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Destruction of Estrogens Using Fe-TAML/Peroxide Catalysis

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Endocrine disrupting chemicals (EDCs) impair living organisms by interfering with hormonal processes controlling cellular development. Reduction of EDCs in water by an environmentally benign method is an important green chemistry goal. One EDC, 17α-ethinylestradiol (EE_2), the active ingredient in the birth control pill, is excreted by humans to produce a major source of artificial environmental estrogenicity, which is incompletely removed by current technologies used by municipal wastewater treatment plants (MWTPs). Natural estrogens found in animal waste from concentrated animal feeding operations (CAFOs) can also increase estrogenic activity of surface waters. An iron-tetraamidomacrocyclic ligand (Fe-TAML) activator in trace concentrations activates hydrogen peroxide and was shown to rapidly degrade these natural and synthetic reproductive hormones found in agricultural and municipal effluent streams. On the basis of liquid chromatography tandem mass spectrometry, apparent half-lives for 17 α- and 17 β-estradiol, estriol, estrone, and EE_2 in the presence of Fe-TAML and hydrogen peroxide were approximately 5 min and included a concomitant loss of estrogenic activity as established by E-Screen assay.

Introduction

The degradation of water-borne estrogens derived from animal excreta was described as long ago as 1976 (*1*). Typically, removal in MWTPs is greater than 90% for the major natural estrogen, 17β-estradiol (17β-E_2, Figure 1), and its more persistent degradation derivative, estrone (E_1, Figure 1) (*2, 3*). Although it has often been assumed that low pollutant concentrations in environmental waters are nonhazardous, EDC effects often follow nonmonotonic dose–response profiles with the low doses causing impairments (*4*). Developmental disruption by exogenous hormones from human activities is occurring. For example, fish are sensitive to estrogens in water even at parts per trillion (ppt) concentrations. Production of a female-specific egg protein (vitellogenin) by male fish exposed to parts per trillion concentrations of 17β-E_2 or EE_2 (Figure 1) has been well-documented for lake (*5*) and laboratory exposures (*6*). In addition, fish reproductive efficacy and gonadal development can be compromised by these same estrogen concentrations (*7–9*). In a report commissioned by the Environment Agency of England and Wales (*10*), predicted no effect concentrations (PNECs) were proposed of 1 ng L^{-1} for 17β-E_2 and 0.1 ng L^{-1} for EE_2, or 1 and 0.1 ppt, respectively. Effluents from conventional MWTPs often contain 17β-E_2 and EE_2 in excess of these minute PNECs (*11, 12*), signaling the need for more effective treatment processes for agricultural and municipal waste streams. To this end, the prototype iron-tetraamidomacrocyclic ligand (Fe-TAML) activator, or Fe-B* (*13*) was evaluated as the foundation of a new peroxide approach for destroying problematic water-borne hormones and estrogenic activity associated with estrogen degradation products (structures of hormones and Fe-B*, Figure 1*)*.

Experimental Section

Materials. Chemicals sources were as follows. Solvents were high-performance liquid chromatography grade from Fisher Scientific, Hampton, NH. Estrone, 17β-E_2, estriol (E_3), epiandrosterone, and testosterone were purchased from Sigma, St. Louis, MO. 17α-Estradiol, EE_2, and 5α-androstane-3,17-dione were purchased from Steraloids, Inc., Newport, RI. ^{14}C 17β-estradiol and testosterone at 0.2 uCi/mg were purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. Carbosorb E was purchased from Perkin-Elmer, Boston, MA. The sodium salt of Fe-B* was prepared by Fisher Scientific UK, Limited, Loughborough, England.

