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Oxidation of 17α-ethinylestradiol with Mn(III) and product identification

Sangpill Hwang, Dong-Ik Lee, Chang-Ha Lee, Ik-Sung Ahn *

Department of Chemical Engineering, Yonsei University, Seoul 120-749, South Korea

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Abstract

With increasing concern about the contamination of aquatic environments by estrogenic pollutants, removal of synthetic estrogens such as 17α-ethinylestradiol (EE2) has been widely studied, especially with respect to the treatment methods. However, the degradation products have rarely been identified. The purpose of this study was to identify structurally the oxidation products of EE2. Mn(III) was used as an oxidizing agent. To obtain sufficient oxidation products for HPLC, LC–MS and NMR spectroscopy, a highly concentrated solution of EE2 (1 mM) was prepared in a mixture of water and a water-miscible organic solvent. From HPLC of the reaction products, a single compound (I) was found to be predominant. From LC–MS, its molecular mass was found to be 294, and two hydrogens were believed to have been removed from EE2 (M.W. 296) to form a C=C double bond. The structure of compound I (position of the double bond) was determined using ^{1}H NMR, ^{13}C NMR, H–H COSY, HSQC and HMBC. As minor products, isomeric dimers (M.W. 590) of EE2, as well as the products (M.W. 588) in which EE2 was coupled to compound I were also formed during the Mn(III)-mediated oxidation of EE2.

Keywords: 17α-Ethinylestradiol; Manganese; Oxidation; Product identification; Endocrine disruptor

1. Introduction

There have been increasing concerns about the contamination of aquatic environments by estrogenic pollutants [1]. Not only industrial chemicals such as pesticides, bisphenol A, PCBs and phthalate, but also natural estrogens and synthetic estrogens such as 17α-ethinylestradiol (EE2) have been found to be potent endocrine disruptors [2,3]. For example, EE2 is mass produced as a major constituent of the human contraceptive pill and postmenopausal hormonal supplements. As a synthetic estrogen, EE2 is more stable in water than natural estrogens. Snyder et al. [4] reported that EE2 remained unchanged after 120 h in activated sludge treatment. Hence, several methods have been studied to remove EE2 from wastewaters.

Vader et al. [5] reported that nitrifying activated sludge degraded EE2 at a maximum rate of 1 μg ((g dry weight of activated sludge)$^{-1}$ h^{-1}). From HPLC, the products were found to be more hydrophilic than EE2, and the authors postulated that nitrifying micro-organisms oxidize EE2 to its hydroxylated derivatives [5]. Even though there have been many studies of biological treatment processes that effectively reduce levels of endocrine-disrupting chemicals in water or wastewater [6–9], the low levels of these chemicals in the effluent are still a concern because they exert physiological effects at very low concentrations [10]. Hence, chemical oxidation, photolysis, and photolysis combined with chemical oxidation have been studied for the removal of EE2 from water and wastewater.

As an example of chemical oxidation methods, ozonation of EE2 has been reported to reduce its estrogenicity by a factor of more than 200 [11]. Ozonation products of EE2 have been indirectly identified using 5,6,7,8-tetrahydro-2-naphthol and 1-ethinyl-1-cyclohexanol, which have the phenolic and the ethinyl moieties of EE2, respectively, as model compounds [11]. MnO_2 granules have been shown to be quite effective for the removal of EE2, not only as a catalyst for oxidation but also as a sorbent [12]. Photolysis using UV light, as well as ozonation have been recognized as promising technologies for advanced water treatment. Hence, photolysis of EE2 has been extensively studied [10,13–15], along with product identification by on-line HPLC–NMR and HPLC–MS [16]. However, more effective degradation of EE2 is obtained when it is combined with chemical oxidation, such as H_2O_2-mediated oxidation [10] or

* Corresponding author. Tel.: +82 2 2123 2752; fax: +82 2 312 6401.
E-mail address: iahn@yonsei.ar.kr (I.-S. Ahn).

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TiO_2-catalyzed photo-oxidation [15]. In spite of such an advantage of chemical oxidation for the effective removal of EE2, the products have not been identified, except indirectly, as reported by Huber et al. [11].

The purpose of this study was to structurally elucidate the products of chemical oxidation of EE2. The Mn(III) ion was used as an oxidizing agent. Chemical oxidation involves the generation of radicals, which leads to the production of numerous products. As multiple on-line analysis instruments such as HPLC–NMR and HPLC–MS were not available in our lab, these products needed to be prepared in sufficient quantities to allow their separate analysis by thin-layer chromatography (TLC), HPLC, LC–MS and NMR spectroscopy. Hence, a highly con-

