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*Ozonation of Tamoxifen and Toremifene – Reaction Kinetics
and Transformation Products*

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Abstract

The oxidation of the two anti-estrogenic pharmaceuticals tamoxifen and toremifene with ozone in water was investigated concerning kinetics, reaction pathway, and transformation product formation. For both compounds a high dependency of second order rate constants and products on pH was determined. In case of full protonation of the amine (cation) ozone attacks with a second order rate constant of $1.57 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for tamoxifen and $4.37 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for toremifene. The neutral tertiary amine has an unexpected high second order rate constant of $3.17 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for tamoxifen and $1.46 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for toremifene. For the reaction of ozone and the tertiary amine only *N*-oxide formation was observed. pK_a values for tamoxifen (9.49 ± 0.22) and toremifene (9.57 ± 0.22) can be reported based on experimental data. Eight transformation products (TPs) were observed and identified based on MS/MS spectra or a reference standard. Products observed derived from Criegee reaction and hydroxylation as well as *N*-oxide formation. Further TPs from reactions with TAM products were combinations of *N*-oxides, Criegee products and hydroxylation products. Thus, reaction pathways can be derived and primary and secondary TPs distinguished for the first time.

Keywords:

Dissociation Constants, Ozone, Transformation Products, Reaction Kinetics, Micropollutants, Tamoxifen, Toremifene

49 TOC Art

51 Introduction

52 Endocrine disruptive compounds are an important group of micropollutants since they
53 can have biological effects already at low concentrations^{1, 2}. Indeed, endocrine
54 effects have been observed in wastewater treatment plant effluents³. Therefore these
55 are a major pathway for micropollutants to enter surface waters since most of the
56 micropollutants are not fully degraded in the biological treatment step⁴. Oxidative
57 processes offer an effective additional treatment to reduce micropollutant discharge
58 into surface waters via wastewater treatment plant effluents. Thus, ozone (O₃) is
59 broadly discussed and tested in large scale⁵⁻⁸. Especially in wastewater treatment O₃
60 is largely consumed by organic matter. Hence, it is often dosed proportional to the
61 dissolved organic carbon (DOC) content of the wastewater⁹. Second order rate
62 constants are an important tool for assessing the degradation efficiency of
63 micropollutants. Rate constants of $> 1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ may result in a complete
64 abatement of pollutants at an ozone dose of 1 g(O₃) per g(DOC)¹⁰. Another oxidant
65 formed during ozonation is the hydroxyl radical ([•]OH), which also contributes to the
66 degradation of micropollutants that react slowly with O₃¹¹.

67 Even though ozonation does not result in a mineralization of pollutants, their
68 biological effect can be removed (e.g., estrogenic activity¹²). However, some
69 biological effects may persist such as anti-estrogenic activity, which was reported to
70 remain partially, even after further biological treatment following ozonation³.
71 Furthermore, ozonation can also result in formation of toxic transformation and by-
72 products such as *N*-nitrosodimethylamin (NDMA) and bromate¹³⁻¹⁷.

73 Tamoxifen (TAM) and its chlorinated derivate toremifene (TOM) are nonsteroidal
74 selective anti-estrogenic estrogen receptor modulators, which are the most effective
75 pharmaceuticals used in therapy to prevent reoccurrence of estrogen receptor-
76 positive breast cancer¹⁸. These compounds are not removed by biological treatment
77 during wastewater treatment and can therefore enter surface waters by wastewater
78 discharge¹⁹⁻²². The highest reported concentration is 369 ng L⁻¹ of TAM in a
79 wastewater effluent and up to 212 ng L⁻¹ reported in the river Tyne²³ exceeding the
80 calculated PNEC value of 81 ng L⁻¹.

