Formation and Effects of Transformation Products during the Ozonation of Tamoxifen

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Oliver Knoop

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Gutachter:	Prof. Dr. Torsten C. Schmidt
	Prof. Dr. Bernd Sures
Vorsitzender:	Prof. Dr. Thomas Schrader

There is an art, it says, or rather, a knack of flying.
The knack lies in learning how to throw yourself at the ground and miss.

from: The Hitch Hiker's Guide to the Galaxy
by Douglas Adams

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Summary

To reduce the amount of micropollutants and their ecotoxicological effects released into the environment via wastewater treatment plant (WWTP) effluents, ozonation showed to be suitable and is broadly discussed as advanced wastewater treatment (AWT) in combination with subsequent biological filtration. Especially endocrine effects can be induced at low concentrations of a broad range of micropollutants. Estrogenic and androgenic effects are diminished due to ozonation and the reactions of compounds inducing these effects are well investigated. In contrast, the corresponding antagonistic effects have recently been reported to be resilient to this treatment strategy and until now, only little information for the reaction of anti-estrogenic compounds with ozone is available. For this reason, this study focuses on the reaction of tamoxifen (TAM), a selective estrogen receptor modulators (SERM), with ozone, the formation of transformation products (TPs), and the simultaneous progression of anti-estrogenic activity and acute ecotoxicological effects.

All experiments were performed in ultra-pure water in presence of radical scavengers to rule out radical reactions and focus solely on the reaction of TAM and ozone, including the dissociation of TAM in dependency of the pH. Reaction kinetics were investigated using a competition kinetics approach over a broad range of pH to determine the second order rate constants for the reactions of ozone with the corresponding species of TAM. Rate constants differed substantially between the two relevant TAM species (k(TAM, O_3) = 3.25×10^8 M^{-1} s^{-1}; k(TAM-H^+, O_3) = 1.57×10^4 M^{-1} s^{-1}), resulting in an apparent second order rate constant of > 10^6 M^{-1} s^{-1} at pH 7. Hence, full abatement by ozonation as AWT can be assumed. However, for the neutral species with a tertiary amine as most reactive moiety towards ozone, the species specific second order rate constant obtained is unexpectedly high.

Five TPs were identified in total using high resolution mass spectrometry and structure elucidation based on fragmentation in tandem mass spectrometry. Two primary TPs, TP 270 (Criegee product) and TP 388 (hydroxylation), are formed during the ozonation of the corresponding acid of TAM. TAM-*N*-oxide is the primary product formed in the reaction of the neutral TAM species and ozone and was unequivocally identified using a reference standard. Further oxidation of the primary TPs by ozone results in the

formation of the two secondary TPs TP 286 and TP 404, the corresponding *N*-oxides of TP 270 and TP 388.

Anti-estrogenic activity using the *Arxula adeninivorans* yeast estrogen screen (A-YES) assay, and acute toxicity for *Daphnia magna* and growth inhibition of the green algae *Desmodesmus subspicatus* were monitored in dependence of the ozone dose, to allow correlation analysis with the formation of TPs. The anti-estrogenic activity was proliferated with increasing ozone dose, even after full abatement of TAM, and the residual effect correlated to the formation of TP 270. The formation of *N*-oxides was on the other hand coherent with a loss of the anti-estrogenic activity. The observed immobilization of *D. magna* was removed at low ozone doses completely. An increase of the algae growth inhibition with an increasing ozone dose was observed, which correlated again with the formation of TP 270, as well as TP 388. At pH 3, ozonation of TAM resulted in a duplication of both effects, anti-estrogenic activity and algae growth inhibition. The main observed TP here was TP 270, which was identified as 4-(Dimethylaminoethoxy)-benzophenone. Due to the high risk potential of TP 270, a new purification strategy was successfully developed for obtaining pure TP270 for further toxicity testing by synthesis. This study elucidated the reaction of the SERM TAM with ozone and the formation of primary and secondary TPs. Additionally, the formation of toxicologically relevant TPs was demonstrated. Therefore, this study might help to understand the persistence of anti-estrogenic effects in ozonation as AVT. To further foster our understanding in that regard, future studies need to further investigate the ozonation of SERMs, including metabolites, formation of TPs in wastewater, and their removal in subsequent biological filtration.

Zusammenfassung

Der Einsatz von Ozon hat sich als geeignet erwiesen, um eine Reduzierung der Mengen an Mikroschadstoffe und deren ökotoxikologischer Effekte zu erreichen, welche über gereinigtes Abwasser in die Umwelt eingetragen werden. Daher wird es in Kombination mit einer anschließenden biologischen Filtration als eine Möglichkeit der erweiterten Abwasserbehandlung in großem Umfang diskutiert. Besonders endokrine (hormonelle) Effekte können bereits durch geringe Konzentrationen von einer Vielzahl von Mikroschadstoffen induziert werden. Östrogene und androgene Effekte können durch den Einsatz von Ozon vermindert werden. Die Reaktionen von Ozon und Mikroschadstoffen, die diese Effekte verursachen, sind bereits gut untersucht. Im Gegensatz dazu wurde kürzlich berichtet, dass die antagonistischen Effekte durch diese Behandlung nicht entfernt werden können. Bisher sind nur wenige Informationen über Reaktionen von anti-östrogenen Verbindungen mit Ozon bekannt. Aus diesem Grund fokussiert sich diese Studie auf die Reaktion von Ozon mit Tamoxifen (TAM), einem selektivem Östrogenrezeptormodulator (SERM), der Bildung von Transformationsprodukten (TPs) und dem gleichzeitigen Verlauf der anti-östrogenen Aktivität und der akuten ökotoxikologischen Wirkung.

Alle Versuche wurden in Reinstwasser in Anwesenheit eines Radikalfängers durchgeführt, um Radikalreaktionen auszuschließen und lediglich die Reaktion von TAM und Ozon, unter Berücksichtigung der Dissoziation in Abhängigkeit des pH, zu betrachten. Reaktionsgeschwindigkeiten wurden mithilfe von Kompetitionskinetiken über einen weiten pH Bereich untersucht, um die speziesspezifischen kinetischen Konstanten zweiter Ordnung für die Reaktion von Ozon und TAM zu bestimmen. Die Geschwindigkeitskonstanten beider Spezies unterscheiden sich substanziell (k(TAM, O_3) = 3.25 x 10^8 M^{-1} s^{-1}; k(TAM-H^+, O_3) = 1.57 x 10^4 M^{-1} s^{-1}). Daraus ergeben sich bei pH 7 kinetische Konstanten von $> 10^6$ M^{-1} s^{-1}. Dementsprechend kann ein vollständiger Abbau von TAM durch den Einsatz von Ozon als erweiterte Abwasserbehandlung angenommen werden. Es ist zu betonen, dass für die neutrale Spezies, bei der das tertiäre Amin die reaktivste Gruppe für die Reaktion mit Ozon ist, eine unerwartet hohe kinetische Konstante zweiter Ordnung beobachtet wurde.