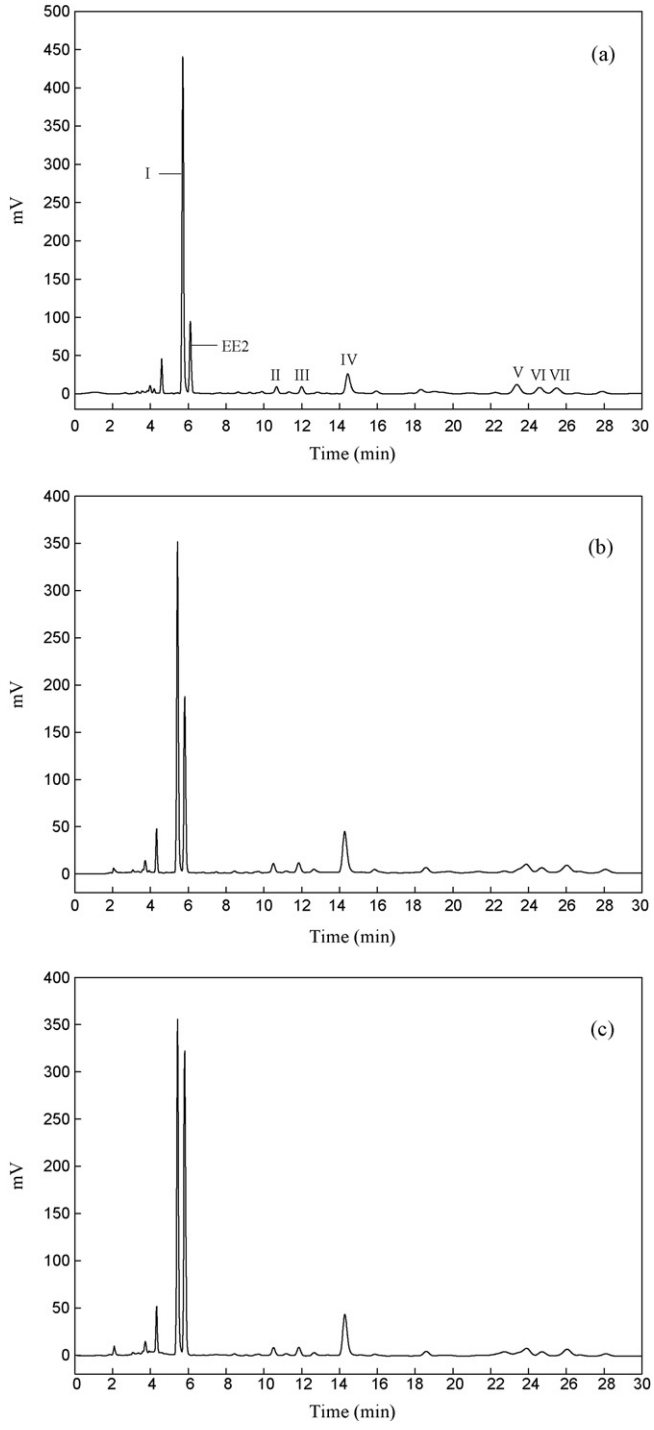

Fig. 1. HPLC chromatograms for the products of the Mn(III)-mediated oxidation of EE2 in the mixture of water and acetonitrile. The content of acetonitrile was 30% (v/v) (a), 50% (v/v) (b), and 70% (v/v) (c). In all cases, 1.0 mM EE2 was reacted with 5.0 mM manganese(III) acetate.

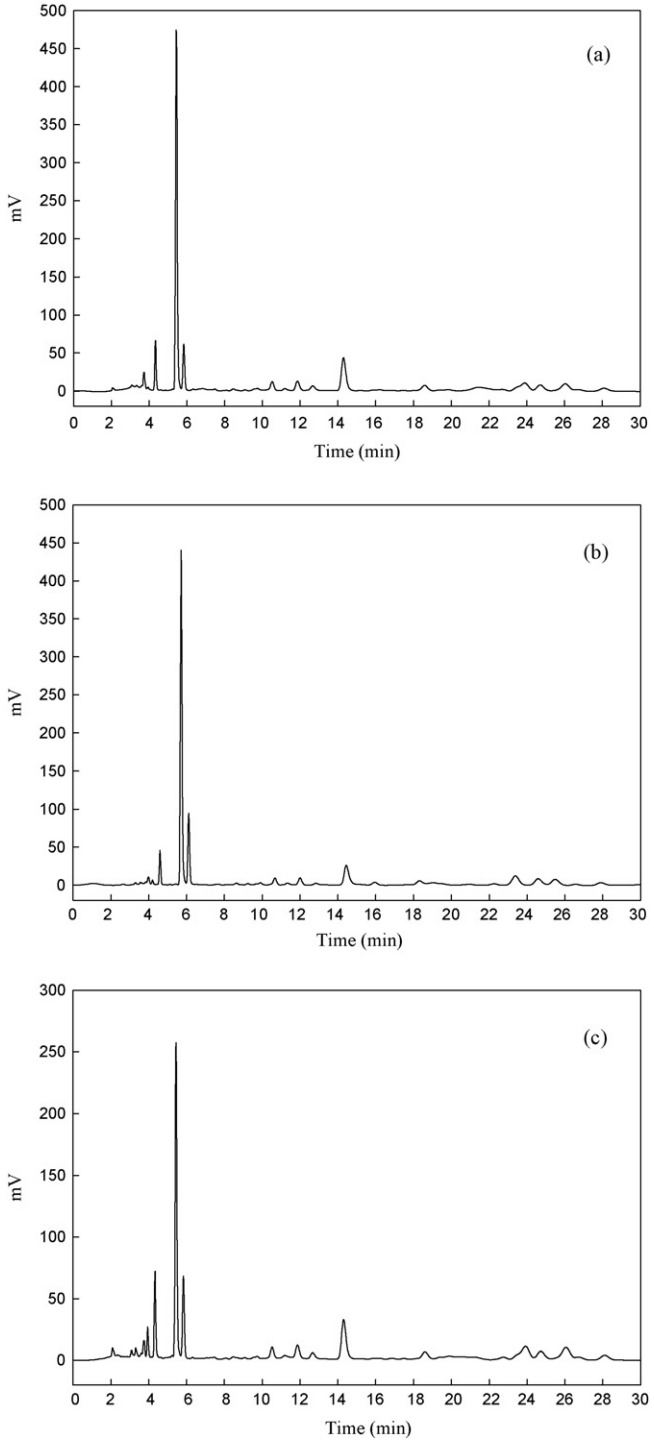

Fig. 2. HPLC chromatograms for the products of the Mn(III)-mediated oxidation of EE2 in the water mixture of acetonitrile (a), acetone (b), and 1,4-dioxane (c). In all cases, the content of the organic solvent was 30% (v/v) and 1.0 mM EE2 was reacted with 5.0 mM manganese(III) acetate.