81 The possibility to degrade TAM in different (advanced) oxidative processes (e.g.,
82 ozonation or peroxone process)^{24, 25} and by sunlight²⁶ were previously described in
83 literature. However, information on rate constants and formation of transformation

products during ozonation is hardly available. Three TPs for the ozonation of TAM have been proposed earlier²⁷ and two further TPs were reported recently²⁸, but without information for validation of reported structures. Another study reported further TPs with proposed structures, reporting MS/MS spectra for one TP²⁵. However, the structures proposed here are very unlikely to be formed in ozone reactions (e.g. hydroxylation of an alkane moiety). Additionally ozone was applied in high excess, but no quantification of the ozone exposure was provided. Based on the reported experimental set up, an investigation of primary TPs was not possible. Hence, a further investigation of the reaction pathway of ozone and TAM/TOM is necessary and to further elucidate the structural identity of TPs formed. Especially since TPs might also have effects on aquatic life⁶. Toxic effects were investigated before and after ozonation of TAM at pH 7 with a bioassay based on the bioluminescence of *aliivibrio fischeri* showing a decreasing but remaining baseline toxicity²⁵, as well as for TPs from photo-transformation of TAM, showing also slightly reduced toxicity compared to TAM²⁶. A recent study also showed that TPs formed during the ozonation of TAM preserve or amplify the anti-estrogenic effect caused by TAM in water²⁸. Therefore investigations on formation of TPs as well as reaction mechanisms are of considerable interest for the ozonation of TAM and TOM.

TAM and TOM reveal two major potential sites of attack, a tertiary amine and an olefin. Hence, the reactivity and the site of attack by ozone is largely controlled by pH in analogy to metoprolol¹⁰. Due to this pH dependency knowledge of the exact dissociation constant (pK_a) is important²⁹, which was not yet determined for TAM and TOM.

The aim of this study is the detailed investigation of pH-dependent reaction kinetics, formation of TPs and degradation pathways during the ozonation of TAM and TOM, including determination of their pK_a values.

Materials and Methods

Chemicals

TAM and TOM were purchased at Alfa Aesar (Karlsruhe). Available metabolites were 4-hydroxy-*N*-desmethyldamoxifen and 4-hydroxydamoxifen (Cayman Chemical Company, Middlesex), propiophen-1-one (Alfa Aesar) and TAM-*N*-oxide (LGC, Ann Arbor, Michigan). 2-Methyl-2-propanol, or tertiary butanol (TBA), and cinnamic acid were purchased from Merck (Darmstadt). Phenol and 2-chlorophenol were

purchased from Sigma-Aldrich (Steinheim). Potassium dihydrogenphosphate and dipotassium hydrogenphosphate (Merck, Darmstadt) were used for phosphate buffer preparation, and sodium hydroxide (VWR, Darmstadt), and hydrochloric acid (Fisher Scientific, Bremen) were used for pH adjustment. Purity of all chemicals was $\geq 98\%$. Ultra-pure water was produced onsite (Purelab Ultra, Elga LabWater, Celle). LC-MS grade methanol (HiPerSolv CHROMANORM, VWR), water (LiChroSolv), and formic acid (Suprapur; Merck) were used as eluents for LC-MS measurements.

Ozone solutions were prepared by bubbling ozone-containing gas, produced onsite with an ozone generator (BMT 802 X, BMT Messtechnik, Berlin; feed gas: O_2 6.0, Linde, Germany), through ultra-pure water at room temperature (O_3 concentration $\approx 0.5 - 0.7$ mM). In case higher ozone concentrations were needed the solution was cooled using an ice bath (ozone concentration $\approx 1 - 1.5$ mM). The concentration of the ozone solution was determined using a UV-1650PC UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) at 258 nm ($\epsilon = 2950 \text{ M}^{-1} \text{ cm}^{-1}$)³⁰. Accuracy of spiking the ozone for batch experiments was demonstrated using the indigo method¹⁰ with an accuracy of $\pm 5\%$. In all experiments added ozone reacted until complete ozone consumption.

Potentiometric titration

Dissociation constants (pK_a) were determined using a Tiamo titrator system (Metrohm, Filderstadt), consisting of an automatic stirrer, 800 Dosino automatic burette, a glass pH-electrode (pH 0-14) and a temperature electrode. The glass electrode was calibrated using pH 4 and pH 9 buffer solutions (Metrohm). All solutions used for pK_a determination contained 0.15 M KCl to maintain a constant ionic strength of 0.3 and titrations were performed at ambient temperature (21.1 ± 1.1 °C) under nitrogen atmosphere in parafilm-covered 100-mL beakers. 0.01 M HCl was used to standardize the titrant NaOH. The second dissociation constant of 0.01 M phosphoric acid ($pK_{a,2}$: 7.2) was determined for validation^{29, 31} using the first derivative to be 7.08 ± 0.22 , giving a pK_a accuracy of ± 0.22 . A cosolvent mixture of equal parts of methanol, dioxane, and acetonitrile (MDM) was implemented due to the low solubility of TAM and TOM. For the titration 20% (v/v) MDM – water mixtures were used containing 0.05 mM of the cationic species of both analytes. The cosolvent dissociation constant (p_sK_a) was determined in triplicates using the second

derivative and the dissociation constant for water (pK_a) was then calculated according to Völgyi, et al.³².