Fünf TPs wurden mit Hilfe von hochauflösender Massenspektrometrie und Tandem-Massenspektrometrie-basierter Strukturaufklärung, identifiziert. Die zwei primären TP, TP 270 (Criegee-Produkt) und TP 388 (Hydroxilierung), werden bei der Ozonierung

der korrespondierenden Säure von TAM gebildet. Tamoxifen-*N*-oxid ist das primäre Produkt, das aus der Reaktion von Ozon mit der neutralen TAM-Spezies gebildet wird und wurde durch einen Referenzstandard identifiziert. Die weitergehende Oxidation der primären TPs durch Ozon führt zu der Bildung von TP 286 und TP 404 als sekundäre TP. Diese sind die korrespondierenden *N*-Oxide von TP 270 und TP 388.

Die anti-östrogene Aktivität wurde mit dem hefebasiertem *Arxula adeninivorans* yeast estrogen screen (A-YES) assay bestimmt. Diese wurde ebenso wie die akute Toxizität für *Daphnia magna* und die Hemmung des Algenwachstums der Grünalge *Desmodesmus subspicatus* in Abhängigkeit von der Ozondosis bestimmt, um daraus den verbleibenden Effekt mit der Bildung von TPs zu korrelieren. Die anti-östrogene Aktivität blieb bei zunehmender Ozondosis, auch nach vollständiger Reduktion der TAM-Konzentration, erhalten. Der verbleibende Effekt konnte mit der Bildung von TP 270 korreliert werden. Die Bildung von *N*-Oxiden konnte hingegen mit einer Abnahme der anti-östrogene Aktivität korreliert werden. Die Immobilisierung von *D. magna* wurde bereits bei geringen Ozondosen komplett aufgehoben. Dahingegen wurde eine Zunahme der Wachstumshemmung von *D. subspicatus* mit steigender Ozondosis beobachtet. Auch hier korrelierte die Effektzunahme mit der Bildung von TP 270, aber auch TP 388. Bei pH 3 resultiert die Ozonierung von TAM in einer Verdopplung der anti-östrogenen Aktivität sowie auch der Hemmung des Algenwachstums. Hier war das Hauptprodukt TP 270, welches als 4-(Dimethylaminoethoxy)-benzophenone identifiziert wurde. Auf Grund des erhöhten Risikopotentials von TP 270 wurde eine neue Aufreinigungsstrategie erfolgreich entwickelt, um TP 270 hoher Reinheit für weitere Toxizitätstests zu gewinnen.

In dieser Studie wurde die Reaktion von Ozon mit dem SERM TAM sowie die Bildung von primären und sekundären TPs untersucht. Zudem konnte die Bildung toxikologisch relevanter TPs nachgewiesen werden. Dadurch kann zu einem verbesserten Verständnis über die Beständigkeit anti-östrogener Effekte beim Einsatz von Ozon in der erweiterten Abwasseraufbereitung beitragen. Weitere Studien zur Ozonierung von SERMs, inklusive deren Metabolite, der Bildung von TPs in Abwasser und deren Entfernung durch nachgeschaltete biologische Filtration werden benötigt, um dieses Verständnis weiter auszubauen.

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1 General Introduction

1.1 Preface

Organic anthropogenic compounds can be found in freshwater systems almost worldwide, including industrial chemicals, household chemicals, personal care products and pharmaceuticals [1-3]. Since observed concentrations are most often in the range of µg L^{-1} or lower, they are referred to as organic micropollutants [4] or trace organic compounds (TOrCs). Many of these micropollutants can already induce considerable toxicological effects in the concentrations found in the environment or bioaccumulate [5]. Not only organisms that are exposed directly in the environment can be effected by micropollutants. It can also be a hazard for humans, since the freshwater systems are the main source for drinking water [6] and long term effects are mostly unknown [7]. However, a direct toxic effect on human health seems unlikely [8] as long as an adequate drinking water treatment is applied. Hence, the increasing contamination of freshwater is one of the key environmental problems humanity is facing [9].

The major sources for micropollutants in the aquatic environment are the release of (treated) wastewater and surface run-offs. Conventional biological wastewater treatment can remove some micropollutants only partly or not at all [10, 11]. Thus, the development and implementation of appropriate and cost effective strategies to minimize the release of micropollutants into the environment is currently a scientific, but also political and societal challenge, including the implementation of advanced wastewater treatment technologies after the biological treatment, minimization of usage, safe disposal, and substitution by more ecofriendly compounds [9, 12, 13].

Technologies from drinking water treatment such as sorption on activated carbon or oxidative processes can be used for a more comprehensive micropollutant removal [14, 15]. Based on removal of residues and also economic aspects activated carbon and ozonation are the most favored advanced treatments [16, 17] and are tested and implemented in full scale for advanced wastewater treatment [18-22]. Even the possibility for direct water reuse was successfully shown [23]. Other investigated advanced treatment options are specified biological reactors, UV-irradiation, and processes aiming at the in-situ formation of hydroxyl radicals, so called advanced oxidation processes (AOPs) [24, 25].

Although oxidative processes are a promising tool for the removal of micropollutant residues [18, 26], there is a crucial aspect that needs to be considered: in the reaction of micropollutant and oxidant new compounds are formed. These are either referred to as by-products or as transformation products (TPs) and only few are known [27]. These unknown TPs cannot be monitored using target chemical analysis and are usually not included in the evaluation of advanced treatments [28]. TPs can proliferate the effect of the original micropollutant or can induce even stronger and/or other toxicological effects [3]. Consequently, the identity of formed TPs, their precursors, and the toxicological potential of the TPs need to be known. The most noted (by-) products formed during ozonation are *N*-nitrosodimethylamine (NDMA) and bromate, both carcinogenic compounds [29, 30]. Hence, their formation potential needs to be investigated if a wastewater treatment plant (WWTP) shall be equipped with ozonation [31].

The evaluation of the efficiency of the advanced wastewater treatment should also be based on the reduction of (eco-)toxicological effects [32]. Here, a wide variety of possible effects can be monitored, including acute and chronic toxicity for different organisms (*in vivo*) and cell based assays (*in vitro*) (i.e., genotoxicity, cytotoxicity, neurotoxicity,…) [33, 34]. *In vitro* assays also might allow an extrapolation of sub-lethal effects as long term effects on organisms and populations. These also include interactions with hormonal systems, called endocrine effects [35]. These are of high interest, since the hormonal system of organisms can be easily disturbed. Additionally, a huge variety of compounds can affect the hormonal system already in low concentrations [36]. The most investigated endocrine effects caused by micropollutants are estrogenic and androgenic effects [37-39]. In the last years, also the corresponding antagonistic effects were monitored for water treatment [40, 41]. Micropollutants with a known estrogenic or androgenic potency were shown to be removed by ozonation, as well as the corresponding estrogenic and androgenic effects [42]. However, anti-estrogenic effects can still be found after the ozonation of wastewater [39] and the transformation of anti-estrogenic micropollutants by ozonation has not been thoroughly investigated.

