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Using Electronic Theory To Identify Metabolites Present in 17α-Ethinylestradiol Biotransformation Pathways

William J. Barr,[†] Taewoo Yi,[§] Diana Aga,[‡] Orlando Acevedo,[∥] and Willie F. Harper, Jr.*[,†]

[†]Department of Civil and Environmental Engineering, Swanson School of Engineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, United States

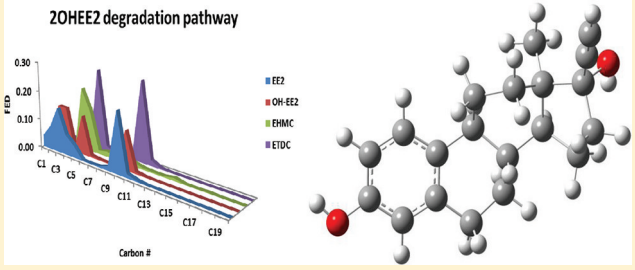
[§]Department of Environmental Science and Engineering, Ewha Womans University, Seoul, 120-750 Korea

[‡]Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, New York 14260, United States

[∥]Department of Chemistry and Biochemistry, Auburn University, Auburn, Alabama 36849, United States

Supporting Information

ABSTRACT: This research used electronic theory to model the biotransformation of 17α-ethinylestradiol (EE_2) under aerobic conditions in mixed culture. The methodology involved determining the Frontier Electron Density (FED) for EE_2 and various metabolites, as well as invoking well-established degradation rules to predict transformation pathways. We show that measured EE_2 metabolites are in good agreement with what is expected based on FED-based modeling. Initiating reactions occur at Ring A, producing metabolites that have been experimentally detected. When OH-EE_2 and 6AH-EE_2 are transformed, Ring A is cleaved before Ring B. The metabolites involved in these pathways have different estrogenic potentials, as implied by our analysis of the log P values and the hydrogen bonding characteristics. The OH-EE_2 and 6AH-EE_2 transformation pathways also show redox-induced electron rearrangement (RIER), where oxidation reactions lead to the reduction of carbon units present along the bond axis. Sulfo-EE_2 appears to be difficult to biotransform. These findings clarify theoretical and practical aspects of EE_2 biotransformation.



INTRODUCTION

The presence of 17α-ethinylestradiol (EE_2) in the aquatic environment continues to be a topic of considerable interest to the water quality community. It is an anthropogenic pollutant that is present in rivers, lakes, and groundwaters,[1−3] and it induces developmental anomalies in wildlife (such as feminized male fish).[4−7] Negative ecological impacts may occur at very low concentrations (i.e., ppb or ppt). EE_2 is primarily introduced into the aquatic environment via domestic wastewater, so sewage treatment processes are critical for eliminating EE_2 from the water cycle. Learning more about the removal of EE_2 is an important priority; we need to discover the transformation pathways, and we also need to learn how to minimize or remove the toxicity of the resulting byproducts.

It is now clear that EE_2 can be removed during the activated sludge process. Numerous studies have biologically degraded EE_2 under aerobic conditions,[8−10]and a number of studies have carried out these studies in the concentration range (i.e., low ug/L or ng/L) expected in real wastewater.[11,12] Some studies have detected metabolites using tools like NMR[8] or LC/MS/MS,[13] but most of the previous research has reported EE_2 removal without reconciling its ultimate fate or identifying byproducts. Recent work has shown that EE_2 is partially mineralized (i.e., converted to carbon dioxide) during aerobic treatment of sewage. For example Yi et al., 2011[14] degraded EE_2 in fed-batch bioreactors and measured 40−60% conversion to carbon dioxide. Khunjar et al., 2011[13] degraded EE_2 in aerobic chemostats, and they measured 13% and 26% EE_2 conversion to carbon dioxide. Both of these reports show that it is possible to mineralize EE_2 and its byproducts from water, but, in each case, a significant fraction of ^{14}C-EE_2 remained in the wastewater either in the aqueous phase or associated with suspended solids. EE_2 can be removed during the activated sludge process, but there are lingering concerns related to the byproducts. This is, therefore, an appropriate moment to discover important components of the biotransformation pathways.