Reaction kinetics

Second order rate constants for the neutral species $k(M, O_3)$ and the protonated, cationic species $k(MH^+, O_3)$ were determined using competition kinetics³³. 100 mmol TBA was used to dissolve 10 μ mol of TAM or TOM in 900 mL ultra-pure water. TBA was simultaneously used as scavenger for hydroxyl radicals formed during ozonation. 10 μ mol of the competitor was added and phosphate buffer (preparation modified after Sørensen³⁴) was added to obtain a 0.5 mM phosphate-buffer concentration. Solutions of HCl and NaOH were used for final pH adjustment. Finally the total volume was adjusted to 1 L, yielding a concentration of 10 μ M for each analyte and competitor, and the pH was checked again. Ozone solution was added using glass syringes (Poulsen&Graf, Wertheim) to 100 mL samples to obtain 0, 2, 4, 6, 8, 10, 12.5, 15, and 20 μ M concentrations. Experiments were performed at pH 3 – 7 and 11 (TAM), and pH 2 – 7 (TOM) in triplicates at ambient temperatures. Cinnamic acid (pK_a 4.44) was used as competitor for TAM at pH 3 and TOM at pH 2 and 3.5 ($k(\text{cinnamic acid}, O_3) = 5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $k(\text{cinnamic acid anion}, O_3) = 3.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$)³⁵. All other second order rate constants were determined using phenol (pK_a 9.9) as competitor ($k(\text{phenol}, O_3) = 1300 \text{ M}^{-1} \text{ s}^{-1} \pm 200 \text{ M}^{-1} \text{ s}^{-1}$, $k(\text{phenolate anion}, O_3) = 1.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \pm 0.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$)³⁶. After addition of ozone, samples were kept overnight at ambient conditions (ozone depleted completely) and were then analyzed using a Kinetex® EVO C18 column (150 x 3.0 mm 5 μ m, 100 Å; Phenomenex, Aschaffenburg), a Shimadzu 10A liquid chromatograph equipped with a diode array detector (Shimadzu, Kyoto), and a methanol / pH 2 water gradient. Recorded chromatograms at $256 \pm 1 \text{ nm}$ and $278 \pm 1 \text{ nm}$ were used for quantification. For further information on the analytical method see SI 2.

Determination of TPs

The same experimental setup as for the reaction kinetics was used (10 μ M TAM/TOM, 100 mM TBA, and 0.5 mM phosphate buffer), except that no competitor was added and sample volume was reduced to 10 mL and gastight syringes (Hamilton, Reno) were implemented for dosing ozone solution. Experiments were performed at pH 2, 7, and 11. Ozone solution was added to gain final concentrations of 0, 2, 4, 6, 8, 10, 15, 20, and 30 μ M. Samples were analyzed with a Kinetex® C8

column (50 x 2.1 mm; 5 μ m; 100 Å; Phenomenex, Aschaffenburg) using an Agilent 1100 Series LC coupled to an 6120 quadrupole LC/MS (Agilent, Waldbronn) and a methanol (+0.1 % (v/v) formic acid) / water (+0.1 % (v/v) formic acid) gradient. Electrospray ionization was operated at 3 kV and a nebulizer pressure of 30 psig. Dry gas flow rate was set to 10 L min⁻¹ and heated to 300 °C. For quantitative / semi-quantitative evaluation selected ion mode was used to monitor the ions 270.1, 286.1, 372.1, 388.2, and 404.2 in TAM experiments and 270.1, 286.1, 406.2, 422.2, and 438.1 in TOM experiments, which were beforehand identified using scan mode. For further information see SI 3.