1.3 Ozone

Ozone is an electrophilic oxidant [43] and reacts quickly with electron-rich moieties such as tertiary amines, olefins, and activated aromatic compounds [44]. It also reacts with the bases of the deoxyribonucleic acid (DNA) and has therefore to be considered mutagenic and genotoxic [45]. Additionally, it can cause cell lysis in microorganisms [46] and hence has been used for disinfection in water treatment since the beginning of the 20th century [44]. With an increasing concern in the beginning of the 21st century on micropollutants in the aquatic environment and therefore also in our drinking water resources , ozone was successfully tested and implemented as oxidative treatment for the removal of micropollutants [21, 26, 47]. However, micropollutants are not fully mineralized at typical ozone doses applied in water treatment but transformation products are formed [27, 48]. Hence reactions of ozone with micropollutants in aqueous solutions are currently intensively investigated.

1.3.1 Ozone reaction in aqueous solutions

Since hydroxide ions can initiate ozone decomposition in water, the pH strongly contributes to the stability of ozone in aqueous solutions [49]. The decomposition of ozone in water leads to the formation of hydroxyl radicals, which are strong and nonselective oxidants and can be used for oxidation purposes as well [50]. Hydroxyl radicals can also be formed during the reaction of ozone and fractions of the dissolved organic matter (DOM) in waters, but can also be scavenged by other fractions of the DOM and do not strongly contribute to the abatement of micropollutants during ozonation of wastewater [44, 49].

As electrophilic oxidant, ozone can react with a wide range of compounds in aqueous solution and via three main reactions: (i) electron-transfer, (ii) substitution/elimination, and (iii) electrophilic addition [51]. For organic micropollutants the reaction of ozone with olefinic, aromatic, nitrogen and sulfur containing moieties are most important and are shown in Figure 1-1 and Figure 1-2. Olefins (i) react according to the Criegee reaction (1+2) [52] and subsequent reaction with water (3), resulting in the formation of ketones and/or aldehydes and hydrogen peroxide [53]. First step in the reaction of ozone with aromatic moieties (ii) is the adduct formation (3), followed by either a Criegee reaction (5) and ring opening (6), formation of a radical cation by dissociation (7), or hydroxylation (8-12). For the hydroxylation either the release of oxygen (8) and subsequent hydrogen shift (9), or hydrogen abstraction (10) and subsequent oxygen release (11) followed by protonation are assumed. The radical cation can also be

formed by direct electron transfer [44, 54, 55]. However, benzene itself reacts quite slowly and hence, aromatic moieties need to be activated by substituents for the reaction with ozone [54]. Nitrogen containing moieties have to be differentiated due to variations of reaction pathways. Ozone reacts with amines (iii) via adduct formation (12). The ozone adduct leads to the formation of an *N*-oxide by either decomposing into radical ions (13), which further react in a cage reaction (14), or by loss of singlet oxygen ($^1O_2$) [56]. *N*-oxides of tertiary amines are stable, for primary and secondary amines a rearrangement to the corresponding hydroxylamines follows quickly [44]. For aliphatic amines the formed amine radical cation can also undergo desalkylation (15,16) [44]. Aromatic amines or anilines (iv) can either react with ozone at the amine moiety or at the activated aromatic ring [57]. However, ring addition is mainly observed for aniline [44]. In addition to the reactions for aromatic moieties (ii) ring opening in the ortho- & meta-position can cause a rearrangement and formation of an *N*-heterocylclic ring (not shown). Ozone addition to the ring can also form a cationic radical (17, 18), which is stabilized at the nitrogen. This radical might also be formed by direct electron transfer. Anilines can also form *N*-oxides corresponding to the reaction with non aromatic amines (12-14). However, nitrosobenzene can also be formed due to ozonation of aniline (19- 21) [57]. For *N*-heterocyclic moieties (v) the main observed reaction leads to the formation of the corresponding *N*-oxides (23) via adduct formation (22), though other reactions such as electron transfer to the ring or the nitrogen, and addition reactions (hydroxylation) to the ring or the Criegee reaction are possible [58]. Sulfur moieties (vi) such as sulfides, disulfides, and sulfuric acids can react with ozone. Here, adduct formation (24) and subsequent oxygen loss yield an oxygen transfer to the sulfur [59]. Fully oxidized sulfur moieties, e.g. sulfates do not react with ozone [44].

Since such electron rich moieties are also present in DOM, most ozone applied for the treatment of surface water and wastewater is consumed in the reaction with DOM. For the abatement of micropollutants the reaction rate has to be high enough to compete with the ozone consumption by the DOM. Hence, the second order rate constants of micropollutants have to be $k > 10^4$ M^{-1} s^{-1} for full abatement at pH 7 with an ozone dosage of 0.5 g O_3/g DOM [18] during wastewater ozonation. Compounds with second order reaction rates $k < 10^2$ M^{-1} s^{-1} are considered refractory to ozonation as advanced water treatment.