Ring cleavage is a key event in the EE_2 transformation pathway because, without rings, the metabolites are easier to assimilate[15] and unlikely to bind to estrogen receptors.[16] Understanding EE_2 ring cleavage would allow us to better understand the intermediates that may be present in wastewater effluents (including those that are difficult to detect analytically). The current metabolite data set has also lead to some apparently conflicting ideas about EE_2 ring cleavage. For example, Yi and Harper, 2007[8] and Khunjar et al., 2011[13] both

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reported metabolites that show that ring A is the first to be cleaved during biotransformation. Their results are in conflict with those of Haiyan et al., 2007,[17] who used Sphingobacterium sp. JCR5 to degrade EE_2, and, based on the daughter products they detected, they proposed that EE_2 is initially oxidized to estrone, followed by ring B (not ring A) cleavage. Collecting more metabolite data and modeling biotransformation reactions can address and resolve questions related to ring cleavage.

Identifying byproducts will also address concerns related to estrogenicity, which in turn, is influenced by chemical structures. For example, Fang et al., 2001[16] analyzed 230 natural and synthetic steroids (with and without phenolic rings), and they found that the number of hydrogen bond donating groups (n_d) correlated negatively with estrogenicity. They also found that the octanol−water partitioning coefficient (log P) was positively correlated with estrogenicity, because compounds with relatively low log P values were more soluble and less likely to interact with hormone receptors. Lipinski et al., 2001[18]found similar results for their analysis of approximately 2500 organic compounds. Schultz et al., 2001[19] developed structure−activity relationships for 120 aromatic compounds, and they found that n_d correlated well with estrogenicity. They found that the number of hydrogen bond accepting groups (n_a) was negatively correlated with estrogenicity. They also found that the hydrophobicity of rings B, C, and D (but not A) was positively correlated with estrogenicity. These parameters (log P, n_d, n_a) can be determined from the chemical structures of EE_2 and its metabolites. Therefore, it is possible to assess the estrogenic potential associated with compounds involved in biotransformation pathways.

The current work aims to apply frontier electron density (FED) theory to explore EE_2 biotransformation. FED calculations can elucidate the fundamental principles governing EE_2 reactivity by predicting which positions on the molecule will most likely undergo electrophilic attack. Of particular importance is the localization of the highest occupied molecular orbital (HOMO), as electrons occupying this frontier orbital are most free to participate in the initiating reactions. The general concept is that an electron-poor molecule will readily attack a position of large electron density. Fukui developed the powerful FED model for describing chemical reactivity via frontier molecular orbital (FMO) theory and pioneered much of the early work connecting FED to chemical reactivity in aromatic hydrocarbons.[20]Wheland and Pauling, 1935[21] successfully used FED to explain the reactivity of substituted aromatics. More recently, Ohko et al., 2002[22] using FED to explain the initiating reactions associated with the photocatalysis of 17β-estradiol, and Ohura et al., 2005[23] showed that air-borne polycyclic aromatic hydrocarbons were abiotically chlorinated in positions that corresponded to high FED. Lee et al., 2001[24] used Fenton oxidation to remove polycyclic aromatic hydrocarbons, and they successfully used FED to predict the order of daughter product production. Although these previous attempts focused on abiotic reactions, they bolster the potential for predicting biological oxidations in the same way. Prior efforts to conduct *a priori* predictions of biodegradation have been very successful when focusing on readily degradable substrates (e.g., glucose) that enter well-characterized metabolic pathways (e.g., glycolysis). FED-based techniques present the promise of predicting biodegradation on complex organics like EE_2; a contribution here can eventually make a significant impact. The specific objective of this work is to compare measured EE_2 metabolites to those predicted by FED-based theory. We intended to gain theoretical and practical insights into EE_2 biotransformation steps as well as the nature of the metabolites that are generated.

■ EXPERIMENTAL SECTION

Overall Approach. We simulated the transformation of EE_2 using FED-based modeling, which consisted of calculating the FED of all of the carbon units and then simulating transformation according to degradation rules. These simulations assume that a wide range of nonspecific enzymes (e.g., oxygenases) are active. We then compared the computational results to the identity of the measured metabolites reported in literature (including those reported from our lab). We also analyzed the results of ^{14}C-EE_2 experiments done in Dr. Willie Harper's lab and used these chromatograms to propose additional metabolites (including a few that had not been previously reported). We used the chemical structures to evaluate estrogenic potential; this is related to (but not the same as) estrogenicity, which also depends on the regulation of complex endocrine pathways.[25] Finally, we restricted the scope of this work to include EE_{z2} transformation steps leading up to (or immediately following) the first ring cleavage. This limitation confined the study to the range where the majority of measured metabolites are located.

