 17β-estradiol (E2), 1-ethinyl-1-cyclohexanol (ECH), 5,6,7,8-tetrahydro-2-naphthol (THN), adipic acid, cyclohexanone, 2-methyl-3-butyn-2-ol, α-hydroxyisobutyric acid, and the methylester of 1-hydroxycyclohexane-1-carboxylic acid were purchased from Sigma-Aldrich (see Figure 1 for chemical structures).

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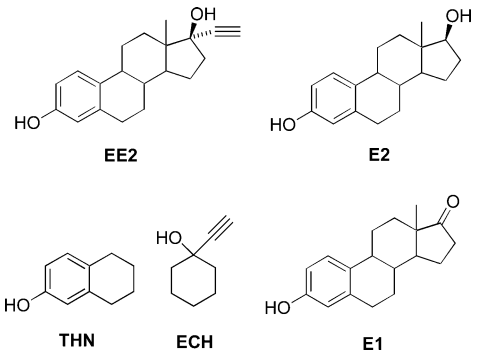



**FIGURE 1. Structures of 17α-ethinylestradiol (EE2), 17β-estradiol (E2), estrone (E1), and the model compounds 5,6,7,8-tetrahydro-2-naphthol (THN) and 1-ethinyl-1-cyclochexanol (ECH).**

The chemicals were of the highest purity available. 17α-Ethinylestradiol (EE2) was provided by Schering/Berlin, Germany. Depending on the experiments, stock solutions of EE2 and the model compounds ECH and THN were prepared either in Milli-Q purified water (Millipore), in *tert*-butyl alcohol (TBA), or in acetone. Stock solutions of E1 and E2 were prepared in TBA or in acetone. All chemicals used for solutions (buffer, eluents, etc.) were reagent grade and were used without further purification. $O_3$ was produced with a Fischer 500 and a Fischer 502 ozone generator by using pure oxygen as feed gas. $O_3$ stock solutions (~1 mM) were produced by sparging $O_3$-containing oxygen through Milli-Q water that was cooled in an ice bath (*15*).

**Determination of EE2.** EE2 was determined with a Hewlett-Packard 1050 series HPLC system equipped with a Nucleosil-100 C18 column (4 × 125 mm, 5 μm) and a fluorescence detector (HP 1064A). The mobile phase consisted of 50% 10 mM phosphoric acid and 50% acetonitrile at a flow rate of 0.6 mL/min. An excitation wavelength of 229 nm and emission wavelength of 309 nm were used for fluorescence detection. Under the experimental conditions, the quantification limit of EE2 was 5 nM (1.5 μg/L) for an injection volume of 100 μL. The error of a single measurement was approximately ±5%. Enrichment by freeze-drying lowered the quantification limit to 0.2 nM (60 ng/L). The EE2 recovery for freeze-dried samples was 70−90%.

**Determination of $O_3$, Hydroperoxides, and Formic Acid.** Dissolved $O_3$ was determined with the indigo method (*15*) or spectrophotometrically by measuring the absorbance at 258 nm ($\epsilon = 3000\ M^{-1}\ cm^{-1}$). Hydroperoxides and hydrogen peroxide ($H_2O_2$) were measured using Allen's reagent as described by Flyunt et al. (*16*). Catalase was applied to selectively quench $H_2O_2$. Formic acid was determined with ion chromatography using a method adapted from (*17*).

**LC-MS/MS Analysis.** The HPLC system consisted of a Merck-Hitachi pump with an Inertsil ODS-3 column (4.6 × 100 mm, 3 μm). Elution was performed with 0.1% acetic acid (A) and acetonitrile (B) at a flow rate of 0.4 mL/min. The gradient was as follows: B started at 20% and was increased with a linear gradient to 80% after 15 min. After 2 min with B at 80%, the system was reequilibrated for 15 min. Mass spectrometry was performed using an API 365 (PE Sciex) triple quadrupole mass spectrometer with turbo-electrospray ionization (ESI). Nitrogen was used as curtain gas, and synthetic air was used as nebulizer gas. Curtain and nebulizer gas flows were both operated with a flow rate of 1.0 L/min. The ESI interface was heated to 400 °C. The HPLC flux was split 1:10 to diminish the flow rate in the ESI.

Full scans as well as precursor and product ion scans were conducted to determine the quasi-molecular ions and the structure of major oxidation products. Before analysis, aliquots of the ozonated solutions (100−250 mL) were freeze-dried. The residual components were resuspended in approximately 2 mL of a water/acetonitrile (20/80, v/v) mixture and filtered with a 0.2 μm filter. The samples were analyzed in the positive as well as the negative mode. Adipic acid, cyclohexanone, 1,2-cyclohexanedione, and α-hydroxyisobutyric acid were quantified using the MRM (multiple reaction monitoring) mode after optimizing the LC-MS/MS for each compound. For quantification, aqueous solutions were analyzed directly without any concentration steps.

**GC/MS Analysis.** After freeze-drying 100−250 mL aliquots of ozonated model-compound solutions, the residual components were resuspended in approximately 2 mL of acetone and filtered with a 0.2 μm filter to remove insoluble components. Aliquots of 0.2 mL were derivatized by adding 0.2 mL of a diazomethane/diethyl ether solution at −20 °C. The samples were incubated for 1 h at −20 °C before diazomethane was quenched with 2−3 drops of acetic acid/acetone solution (1:10, v/v). Subsequently, the volume of the samples was reduced to 200 μL by a gentle nitrogen stream. Diazomethane is highly toxic and carcinogenic. All work has to be conducted in a fume hood, and skin contact has to be strongly avoided.

Separation and detection were accomplished with a Varian GC 3400 coupled to a Varian Saturn 4D mass spectrometer. The gas chromatograph was equipped with a PTV injector and a Restek XTI-5 column (30m × 0.25 mm × 0.25 μm). GC injection parameters: 5 μL, splitless; 50 °C; 100 °C/min to 300 °C, 300 °C isothermal 10 min. Oven temperatures: 50 °C isothermal for 2 min, 2.8 °C/min to 120 °C, 15.4 °C/min to 290 °C, and 290 °C isothermal for 10 min.