For MS/MSⁿ measurements samples were diluted 1:1 with methanol, containing 0.1 % (v/v) formic acid, and analyzed with an Amazon Speed Ion Trap MS (Bruker, Billerica) via direct injection with a 500 μ L syringe (Hamilton, Reno) at 7.5 μ L min⁻¹ with electrospray ionization (4.5 kV, 10 psi) and 5.7 L min⁻¹, 350 °C dry gas. MS/MS-spectra were recorded using samples ozonated at pH 2 for TP 388 and TP 422, and samples ozonated at pH 11 for TAM-*N*-oxide and toremifen-*N*-oxides. These samples were checked beforehand by LC-MS measurements to ensure clean MS/MS spectra. Exact masses were determined using a QExactive Orbitrap MS. Settings and results are given in SI 3.2.

Results and Discussion

Potentiometric titration

Determined p_sK_a for TAM and TOM are 8.96 ± 0.11 and 9.04 ± 0.10. The pK_a was then calculated using the calibration for acids from determined p_sK_a values³², resulting in pK_a values for TAM and TOM of 9.49 ± 0.22 and 9.57 ± 0.22 respectively. Further information is given in SI 1. The determined dissociation constants show a significant deviation from the predicted pK_a of 8.76 for TAM and TOM³⁷.

Reaction kinetics

The determined second order rate constants are summarized in Table 1 and pH dependency is shown in Figure 1. For TOM higher variations of determined second order rate constants were observed (see SI 2) which have to be considered in data interpretation. Due to the low solubility in pure water for both compounds high TBA concentrations (100 mM) during all experiments were applied. This concentration corresponds to a >1000 fold excess over TAM, TOM and the corresponding competitor. Even in case the compounds under study and/or the competitors react

215 with hydroxyl radicals at diffusion controlled rates the excess of TBA will be sufficient
216 to scavenge > 95% of hydroxyl radicals ($k(\text{OH-radical} + \text{TBA}) = 4.2\text{--}7.6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$
217 ³⁸). Hence, interferences by hydroxyl radicals can be ruled out.

218 As expected, the observed second order rate constants increase with pH due to an
219 increasing fraction of the neutral deprotonated amine. For both compounds the
220 second order rate constants of the neutral species are in the order of $10^8 \text{ M}^{-1} \text{ s}^{-1}$
221 which corresponds to the reaction of ozone at the free amine, although second order
222 rate constants for amines are usually $< 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ^{39, 40}. The species specific second
223 order rate constants of TAM and TOM were calculated using a data fitting analysis
224 based on Equation S3. The same procedure was performed using the
225 minima/maxima of experimental second order constants to estimate the accuracy of
226 the regression, stated as error for TAM and TOM in table 1. Second order reaction
227 rates for TAM were also experimentally determined using 2-chlorophenol and phenol
228 at pH 11 (see SI 2.1, Table S3). However, due to incomplete recovery in some
229 experiments (data not shown), rate constants determined at pH 11 have to be
230 considered with care and were therefore not implemented in the determination of the
231 species specific rate constant.

232 The cation (protonated amine) probably reacts with ozone at the olefin moiety. The
233 TAM cation reacts by a factor of three faster than the TOM cation. This can be
234 explained by a reduced electron density of the olefin moiety due to the chlorine
235 substituent in TOM, which is a major factor influencing ozone reaction with olefins⁴¹.
236 The reactivity $\text{p}K_{\text{a}}$ is defined as the pH where the reactions of ozone with each of the
237 species present contribute equally to the apparent second order rate constant¹⁰. For
238 TAM and TOM this is the case at pH 5.18 and pH 5.05, respectively. The connectivity
239 of apparent and species specific second order rate constants and the pH is given in
240 SI 2, Equation S3-S5.

241 A good correlation of determined kinetic data with the new determined $\text{p}K_{\text{a}}$ values
242 and consistency with a former reported second order rate constant at pH 7.1 ²⁴ can
243 be observed. Based on the structural similarity the species specific second order rate
244 constants for the neutral species of TAM and TOM can be expected to be similar,
245 which is confirmed in this study. However, the second rate constants deviate from the
246 reported species specific second order rate constants of several other tertiary amines
247 such as trimethylamine ($4.1 - 5.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)^{36, 40}, triethylamine ($2.1 - 4.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)^{36, 40}.