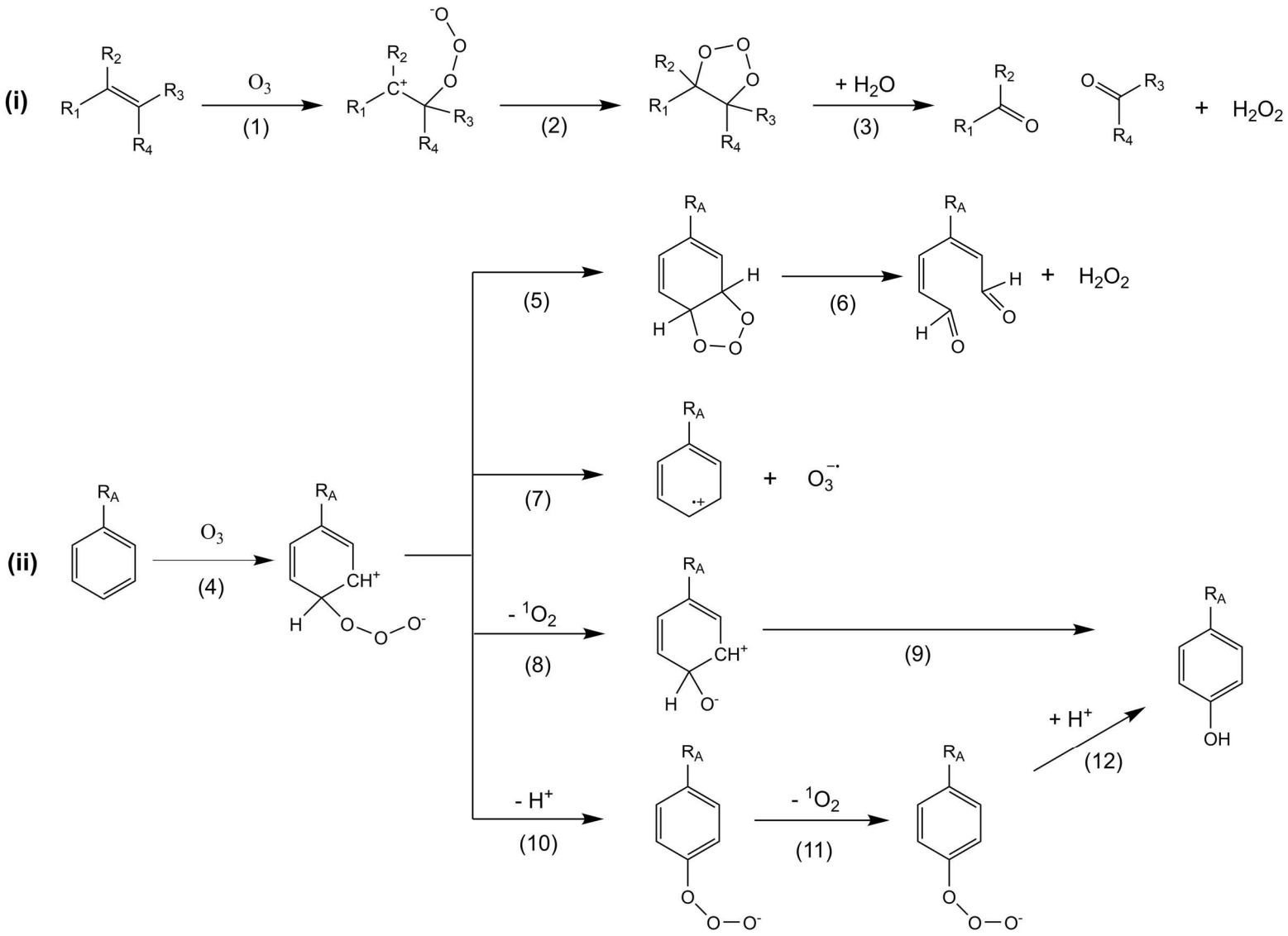

Figure 1-1: Reactions of ozone with (i) olefins, (ii) aromatic moieties.

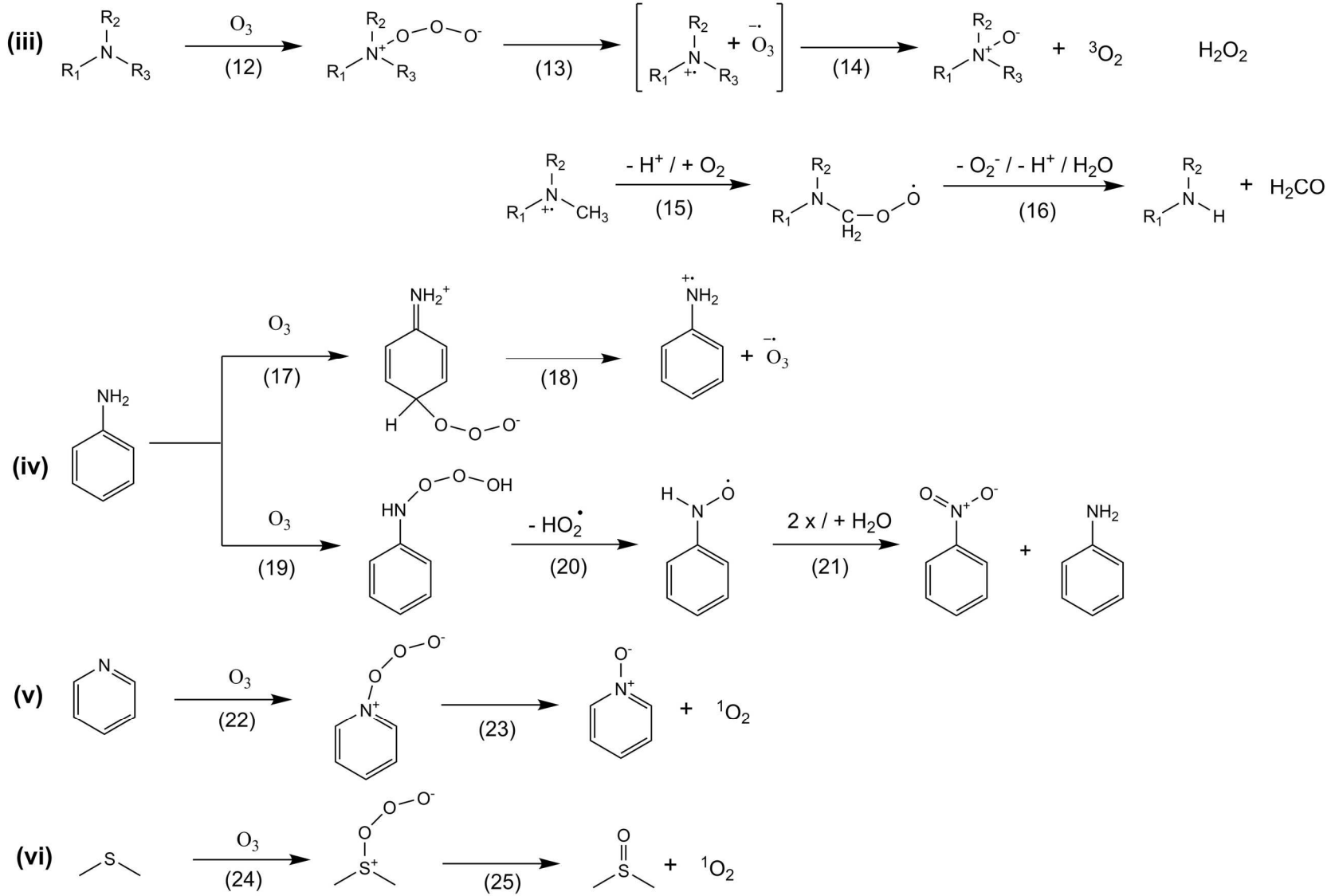

Figure 1-2: Reactions of ozone with (iii) amines, (iv) aromatic amines, (v) N-heterocyclic moieties, and (vi), sulfides.

The reaction kinetic for each moiety is strongly influenced by substituents that increase (activating) or reduce (deactivating) the electron density [53] of the moiety and therefore increase or decrease the energy of the highest occupied molecular orbital (HOMO) of the reactive moiety [60], respectively. The influence of substituents on the activation of aromatic rings can be estimated based on Hammett constants [43, 61].

In addition to the substituents, the pH strongly influences the reaction rate for the reaction of a compound with ozone, if the speciation of the reactive moiety itself (e.g. amines) or the electron donating substituent (e.g. phenol) is pH dependent [62]. In general, protonation of a compound decreases the reactivity towards ozone since the electron density is decreased due to the formation of a covalent bond with the proton. For example, triethylamine has a second order rate constant *k* of 4.1 – 5.1 x 10^6 M^{-1} s^{-1} [56, 63] whereas the second order rate constant *k* of the corresponding acid is 5 ± 4 M^{-1} s^{-1} [63]. The second order rate constant of phenol is also 6 orders of magnitude lower than for the corresponding phenolate ion [62]. A short overview of typical ranges for the above mentioned moieties is given in Table 1-1.

Table 1-1: Ranges for second order reaction rates of various moieties according to Sonntag & von Gunten (2012) [44].