**Recombinant Yeast Estrogen Screen (YES).** The YES was conducted as described by Routledge and Sumpter (*13*). Briefly, the recombinant yeast strain used in the screen expresses the human estrogen receptor (hER), which can interact with estrogenic compounds. Upon binding an active compound, the hER triggers the expression of the reporter gene lac-Z, which promotes the production of the enzyme β-galactosidase. The enzyme metabolizes the yellow dye chlorophenol red-β-D-galactopyranoside (CPRG) into a red product that can be measured spectrophotometrically.

Depending on the expected EE2 concentrations, aqueous samples were freeze-dried and resuspended in ethanol or directly diluted in ethanol to obtain appropriate concentrations for the YES. Based on these solutions, series of 6 or 12 dilutions (1:1) were prepared in ethanol. Of each dilution, 20 μL was added to a 96-well microtiter plate. Besides the samples, each plate contained a standard curve with E2 in ethanol (final concentration dissolved in assay medium: $2 \times 10^{-8}$ to $1 \times 10^{-11}$ M) and a row of blanks. After ethanol was evaporated to dryness, the yeast cells were added together with the assay medium. The color development was measured after incubating the microtiter plate for 72 h at 30 °C.

To evaluate the data, the sigmoidal concentration−response curve of the E2 standard was fitted to a symmetric logistic function (eq 1) using the software Prism (GraphPad, San Diego, CA).

$$\text{response} = a + \frac{b - a}{1 + 10^{(\log \text{EC50} - \log c)\cdot m}} \qquad (1)$$

where $a$ is the baseline response (bottom), $b$ is the maximum response (top), $c$ is the concentration, $m$ is the Hill slope, and EC50 is the concentration provoking the half-maximal response.

With the values received for $b$ (top) and $a$ (bottom), the response of the standard and the samples was expressed as the percent of maximum response evoked by E2. To determine the EC50 and the Hill slope $m$ of the E2 standard curve, the concentration−response curve was fitted again to eq 1 with the response expressed in percent whereby $a$ and $b$ were held constant at 0% and 100%, respectively. To

calculate the EC50 values for the samples, the response in percent was fitted to eq 1 whereby *a* (0%), *b* (100%), and *m* were held constant. The concentration *c* in eq 1 was replaced by the concentration factor. The estrogenic activity of a sample expressed in 17$\beta$-estradiol equivalents (EEQs) was then calculated as the ratio of the EC50 for E2 to the EC50 for the sample.

**Ozonation Experiments for YES.** If not stated otherwise, the reaction solutions consisted of Milli-Q purified water spiked with 1 or 10 $\mu$M EE2 and were buffered to pH 8 with 5 mM phosphate buffer. Furthermore, the solutions contained 5 mM TBA as an $^{\bullet}OH$ scavenger.

*Substoichiometric $O_3$ Doses.* Experiments with substoichiometric $O_3$ doses were carried out as follows: To a series of identical reaction solutions (20 mL) with 10 $\mu$M EE2, $O_3$ doses ranging from 5 to 24 $\mu$M were added under vigorous stirring. Immediately after the addition of $O_3$, 10 mL of the solution was removed from the reaction vessel and stored for 2−3 days at room temperature. Of the 10 mL, 0.5 mL was taken for HPLC analysis and 9.5 mL was freeze-dried. After freeze-drying, the residual components were redissolved with the help of sonication in 0.95 mL of ethanol. Insoluble phosphate salts were allowed to settle before the liquid phase was removed and stored at −20 °C until the YES was conducted. Samples with EE2 > 0.5 $\mu$M were diluted directly with ethanol without freeze-drying.

*Reduction of Estrogenicity as a Function of $O_3$ Exposure.* To quantify the reduction in estrogenicity as a function of $O_3$ exposure**,** a reaction solution (500 mL) spiked with 1 $\mu$M EE2 was thermostated at 10 °C. After adding 20 $\mu$M (1 mg/L) $O_3$, a series of 10 mL samples was taken over the course of the reaction. Thiosulfate was used to quench $O_3$ in the samples. The corresponding $O_3$ exposures of the samples were calculated on the basis of an $O_3$ decay curve, which was determined in a preliminary experiment under conditions identical to those described in ref *18*. In an experiment with a slightly different setup, 20 $\mu$M (1 mg/L) $O_3$ was added to a series of 250 mL solutions. Samples of 100 mL were taken after the desired time intervals and immediately quenched with thiosulfate. These experiments allowed for the quantification of EE2 concentration < 1 nM with HPLC due to a higher enrichment during freeze-drying. After quenching $O_3$, samples of both experiments were stored for 3 days at room temperature before they were freeze-dried. With the help of sonication, the residual components were redissolved in 1 and 2 mL of ethanol, respectively.

**Kinetics of Reappearance of EE2 after Ozonation.** The reappearance kinetics of EE2 was investigated at two different $O_3$ concentrations (50 and 100 $\mu$M), which were added to two reaction solutions spiked with 10 $\mu$M EE2. After 5 min, 2 mL of the solutions was withdrawn and transferred into an amber HPLC vial where $O_3$ was immediately quenched with thiosulfate. Each of the samples was repeatedly analyzed over the next 165 h.

To investigate the pH dependence of the EE2 reappearance, reaction solutions were buffered to pH 5, 6, 7, 8, and 9. The experiments were performed as described for the reappearance kinetics except that samples were only analyzed after 3 days.

**Investigation of Product Formation.** The oxygen stream containing 2% $O_3$ was continuously bubbled through a 1-L amber glass bottle, which contained the reaction solution. The solution was stirred with a magnetic stir bar at 80 rpm. Reaction solutions of the model compounds THN (0.2 mM) and ECH (5 mM) as well as of 2-methyl-3-butyn-2-ol (5 mM) were prepared by dissolving the pure chemicals in acetone or TBA and spiking them to Milli-Q water. The reaction solutions containing the model compounds were ozonated during 5−40 min. The experiments that produced the highest yield with respect to the quantified oxidation product were repeated at pH 7 (5 mM phosphate buffer) with the corresponding ozonation time. Reaction solutions containing the estrogens consisted of EE2 (0.02 mM), E2 (0.005 mM), or E1 (0.005 mM) dissolved in Milli-Q water and either acetone or TBA as $^{\bullet}OH$ scavenger. Due to the low solubility of the estrogens in aqueous solutions, only low starting concentrations could be selected. In a first step, estrogen solutions were ozonated for 0.5 min (E1, E2) to 2 min (EE2). To increase the product yield, the ozonated solution was spiked again with the corresponding estrogen and subjected to ozonation for an identical time interval. Overall, this procedure was repeated 10 times. The fact that oxidation products were more polar than the parent compound prevented the precipitation of oxidation products.