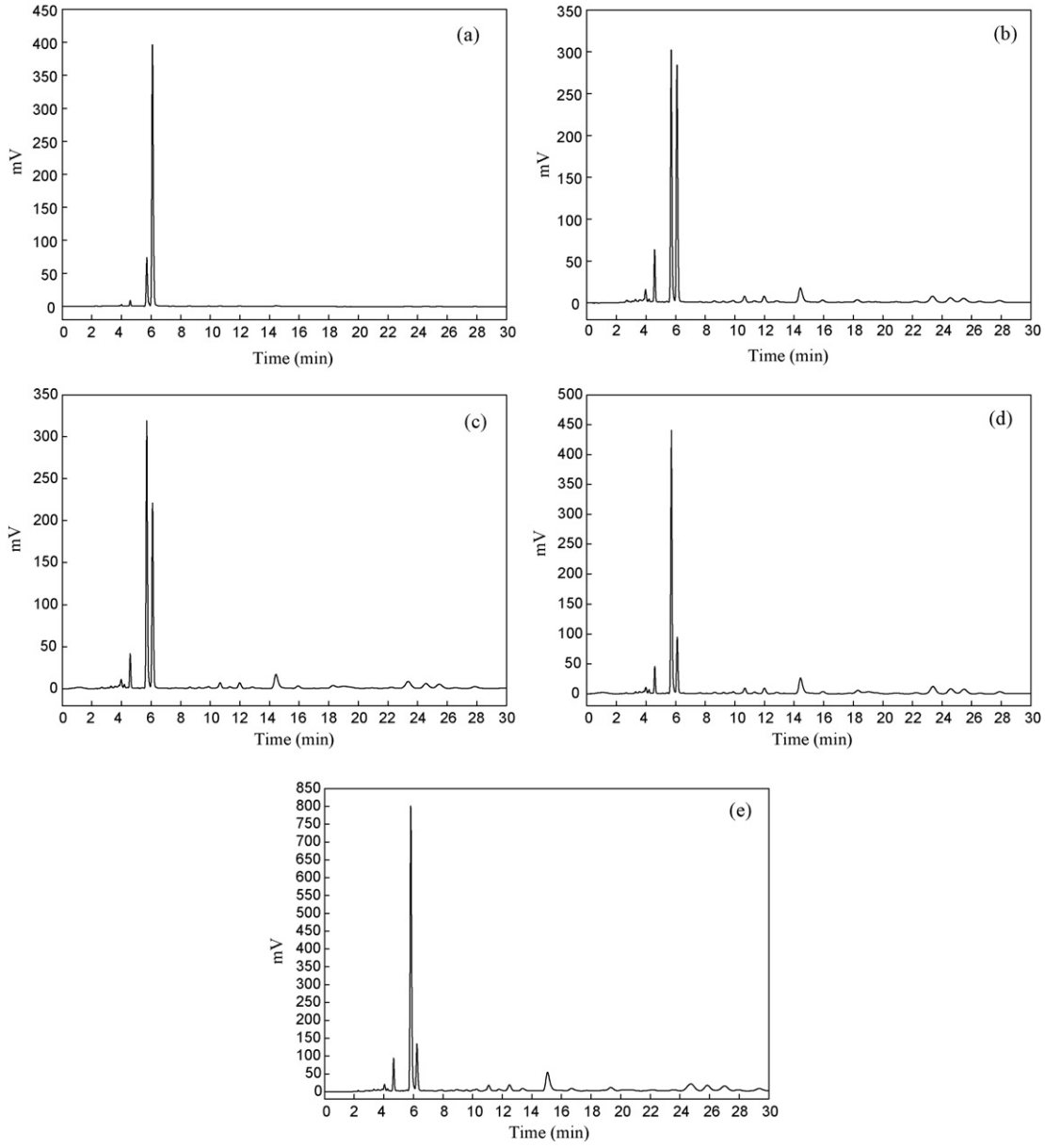

Fig. 3. HPLC chromatograms for the products of the Mn(III)-mediated oxidation of EE2 at different concentrations of manganese(III) acetate: 0.5 mM (a); 1.0 mM (b); 3.0 mM (c); 5.0 mM (d); 7.0 mM (e).

centrated solution of EE2 (1 mM) was prepared in a mixture of water and a water-miscible organic solvent. Mn(III) is generated from the oxidation of Mn(II) by manganese peroxidase of white-rot fungi [17], therefore, the oxidation of EE2 by Mn(III) implies the feasibility of biological removal of EE2 by white-rot fungi.

Table 1
Molecular mass of Mn(III)-mediated oxidation products of EE2

Peaks	RT (min)	$[M+H]^+$	$[M+H-H_2O]^+$
EE2	6.0	297	279
I	5.8	295	277
II	10.5	591	573
III	12.1	591	573
IV	14.4	591	573
V	23.2	589	571
VI	24.3	589	571
VII	25.2	589	571

2. Materials and methods

2.1. Mn(III)-mediated oxidation of EE2

In the mixture of a water-miscible organic solvent and water, EE2 (Sigma) was dissolved to a concentration of 1.0 mM. Acetonitrile, 1,4-dioxane and methanol were tested as model organic solvents. The oxidation reaction was initiated by the addition of manganese(III) acetate (Aldrich). After 2 h reaction at room temperature, the organic solvent was removed by evaporation at 50 °C under reduced pressure. Then, the reaction mixture was extracted three times with ethyl acetate. The organic layers were

withdrawn, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. Extracts were analyzed by HPLC and purified by TLC. The purified products were then identified using LC–MS and NMR spectroscopy.