Reactions. Reactions were performed for each time point in triplicate using the following conditions: hormone (80 μM), Fe-B* (83 nM), with or without H_2O_2 (4 mM), potassium phosphate buffer (0.01 M, pH 8 or 10), ca. 22 °C, ca. 120 cycles min^{-1} in a shaker (incubation times indicated in Figure 2). Although the concentration of hormone chosen was higher than environmentally relevant, it was required because of detection limits for LCMS/MS and NMR analyses without the complication of sample extraction. One reaction per time point was used for testosterone, Fe-B* concentrations as shown. At the termination of reactions, catalase (60 times the concentration capable of destroying 4 mM H_2O_2 in 1 min) was added, followed by acidification of the samples (pH ca. 3.3; HCl) which inactivates Fe-B* through acid-induced demetalation (*14*).

LCMS/MS. Samples were analyzed by liquid chromatography tandem mass spectrometry (LCMS/MS) as described in the Supporting Information. The concentrations of compounds were calculated on the basis of concurrently run standards, summing peak areas for the ionized parent and two fragments (see the Supporting Information, Figure S4). Sixty minute reactions with and without H_2O_2 were also examined for degradation of the Fe-B* complex by MS-MS, as well as for other degradation products by MS, by scanning 100–500 m/z over the entire chromatographic run.

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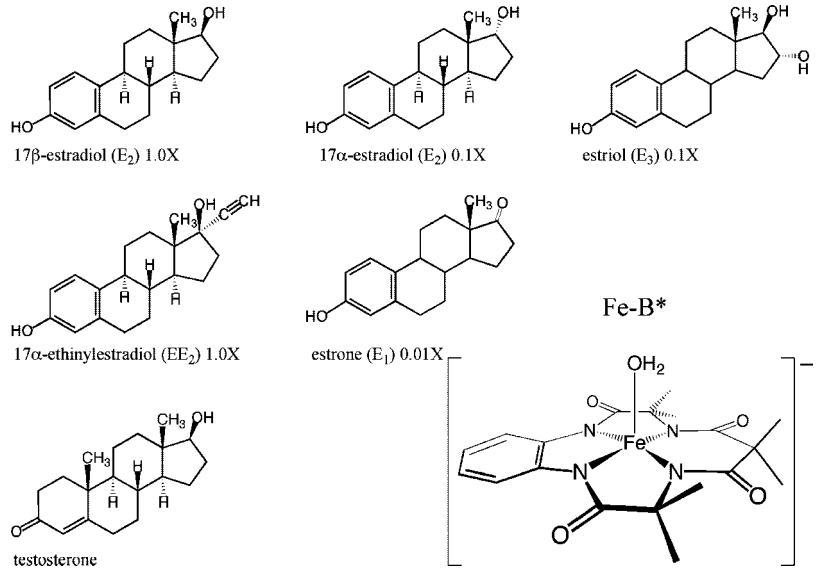

FIGURE 1. Chemical structures of Fe-TAML activator (Fe-B*) and steroid hormones with their relative estrogenic activity compared to 17β-E_2.[15]