1 s^{-1})^{40, 42}, and the pharmaceutical tramadol ($1.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)⁴³, which are two orders of magnitude lower. However, other amines indeed are reported to have similar reactivity as TAM and TOM, i.e., dimethylethanolamine ($k(\text{O}_3)$: $1.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, pK_a : 9.2)⁴⁴. 3-(dimethyl-aminomethyl)-indole ($k(\text{O}_3)$: $3.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, pK_a : 10.0) is reported with an even higher species specific second order rate constant than TAM and TOM⁴⁴. Although this compound also contains an aromatic moiety that could react with a similar second order rate constant, the pH dependency of the reaction, the pK_a , and dimethylamine as main product indicate that the tertiary amine and not the aromatic moiety reacts with ozone⁴⁴. Hence, aniline like behavior of 3-(dimethyl-aminomethyl)-indole can be ruled out. Affirmation of the high species specific second order rate constants was corroborated in this study by good agreement of the calculated data for TAM and TOM. The good match of the second order rate constant of TAM with literature data (Figure 1 A) is a further confirmation of our results. Additional competition kinetic experiments were done using tramadol, as structurally similar compound. These experiments showed a strong degradation of TAM and only small degradation of tramadol though no significant degradation of tramadol was observable at the ozone dosages 1, 2, 3, and 5 μM (see SI, Chapter S5). Additionally the competition kinetics experiments of TAM vs. tramadol revealed a very poor linear correlation of the double logarithmic plot for most ozone dosages (1-5 μM) (Figure S10 B), presumably due to the large difference in reaction kinetics. The slope of the linear regression is 2.47 ± 1.09 (44 %) and barely shows a correlation ($R^2 = 0.270$). Only when the data of the highest two ozone dosages are included (7.5-10 μM) a suitable correlation can be observed ($R^2 = 0.830$) (Figure S10 A). The competition kinetics experiment has shown that TAM reacts much faster than tramadol, however, the poor correlation (especially for the part shown in Figure S10 B) barely allows quantitative evaluation of the data (further details cf. SI).

Table 1: Dissociation constants, species specific, and apparent second order rate constants for the reaction of ozone with of TAM and TOM; (neutral species: $k(\text{M}, \text{O}_3)$), cationic species: $k(\text{MH}^+, \text{O}_3)$, apparent rate constant at pH 7: $k(\text{O}_3, \text{pH } 7)$, ratio of $k(\text{MH}^+, \text{O}_3) / k(\text{M}, \text{O}_3)$, and reactivity pK_a . *Estimated values.

	Tamoxifen			Toremifen		
pK_a	9.49	±	0.22	9.57	±	0.22

$k(\text{M}, \text{O}_3) / \text{M}^{-1} \text{s}^{-1}$	$3.17 \times 10^8 \pm 1.19 \times 10^8$	$1.46 \times 10^8 \pm 2.71 \times 10^7$
$k(\text{MH}^+, \text{O}_3) / \text{M}^{-1} \text{s}^{-1}$	$1.57 \times 10^4 \pm 1.63 \times 10^3$	$4.37 \times 10^3 \pm 1.62 \times 10^3$
$k(\text{O}_3, \text{pH } 7) / \text{M}^{-1} \text{s}^{-1}$	$1.04 \times 10^6 \pm 3.83 \times 10^5$	$3.75 \times 10^5 \pm 2.13 \times 10^4$
$k(\text{MH}, \text{O}_3) / k(\text{M}, \text{O}_3)$	4.94×10^5	2.99×10^5
reactivity pK_a	5.18	5.05

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279 For the reaction of ozone at the olefinic moiety of TAM and TOM (cationic species)
280 second order rate constants in the same order of magnitude as other micropollutants
281 containing olefinic groups ($10^3 - 10^5 \text{ M}^{-1} \text{ s}^{-1}$) such as carbamazepine, cephalexin, and
282 anatoxin-a^{6, 45-47} were found. For real water treatment (pH 7-8)¹⁰ TAM and TOM have
283 apparent second order rate constants $> 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and can thus be considered to be
284 readily transformed during wastewater ozonation; ($k(\text{TAM}, \text{app.}, \text{O}_3)$: $1.04 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$,
285 $k(\text{TOM}, \text{app.}, \text{O}_3)$: $3.75 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, at pH 7).

286 The reaction kinetics of tertiary amines have a surprisingly large range of second
287 order reaction rates constants, which cannot be readily explained, yet. This shows
288 that further research is needed regarding ozone reactions with nitrogen containing
289 compounds.

Figure 1: Individual Determined and calculated second order rate constants for ozonation of tamoxifen (A) and toremifene (B). - - $[MH^+]$, — — $[M]$, — — $k(O_3, \text{calculated})$ with error as dotted line in grey, $k(O_3, \text{determined})$ with different competitors: crosses: cinnamic acid, diamonds: phenol, closed square: second order rate constants of TAM from ²⁴.