2.2. Analytical methods

For TLC, samples were loaded onto a silica gel 60 F_{254} TLC plate (Merck) and developed with a mixture of hexane, benzene and acetone (50:30:20). Bands detected by UV at 254 nm were scratched off and extracted with ethyl acetate.

Ethyl acetate extracts and TLC-purified products were analyzed by HPLC (Waters 510) equipped with a UV absorbance detector at 254 nm (Waters 486). Separation was performed on a reverse-phase C-12 column (4-μm particle size, 150 mm × 4.6 mm, 80 Å pore size; Phenomenex). Samples were eluted under isocratic conditions with 60% acetonitrile and 40% water at 40 °C and a constant flow rate of 1.0 ml/min.

LC–MS was performed to determine the molecular mass of TLC-purified products and recorded using an LC–ESIMS (Triple Quadrupole Tandem Mass Spectrometer). For the structural identification of products, NMR spectra were recorded on a Bruker Advance 500-MHz NMR spectrometer (Bruker BioSpin).

3. Results and discussion

3.1. Effect of organic solvents on the oxidation of EE2

When the water mixture of acetonitrile was used as a solvent for dissolving EE2, HPLC chromatograms of reaction products, depending on its content, are shown in Fig. 1. The retention time (RT) for EE2 was 6.0 min. Among the seven recognizable products (see roman numbers in Fig. 1a), the compound for peak I, whose RT was 5.8 min, was most dominant. There is no single predominant product in the photodegradation of EE2 [16]. As seen from the height of the EE2 peak, the degree of EE2 removal was lower at the higher concentration of acetonitrile. It is believed to have been caused by the lower activity of

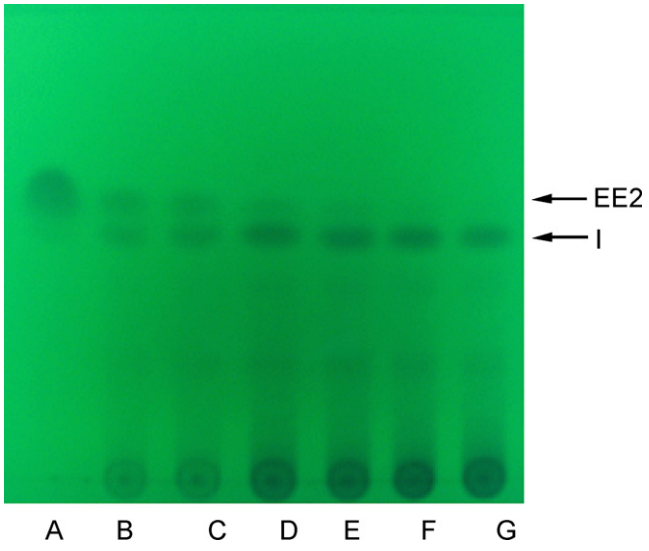

Fig. 4. TLC chromatograms for the products of the Mn(III)-mediated oxidation of EE2 at different concentrations of manganese(III) acetate: 0 mM (lane A); 0.5 mM (lane B); 1.0 mM (lane C); 3.0 mM (lane D); 5.0 mM (lane E); 7.0 mM lane F and G.