Moiety	**Speciation**	**k / M^{-1} s^{-1}**
olefins		10^3 – 10^6
aromatic rings		10^{-1} - 10^9
phenols	neutral	10^3 - 10^5
	anionic	10^7 – 10^9
nitrogen containing comp.		10^1 - 10^7
anilines	neutral	10^5 - 10^9
amines	neutral	10^3 - 10^7
	protonated	< 1
sulfur containing comp.		10^3 - 10^6
C-H functions		< 100

The apparent second order rate constant $k_{\mathrm{O_3}+M_{\mathrm{total}}}$ can be calculated based on the species specific second order rate constant $k_{\mathrm{O_3}+\mathrm{i}}$ and the fractions f of all species i present according to Equation 1-1 [62].

Equation 1-1:

$$k_{O_3+M_{\text{total}}} = \sum_{i=1}^{n} (f_i * k_{O_3+i})$$

Due to the high variations of the species specific second order rate constants for a moiety, e.g. phenol, the dissociation of a compound can determine the degradation rate during water treatment and, in case of multiple reactive moieties present in the compound, also the reaction pathway [64, 65]. Hence, knowledge of the exact dissociation constant K_a of an ionizable compound is essential [66].

Though many reaction pathways of ozone reactions in aqueos solution have been thoroughly investigated, reactions of ozone and some moieties are still under investigation [67, 68]. However, prediction of micropllutant depletion has become highly reliable [60, 69] and the knowledge of ozone reactions enhances the identification of ozonation derived TPs and their structural validation [65, 70]. For several compounds the reaction kinetic and the formation of TPs has been investigated [64, 65, 70-78] to predict degradation during water treatment and the proliferation of (eco)-toxicological effects by TPs. The ozonation of antimicrobial compounds can reduce the antimicrobial activity [72, 79-81] and other biologically active functional groups [82] though also the simultaneous formation of bioactive TPs has been reported [73, 80]. Hence, the formation of TPs may cause toxicological effects that are not induced by the parent compound [27] and prediction of effect removal based on the removal of the parent compound only covers specific effects with well-known mechanisms.

The ozonation of endocrine active compounds has been widely reported but is till now mainly focused on estrogenic compounds [71, 83-85]. Here, the reaction with ozone causes a removal of the estrogenic effect due to the high reactivity of the phenolic moieties found in these compounds, which is essential for the interaction with the estrogen receptor (ER) [86]. Until now, only few data on the ozonation of selective estrogen receptor modulators (SERMs) or anti-estrogenic compounds are reported. For tamoxifen (TAM) a single apparent second order rate constant of 3.56×10^6 M^{-1} s^{-1} has been reported [87] but no species specific reaction rates were determined. Another study investigated the formation of TPs [88]. However, the TP structures suggested in [88] are not feasible since the reactivity of the tertiary amine has been completely neglected, and the experimental set up did not allow a thorough

investigation of reaction pathways for ozonation. Knowledge of the reaction pathways is essential to understand the progress of the endocrine effects in the ozonation of TAM.

1.3.2 Ozone for wastewater treatment

Full scale ozonation for wastewater treatment as additional treatment step after the biological treatment is already implemented at several WWTPs in various countries [21, 23, 28, 89]. Since the load of DOM is in general higher than the load of micropollutants the majority of the applied ozone is consumed by reactions with ozone. For this reason, the applied ozone dose is in general adapted to the DOM load, measured as dissolved organic carbon (DOC) to obtain a ratio of 0.4 – 1.2 g O_3/ g DOC to gain a sufficient reduction of the non-ozone-refractory micropollutants [18, 20, 42, 90]. Hydroxyl radicals formed in the reaction of ozone and DOM are short lived and hence for less than 30 s available for reacting with micropollutants [85]. Only a minor fraction of the micropollutant attenuation is related to the degradation by hydroxyl radicals [44]. Ozonation of DOM and micropollutants leads to the formation of biologically more accessible TPs and by-products, such as aldehydes, ketones and organic acids [91]. Also for full-scale ozonation the formation of bioactive TPs [20, 89] and toxic by-products such as NDMA and bromate are reported [28-30]. Subsequent biological active filtration such as sand filtration or biological activated carbon (BAC) removes biodegradable TPs and is hence implemented after full-scale ozonation [19, 20, 92]. Nevertheless, in some cases persistent TPs can be formed during ozonation which are then released into receiving surface waters [27]. Due to the formation of mostly unknown TPs and a possible proliferation or formation of toxicological effects due to ozonation, these effects need to be monitored for a comprehensive evaluation of the wastewater treatment [31, 32, 93].

The estrogenic and androgenic activity in wastewater can be reduced by ozonation and subsequent biological filtration [42, 83]. However, antagonistic effects can be resilient through the whole treatment [39]. Itzel et al. split samples after enrichment into different fractions by liquid chromatography and showed that the anti-estrogenic effect before and after ozonation was induced by different fractions of the samples. They concluded that the original compounds inducing the anti-estrogenic effect were degraded and formed TPs are responsible for the remaining effect [89]. Though the anti-estrogenic compound tris(1,3-dichloro-2-probpyl)phosphate (TCEP) was identified

in this study, formation of this compound due to ozonation is highly unlikely. In fact, identification of endocrine antagonistic TPs has not been investigated yet.

1.4 Ecotoxicological evaluation

Micropollutants can induce a wide range of different ecotoxicological effects. Acute species specific toxicities are most often known for these compounds though concentrations in the environment and wastewaters are often too low to induce acute toxic effects [94, 95]. However, acute toxic effects can be induced by a broad range of modes of actions and are hence rather unspecific. Specific and mostly sub-lethal effects, such as genotoxicity, mutagenicity, endocrine effects, neurotoxicity, and antibiotic effects can either be investigated based on biomarkers from exposed organisms or in *in vitro* assays [33, 34]. These sub-lethal effects, including endocrine activity, are also of high interest due to the long-term exposure for aquatic organisms [96]. Endocrine effects may therefore not solely affect individual organisms, but can harm the overall population due to changes in sex ratio and reproduction, as reported for estrogenic compounds. Here, in a 7 year study Kidd et al. showed that fish populations can be heavily affected by being exposed with endocrine active compounds at the lower ng L^{-1} range [96]. The fathead minnows (*Pimephales promelas*) population of a lake was near extinction by exposure of 17α-ethinylestradiol by affecting reproduction in females and feminization of males. This highlights the risk of endocrine active compounds onto aquatic populations.