Determination of TPs

In the following, TPs will be denoted according to the observed m/z ratios, if these were not verified by reference standards. For TAM and TOM two mutual TPs, i.e., TP 270 and TP 286, were determined. Further observed TPs of TAM were TP 388, TP 404, and tamoxifen-*N*-oxide (TAM-*N*-oxide) and for TOM TP 422, TP 438, and TP 422-*N*. The last can be assumed to be toremifene-*N*-oxide, based on retention time shifts similar to those found for TAM-*N*-oxide and MS/MS spectra. Structures of all TPs are derived from MS/MS spectra and exact masses (see SI 3, Table S5). Based on the m/z values one TP for the ozonation of TAM was reported in a previous study, but without information on structural identity²⁸. TP 286 and TP 388 have also been

reported with the same m/z ratio in another study and three further TPs (TP 106, TP214, and TP 224). Here other structures were proposed for the common TPs²⁵.

Reaction pathways

Based on the observed transformation products the same reaction pathways can be assumed for TAM and TOM as shown in Figure 2. The reaction of ozone with the olefin leads to formation of an intermediate ozonide according to the Criegee mechanism in water (reaction 1) at pH 2. A subsequent reaction with water (reaction 2) leads to formation of [4-[2-(Dimethylamino)ethoxy]phenyl](phenyl)methanone (TP 270), propiophen-1-one (TP A) for TAM or 3-chloropropiophen-1-one (TP B) for TOM, and hydrogen peroxide⁴¹. TP 270 and TP A are also formed during photo-transformation of TAM, although propiophen-1-one is unstable under oxidative conditions²⁶ and was not observed in this study. The alkoxybenzene ring is activated (σ_P (ethoxy group) = -0.24)⁴⁸ and can be hydroxylated by ozone (reaction 3). However, the reaction should be less favored compared to the double bond and the amine since alkoxybenzenes as anisole ($k(O_3)$: 290 M⁻¹ s⁻¹) or 1-phenoxy-2-propanol ($k(O_3)$: 320 M⁻¹ s⁻¹) react slowly with ozone^{10, 49}. Even though the alkoxybenzene ring of TAM and TOM is hardly activated by the olefinic group (σ_P (styrene) = -0.07)⁴⁸ product analysis showed that the Criegee product was formed. This indicates that the Criegee reaction at the olefinic group is favored over the reaction with the alkoxybenzene ring of TAM and TOM at pH < 5. For the hydroxylation of the benzene ring first a zwitter-ionic ozone adduct is formed, which is probably stabilized at the ortho-position, followed by either an oxygen transfer (reaction 4) and hydrogen shift (reaction 5) or re-aromatization by proton cleavage (reaction 6) followed by an oxygen transfer (reaction 7) and subsequent protonation of the phenolate ion (reaction 8)⁴⁹. Since reaction 6 is less observed under acidic conditions, reactions 4 and 5 may be favored. Here a ring opening reaction according to the Criegee mechanism is also conceivable⁵⁰, but no corresponding transformation products were observed. Supposedly stabilization of the cation favors the formation of the hydroxylation products, TP 388 / TP 422, which are observed during ozonation experiments.

Reactions of ozone with tertiary amines (reactions 9-12) result in *N*-Oxide formation⁴⁰ either by direct electron transfer forming an ozonide radical anion and an amine radical cation (reaction 9) or by adduct formation (reaction 10) followed by radical

formation (reaction 11). The retention time of the *N*-centered radicals and the ozonide radical located side by side in the solvent cage favors their bimolecular reaction resulting in the *N*-oxide (reaction 12)^{10, 43, 51, 52}. The corresponding *N*-oxides are also known to be formed as human metabolites that can be reduced back to TAM / TOM in their interaction with enzymes⁵³. The reactivity pK_a indicates that at $pH > 5$ ozone mainly reacts with the amine. Hence *N*-oxide formation is promoted with increasing pH while the Criegee mechanism and hydroxylation are the major reactions at pH -values ≤ 5 . Due to the excess of ozone under typical ozonation conditions further oxidation of the transformation products is possible. Similar reaction pathways (reaction 13-16) lead to the secondary TPs (TP 286, TP 404 / 436) (Figure 2). Products with hydroxylation of the benzene ring and ketone formation were not observed, since the carbonyl group has a deactivating effect (σ_p (benzaldehyde) = 0.43, σ_m (benzaldehyde) = 0.34, σ_p (CHO) = 0.42, σ_m (CHO) = 0.35)⁴⁸. TP A and TP B, expected TPs for the ozone-olefin reaction (1, 2; see Figure 2), were not detected. The confidence of the proposed structure according to Schymanski et al. are level 1 for TAM-*N*-oxide and level 2 (b) for TP 270, TP 286, and TP 422-*N*. For TP 388, TP 404, TP 422, and TP 438, the position of the hydroxyl group remains speculative (confidence level 3)⁵⁴.