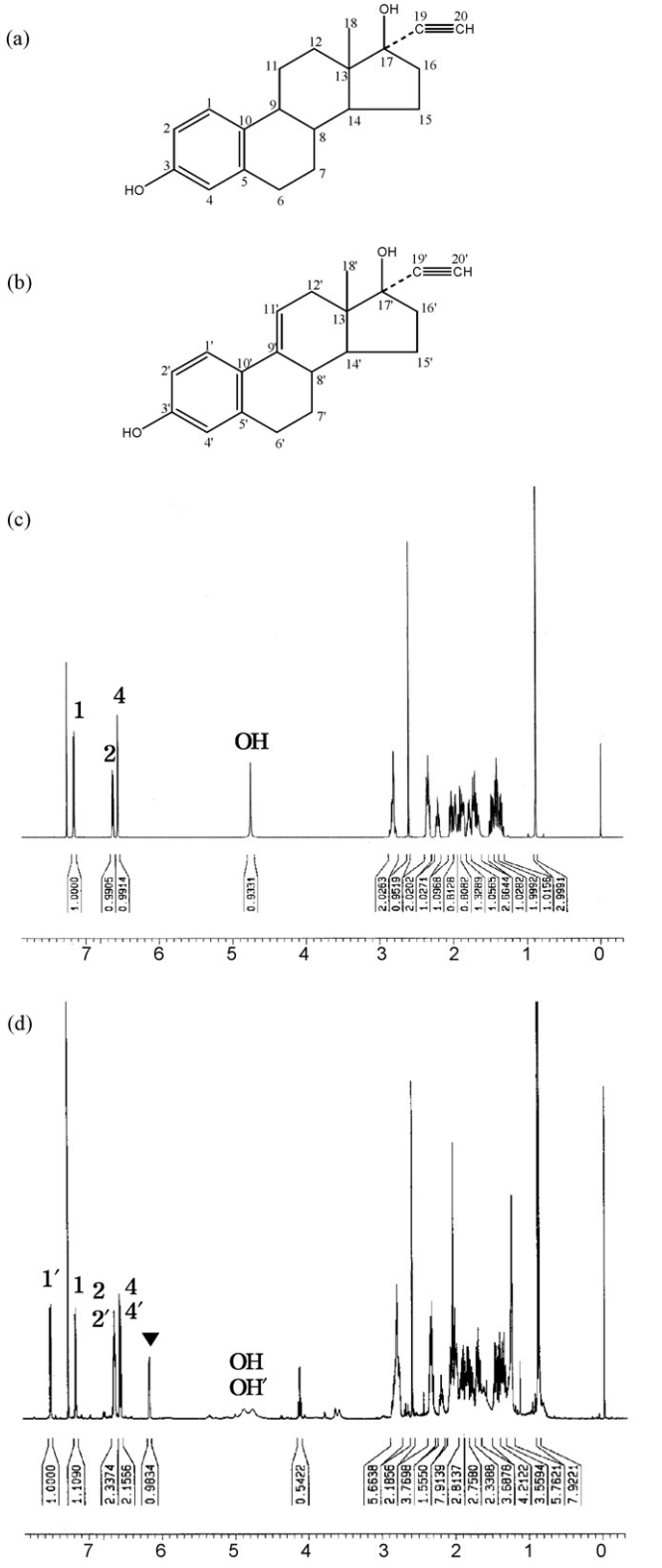

Fig. 5. Chemical structures and ^{1}H NMR spectra of pure EE2 (a and c) and the mixture of EE2 and compound I (b and d).

Mn(III) ions at the higher concentration of acetonitrile. Solutions of EE2 were prepared in a water mixture of other organic solvents (30%, v/v) and oxidized with manganese(III) acetate. As shown in Fig. 2, the degree of EE2 removal was not affected by the organic solvent used. For further experiments, 30% (v/v) acetonitrile was used as a solvent for a 1 mM EE2 solution.

The effect of Mn(III) content on the oxidation of EE2 (1 mM) was estimated by varying the initial content of manganese(III) acetate from 0.5 to 7.0 mM (Fig. 3). The degree of EE2 removal increased as the content of Mn(III) increased from 0.5 to 5.0 mM (Fig. 3a–d). Comparing Fig. 3d and e, it can be seen that the degree of EE2 removal reached a maximum when the ratio of

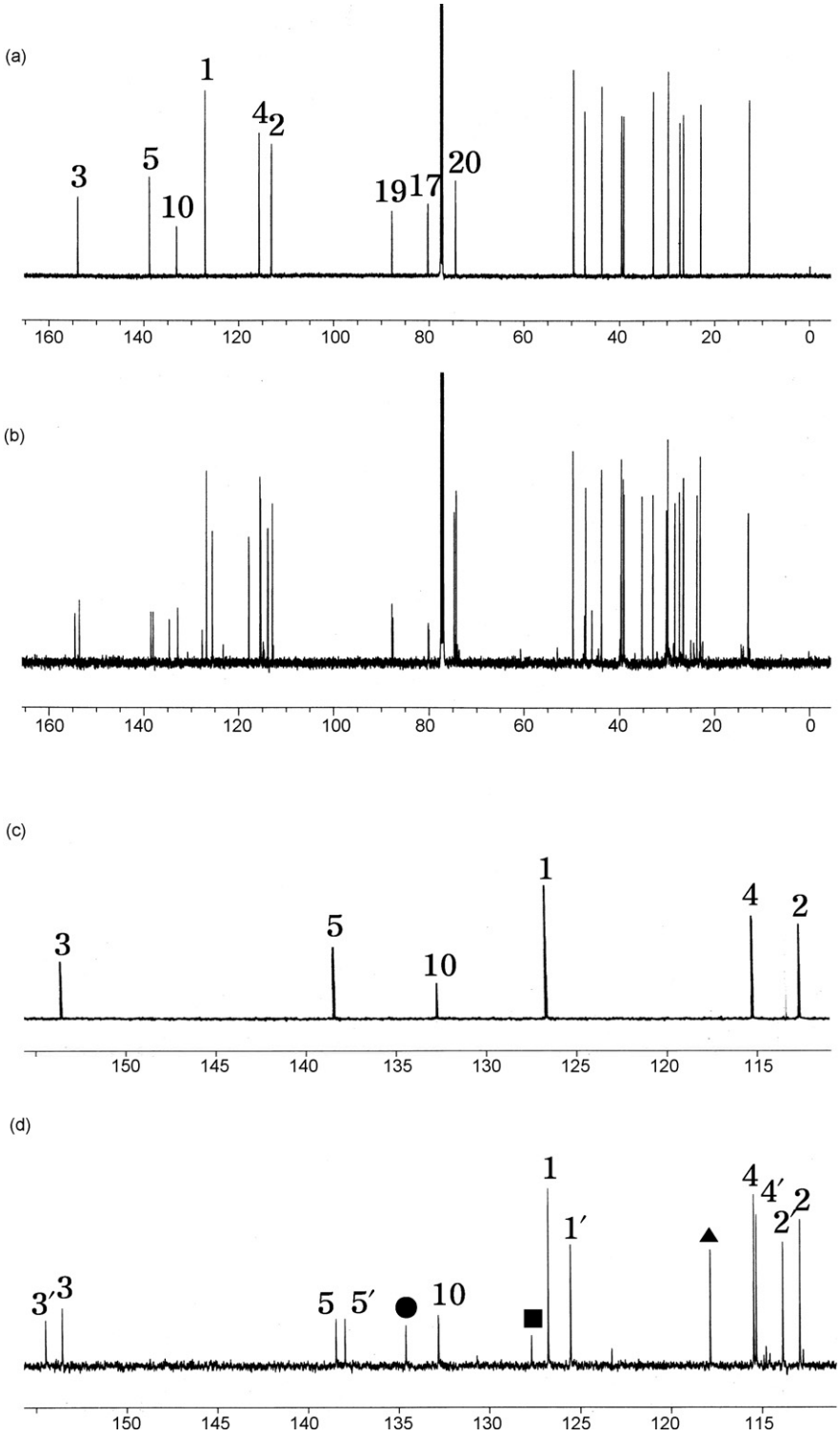

Fig. 6. ^{13}C NMR spectra of pure EE2 (a and c) and the mixture of EE2 and compound I (b and d). The ^{13}C NMR spectra at a chemical shift of 100 and higher are magnified in (c) and (d).