^{14}C-EE_2 Experiments and Metabolite Identification. These studies were conducted with ^{14}C-labeled ethinylestradiol (^{14}C-EE_2) (99% pure; American Radiolabeled Chemicals, St. Louis, MO) at room temperature. Biomass was retrieved from parent bioreactors and used to seed two 500 mL fed-batch bioreactors (FBBRs) (i.e., the bioreactors were fed continuously with substrate, but reactor volume was discharged only during sampling periods). At the beginning of the experiment, the FBBRs were each spiked with ^{14}C-EE_2 at an initial concentration of 24.5 μg/L. Aqueous samples were retrieved at three time points (1 h, 24 h, 48 h) and then subsequently delivered to Dr. Diana Aga's laboratory for metabolite identification. The performance of these FBBRs has been discussed previously.[14] The water samples retrieved from the ^{14}C experiments were analyzed by liquid chromatography/mass spectrometry (LC/MS) and LC/radiochromatographic detection as described previously.[14] Since all samples contained ^{14}C-labeled EE_2, the analysis was performed using an Agilent 1100 HPLC equipped with an online radiochromatographic detector (IN/US Systems, Inc., Tampa, FL) as described previously.[26] After determining the retention times of the radioactive peaks, we reinjected an aliquot of sample into the LC column with the eluate being split between the radioactive detector and a triple quadrupole mass spectrometer (Agilent 6410 MSD); the splitter was put in place to ensure that the LC/MS data corresponded with the radioactive peaks. We used LC/MS in conjunction with a radioactive detector to determine the m/z ratios, which were the basis for proposed metabolite structures.

FED Analysis. Frontier electron density (FED) analyses were performed to determine the electron density profile for EE_2 and for relevant metabolites. The Unrestricted Hartree−Fock (UHF) method and STO-3G basis set were employed for initial structure optimizations using the program Gaussian 03.[27] UHF/6-31G(d) calculations were used for final geometry optimizations, computing vibrational frequencies, and in calculating the electron density of each compound. The FED for all carbon atoms were computed using the following

equation

$$fr = [2^* \sum_i (\text{Cri HOMO})^2] \quad (1)$$

For an electrophilic reaction, the highest occupied molecular orbital (HOMO) densities are normalized by the energy of the frontier molecular orbitals at ground state. The coefficient of each atomic orbital, Cri, is used to produce the frontier movement, where r is the number of carbon atoms in i: 2s, 2px, 2py, and 2pz orbitals.[24] In general, the highest *f*r value indicates the most reactive position. We also determined geometries, energies, and FEDs of carbon atoms for all compounds using density functional theory (DFT) at the B3LYP/6-31G(d) theory level[28,29]for comparison. All simulations were performed on computers located at the Pittsburgh Supercomputer Center. FED values for EE_2-related carbons were typically between 0 and 0.3.

Degradation Rules. FED values were used to determine the location of the most reactive part in the model compounds. To determine what happens at the reactive position, we invoke six *degradation rules* relevant for biologically mediated electrophilic reactions: [30−39]

Rule 1 − The enzyme attacks the carbon atom at the highest FED. The carbon atom being oxidized must be bound to a −H, =O, or −OH group.

Rule 2 − The phenol ring is cleaved after being oxidized to catechol. Oxygenolytic cleavage of the phenol ring occurs via *ortho*- or *meta*-cleavage. Ring cleavage takes place between the hydroxylated carbon with highest FED value and carbon with higher FED out of two adjacent carbons.

Rule 3 − The cyclohexane and cyclopentane rings are opened after oxidation to cyclohexanone and cyclopentanone, respectively. Ring cleavage of either cylcohexanone or cyclopentanone is determined by the same rule with phenol ring cleavage.

Rule 4 − After ring cleavage, carbon chains are degraded to hydroxyl-, ketone, and carboxylic acid, followed by a decarboxylation step.

Rule 5 − Resonance can cause the phenol ring to be converted to a semiquinone tautomer, which can be oxidized according to degradation rules 1−4.

Rule 6 − If the degradation rules are not applicable to rules 1 through 4, enzymatic attack proceeds at the carbon atom with the second highest FED value.

Estrogenicity. We examined estrogenic characteristics by calculating the log P and the number of hydrogen bond donors and acceptors. We calculated both of these parameters using the atom-based additive approach.[40]

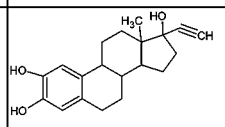
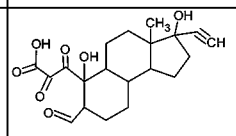
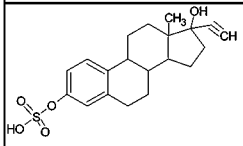
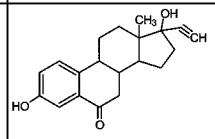
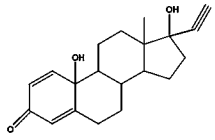
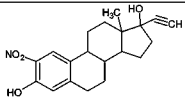
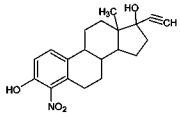
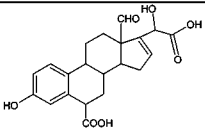
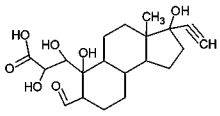
■ RESULTS