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Figure 2: Reaction pathways for formation of primary TPs of tamoxifen (R1: CH₃) and toremifene (R2: CH₂Cl) by ozonation and formation of secondary TPs. Reactivity pK_a at pH 5. Measured TPs highlighted in dotted boxes, intermediates and TPs which have been hypothesized are marked with #.

TP 286 was reported as the major formed TP by Ferrando-Climent et al. 2017²⁵. Here, the simultaneous cleavage of the olefin and hydroxylation of the benzene ring is proposed after the formation of the ozonide. However, this pathway of simultaneous ketone formation at the olefin moiety and hydroxylation of the aromatic ring is unlikely when compared to known reactions with comparable structures such as cinnamic acid³⁵. It seems much more likely that at pH 8 ozone attacks the amine moiety resulting in *N*-oxide formation as supposed in our study via reactions 9 – 12, and *N*-oxide formation has been shown for the ozonation of several micropollutants containing amine moieties^{55, 56}. We assume that due to their experimental setup Ferrando-Climent et al. 2017 were not able to detect primary TPs we observed in this study and therefore they suggested the simultaneous oxidation at two neighboring moieties as one oxidation process. Additionally the proposed structure for TP 388, a hydroxylation of the alkane, as also proposed by Ferrando-Climent et al., is unlikely to be formed in ozonation. However, it was only observed in a combined treatment using UV and ozone. In this process photolysis of ozone yields highly reactive hydroxyl radicals which may have formed the structure suggested by Ferrando-Climent et al. for TP 388. However, no MS/MS data have been reported here²⁵.

(Semi)-quantitative evolution of target compounds and TPs

Quantification via external calibration was performed for TAM, TOM, and TAM-*N*-oxide. For the other observed TPs no reference standard were accessible and therefore only semi-quantitative evaluation was performed based on measured peak area. Degradation of TAM and TOM as well as corresponding product formation in dependence of the ozone dose is shown in Figure 3. The estimation of the ozone consumption is shown in SI 4.

Tamoxifen

At pH 2 TAM was completely degraded at an ozone dose of 15 μM . The main TP formed was TP 270 followed by TP 388. Ozone in excess leads to degradation of TPs 270 and 388 as well as TP 286 and TP 404 as secondary products. Due to the linear correlation (Figure S9 A), no dominant interferences can be assumed and therefore an ozone consumption of 1.3 mol ozone per mol TAM degradation at pH 2 could be determined. During ozonation at pH 7 only 99% of TAM was removed with an ozone dose of 30 μM maybe due to ozone depletion by subsequent oxidation of primary TPs. The ozone consumption per mol TAM here is 2 mol O_3 . TAM-*N*-oxide is

formed as main TP with a maximum of 2.19 μM at a dose of 10 μM of ozone, but oxidized to TP 286 and TP 404 at higher ozone dose. The other primary TPs, TP 270 and TP 388, had maximum peak areas an order of magnitude below those during ozonation at pH 2. All three primary TPs were almost depleted at the final ozone dose of 30 μM and TP 286 and TP 404 were continuously formed as secondary products. The decrease of TP 404 indicates a further transformation process which could be explained with the activation of the aromatic ring by the hydroxyl group¹⁰ ($\sigma_p(\text{OH}) = -0.37$)⁴⁸. Higher ozone doses might cause abatement of TPs containing phenolic moieties, but ozone doses applied in this study might have been fully consumed by the reactions stated in Figure 2. However, further TPs were not detected using reversed phase liquid chromatography, as products formed due to ring opening show increased polarity and therefore might have escaped detection. Hence, minor fractions of the TPs containing the phenolic moiety might indeed have been further oxidized by ozone at the studied ozone doses. TP 286 seems to be reacting slowly with ozone, since it was also found after very high ozone dose²⁵. Low solubility ($c(\text{TAM}) < 1 \mu\text{M}$) at pH 11 does not allow to monitor the TAM degradation, but TP formation can be observed, as 0.5 μM TAM-*N*-oxide was formed. No ratio for the consumption of ozone could be calculated. Nevertheless signals for the further oxidation of the TAM-*N*-oxide to TP 286 and TP 404 can be observed, since ozone is present in excess to the TAM concentration, but these are an order of magnitude below the signal intensities found at pH 7.