Mn(III) to EE2 was 5.0, and any further increase in Mn(III) concentration did not increase the oxidation of EE2.

3.2. Identification of oxidation products

Seven products that were analyzed by HPLC (Fig. 1a), were further analyzed by MS and their molecular masses are summarized in Table 1. The molecular mass of compound I was 294. As the molecular mass of EE2 was 296, compound I is believed to have been formed by the removal of two hydrogens from EE2. Compounds II, III and IV have the same molecular mass of 590. Hence, they are believed to be isomeric dimers of EE2 formed via C–C or C–O coupling of phenoxy radicals of EE2. These isomeric dimeric products have been reported to be formed by the photolysis of EE2 [16]. Compounds V–VII have the same molecular mass of 588 and are believed to have been formed by radical coupling of EE2 and compound I. As shown by HPLC (Figs. 1–3), the level of compounds V–VII was similar to that of compounds II–IV. However, the formation of compounds V–VII during the oxidation of EE2 has not been reported elsewhere.

The results of TLC analysis of the products are shown in Fig. 4. Samples were taken from the experiments investigating the effect of Mn(III). UV absorbance of EE2 spot decreased as the concentration of Mn(III) increased, which agrees with the HPLC results shown in Fig. 3. UV absorbance of the spot just below the EE2 spot increased as the concentration of Mn(III) increased. Hence this spot corresponds to compound I.

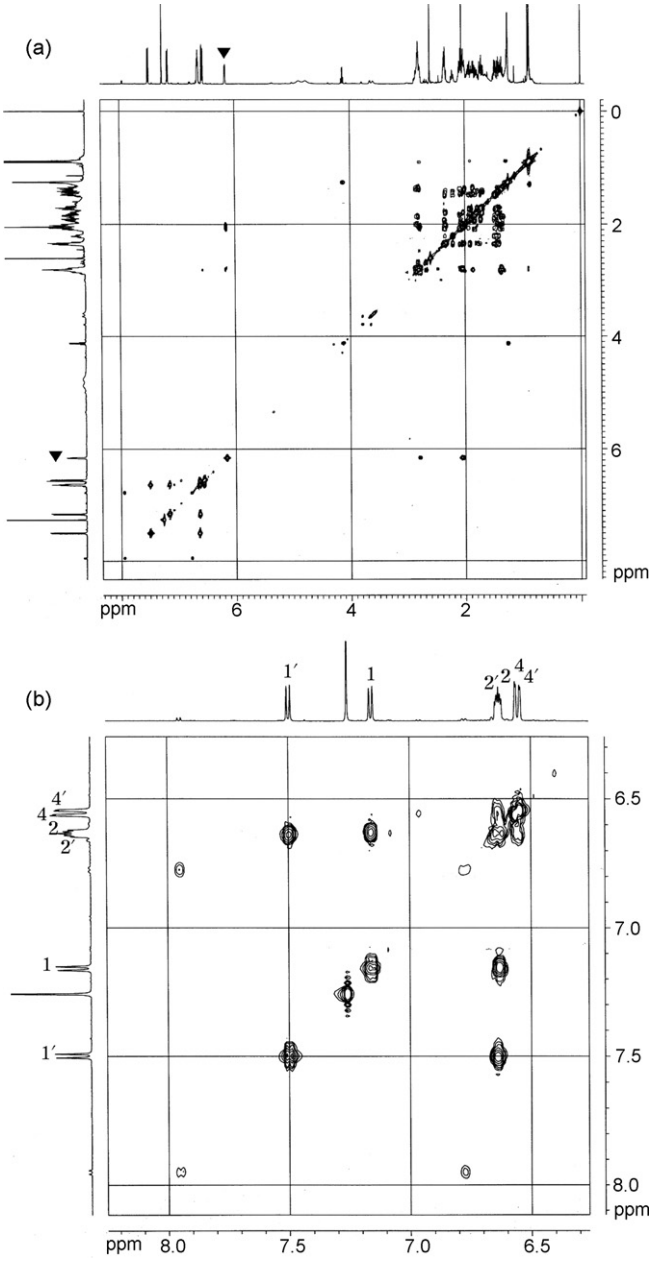

Fig. 7. H–H COSY spectra for the mixture of EE2 and compound I at a chemical shift of 0.0–8.0 ppm (a) and 6.3–8.0 ppm (b).