Proposed structures for the metabolites detected from the ^{14}C experiments are shown in Table 1. Two of the quickly produced (i.e., after 1 h of reaction time) metabolites are M312 and M376 (corresponding to *m*/*z* 311 and *m*/*z* 375 peaks in negative ESI MS), and their proposed identities are OH-EE_2 and SO_4-EE_2 (Sulfo-EE_2) respectively. These structures suggest hydroxyl- and sulfo-transfer initiating reactions that can be carried out by a wide range of common oxygenase and sulfotransferase enzyme.[15,41] The identities of these initial metabolites are supported by Yi and Harper, 2007,[8] who detected both of them from a nitrifying membrane bioreactor using thin layer chromatography and NMR. The metabolite M310 (*m*/*z* 309) is EDMO, a byproduct that shows the presence of a ketone group on ring B. M385 is DOEF, which shows that the ethinyl group has been converted to a carboxylate group, and that a carboxylation reaction takes place at ring B. The proposed structure for M314 (*m*/*z* 313) is 6AH-EE_2; this metabolite has been measured by Della-Greca et al., 2008,[42]and its formation is not surprising because the C10 carbon unit has high frontier electron density (C10 = 0.38) and it is therefore an attractive location for electrophilic modification. Finally, M341 (*m*/*z* 340) is either 2 Nitro-EE_2 or 4 Nitro-EE_2; both formed by way of an abiotic nitration reaction.[11]This collection of metabolites is largely consistent with what has been detected previously from nitrifying mixed cultures.[8,13,26]

We now turn our attention to FED-based prediction of EE_2 metabolism. Figure 1 shows EE_2 and three initial metabolites. The chemical convention used here denotes rings A through D as shown in the parent structure in the upper left-hand corner of Figure 1. Ring A of EE_2 contains several high FED C units (1−5 and 10), making it the most attractive location for electrophilic modification. The reactions reflected by the structure of these initial metabolites are consistent with ring A modifications. OH-EE_2, detected in the current study and in two previous reports, is hydroxylated at carbon unit 2 (C_2 FED = 0.1), while both Sulfo-EE_2 and EHMD are modified at C3 (FED = 0.16). These initiating reactions support the idea of using FED theory to explore biologically mediated initiating reactions, and we also extract two additional points from Figure 1. First, EE_2 C10 has the highest FED value, but initial hydroxylations are unlikely to occur at this site because C10 is not bound to an −OH, =O, or −H group (see Rule 1). Second, the two levels of theory both point to the same carbon units as likely reaction sites. This is an important insight. UHF theory is among the most common methods for determining FED. The disadvantage of this method is that it uses a crude central field approximation to account for electron−electron interactions, rendering it more inaccurate. Density functional theory accounts for electron−electron interactions more rigorously. Our results show that UHF can provide useful information related to EE_2 initiating reactions.