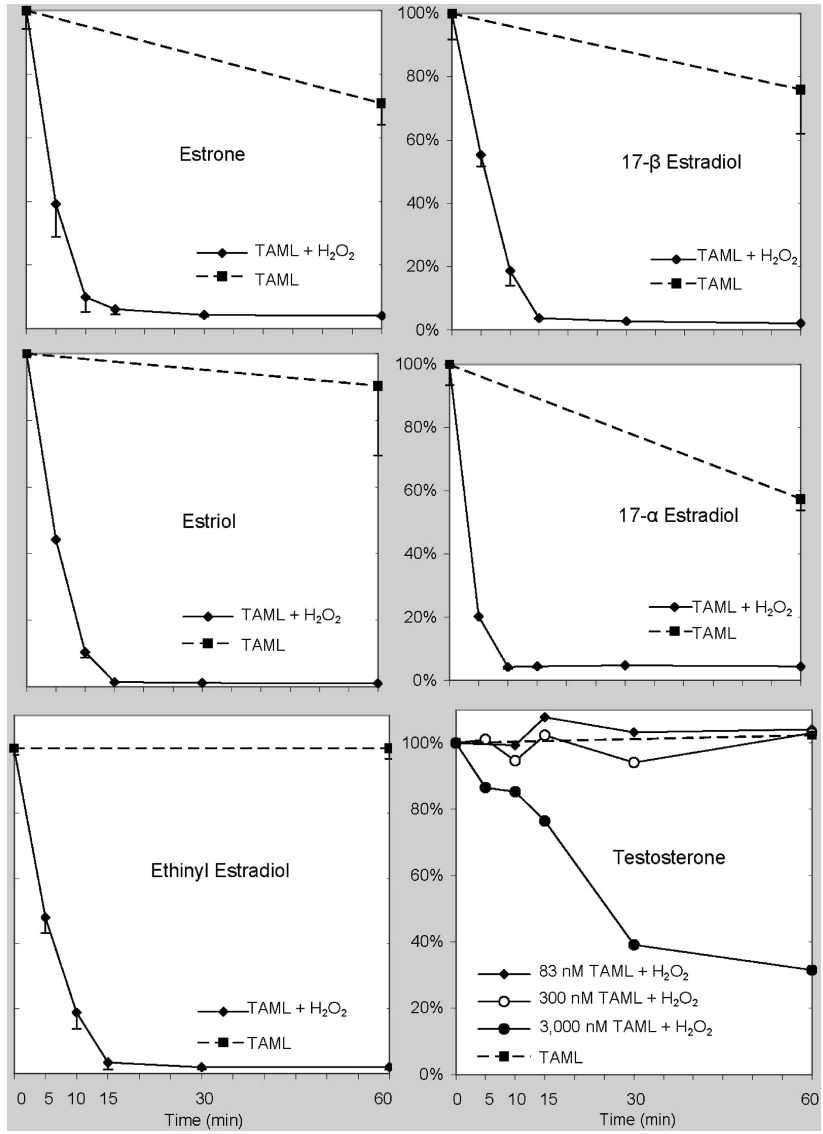

FIGURE 2. Degradation of steroids by Fe-B*/H_2O_2, analyzed by LCMS/MS. Nominal T0 concentration of steroids was 80 uM, and Fe-B* was 80 nM for all except testosterone, which varied as indicated. Mean percent of time 0 concentration, $n = 3$ reactions per time point for estrogens, $n = 1$ for testosterone.

TABLE 1. Rate Coefficients[a] for Estrogenic Compounds with Fe-TAML and H_2O_2 and Percent Degradation after 60 min

	estrone	estradiol 17α-	estradiol 17β-	estriol	ethinyl- estradiol
k' Fe-B*/H_2O_2 (min)	0.199	0.313	0.149	0.200	0.164
k_C Fe-B*/H_2O_2 (min^{-1})	0.0057	0.0099	0.0047	0.0020	n/a
s.e.	0.011	0.067	0.021	0.032	0.017
n	3	3	3	3	3
R^2	0.99	0.99	0.97−0.99	0.99	0.99
$t_{1/2}$ (min)[b]	3.5	2.2	4.7	3.4	4.2
% T_0 at T_{60} by LCMS/MS[c]	4% ± 0.4	4% ± 0.3	2% ± 0.2	1% ± 0.2	2 ± 0.7
vs E-Screen activity[d]	5% ± 3.8	7% ± 2.4	4% ± 1.2	2%[e]	7%[e]

[a] Mean, 3 reactions per time point, 80 μM estrogen concentration. [b] On the basis of the intrinsic decay coefficient (k). [c] Mean ± SD of triplicate experiments. [d] Mean ± SD of triplicate experiments, six wells per reaction, duplicate or triplicate reactions per time point, 80 μM estrogen concentration. [e] As percent degradation in duplicate E-Screen experiments differed by only 1% a third set of experiments was not performed for these compounds.