Toremifene

The lower solubility of TOM did not allow to determine the ozone consumption (non-linear correlation, Figure S9 B). Nevertheless one can see that a higher ozone dose is necessary for the complete abatement of TOM than for TAM at pH 2 (compare Figures 3 A and D). For TOM, TP formation at pH 2 is similar to the oxidation of TAM. The same signal intensity was found for TP 270 as during the oxidation of TAM and as for TP 422. In contrast, TP 438 as secondary TP was found here as well. A larger difference to the ozonation of TAM can be found at pH 7 since TOM is only degraded by 65 % even after addition of ozone in excess. The formed *N*-oxide is further degraded to secondary TPs and may thus have competed with TOM for ozone. This effect is less pronounced for TAM since the olefin moiety of TOM is less reactive towards ozone (see second order rate constants, Table 1). Signal intensity for TP 286 is 3 times higher than for TP 438, but compared to the ozonation of TAM only

half of the signal intensity was found. At pH 11 the low solubility also inhibits the investigation of the degradation of TOM and product formation. TP 422-N was the only TP observed at pH 11, also with low signal intensities compared to pH 2 and 7. Since *N*-oxide metabolites are known to be slightly less polar than their parent compounds⁵⁵, low solubility has to be considered here as well. Hence, the formation of TP 422-N might be underestimated.

Figure 3: Degradation of TAM at pH 2 (A), pH 7 (B), and pH 11 (C) and TOM at pH 2 (D), pH 7 (E), and pH 11 (F) during ozonation and formation of TPs, semiquantitative. ○ - TP 270, □ - TP 286, ◇ - TP 388, ▼ - TP 404, ◆ - TAM, ● - TAM-N-oxide, x - TP 422-N

440 422, + - TP 438, ▲ - TOM, and ■ - TP 422-*N*. Initial nominal concentration c_0 of
441 TAM/TOM: 10 μM .

442 *Estimation of risk reduction*

443 Based on pH during ozonation of wastewater (~pH 7-8) mainly *N*-oxides are formed
444 from TAM and TOM in wastewater and in general, *N*-oxides showed to be persistent
445 in biological post treatments but can be partially removed by sorption to activated
446 carbon⁵⁷. Only under anaerobic conditions in the environment *N*-oxides can be
447 transformed back to their parent compounds^{6, 55, 58, 59}. After ozonation though, oxic
448 conditions are usually maintained during further treatment steps. Hence, ozonation
449 may not be a final barrier for removing TAM and TOM. This emphasizes the
450 importance to further study the environmental behavior of TAM and TOM and their
451 TPs.

452 Concerning toxicity of the TPs, only for TP270 data are available with an EC_{50} of
453 0.89 $\mu\text{g L}^{-1}$ for *Ceriodaphnia dubia*²⁶. Nevertheless Ferrando-Climent et al. showed
454 that the effect on *vibrio fischeri* can still be observed after full degradation of TAM and
455 simultaneous formation of TP 286²⁵. The anti-estrogenic activity of TAM was
456 monitored after ozonation in another study and showed that the anti-estrogenic effect
457 can be preserved or even be amplified by roughly the factor 2 due to the formation of
458 TP 270²⁸. This demonstrates the significance of monitoring toxicity of TPs formed
459 during oxidative processes, but also the necessity of testing the toxicity with multiple
460 organism species to allow a comprehensive environmental risk assessment
461 concerning the success of toxicity removal of micropollutants by ozonation.

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Supporting information

Additional information on pK_a determination (SI 1), Reaction kinetics (SI 2), transformation products (SI 3), ozone consumption (SI 4), and comparison of the degradation of TAM and tramadol by ozone (SI 5).

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