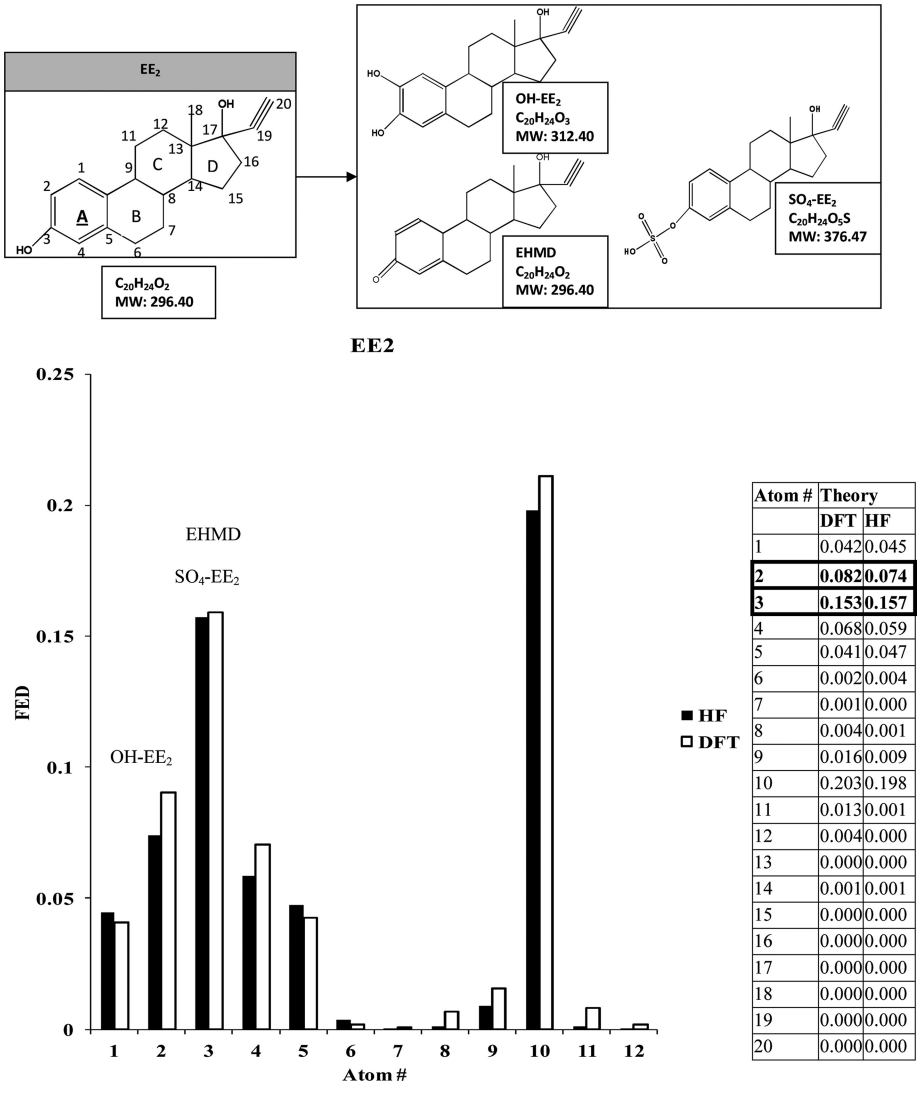
The initial metabolites can be further degraded to other byproducts. Figure 2 shows a metabolic pathway from EE_2 to ETDC. Ring A cleavage occurs between C2 and C3 because of oxygenolytic activity typically carried out by dioxygenases,[43] and this ring cleavage step causes a dramatic extraction of electrons, decreasing the FED of C2 (0.15 to 0.005), C3 (0.14 to >0.001), and C10 (0.13 to 0.013). There is also an interesting increase in the FED of C4 (<0.001 to 0.1), consistent with the idea that removing electrons can cause the electron density along the C−C bond to increase. This is computationally possible because the nucleophilic Fukui function (the mathematical underpinning of FED-based calculations) can take on negative values (in contrast to the basis of tradition, simple frontier molecular orbital theory). The nucleophilic Fukui function is a key indicator of redox-induced electron rearrangement (RIER), where oxidation of a particular molecule can lead to the reduction of a specific region of the molecule (usually along the bond axis, between the carbon atoms). Melin et al., 2007[44] documented similar observations while studying the oxidation of substituted acetylenes. Overall, these results make three important points. First, they show an example of a synthetically generated degradation pathway that predicted two measured metabolites (OH-EE_2, ETDC). Second, they demonstrate that

Table 1. Metabolites Identified in Current Study

m/z	Proposed structure	IUPAC name	Abbreviation	Analytical conformation	Reported by others
311		17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-2,3,17-triol	OH-EE_2	dansyl chloride derivatization - Positive	Yi and Harper, 2007; Skotnicka-Pitak *et. al.*, 2009
375		3-(3-ethynyl-7-formyl-3,6-dihydroxy-3a-methyl-1,2,4,5,5a,7,8,9,9a,9b-decahydrocyclopenta[a]naphthalen-6-yl)-2,3-dioxo-propanoic acid	O-DNDPA	dansyl chloride derivatization - Negative	NO
		(17-ethynyl-17-hydroxy-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthren-3-yl) hydrogen sulfate	Sulfate-EE_2	NO	Yi and Harper, 2007
309		17-ethynyl-3,17-dihydroxy-13-methyl-7,8,9,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-6-one	OPO	dansyl chloride derivatization - Positive	NO
313		17-ethynyl-10,17-dihydroxy-13-methyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-3-one	6AH-EE_2	No	Della-Greca et. al. 2008
340		17-ethynyl-13-methyl-2-nitro-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol	2 Nitro-EE_2	dansyl chloride derivatization - Positive	Skotnicka-Pitak *et. al.*, 2009: Gaulke *et. al.*, 2008
		17-ethynyl-13-methyl-4-nitro-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol	4 Nitro-EE_2	dansyl chloride derivatization - Positive	Skotnicka-Pitak *et. al.*, 2009; Gaulke *et. al.*, 2008
385		17-(1,2-dihydroxy-2-oxo-ethyl)-13-formyl-3-hydroxy-6,7,8,9,11,12,14,15-octahydrocyclopenta[a]phenanthrene-6-carboxylic acid	OPCA	dansyl chloride derivatization - Positive	Skotnicka-Pitak *et. al.*, 2009
379		3-(3-ethynyl-7-formyl-3,6-dihydroxy-3a-methyl-1,2,4,5,5a,7,8,9,9a,9b-decahydrocyclopenta[a]naphthalen-6-yl)-2,3-dihydroxy-propanoic acid	DNDPA	dansyl chloride derivatization - Positive	NO
293	Unknown	Unknown	Unk.	dansyl chloride derivatization - Positive	NO