Initially, samples were tested in all buffer components separately by LCMS/MS. A problem arose in that the ionization potential changes depending on the environmental milieu. Ionization was greater in the presence of H_2O_2 than in the presence of buffer, and even less in the presence of Fe-B*−time 0 (MS infusion study, see the Supporting Information, Figures S2 and S3 and Table S1). To compensate for this, the compromise was made to use Fe-B* alone for T0.

Estrogenic Activity. Destruction of estrogenic activity was assessed by E-Screen assay as previously described (*15, 16*). This assay measures estrogen-dependent proliferation of a human mammary epithelial cell line in the presence of sample, relative to estradiol standards. Reactions were conducted as described above, for 0 and 60 min, then a series of dilutions were assayed by E-Screen to obtain data within the linear range for the test compound (1:15 000 to 1:24 million). Controls reactions (Fe-B* plus H_2O_2 diluted 1:15 000) were evaluated for estrogenic activity and toxicity.

NMR. Further confirmation of chemical degradation was provided by NMR analysis of 17β-E_2. One half of the tested reaction media was taken to dryness by vacuum centrifugation and was suspended in CD_3OD. (See the Supporting Information for details).

Results and Discussion

Chemical Evaluation, LCMS/MS. In initial testing, 80 μM 17β-E_2 and testosterone were incubated at pH 10 with Fe-B* (83, 300, and 3000 nM) and H_2O_2 (4 mM). Although the 3000 nM Fe-B* resulted in testosterone degradation (Figure 2), the rate of reaction for 17β-E_2 was so rapid that after just 5 min residual 17β-E_2 was detectable by LCMS/MS for only the 83 nM Fe-B* reaction (not shown). Treatment at pH 10 of ^{14}C-labeled E_2 and testosterone with Fe-B* (83 nM) and peroxide (4 mM) confirmed these results (see the Supporting Information, Figure S1).

Further reactions were conducted at pH 8, more closely reflecting the pH of most wastewater. Degradation of both natural and synthetic estrogens was rapid with Fe-B*/H_2O_2 (Figure 2), with rapid decay requiring both Fe-B* and H_2O_2. After 15 min, ∼ 95% of the original estrogens had been degraded. A background loss in the presence of buffer alone was found for the natural estrogens (not for EE_2) which was not significantly enhanced and sometimes reduced by addition of peroxide without Fe-B*. Therefore, hormone stability was always assessed by incubation (0 and 60 min) with buffer and Fe-B* without H_2O_2 (Figure 2). This instability was supported by MS infusion studies (see the Supporting Information, Table S1) where the total sum of fragments and parent ions of 17β-E_2 decreased by ∼50% after 60 min in the presence of water:acetonitrile; acetonitrile potassium phosphate buffer; Fe-B*; or H_2O_2. This was consistent with E-Screen results for both 17β- and α-E_2 incubated in the same manner. The differential stability of the hormones is indicated by the T60 differences.

Assuming first-order decay kinetics, time-dependent estrogen concentrations in a batch reactor can be expressed as

$$\frac{C}{C_0} = \exp\{-(k + k_C)t\} = \exp\{-k' t\} \quad (1)$$

where k is the intrinsic decay coefficient of estrogen with Fe-B* and H_2O_2 (min^{-1}); k_C is the background decay coefficient of estrogen in the absence of H_2O_2 (min^{-1}), $k_C = (C_{60}/C_0)/60$; k' is the apparent decay coefficient of estrogen with Fe-B* and H_2O_2 (min^{-1}), $= k + k_C$; C_0 is the initial estrogen concentration (M); C_{60} is the [estrogen] remaining after 60 min with Fe-B* only (M); C is the time-dependent estrogen concentration with Fe-B* + H_2O_2 (M); and t is time (min).