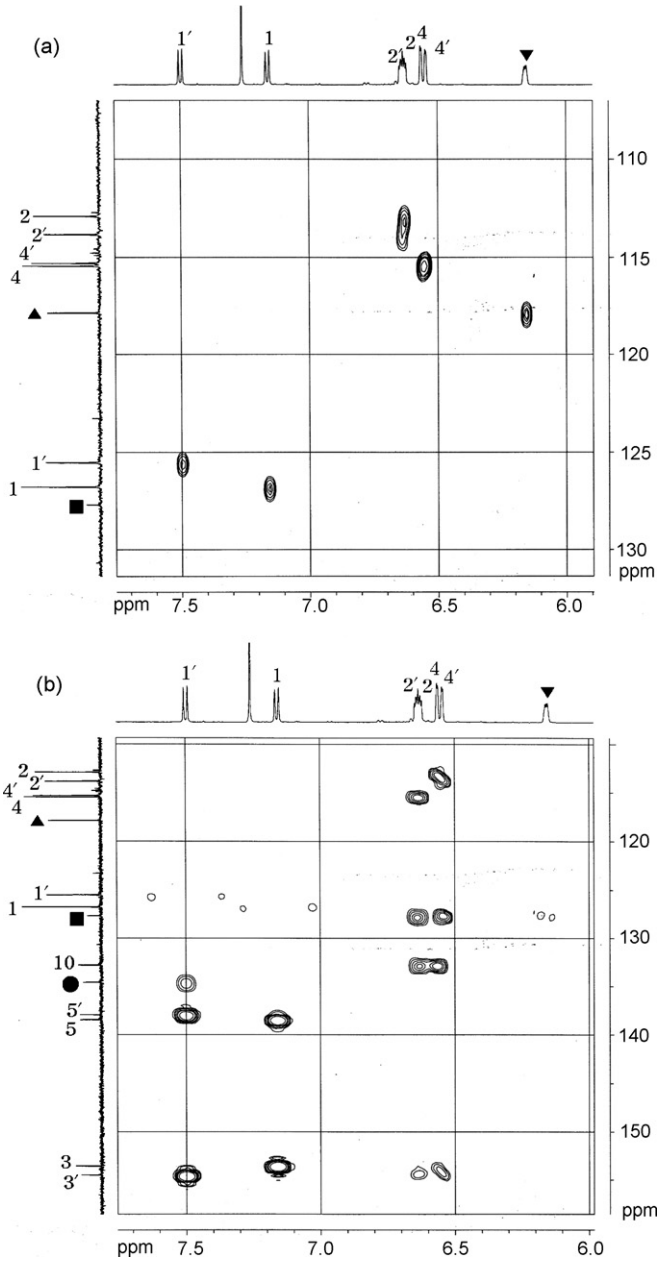

Fig. 8. HSQC (a) and HMBC (b) spectra for the mixture of EE2 and compound I.

To obtain a sufficient quantity of compound I for NMR analysis, preparatory TLC was performed with a large loading of reaction products. Unlike the TLC separation with the small loading shown in Fig. 4, two spots of EE2 and compound I overlapped and NMR analysis was performed with the mixture of EE2 and compound I. ^{1}H NMR spectra of pure EE2 and a mixture of EE2 and compound I are shown in Fig. 5, along with the known chemical structure of EE2 and a suggested structure for compound I. Comparing Fig. 5a and b, the NMR spectrum for compound I is believed to be identical to that for EE2, except for the peak for proton H1′ in the aromatic ring, which was shifted from 7.2 to 7.5 ppm, and the appearance of a new peak at 6.2 ppm. The shift of the H1′ peak implies that a C=C double bond was formed near the aromatic ring, and was located between C9′ and C11′ or between C6′ and C7′. The appearance of only one singlet peak at 6.2 ppm excluded the possibility of the double bond between C6′ and C7′. ^{13}C NMR spectra of pure EE2 and the mixture of EE2 and compound I are shown in Fig. 6a and b, respectively. Six peaks for carbons in the aromatic ring (C1, C2, C3, C4, C5 and C10) and three peaks for carbons with a C≡C triple bond (C17, C19 and C20) are clearly seen in the spectrum of pure EE2 (Fig. 6a) [18]. Almost twice as many peaks were seen in the spectrum of the mixture of EE2 and compound I (Fig. 6b). To verify the formation of a C=C double bond in compound I, the ^{13}C NMR spectra of pure EE2 and the mixture of EE2 and compound I at a chemical shift of 100 and higher, were magnified and are shown in Fig. 6c and d, respectively. Compared to Fig. 6c, five peaks in Fig. 6d could easily be assigned to carbons in the aromatic ring of compound I (C1′, C2′, C3′, C4′ and C5′). For three new peaks at 134.6 (●) ppm, 127.8 (■) ppm and 117.9 (▲) ppm, one was for a carbon in the aromatic ring (C10′) and the other two peaks must have been for the carbons forming a double bond near the aromatic ring.

Contour plots of H–H correlation spectroscopy (H–H COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) were used to determine the location of a new C=C double bond in compound I. In the H–H COSY spectrum (Fig. 7a), no contour was seen between the singlet at 6.2 ppm (▼) and any peak of protons in the aromatic ring (H1′, H2′ and H4′). Therefore, the hydrogen bound to the carbon in the double bond was not adjacent to the aromatic ring, which means that the double bond was not formed at C6′. From the H–H COSY spectrum shown in Fig. 7b, it can be seen that the peaks for protons in the aromatic ring were correctly assigned.

HSQC and HMBC spectra for the mixture of EE2 and compound I are shown in Fig. 8a and b, respectively. As shown in the HSQC spectrum, the carbon peak at 117.9 ppm (▲) was correlated with the proton peak at 6.2 ppm (▼), which means that the carbon peak at 117.9 ppm (▲) was for a carbon forming the C=C double bond near the aromatic ring. No correlation with a proton peak was observed for the carbon peaks at 134.6 (●) ppm and 127.8 (■) ppm (data not shown), which means they were for C10′ and a carbon forming the C=C double bond without a hydrogen bond. Also, from the HSQC spectrum, it can be seen that the peaks for carbons in the aromatic ring were correctly assigned. The HMBC spectrum is used to correlate ^{1}H and ^{13}C peaks for atoms separated by multiple bonds (usually two or three). In aromatic rings, the most common correlations seen in HMBC spectra are three-bond correlations because they are typically 7–8 Hz, which is the value for which the experiment is optimized. In the aromatic ring of EE2 (Fig. 5a), (H1, C3), (H1, C5), (H2, C4), (H2, C10), (H4, C2) and (H4, C10) were the pairs of hydrogen and carbon atoms separated by three bonds, which correspond with the positions of contours in the HMBC spectrum (Fig. 8b). For compound I, contours were observed at (H1′, C3′), (H1′, C5′), (H2′, C4′) and (H4′, C2′), as expected. As the carbon peak at 127.8 ppm (■) was found to be correlated with H2′ and H4′, it must have been the peak for C10′. The carbon peak at 134.6 ppm (●) was for a carbon forming the C=C double bond without a hydrogen bond. As this carbon peak was correlated with H1′, it must have been the peak for C9′. Therefore, it can be concluded that the double bond was formed between C11′ and C9′, and compound I had the chemical structure shown in Fig. 5b.