ring A is cleaved before ring B, and third, these results support RIER, which allows the Fukui function to be negative and the FED of nearby carbon units to increase during oxidation reactions.

Figure 1S also shows the metabolic pathway from EE_2 to EDMC via 6AH-EE_2. The first step is a tautomerization at C3 (FED = 0.153), which has one of the highest FED values on the parent compound. The second step is a hydroxylation at C10, resulting in a metabolite (6AH-EE_2) that was generated by an enriched Sphingomonasculture.[42] During this step, the FED decreased in each carbon unit, except for C5, which more than doubled (0.018 to 0.037). Ring A is cleaved between C2 and



Atom #	Theory	
	DFT	HF
1	0.042	0.045
2	**0.082**	**0.074**
3	**0.153**	**0.157**
4	0.068	0.059
5	0.041	0.047
6	0.002	0.004
7	0.001	0.000
8	0.004	0.001
9	0.016	0.009
10	0.203	0.198
11	0.013	0.001
12	0.004	0.000
13	0.000	0.000
14	0.001	0.001
15	0.000	0.000
16	0.000	0.000
17	0.000	0.000
18	0.000	0.000
19	0.000	0.000
20	0.000	0.000

Figure 1. FED profile for EE_2. Three initial metabolites are shown at the corresponding reaction sites.

C3 after oxidation at C2, and then the degradation of the carboxylic groups in CEDM accounts for the production of the last metabolite shown in this pathway. This pathway supports the notation that ring A is cleaved before ring B, and it provides another example of RIER.

The third initial metabolite of interest in this study is Sulfo-EE_2 (Figure 2S). This metabolite has been identified in the current study and in two previous studies that transformed EE_2 using mixed cultures of activated sludge. The FED profile of Sulfo-EE_2 shows that the highest FED value resides at C10 (0.2). Hydroxylation of C10 is possible in principle, but the ensuing transformation at ring A cannot be theoretically predicted at this time because there are no established biological degradation rules that account for the presence of the sulfate group at C3. Further, redox reactions involving the sulfur unit are precluded from the current approach because of its low FED value (<0.01). In situ, there are sulfotransferase and sulfatase enzymes that can, in principle, transform Sulfo-EE_2 (or related structures). However, these activities are not predicted using FED-based theory. It is also interesting to note that Sulfo-EE_2 appears to be more recalcitrant than OH-EE_2 or 6AH-EE_2. Khunjar et al., 2011[13] recently found that Sulfo-EE_2 produced by a nitrifying culture was not degraded further by a heterotrophic culture that was placed in series. Their findings imply that Sulfo-EE_2 may be more difficult to degrade. Our study corroborates their observations, because the degradation of Sulfo-EE_2 is not elucidated well by known degradation rules or by FED-based theory.

We now examine estrogenic potential using the number of hydrogen bond donating and accepting groups and the log P. Figure 3S shows these values for EE_2 and the initial metabolites. EHMD has lower estrogenic potential than EE_2 because the n_a is greater (3 vs 2), the n_d is unchanged, and the log P is smaller than that of EE_2 (2 vs 3.7). Sulfo-EE_2 also appears to have lower estrogenic potential than EE_2 for similar reasons. OH-EE_2 has a lower log P (3.4 vs 3.7) and higher n_a (3 vs 2) compared to EE_2, but it also has an additional hydrogen bond donating group, a fact that may counterbalance the changes in log P and n_a. Thus, in this case the relative estrogenic potential is not as clear; however, we hypothesize that OH-EE_2 has less *estrogenicity* than EE_2 because previous work has shown that 2OH-E_2 (not 2OH-EE_2) is less estrogenic than E_2.[45] If hydroxylation at C2 reduces estrogenicity for E_2, it seems reasonable to expect the same for EE_2.