All estrogens dissipated exponentially (eq 1; see the Supporting Information, Figure S6). The values of the intrinsic decay coefficients (k) were determined by nonlinear multiparameter regression of the data and are reported along with k_C, R^2, and $t_{½}$ in Table 1. Although an appreciable amount of background decay was observed for all but the synthetic estrogen EE_2, the decay rate was increased 32−100 times in the presence of both H_2O_2 and Fe-B*. The half-lives for all estrogens are less than 5 min, determined on the basis of the intrinsic decay coefficients.

The LCMS chromatograms from T60 reaction samples with and without H_2O_2 were evaluated for breakdown products. No major peaks were observed with the exception of the demetalated activator and/or residual parent estrogen (Supporting Information, Figure S5). Because ortho-phenylenediamine could potentially be formed by degradation of the Fe-B* activator, chromatograms were processed for this known carcinogen and for two oxidative breakdown products, $C_6H_4(NH_2)(NO_2)$ and $C_6H_4(NO_2)_2$. Although compounds may have been formed that were neither detected (ionization state dependent) nor eluted under the employed chromatographic conditions, none of these breakdown species were detected in any estrogen degradation reaction.

Loss of Estrogenic Activity. E-Screen results (Table 1) provided support for the extensive estrogen degradation indicated by the LCMS/MS technique. After treatment with Fe-B*/H_2O_2 (60 min), estrogenic activity was reduced by approximately 95% for all estrogens examined. This confirmed that most of the parent compounds were destroyed and that any breakdown products had little to no estrogenic activity. No toxicity of either Fe-B*, Fe-B*/H_2O_2, or products formed from the catalytic treatments was found at the concentrations used in cellular incubations (1: ≥15 000 dilution).

NMR Confirmation. NMR studies further substantiated these degradation results. ^1H NMR examination of 17β-E_2

samples treated with Fe-B*, H_2O_2-alone, and FeB*/H_2O_2 (60 min) revealed that both Fe-B* and peroxide are required for loss of the signals for aromatic (~7.1 and ~6.5 ppm), methylene (~2.8 to 1.0 ppm) and methyl protons (0.7 ppm).

Alternative Degradation Techniques. Various techniques such as photolysis, ozonation, chlorination, and microbial degradation have shown some degree of estrogen destruction; however, none of these techniques are able to destroy estrogens as effectively as Fe-B*/ H_2O_2. The fluorescent signal of EE_2 is reduced by 80% after 1 h exposure to UV light (254 nm) (*17*) and the photodegradation of E_1 and 17β-E_2 has been reported (*18*). It is clear from our LCMS/MS data and those of others (*19*), that significant degradation of estradiols can occur without "treatment". Rapid degradation of 17β-E_2 and EE_2 was reported by ozonation in a time frame similar to that required by Fe-B*/H_2O_2 (*20, 21*), but ozone treatment is expensive. Although much cheaper, chlorination is less effective than ozonation (*20*) and complicated by toxic as well as estrogenic byproducts. Chlorine treatment required at least two hours to remove estrogenic activity from 17β-E_2 solutions, while activity in EE_2 solutions was still elevated after 24 h (assessed by E-Screen) (*20*). Moreover, estrogenic activity was actually found to increase during the chlorination process for both 17β-E_2 and E_2 (*20*). Other researchers found chlorination yielded limited degradation of estradiol in 10 min assessed by LCMS/MS, Yeast Two-Hybrid assay for estrogenic activity, and estrogen receptor (human α and β) Binding Assay (*22*). In addition to limited degradation, chlorination was found to form estrogenic byproducts from bisphenol A (BPA, a major xenoestrogen) and estrogens (*22–24*). Chlorine treatment resulted in chlorinated E_1, E_2, and EE_2 that were 1×10^3 to 1×10^4 more estrogenic than BPA as assessed by yeast two-hybrid assay, with the relative potency depending on the site of chlorination and the source of estrogen receptor (human or fish; medaka). Bacterial degradation of estrogens has been documented (*25*) and recent research has focused on identifying estrogen-degrading strains (*26*). Although microbes efficiently degrade estradiol to estrone, the latter is more persistent (*26*). However, some bacterial strains isolated from activated sludge were found to be capable of degrading estrone (*27*) and might prove useful as probiotics in waste digesters, but they would first need to be evaluated for strain safety, including assessment of toxicity and toxic product formation.