The toxicity of compounds is stated as effect concentration EC_X, with X equaling the percentage of the observed effect, resulting from toxicity testing using multiple concentrations. Ideally, concentrations used in the test range from no effect induction to full effect induction. The effect concentration 50 (EC_{50}) is most often stated, as it represents the concentration with an effect of 50 %, e.g. for acute toxicity 50 % of the tested organisms die. The no observed effect concentration (NOEC) is the highest actually tested concentration at which no effect significantly deviating from the control is observed and is hence not calculated. Therefore, the NOEC is strongly dependent on the tested concentrations and therefore the test design. Nevertheless, NOEC values are most important for the ecotoxicological assessment of compounds, as they can be used to derive limits of concern. To evaluate the ecotoxicological potential of a compound, determining the toxicity for single biological species is insufficient [97], but testing the toxicity for all species is simply not feasible, due to the high variety of species. Hence, the ecotoxicological assessment is based on the lowest reported

NOEC value for all species obtained during long term tests ($NOEC_{lowest}$) and is divided by an assessment factor according to Equation 1-2. The risk assessment factor (RAF) depends on the trophic levels (primary producer/ invertebrates/ vertebrates) covered with ecotoxicological data. For NOEC values available only for one trophic level a factor of 100 is implemented, a factor of 50 for two trophic levels, and a factor of 10 if NOEC values are available for all three levels [97]. This concentration is then stated as predicted no effect concentration (PNEC) for the corresponding compound. At environmental concentrations below the PNEC, no effects in the environment are expected.

Equation 1-2:

$$PNEC = \frac{NOEC_{lowest}}{RAF}$$

The risk characterization of a substance is then based on the ratio of actually measured or predicted environmental concentration (MEC or PEC) and PNEC (MEC/PNEC or PEC/PNEC) for each compartment (water/sediment/soil) [97].

Next to the toxicity of a compound also the persistency, bioaccumulation, and mobility, need to be considered for an ecotoxicological risk assessment [97].

However, data for an ecotoxicological characterization of individual micropollutants are often limited in the literature and monitoring of all micropollutants is not feasible [34]. For this reason, effect-based monitoring tools are considered for implementation in the European regulation of water quality assessment [98] and a variety of bioanalytical test batteries were developed for a more comprehensive ecotoxicological water quality monitoring [31-34, 95, 99, 100].

The investigation of product formation during degradation processes and the risk assessment of the formed TPs is important for a more comprehensive risk assessment of the parent compounds [101]. Shortly, two different approaches are considered: (i) TPs known to be formed with yields of more than 10% of the signal of the precursor in a transformation process should be tested for toxicity, or (ii) the toxicity of mixtures is assessed to determine the overall contribution of TPs to the environmental risk. In case of toxicity induced by TP mixtures, effect inducing TPs can be identified by subsequent chemical analysis. This approach is the so called effect directed analysis (EDA) [102] and is most effective to identify TPs proliferating toxicological effects of

the parent compound. EDA has been successfully applied to a wide variety of compounds, transformation processes and complex environmental samples [72, 73, 80, 84, 95, 101, 103]. On the other hand it has to be highlighted that effects of micropollutants at environmental concentrations can only be observed using assays focusing on specific effects [103]. Acute toxic effects are normally not induced at these concentrations. Therefore, in environmental samples EDA is solely applicable for specific effects such as endocrine activity or genotoxicity [104].

1.5 Selective Estrogen Receptor Modulators

Various chemicals of different origin are known to interact with the estrogen receptor (ER) and can either induce or block the transcription of the estrogen gene [105]. One group of pharmaceuticals, the so called selective estrogen receptor modulators (SERMs), has been developed to intentionally either induce or inhibit the ER depending on the tissue and receptor type (α/β) [106]. SERMs can be used to inhibit the regrowth of tumorous breast cells and represent the standard treatment in ER-positive breast cancer therapy. Here, SERMs are usually applied over periods of 5 to 10 years [107, 108].

1.5.1 Tamoxifen and derivates

The nonsteroidal SERM tamoxifen (TAM) is considered as the ‘gold standard’ or ‘first-line treatment’ for breast cancer therapy in women [106, 108]. Various derivates were developed based on the triphenylethylene structure of TAM, such as toremifene (TOM), ospimefene, droloxifene, idoxifene, and clomifene with minor deviations to the structure of TAM [107, 108], as shown in Figure 1-3.

The pharmacokinetic of TAM has been summarized by Morello et al. in 2003 [108] and will only be stated here shortly. TAM is nearly 100 % bioavailable due to the rapid absorption in the gastrointestinal tract based on the high lipophilicity of the substance. During phase I metabolism in the liver, 4-hydroxytamoxifen (4-OH-TAM) and *N*-desmethyltamoxifen are formed by cytochrome P450 (CYP) enzymes. *N*-desmethyltamoxifen can be further metabolized by CYP2D6 to 4-hydroxy-*N*-desmethyltamoxifen (endoxifen). 4-OH-TAM and endoxifen are considered as the activated metabolites of TAM because their antagonistic activity is approximately 100 fold more potent [108]. However, plasma concentrations of 4-OH-TAM in patients are one to two orders of magnitude lower than those of TAM [109] and only endoxifen is found in similar concentrations in the serum compared to TAM [110, 111].

Unfortunately, CYP2D6 is strongly dependent on the individual human genotype and therefore the genotype predetermines the efficiency of TAM therapy in individuals [110, 112].

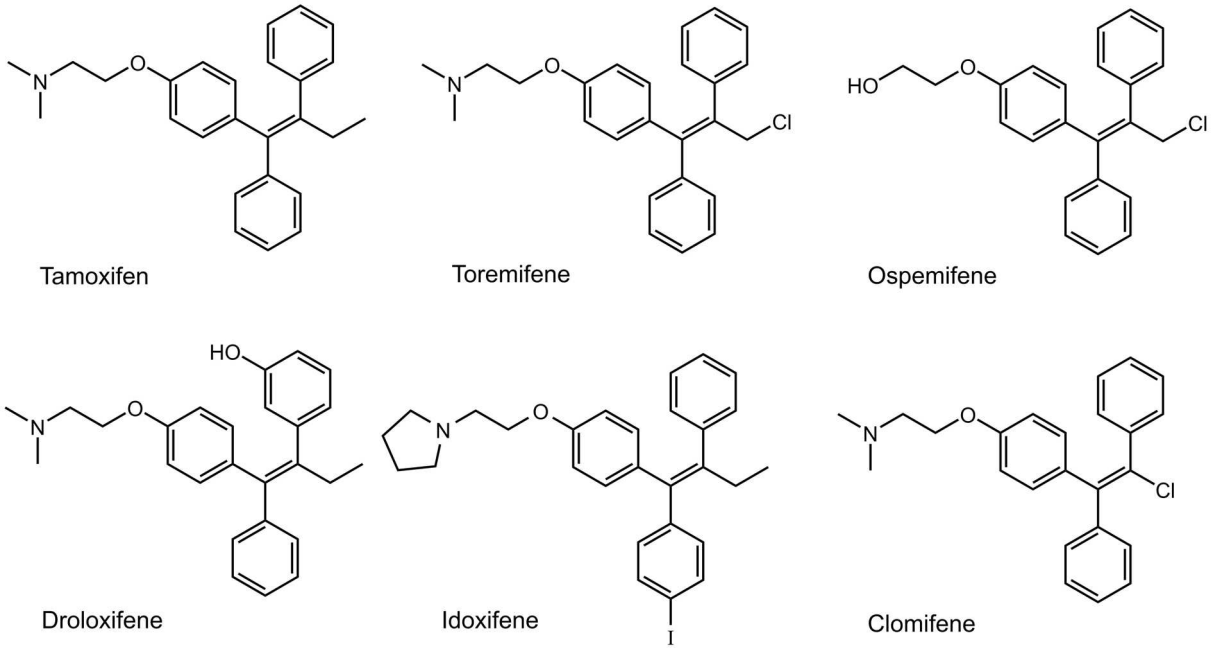

Figure 1-3: Structures of the triphenylethylene based SERMs TAM, TOM, ospimefene, droloxifene, ioxifene, and clomifene.