## Results and Discussion

The second-order rate constant ($k_{O3}$) for the reaction of 17α-ethinylestradiol (EE2) with ozone ($O_3$) is extremely high (at pH 7, $k_{O3} = 3 \times 10^6$ $M^{-1}$ $s^{-1}$ (*5*)), resulting in a half-life of approximately 10 ms for an $O_3$ concentration of 1 mg/L. $O_3$ attack must take place on the phenolic moiety of EE2, because phenols are well known to react fast with ozone at neutral or basic pH (*19*). In this pH range, the reaction of $O_3$ with the phenolate anion of EE2 is the predominant reaction, whereas the reaction with neutral EE2 is negligible. Consequently, the reaction of EE2 ($pK_a = 10.4$) with $O_3$ is strongly pH-dependent (the apparent $k_{O3}$ at pH 8 is 10 times higher than at pH 7). The second reactive group of EE2, the ethinyl group, has a considerably lower reactivity toward $O_3$. Its $k_{O3}$ is estimated to be close to the $k_{O3}$ of ECH, which was determined in this study to be 200 $M^{-1}$ $s^{-1}$ at 20 °C. The corresponding half-life is approximately 3 min for an $O_3$ concentration of 1 mg/L. On the basis of these kinetic considerations, it is clear that EE2 disappears very fast during ozonation processes. However, if ozonation is applied to treat EE2-containing water, the removal of estrogenic activity has to be assessed in addition to the disappearance of EE2. To test whether ozonation reduces estrogenic activity, aqueous solutions of EE2 were treated with $O_3$ and subsequently tested for estrogenic activity using a yeast estrogen screen (YES).

**Reduction of Estrogenicity with Substoichiometric $O_3$ Doses.** The high $k_{O3}$ for the oxidation of EE2 refers to the first transformation step. On the basis of a study on the ozonation of phenol (*20*), it can be assumed that some of the important subsequent oxidation steps of EE2 proceed as fast as the first transformation step. This sequence of fast transformation steps will lead to a modification and partly to a cleavage of the phenol ring in EE2. To test whether this results in a substantial removal of estrogenicity, experiments were performed with substoichiometric $O_3$ doses. Under these conditions, only the fast initial transformation steps take place.

Figure 2a illustrates the correlation between decrease in estrogenicity and the concentration of EE2 as a function of $O_3$ dosages. The estrogenicity expressed in 17$\beta$-estradiol equivalents (EEQs) and EE2 concentrations were normalized to the values of the solution without $O_3$ ($[EE2]_0 = 10$ $\mu$M). Approximately 1.8 mol of $O_3$ was consumed per mole of EE2. The stoichiometry is higher than 1 because oxidation products of the first oxidation step obviously underwent further reactions with $O_3$. Adding 19 $\mu$M $O_3$ reduced the estrogenicity of the solution to 1.5%. The reduction in estrogenicity was proportional to the decrease in EE2. This indicates that the estrogenicity of the intermediates of the first transformation step is much lower than that of EE2. In Figure 2b, the estrogenicity expressed in EEQ is plotted versus the EE2 concentration. The fact that the data form a straight line with a slope of approximately 1 demonstrates that the parent compound EE2 is responsible for the observed estrogenicity. Even at the two lowest EE2 concentrations, accounting for

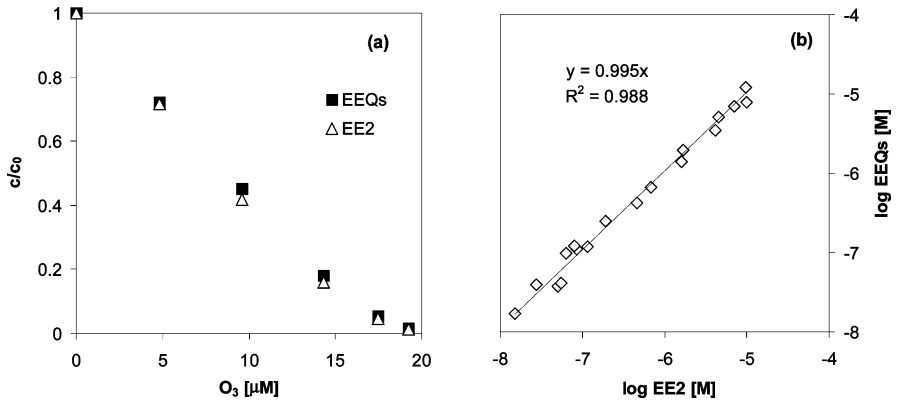



**FIGURE 2. The effect of substoichiometric $O_3$ doses on the estrogenicity of aqueous solutions of EE2. Experimental conditions: $[EE2]_0 = 10\ \mu M$, pH = 8, $T$ = RT, [TBA] = 5 mM. (a) Relative decrease of estrogenicity and EE2 concentration as a function of $O_3$ dose. (b) Logarithmic plot of estrogenicity in 17$\beta$-estradiol equivalents (EEQs) versus EE2. Data are from Figure 2a and an additional experiment.**

0.12% and 0.28% of the initial EE2 concentration, no significant deviation from the straight line occurs. This means that the sum of the estrogenic activity of the intermediates is at least 200 times lower than the estrogenicity of the original EE2 solution.

These findings were confirmed with an estrogen receptor competitive-binding assay adapted from Blair et al. (*21*). In this assay, the estrogen receptor can directly interact with the investigated compounds. Because no living organism is used, biological processes associated with test organisms do not influence the response of the assay. After ozonation, EE2-containing solutions did not displace radiolabeled E2 from the rat estrogen receptor, whereas untreated solutions displaced it completely. This means that the oxidation products of EE2 do not bind efficiently to the estrogen receptor.

The YES experiments described above were designed to yield a reduction of estrogenicity by a factor of 1000−10 000. However, the observed reduction was lower due to a slow reappearance of EE2 after oxidation. The reappearance of EE2 will be discussed later. To achieve concentrations lower than 0.1 $\mu$M with nearly stoichiometric $O_3$ doses, the reaction solutions had to be spiked with a second $O_3$ dose after storing the solutions for 3 days at room temperature. The second $O_3$ dose was kept as low as possible to avoid significant changes in the product distribution of the fast initial transformation steps.