Due to the double bond, compound I is believed to be more easily oxidized or hydrolyzed in the further treatment (e.g. activated sludge) than the parent compound. Although the product identification has not been reported for the other oxidation of EE2 including ozonation, photolysis, and photolysis combined with ozonation, compound I would be also the intermediate in those oxidation reactions.

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References

[1] H.M. Coleman, B.R. Eggins, J.A. Byme, F.L. Palmer, E. King, Photocatalytic degradation of 17-β-oestradiol on immobilized TiO_2, Appl. Catal. B: Environ. 24 (2000) L1–L5.
[2] R. White, S. Jobling, S.A. Hoare, J.P. Sumpter, M.G. Parker, Environmentally persistent alkylphenolic compounds are estrogenic, Endocrinology 135 (1994) 175–182.
[3] C. Desbrow, E.J. Routledge, G.C. Brighty, J.P. Sumpter, M.J. Waldock, Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening, Environ. Sci. Technol. 32 (1998) 1549–1558.
[4] S.A. Snyder, T.L. Keith, D.A. Verbrugge, E.M. Snyder, T.S. Gross, K. Kannan, J.P. Giesy, Analytical methods for detection of selected estrogenic compounds in aqueous mixtures, Environ. Sci. Technol. 33 (1999) 2814–2820.
[5] J.S. Vader, C.G. van Ginkel, F.M.G.M. Sperling, J. de Jong, W. de Boer, J.S. de Graaf, M. van der Most, P.G.W. Stokman, Degradation of ethinyl estradiol by nitrifying activated sludge, Chemosphere 41 (2000) 1239–1243.
[6] H. Tanaka, Y. Yakou, A. Takahashi, T. Higashitani, K. Komori, Comparison between estrogenicities estimated from DNA recombinant yeast assay and from chemical analyses of endocrine disruptors during sewage treatment, Water Sci. Technol. 43 (2001) 125–132.
[7] H.B. Lee, D. Liu, Degradation of 17β-estradiol and its metabolites by sewage bacteria, Water Air Soil Pollut. 134 (2002) 353–368.
[8] K. Fujii, S. Kikuchi, M. Satomi, N. Ushio-Sata, N. Morita, Degradation of 17β-estradiol by a Gram-negative bacterium isolated from activated sludge

in a sewage treatment plant in Tokyo, Japan, Appl. Environ. Microbiol. 68 (2002) 2057–2060.

[9] M. Nasu, M. Goto, H. Kato, Y. Oshima, H. Tanaka, Study on endocrine disrupting chemicals in waste water treatment plants, Water Sci. Technol. 43 (2001) 101–108.

[10] E.J. Rosenfeldt, K.G. Linden, Degradation of endocrine disrupting chemicals bisphenol A, ethinyl estradiol, and estradiol during UV photolysis and advanced oxidation processes, Environ. Sci. Technol. 38 (2004) 5476–5483.

[11] M.M. Huber, T.A. Ternes, U. von Gunten, Removal of estrogenic activity and formation of oxidation products during ozonation of 17α-ethinylestradiol, Environ. Sci. Technol. 38 (2004) 5177–5186.

[12] J. de Rudder, T. van de Wiele, W. Dhooge, F. Comhaire, W. Verstraete, Advanced water treatment with manganese oxide for the removal of 17α-ethinylestradiol (EE2), Water Res. 38 (2004) 184–192.

[13] B. Liu, F. Wu, N.S. Deng, UV-light induced photodegradation of 17α-ethinylestradiol in aqueous solutions, J. Hazard. Mater. B98 (2003) 311–316.

[14] X.L. Liu, F. Wu, N.S. Deng, Photodegradation of 17α-ethinylestradiol in aqueous solution exposed to a high-pressure mercury lamp (250 W), Environ. Pollut. 126 (2003) 393–398.

[15] H.M. Coleman, M.I. Abdullah, B.R. Eggins, F.L. Palmer, Photocatalytic degradation of 17β-oestradiol, oestriol and 17α-ethinylestradiol in water monitored using fluorescence spectroscopy, Appl. Catal. B: Environ. 55 (2005) 23–30.

[16] B.E. Segmuller, B.L. Armstrong, R. Dunphy, A.R. Oyler, Identification of autooxidation and photodegradation products of ethinylestradiol by on-line HPLC–NMR and HPLC–MS, J. Pharm. Biomed. Anal. 23 (2000) 927–937.

[17] H. Wariishi, L. Akaleswaran, M.H. Gold, Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: spectral characterization of oxidized states and the catalytic cycle, Biochemistry 27 (1988) 5365–5370.

[18] K. Moriyama, H. Matsufuji, M. Chino, M. Takeda, Identification and behavior of reaction products formed by chlorination of ethinylestradiol, Chemosphere 55 (2004) 839–847.