Further SERMs are benzothiophene derivates, such as raloxifene or arzoxifene, and are less similar to the structure of TAM [108, 112].

During phase II metabolism glucuronidation by UDP-glucuronosyltransferases (UGTs) or sulfation by sulfotransferases (SULTs) of phase I metabolites enables excretion in the bile [110, 113]. Hence, the main elimination route is via feces [109, 110, 112] and also the unchanged parent compound can be observed in small fractions within the feces [114]. Further, tamoxifen-*N*-oxide (TAM-*N*-oxide) is formed during phase I metabolism, which can be reduced back to TAM by CYP enzymes [115] and accounts to less than 15 % of TAM in the patient's blood plasma [111]. It has to be stated that 4-hydroxlated metabolites undergo isomerization to the respective cis isomers, cis-4OH-TAM and cis-endoxifen, both acting as weak estrogenic antagonists [110]. A further phase I metabolite observed is alpha-hydroxytamoxifen (alpha-OH-TAM) which is sulfonated during phase II metabolism. This sulfonated metabolite can form DNA-adducts [110] and is hence genotoxic. In long term patients an increased risk of endometrial cancer has been observed. However, this risk is still outweighed by the benefit of tamoxifen therapy for breast cancer patients [116]. Phase II metabolism

results in elimination of the anti-estrogenic activity [113]. A comprehensive overview of the human metabolism pathways for TAM is given in Figure 1-4.

1.5.2 Interaction with the Estrogen Receptor

Depending on the target tissue and the ER type (ERα and ERβ), SERMs can either act as agonist or antagonist due to the formation of different ligand-ER-complex structures, cofactors, and target genes in the respective cell type [86, 107, 117, 118]. To form ligand-ER-complexes, SERMs have to be structurally similar to natural estrogens, which are small, unpolar, and contain a terminal phenol moiety, such as estradiol. The phenol moiety binds to the ligand binding domain (LBD) located within a hydrophobic cavity of the ER [119]. Each ligand induces a distinct conformational change of the helix H12 covering both, the LBD cavity and the ligand. This conformational change enables binding of a variety of cofactors at the activation function 2 (AF-2) area of the ER in a ligand specific manner [105]. The bound cofactors, or coregulators, then induce the ER-regulated DNA-transcription [106]. The side chains of SERMs can either physically block the conformational change of H12 [119] and/or due to the terminal basic moiety present as cationic species at pH 7 form an ionic bond to aspartate 351 (ASP 351) of the ER. ASP 351 is in case of agonist binding required for stabilization of the conformational change of H12 and thereby for enabling the AF-2 [118, 120]. Hence, AF-2 is blocked in presence of an antagonist, but a stabilized ligand receptor is formed and no agonist can bind to the ER, resulting in the inhibition of estrogenic activation.

The difference for agonist and antagonist binding to the ER is illustrated in Figure 1-5 with the ligands estradiol as agonist and TAM as antagonist. Since the LBD of the ER is specific for phenol moieties, the weak antagonistic effect of TAM itself and the metabolic activation by hydroxylation in the 4 position in 4-OH-TAM [110] can be explained due to highly different affinities to the ER [121].