**Reduction of Estrogenicity as a Function of $O_3$ Exposure.** In drinking-water treatment, $O_3$ exposures are much higher than in the experiments with substoichiometric $O_3$ doses, in which $O_3$ was immediately consumed. To test whether higher $O_3$ exposures result in further removal of estrogenicity, aqueous solutions of EE2 at 10 °C and pH 8 were subjected to $O_3$ exposures ranging from 0.5 to 20 $mgL^{-1}$ min. Figure 3 shows the decrease of the EE2 concentration together with the EEQ reduction. As expected, the results showed a drop of the estrogenicity by a factor of 200−500 for the lowest measured $O_3$ exposure, confirming the results of the experiments with substoichiometric $O_3$ doses. However, at higher $O_3$ exposures, the further decrease of estrogenicity was very slow. To reduce estrogenicity by a factor of 1000, an $O_3$ exposure of approximately 10 $mgL^{-1}$ min was necessary.

The $O_3$ exposures reported above can be related to $O_3$ exposures applied for disinfection, which is often the primary objective of ozonation. $O_3$ exposures required to achieve certain levels of inactivation for specific microorganisms were calculated on the basis of inactivation rate constants and activation energies compiled by von Gunten (*22*). At an $O_3$ exposure of 0.5 $mgL^{-1}$ min and 10 °C, the inactivation of *Giardia muris* cysts is approximately 2 log units. Under the same conditions, inactivation for *E. coli* is more than 6 log

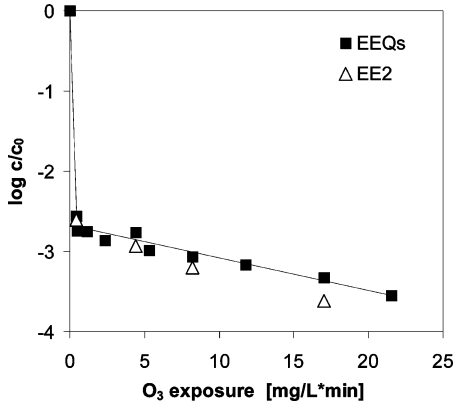



**FIGURE 3. Reduction of estrogenicity and the EE2 concentration as a function of $O_3$ exposure. Experimental conditions: $[EE2]_0 = 1\ \mu M$, $[O_3]_0 = 1$ mg/L, pH = 8, $T$ = 10 °C, [TBA] = 5 mM.**

units. A much larger $O_3$ exposure of approximately 9 $mgL^{-1}$ min is required for a 2-log inactivation of *Cryptosporidium parvum* oocysts. On the basis of this inactivation data, it can be predicted that EE2-based estrogenicity is reduced at least by a factor of 200, if an ozonation processes achieves a 2-log inactivation of *Giardia muris* cysts or by a factor of 1000 if a 2-log inactivation of *Cryptosporidium parvum* oocysts is attained.

To quantify how much of the residual estrogenicity was caused by EE2 itself, EE2 concentrations were measured with HPLC and fluorescence detection as well as LC-MS/MS for confirmation. The results show that up to $O_3$ exposures of approximately 10 $mgL^{-1}$ min, residual EE2 accounted for most of the estrogenicity. At higher $O_3$ exposures, EE2 concentrations were close to the quantification limit of the HPLC method (0.2 nM). Therefore, on the basis of the current data, it cannot be excluded that other compounds are responsible for a part of the estrogenicity observed at higher $O_3$ exposures. A possible explanation for the formation of estrogenic products will be given later.

**Reappearance of EE2.** The presence of EE2 in solutions that were treated with high $O_3$ exposures was unexpected. For an $O_3$ concentration of 1 mg/L, the half-life of EE2 at pH 8 is approximately 1 ms. Consequently, EE2 should disappear below the detection limit in less than 1 s. To assess the reappearance of EE2, the kinetics of this process was investigated. Figure 4 shows the reappearance of EE2 after ozonation as a function of time. The EE2 concentration rose quickly during the first hours after the experiments. After this initial step, the concentrations increased more slowly over several days. As compared to an $O_3$ dose of 50 $\mu$M, the reappearance at 100 $\mu$M $O_3$ was less pronounced but still

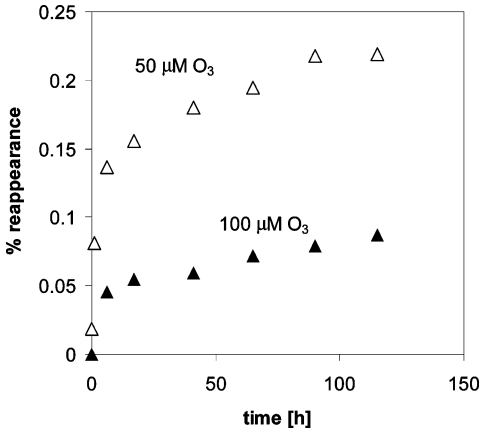



**FIGURE 4. Reappearance of EE2 as a function of $O_3$ dose and time. Experimental conditions: $[EE2]_0 = 10\ \mu M$, pH = 8, $T$ = RT, [TBA] = 5 mM. $O_3$ was quenched with thiosulfate after 5 min. An $O_3$ dose of 50 $\mu$M resulted in an $O_3$ exposure of 4−5 $mgL^{-1}$ min, whereas 100 $\mu$M $O_3$ yielded an approximately 4 times higher $O_3$ exposure of 15−20 $mgL^{-1}$ min.**

significant. The same pattern of reappearance was also detected for THN and E2 (data not shown). This observation was not limited to the selected experimental conditions with pure water. Experiments with pretreated water from the River Seine in Paris, France, showed the same behavior of EE2. Furthermore, the dependence on EE2 concentration seemed to be small. Starting with 10, 1, and 0.1 $\mu$M EE2 resulted in comparable percentages of reappearance (0.05−0.2%) for similar $O_3$ doses.

The reappearance of EE2 can only be explained if a small fraction of EE2 is "protected" from $O_3$ attack. We hypothesize that such "protected" forms of EE2 consist of hydroperoxides formed by the fast reaction of phenoxyl radicals with superoxide anion. This reaction was investigated in detail for radiolytically generated phenoxyl-type radicals by d'Alessandro et al. (*23*). It can be expected that such hydroperoxides would not be highly reactive toward ozone. Because they revert to phenols in a slow reaction by eliminating dioxygen, as shown in the study mentioned above, the formation of hydroperoxides provides a plausible explanation for the slow reappearance of EE2 observed in the present study.