Estrogenic potential changes during the course of the transformation pathways. For example, during the EE_2-to-EDMC pathway (Figure 4S), there are clear indications that estrogenic

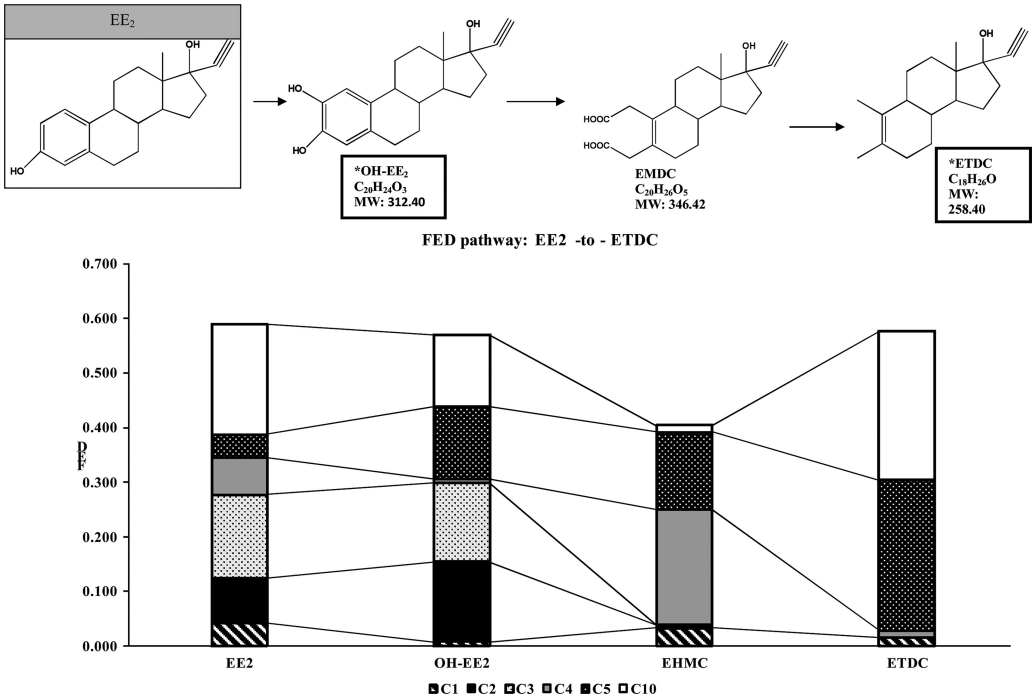


Figure 2. Predicted pathway for the degradation of EE_2 to ETDC. Measured metabolites are indicated with a bold highlight, and metabolites identified as part of this study are marked by an asterisk. Note the dramatic increase at carbon 4 as ring cleavage occurs (2OH to EHMC).

potential decreases during the steps leading to ring cleavage; the log P decreases and the n_a increases. The last compound in the pathway (EDMC) is without the active phenolic ring and is therefore likely to have lower estrogenic potential. There are, however, two predicted metabolites (i.e., ETMD and CEDM) that have a higher n_d (3 and 4 respectively) than EE_2. These two compounds should probably be tested for estrogenicity in future efforts. During the EE_2-ETDC pathway, there are also indications that estrogenic potential is reduced (Figure 5S). OH-EE_2 (as mentioned earlier) is likely less estrogenic than EE_2, and EMDC has less estrogenic potential than OH-EE_2 (or EE_2) because it has lower log P and higher n_a. The last compound in this pathway (ETDC) has lost the active ring and likely has lower estrogenic potential than EE_2. Finally, we hypothesize that Sulfo-EE_2 has less estrogenic potential than EE_2 because Sulfo-EE_2 has a lower log P than EE_2 (i.e., 3.0 < 3.7, Figure 6S) and Sulfo-EE_2 has a higher n_a (5 > 2, Figure 6S).[42,43] Sulfate conjugation does not change n_d.

■ DISCUSSION

Theory. These results show that FED-based calculations can be useful for understanding the transformation of EE_2. It is interesting to observe that UHF-based FED solutions provide guidance with respect to where electrophilic initiating reactions take place, despite the fact that it does not rigorously account for electron−electron interactions. The reason is because UHF provides a broad accounting for electron−electron interactions using the central field approximation. This approach allows electron−electron interactions to be accounted for in a way that is independent of angular coordinates (i.e., the precise location of an electron associated with a particular orbital). The HF solutions to the atomic wave function contain an electron−electron interaction "correction" imbedded into the result, which leads to numerical trends that are largely in line with DFT. UHF is accurate enough for those interested in the initiating reactions associated with EE_2.