Iron TAML/H_2O_2's exceptional efficacy in degrading estrogens has been demonstrated for the commonly reported 17β-E_2 and estrone, as well as 17α-E_2, typically associated with dairy cattle (*28*), estriol (an estrogenic estradiol degradation product, Figure 1), and the birth control pill's EE_2, which often eludes complete destruction in MWTPs. The environmental persistence of EE_2 and estrone and their resistance to bacterial degradation indicate a particular usefulness for the Fe-TAML approach, since neither are resistant to Fe-B*/H_2O_2. The greatest potential probably lies not in Fe-B*/H_2O_2's use on raw untreated wastewater, but instead in a later step, such as on secondary or tertiary effluents, where most of the organic material has already been removed. If Fe-B* was to be so-employed at 80 nM, a concentration demonstrated here to be effective in rapidly eliminating 1000-fold estrogens, then 1 kg of Na[Fe-B*] would be sufficient to treat more than 20 000 tons of water. In a preliminary experiment (data not shown), incubations with a municipal wastewater sample (obtained prior to chlorination) resulted in >99% removal of estradiol as assessed by ELISA. Current analytical methods required the use of higher concentration of estrogens for assessment by LCMS/MS and NMR, but the highly sensitve E-Screen would be appropriate for future research optimizing conditions using actual wastewater effluents and environmentally relevant concentrations of estrogens. Optimization for industrial removal of

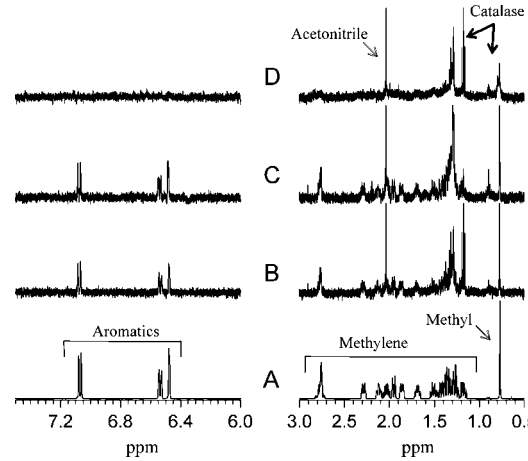

FIGURE 3. ^{1}H NMR spectra of 17β-E_2 reaction mixtures in CD_3OD. (A) Pure 17β-E_2 (no buffer). (B) 17β-E_2 + Fe-B*. (C) 17β-E_2 + H_2O_2 (60 min). (D) 17β-E_2 + Fe-B* + H_2O_2 (60 min). Assignments based on Fujii et al. (*30*).

estrogens by MWTP treatment may result in use of lower concentrations of H_2O_2 (currently used to remove odor-causing hydrogen sulfide), as estrogen concentrations would be lower than used here. The need for such MWTP effluent treatments could increase with use of the "no period pill", which contains the same dose of EE_2 found in traditional birth control pills, but requires a 25% increase in duration of dosing (28 of 28 days vs 21 of 28 days). The target population for this product could also prove to be larger. On the farm, flushing of barns with agricultural "gray" water (recirculated from lagoons) may result in EDC-induced reproductive problems in livestock from exposure to estrogenic hormones in the flush; Fe-B*/H_2O_2's use could provide a simple remedy. The capacity of Fe-TAML/peroxide to destroy bacterial spores, the hardiest of microbes (*29*), as well as numerous other chemicals that elude our current wastewater treatment systems, indicates the potential for multipurpose applications in wastewater treatment.

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Supporting Information Available

Details pertaining to methods, radiolabeled estradiol and testosterone experiments, Fe-B* LCMSMS, and kinetic models (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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