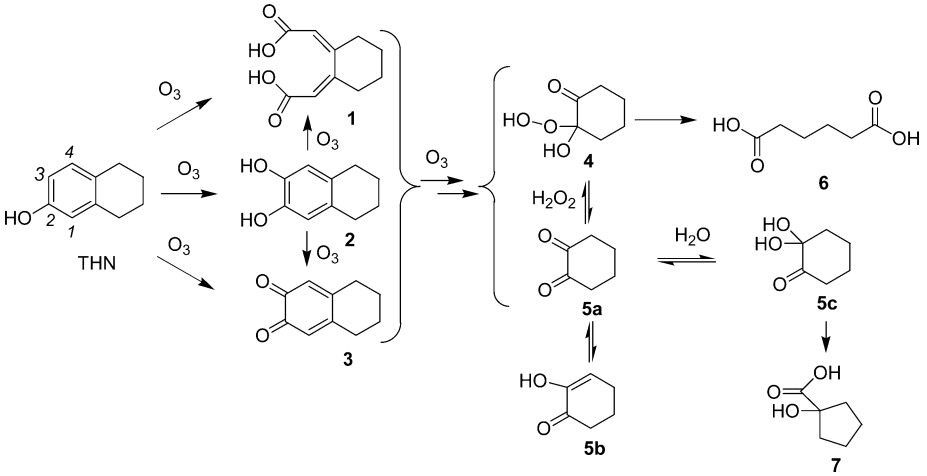
These hydroperoxides would be "protected" from the fast oxidation of the phenolic moiety, but $O_3$ could still attack on the ethinyl group. On the basis of the reaction of $O_3$ with ECH, the second-order rate constant for the $O_3$ attack on the ethinyl group is estimated to be 160 $M^{-1}\ s^{-1}$ at 10 °C. The slow decrease of EE2 in Figure 3 corresponds to a second-order rate constant of approximately 150 $M^{-1}\ s^{-1}$ at 10 °C. Therefore, it seems to be plausible that the $O_3$ attack on the ethinyl group of "protected" EE2 contributes to the disappearance of EE2. This reaction would result in the formation of products for which the phenolic moiety of EE2 can be reformed after ozonation. It can be expected that these products still exhibit estrogenic activity. Therefore, we assume that they could be responsible for the remaining estrogenicity that seemed not to be caused by EE2.

Despite the fact that a reappearance of EE2 has been observed, it has to be emphasized that it accounts only for 0.1−0.5% of EE2 oxidized. We assume that this phenomenon will have little relevance in practice where a removal of 99.5% estrogenicity should be sufficient in most cases.

**Identification of Oxidation Products.** In the second part of this study, oxidation products formed during the ozonation of EE2 were identified. As mentioned earlier, EE2 has a highly ozone reactive phenolic moiety ($k_{O3} = 3 \times 10^6\ M^{-1}\ s^{-1}$ at pH 7, $t_{1/2} \approx 10$ ms for 1 mg/L $O_3$) and a significantly less reactive ethinyl group ($k_{O3} \approx 200\ M^{-1}\ s^{-1}$, $t_{1/2} \approx 3$ min for 1 mg/L $O_3$). On the basis of these rate constants, it can be assumed that both reactive moieties will be attacked by $O_3$ during drinking water treatment. To facilitate the identification of oxidation products, two model compounds were chosen that represent the two different reactive moieties. The selected compounds were 5,6,7,8-tetrahydro-2-naphthol (THN) for the phenolic moiety and 1-ethinyl-1-cyclohexanol (ECH) for the ethinyl group (Figure 1).

**Ozonation of the Model Compound THN.** Aqueous solutions containing THN were ozonated and subsequently analyzed for oxidation products with LC-MS/MS. Scheme 1 shows the suggested reaction mechanism and the identified products. The chemical structure of two oxidation products could be determined with high certainty. The first product was identified as adipic acid (**6**) using an authentic standard for LC-MS/MS analysis. The second oxidation product was identified as 1-hydroxy-cyclopentanecarboxylic acid (**7**). No standard was available for this compound. After derivatization with diazomethane, the fragmentation in the GC/MS spectrum (Figure 5a) was very similar to the fragmentation found in the spectrum of the commercially available methylester of 1-hydroxy-cyclohexanecarboxylic acid (**9**) (Figure 5b). The spectrum of methylated **7** corresponded to the spectrum of methylated **9** shifted 14 Da ($CH_2$) to lower masses, suggesting that **7** consisted of a five- instead of a six-membered ring. The proposed structure of **7** is further supported by the loss of 46 ($CH_2O_2$) from the quasi molecular ion $[M - H]^-$ $m/z = 129$ in LC-MS/MS experiments performed in the negative mode (Table 2). Normally, carboxylic acids lose 44