Table 1 included four biologically produced structures that do not appear in the transformation pathways presented in this manuscript. This may reflect a need to investigate alternative degradation rules, or it may point to limitations associated with FED-based predictions. For example, the structures of DNDPA (m/z = 379) and O−DNDPA (m/z = 375) show hydroxyl groups and a double bond at C4. It is possible that these structures could appear in a pathway similar to the 6AH-EE_2 pathway shown in this paper. Specifically, if 6AH-EE_2 is oxygenated at C4 (which has the second highest FED value), the result is a chemical structure that is a precursor for the two DNDPA structures. Table 1 also shows OPCA (m/z = 385) and OPO (m/z = 309), which show chemical modifications at low FED carbon units. Predicting these structures may require other reactivity indices.

The current results show that FED values increased at C4, which is adjacent to the carbon atoms being attacked on ring A. This result seems counterintuitive, because oxidation reactions remove an electron from the highest molecular orbital, and would presumably decrease the amplitude of the square of the HOMO and therefore the FED (according to traditional frontier molecular orbital theory). However, it is possible to observe a local increase in electron density in a compound that is subject to oxidation. This is RIER, which asserts that, when an electron is removed from the highest molecular orbital, nearby orbitals may "relax" and reconfigure so that the FED of a particular carbon atom may increase. This idea had been computationally demonstrated previously,[48] but it gained its strongest support from recent experiments by Melin et al., 2007,[44] who oxidized dinuclear cobalt and simultaneously reduced the chloranilate linker connecting the cobalt complexes. What appeared as a computational anomaly now had stronger experimental

support. The presence of orbital relaxation should encourage the use of higher levels of electronic theory when exploring full transformation pathways because electron−electron interactions need to be rigorously accounted for.

Application. Practical aspects of EE_2 metabolism have taken shape. The first is related to ring cleavage, which is a key metabolic event because it produces metabolites that are easier to biodegrade. When EE_2 is degraded, ring A is cleaved first. This is supported by the identities of most of the measured metabolites presented in the literature, and it is also supported by the FED analysis presented here. Haiyan et al., 2007 proposed that ring B is cleaved first, based on the identity of metabolites produced by Sphingobacterium sp. JCR5, but their results appear to be the exception, rather than the rule.[15] A second practical issue is related to Sulfo-EE_2. This metabolite has been independently identified from mixed cultures in three different studies, including this current effort. Khunjar et al., 2011 recently found that Sulfo-EE_2 was not degraded by heterotrophic cultures that were otherwise active. Our results support the notion that Sulfo-EE_2 is fairly resistant to biodegradation because of the presence of the sulfate group in ring A.[13] Further, sulfate conjugation appears to cause recalcitrance for estrone, estradiol, estriol, which are similar to EE_2 in structure.[46,49] Sulfo-EE_2 may either be a "dead-end" metabolite, or it may be transformable after desulfurization, which may be a slow process.[49,50] Wastewater treatment plants that are interested in EE_2 should look for Sulfo-EE_2 in secondary effluent.

The broader application of FED-based theory is another issue of practical significance. A new tool enabling the *a priori* prediction of organic metabolites would be a valuable resource for environmental professionals, and there are subtle indications that the FED-based approach can help explain other transformations of interest. Estrone, estradiol, and estriol are conjugated at the carbon units that have high FED values.[49,51−53] Kurisu et al., 2010 proposed an estradiol degradation pathway that is consistent with FED-based theory,[54] and Yi et al.[55] used NMR to identify trimethoprim byproducts that are in agreement with FED-based modeling. It is now possible to duplicate the current effort for other compounds, but it also clear that future research can extend and improve FED-based modeling. For example, other approaches may better address resonance stability, which can cause high FED carbon units to be unreactive.[56] We used Rule 5 to account for this, but alternative approaches may improve model performance. The major challenge for future efforts will concern the proper incorporation of kinetics. FED-based techniques must account for the interactions between nonspecific enzymes (e.g., oxygenases, dehydrogenases, sulfotransferases) and high FED carbon sites, so that binding characteristics can be determined and reaction rates can be computed. A kinetically based FED approach can also reveal the impact of steric effects and shed light on the reversibility of these reactions. This type of model prediction needs to be judged against experimental data that quantifies metabolite concentrations as a function of time, and, at present, this kind of information is in very short supply. Fortunately, there is incentive to fill this data gap, as a kinetically based FED model can tell us the relative yield of different metabolites and help us discover others that are highly estrogenic.

■ ASSOCIATED CONTENT

Supporting Information

More details on the generation of metabolic pathways and estrogenic potential calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (412) 624-9548. Fax: (412) 624-0135. E-mail: wharper@pitt.edu.

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