**SCHEME 1. Reaction of $O_3$ with the Model Compound THN**



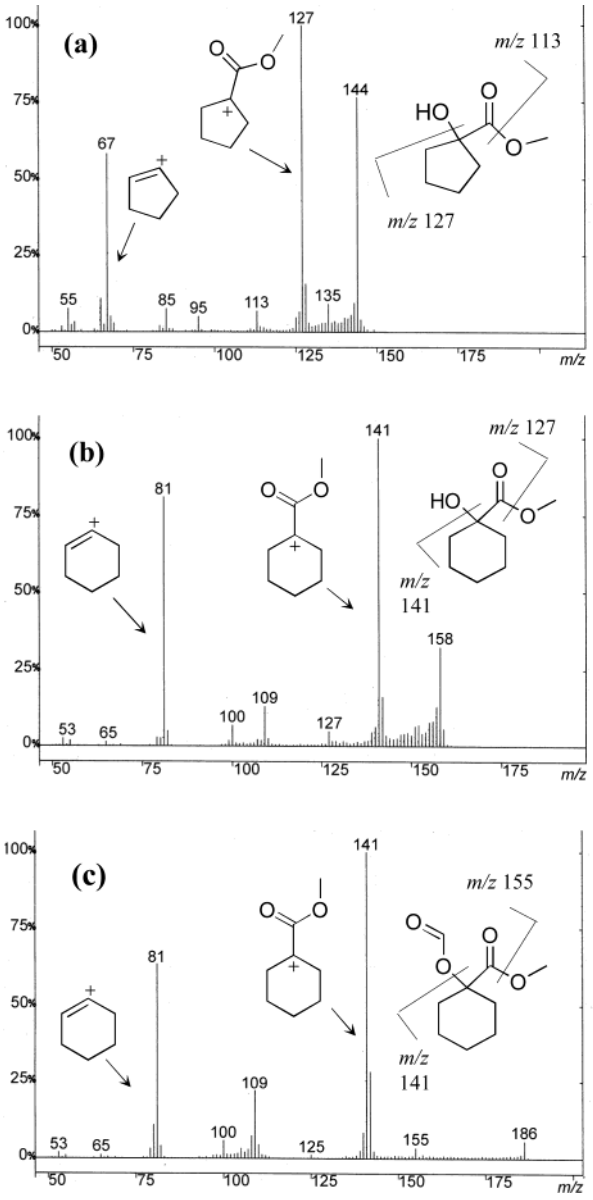



**FIGURE 5. GC/MS spectra of derivatized oxidation products formed during the ozonation of THN (a) and ECH (b,c). Methylester of (a) 1-hydroxycyclopentane-1-carboxylic acid (7), (b) 1-hydroxycyclohexane-1-carboxylic acid (9), (c) 1-hydroxycyclohexane-1-carboxylic acid, formate (10).**

($CO_2$) in the negative mode (*24*), whereas the loss of 46 seems to be characteristic of α and *β*-hydroxy acids (*25*).

A third product with MW = 176 (not shown in Scheme 1) was only detected with LC-MS/MS. The MS/MS spectrum in the negative mode showed the loss of 18, 44, 46, and 62 from $[M - H]^-$. The compound was tentatively identified as 2-hydroxyheptanedioic acid ($C_7H_{12}O_5$) because the loss of 44 and 46 suggests that the compound contains a conventional and an α or *β*-hydroxy carboxylic acid. A forth major peak in the LC-MS chromatogram with MW = 154 could not be identified.

Scheme 1 shows the suggested reaction mechanism for $O_3$ attack at position 3 of the phenol ring. On the basis of a study on the ozonation of phenol (*20*), it can be assumed that the fast first reaction step leads to the muconic acid derivative **1**, 5,6,7,8-tetrahydro-2,3-naphthalendiol (**2**), and 2,3-nathphalendione (**3**) as major intermediates. These intermediates are still reactive to $O_3$ and consequently undergo further reactions, which might result in the formation of the hydroperoxide **4** and/or 1,2-cyclohexanedione (**5a**). Product **4** can be categorized as α-hydroxy or α-keto-hydroperoxide. It can react through hydrogen peroxide elimination (*26*), leading to the formation of **5a**. A second reaction is a rearrangement by cleavage of the bond between the hydroperoxy and the keto group (*27*). The latter reaction results in the formation of adipic acid (**6**), which was identified as a major oxidation product. LC-MS/MS analysis indicated that if **5a** is formed during the reaction, it accounts for less than 10% of THN transformed. However, the fact that **5a** is almost entirely hydrated in aqueous solution (**5c**) and additionally slowly transformed into the enol form **5b** (*28*) makes analysis difficult, and it cannot be excluded that some of the species of **5** were missed in the analysis. The formation of **7** must be the product of a benzilic acid rearrangement (*29*) of **5a** via **5c**. Ring contractions of six-membered cyclic diketones to α-hydroxy acids have been reported by several studies dealing with steroid synthesis (e.g., ref *30*). Usually, these experiments were performed under alkaline conditions in solvent/water mixtures. In aqueous solution, a study on the enolization of **5a** (*31*) explained its slow irreversible disappearance and the formation of an acidic compound by the formation of **7** as well. On the basis of these considerations, we assume that **5** was at least partly transformed into **7** in a relatively slow reaction after ozonation.

Scheme 1 refers to the initial $O_3$ attack at position 3 of the phenol ring. $O_3$ can also attack at position 1. The corresponding first step intermediates will be formed. Further oxidation by $O_3$ might proceed in a different way and yield different products due to the different positions of the double bonds. However, it is possible that products **6** and **7** were partly formed by this way as well.

**Ozonation of the Model Compound ECH.** The ethinyl group represents the reactive moiety of the second model compound 1-ethinyl-1-cyclohexanol (ECH). Even if acetylenes react considerably more slowly with $O_3$ than olefins, it can be assumed that $O_3$ attack on the ethinyl group proceeds analogous to the well-established Crigee mechanism for double bonds (*32*). According to this mechanism, $O_3$ attack on the ethinyl group of ECH results in the formation of the primary ozonide **8**, which quickly decomposes into the hydroxyhydroperoxides **9a** and/or **9b** (Scheme 2). The fact that less than 2 min after the completion of the reaction no hydroperoxides except hydrogen peroxide could be determined demonstrates that **9a** and **9b** are only short-lived intermediates. The $H_2O_2$ yield was 65% relative to the added $O_3$ concentration. This indicates that the keto aldehyde **10** is the major product of this reaction (in aqueous solution **10** may be hydrated).

With LC-MS/MS analysis, three further oxidation products were identified. The first product was identified as 1-hydroxycyclohexanecarboxylic acid (**11**). After derivatization with diazomethane, its GC/MS spectrum (Figure 5b) was identical to the spectrum of the commercially available methylester of the compound. The identification of **11** is in agreement with the detection of formic acid immediately after completion of the reaction of $O_3$ with ECH. For the second product **12**, no standard was available. The GC/MS spectrum of derivatized **12** (Figure 5c) was virtually identical to the spectrum of **11** except that the molecular ion was *m*/*z* 186 instead of *m*/*z* 158 and that the loss of 31 ($OCH_3$) from the molecular ion resulted consequently in a peak at *m*/*z* 155 instead of *m*/*z* 127. The difference of 28 Da between **12** and **11** and the associated derivatives can be explained best by the presence of an additional CO group in **12**. In both spectra, the base peak is at *m*/*z* 141, demonstrating that methylated **12** lost 45 (OCOH) instead of 17 (OH) as did **11**. The further fragmentation of the base peak (*m*/*z* 141) was identical for both molecules. Obviously, the CO group has to be attached to the tertiary alcohol group. Additionally, it could be shown

**SCHEME 2. Oxidation Products Formed by the Reaction of $O_3$ with the Model Compound ECH**

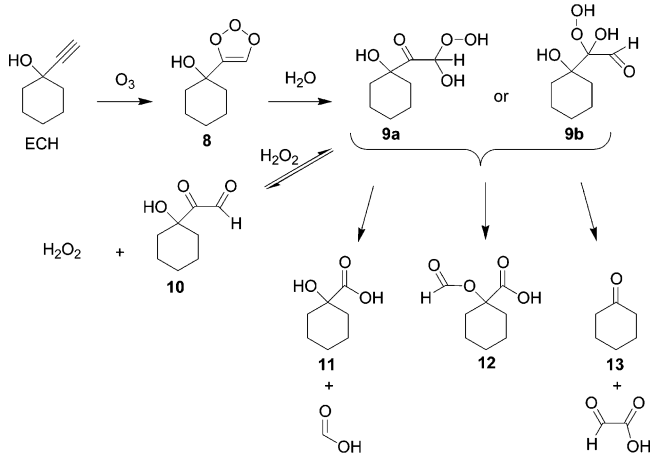



**TABLE 1. Ozonation Products of EE2 Identified with the Model Compounds THN and ECH**

| Products THN (yield) \ Products ECH (yield) | (15%) MW=98 **13** | (20%) MW=144 **11** | (not quantified) MW=172 **12** |
|---|---|---|---|
| (<24%) MW=146 **6** | MW=268 **14** | MW=314 **15** | MW=342 **16** |
| (not quantified) MW=130 **7** | MW=252 **17** | MW=298 **18** | MW=326 **19** |

with LC/MS that **12** hydrolyzes at pH 12 to **11**. The third oxidation product was identified as cyclohexanone (**13**) using an authentic standard for LC-MS/MS analysis. Due to analytical problems, the formation of glyoxylic acid, which should be produced in an equivalent amount, could not be confirmed.

Product **10** was not detected with LC-MS/MS analysis. Because $H_2O_2$ was not destroyed in the samples and the formation of **10** from the hydroxyhydroperoxides **9a** and/or **9b** is a reversible process, **10** was slowly converted into **11**, **12**, and **13** during sample storage. The half-life time for **10** is estimated to be <40 h for 1 mM $H_2O_2$ at pH 7 ($k_{observed}$ = 5−7 $M^{-1}$ $s^{-1}$ at pH 10 and 20 °C). At pH 12, the base-catalyzed reaction of **10** with $H_2O_2$ yielded less than 30% formic acid, indicating that **12** and not **11** or **13** is the major product (glyoxylic acid quickly decomposes to formic acid under these conditions). The slow conversion of a very similar keto aldehyde into the corresponding formate by Baeyer−Villiger-type oxidation in aqueous solution has already been reported in (*33*). In the absence of $H_2O_2$, **10** seems to be fairly stable. A degradation product of hydrocortisone with a moiety analogous to **10** exhibited a half-life > 40 d at pH 8 for base-catalyzed degradation (*34*). Therefore, **10** might be a more important product than **12** under realistic treatment conditions where $H_2O_2$ concentrations are significantly lower.

**Quantification of Product Formation.** The formation of adipic acid (**6**) could be quantified with LC-MS/MS, because an authentic standard was available. Product **6** accounted for up to 24% of THN transformed during ozonation. The second THN product **7** could not be quantified.

Based on measurements of $H_2O_2$ and formic acid, ozonation of ECH yielded 65% **10** and 20% **11**. Cyclohexanone (**13**) accounted for 15%. This compound was quantified with LC-MS/MS, and the result might have been influenced by the storage time. Ozonation of the similar ethinyl compound 2-methyl-3-butyn-2-ol yielded 25% α-hydroxyisobutyric acid at pH 7. This reaction is equivalent to the formation of **11** from ECH, and the 25% yield agrees with the yield determined for **11**. The mass balance for ECH seems complete, whereas for THN the identified products probably do not account for more than 50% of THN transformed.

**TABLE 2. LC-MS/MS Spectra for Oxidation Products of Model Compounds and Steroid Hormones in the Negative Mode[a]**

| N° | MW | $[M - H]^-$ *m/z* | $-H_2O$ Δ18 | $-CO$ Δ28 | $-CO_2$ Δ44 | $-CH_2O_2$ Δ46 | $-H_2O-CO_2$ Δ62 |
|---|---|---|---|---|---|---|---|
| | | | | **THN** | | | |
| 7 | 130 | **129** | 111(13%) | | | 83(84%) | |
| 6 | 146 | 145(32%) | 127(25%) | | **101** | | 83(95%) |
| | 176 | **175** | 157(12%) | | 131(54%) | 129(13%) | 113(57%) |
| | | | | **ECH** | | | |
| 11 | 144 | **143** | | | | 97(57%) | |
| 12 | 172 | 171(0%) | | **143** | | | |
| | | | | **EE2** | | | |
| 14 | 268 | 267(7%) | 249(1%) | | 223(44%) | | **205** |
| 15 | 314 | **313** | 295(4%) | | | 267(10%) | 251(13%) |
| 16 | 342[b] | 341(64%) | | **313** | | | |
| 17 | 252 | **251** | 233(6%) | | | 205(12%) | |
| 19 | 326[b] | 325(41%) | | **297** | | 279(38%) | |

[a] For each spectrum, the *m/z* values for the quasi-molecular ion $[M - H]^-$ and the major fragment ions are given. Bold values correspond to the base peak. The intensity of the ions relative to the base peak is reported in brackets. [b] Full-scan spectra.

**Oxidation Products of EE2.** Table 1 lists the oxidation products of EE2 which were derived from the oxidation products of the model compounds detected with LC-MS/MS. Five out of six possible oxidation products of EE2 could also be detected with LC-MS/MS. Product **18** (Table 1) could not be unambiguously assigned to a peak of the LC/MS chromatogram due to interferences with fragment ions from other products. Table 2 reports the major mass fragments of the MS/MS spectra recorded in the negative mode. The suggested structures of the oxidation products are supported by the loss of characteristic masses depending on the functional groups present in the molecules. Molecule **14** (Table 1) primarily lost 44 Da ($CO_2$) as did the model-compound product adipic acid (**6**). In the negative mode, the loss of $CO_2$ is typical for carboxylic acids (*24*). In contrast, the α-hydroxy acid **17** lost 46 Da ($CH_2O_2$) instead of 44 Da. The same behavior was observed for the model-compound products **7** and **11**. As mentioned earlier, the loss of 46 Da seems to be characteristic for α or *β*-hydroxy acids (*25*). Product **15**, which has two conventional carboxyl groups and one α-hydroxy acid group, lost 46 Da but not 44 Da. However, the loss of 62 Da ($CO_2 + H_2O$) indicated the presence of the two conventional carboxyl groups, because the same fragmentation was also observed for **14** and **6**. The molecules **16** and **19** exhibiting a formate group primarily lost 28 Da (CO) and had the base peak at $[M - 1 - 28]$. The same fragmentation was observed for the model-compound product **12**.

The oxidation products shown in Table 1 represent the stable oxidation products detected with LC-MS/MS. Experiments for the quantification of hydroperoxides and mechanistic considerations demonstrated that some of these oxidation products derive from less stable intermediates. Due to time-consuming sample preparation and the presence of $H_2O_2$ in the samples, these less stable intermediates de-

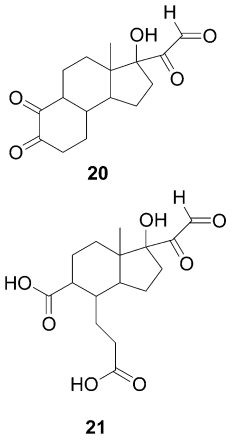
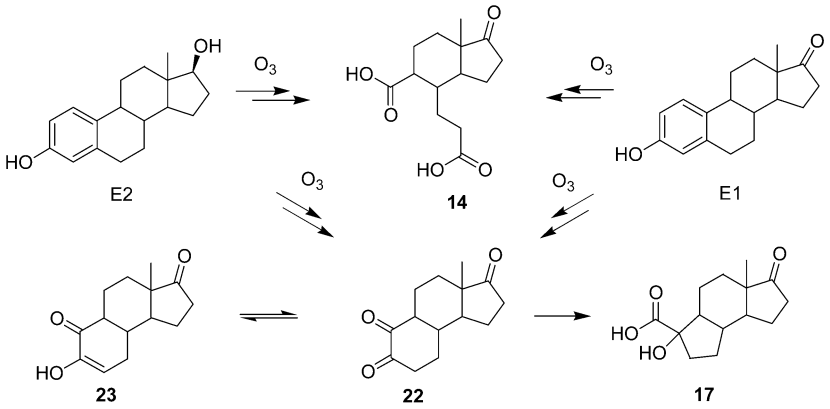



**FIGURE 6. Major intermediates formed by the reaction of $O_3$ with EE2. These compounds were not detected with LC-MS/MS. However, mechanistic considerations provide strong evidence that the detected products in Table 1 were partly formed via 20 and 21. By superimposing the cyclohexanedione moiety of 20 on products 14, 15, and 16 (Table 1), three further products are obtained, which also may be formed. Product 20 can also be transformed into its enol forms as shown in Scheme 1.**

**SCHEME 3. Oxidation Products Formed by the Reaction of $O_3$ with E2 and E1**



composed and were not detected with LC-MS/MS. Figure 6 depicts two major transient oxidation products which must have formed but escaped detection. It is expected that these intermediates persist for a couple of hours to a few days in the water before they are transformed into the more stable products shown in Table 1.

The products listed in Table 1 and Figure 6 are formed at relatively high $O_3$ exposures as applied in ozonation of drinking water. At lower $O_3$ exposures, $O_3$ does not react with the ethinyl group. As a consequence, only the phenolic moiety will be transformed. Despite the lower $O_3$ exposure, it can be expected that the phenol moiety is, at least partly, transformed in the same way as for higher doses because the first reaction steps are fast.

**Oxidation Products of 17$\beta$-Estradiol (E2) and Estrone (E1).** The oxidation products formed during the ozonation of the natural hormones E2 and E1 were investigated as well. Instead of an ethinyl group, E2 and E1 exhibit an alcohol and a keto group at the 17 position (Figure 1). As carbonyl and alcohol groups are much less reactive to $O_3$ than an ethinyl group, E2 and E1 were expected to react only at the phenolic moiety. Surprisingly, the experiments yielded the same two major products for E2 and E1. With LC-MS/MS, they were identified as **14** and **17**, which were also formed during the ozonation of EE2 (Scheme 3). This was expected for E1, because these oxidation products of EE2 have a carbonyl group at the 17 position identical to that of E1. The corresponding alcohol group of E2 was unexpectedly oxidized to the carbonyl group under the applied conditions and yielded the same products as E1. Product **17** must be a decomposition product of the intermediate **22**. As shown in Scheme 1, the cyclohexanedione moiety can be hydrated and is partly present in the enol form **23**.

**Relationship between the Chemical Structure and the Estrogenic Activity of Oxidation Products.** The identified oxidation products of EE2 are expected to form at an $O_3$ exposure of 5−10 $mgL^{-1}$ min at 10 °C. In the experiment shown in Figure 3, the corresponding $O_3$ exposure resulted in a reduction of estrogenicity by a factor of 5000−10 000 if the estrogenicity caused by reappeared EE2 is subtracted. Most probably, the observed reduction of estrogenicity can be attributed to the cleavage of the phenolic moiety of EE2 because the 3-hydroxy group and the aromatic ring of the phenolic moiety is of particular importance for the binding of estrogens to the estrogen receptor (*35*). As demonstrated by our investigation, the phenol rings of EE2, E2, and E1 were cleaved in all of the identified products. However, the fact that the fast initial transformation steps, which result only in a partial cleavage of the phenol ring (Scheme 1), reduced the estrogenicity by a factor of 200−500 indicates that only small modifications of the phenol ring may be required to reduce the estrogenicity of EE2 substantially. Overall, the present study provides direct evidence that the selective oxidation of the phenolic moiety efficiently reduces the estrogenicity of EE2-containing solutions.

For the first time, the oxidation of a pharmaceutical compound during ozonation has been investigated in a comprehensive manner, including oxidation kinetics, product formation, and the pharmacological effects of the oxidation products. The results demonstrate that $O_3$ doses applied for the disinfection of drinking water can efficiently remove EE2, E2, and E1 and the estrogenicity associated with their presence in the water. Due to the selective oxidation of estrogens by $O_3$, ozonation is also a promising tool for the control of estrogenicity in effluents of sewage treatment plants. This has already been confirmed by ozonation experiments using a pilot plant, which was operated with real wastewater (*36*). When the disinfection of wastewater is a legal requirement, ozonation, therefore, may be a viable alternative to other disinfection processes.

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