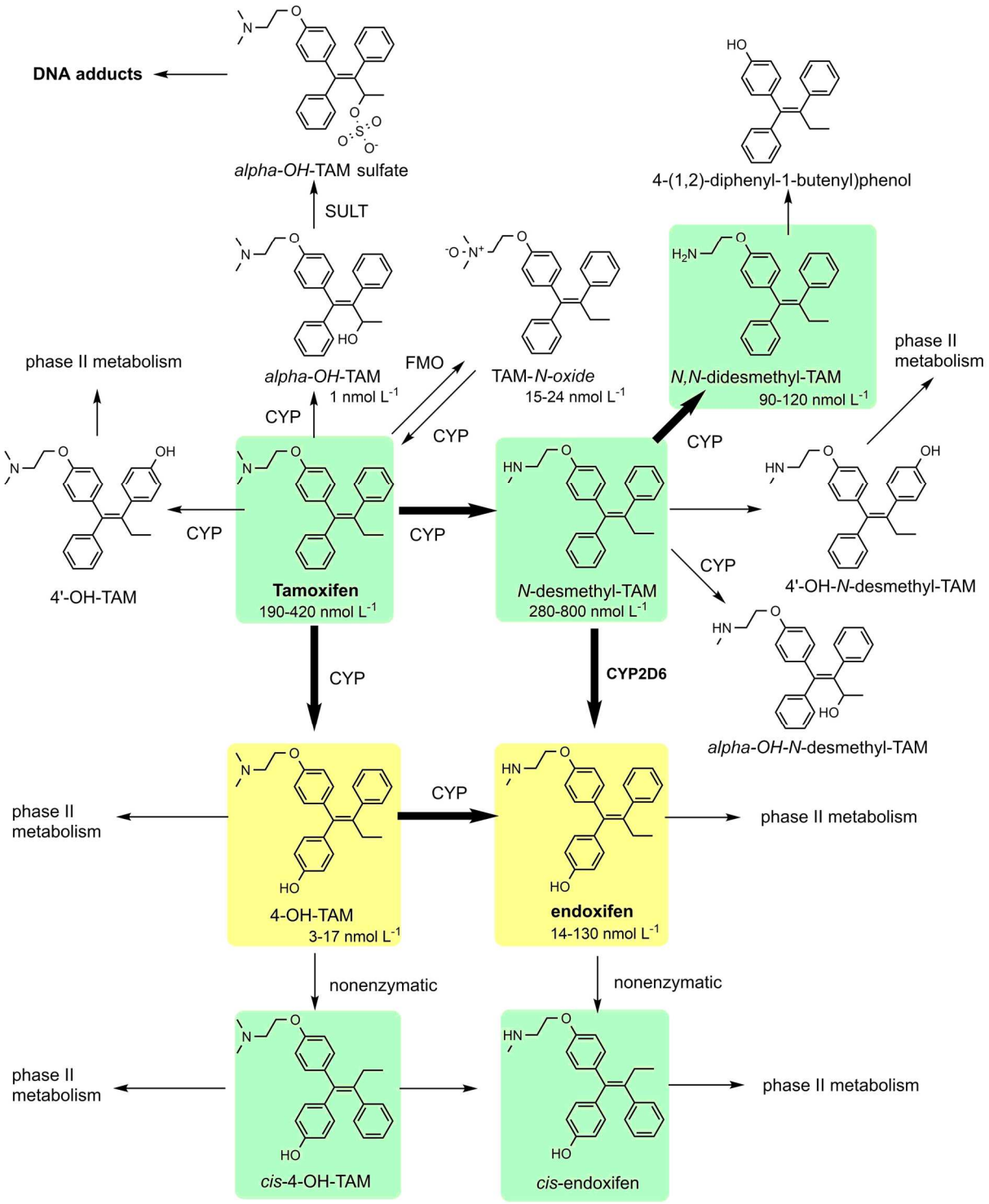

Figure 1-4: Metabolic transformation pathways for tamoxifen (TAM) in humans according to Brauch et al. [110]. Major pathways are highlighted with bold arrows. Weak anti-estrogenic active compounds are highlighted in green, strong antagonists highlighted in yellow. For not highlighted metabolites no interaction with the ER is known or reported. If available, mean plasma concentrations in treated patients are given next to the compound name. 4-OH-TAM: 4-hydroxytamoxifen; CYP: cytochrome P450; FMO: flavin-containing monooxygenase; SULT: sulfotransferase.

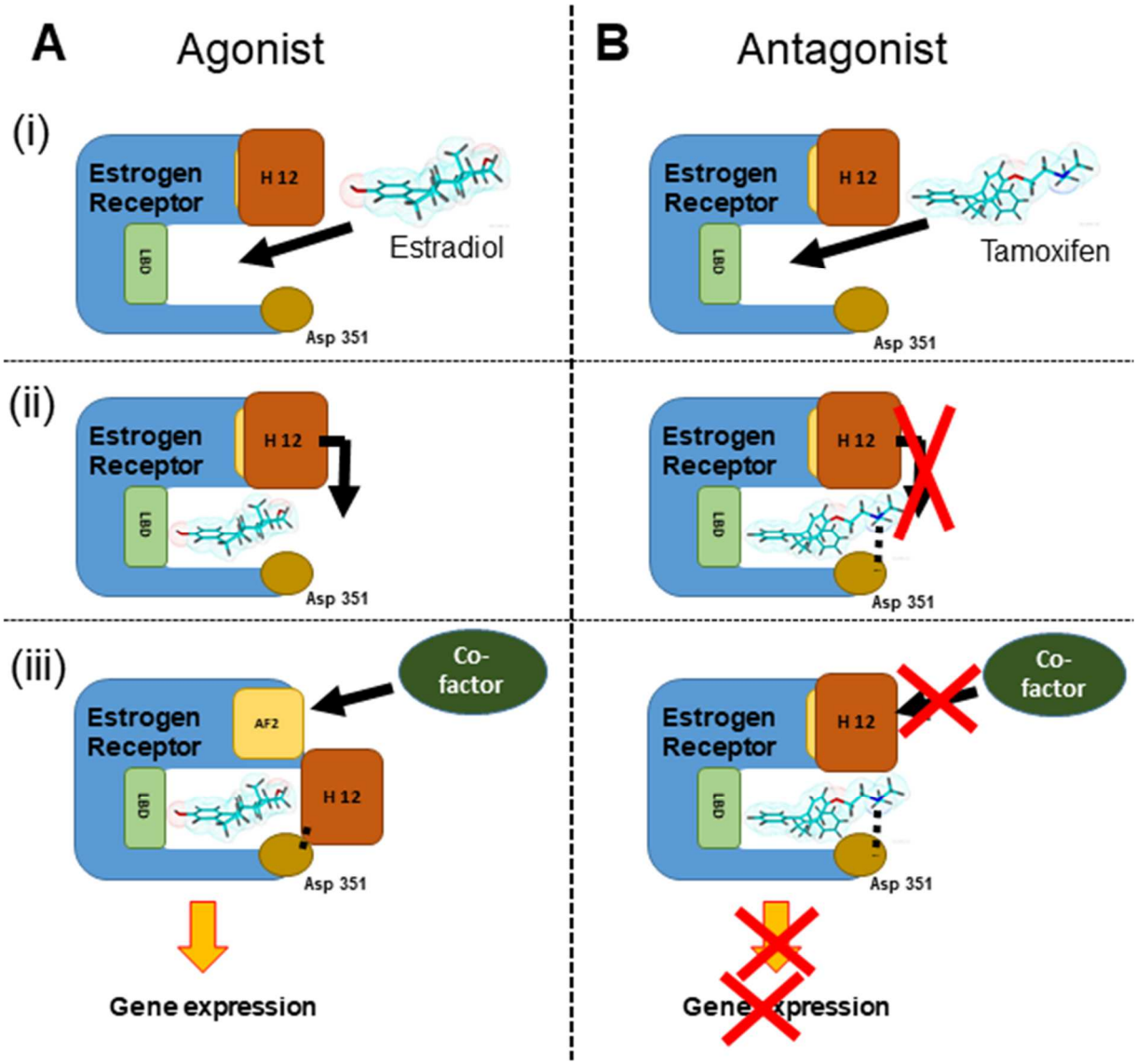

Figure 1-5: Interaction of (A) an agonist and (B) an antagonist and the estrogen receptor. For agonist: (i) binding to the ligand binding domain (LBD), (ii) conformational change of the helix H12 and stabilization by ASP 351, (iii) binding of cofactor to activation function-2 (AF-2) region and subsequent induction of gene expression. For antagonist: (i) binding to the LBD, (ii) conformational change of H12 is blocked and stabilization of antagonist by formation of an ionic bond to ASP 351, (iii) stable ligand-receptor complex, AF-2 region not accessible for cofactor, no induction of gene expression.

1.5.3 Sources and occurrence in the environment

Though only minor fractions of TAM are excreted unchanged by humans [114], tamoxifen can be found in WWTP influents [10, 122, 123], treated wastewater (WWTP effluents) [124, 125], and in surface waters [126-132]. Municipal, manufacturer and hospital wastewaters can be considered the major sources for TAM into the

environment since TAM passes conventional wastewater treatment [123]. In more than 120 countries TAM has been used for breast cancer therapy [112] and can sometimes be observed in the majority of surface water samples [132], if monitored. However, only few monitoring studies include TAM as target, although it was highlighted for monitoring of hospital effluents in 2012 due to its' bioaccumulation potential (>5000 at pH 7) and endocrine activity [133]. A short overview of all reported concentrations for the environment and WWTP effluents are given in Table 1-2.

Table 1-2: Published measured and predicted concentrations of tamoxifen in surface waters (mainly WWTP effluent receiving rivers), and WWTP effluents. * detected in karst aquifer.

	Surface water		**WWTP effluent**		
	TAM concentration / ng L^{-1}		TAM concentration / ng L^{-1}		
Country	measured	predicted	measured	predicted	Source
Sweden		36			[134]
Sweden	13		5 – 210		[127]
England	< 10	63	42		[124]
England	13 – 71				[128]
England	< LOD		0.2 – 0.7		[125]
England	27 – 212		146 – 369		[126]
Norway	66				[129]
France	22 – 25	7	53 – 102	22	[130]
France		9			[131]
Spain	12 – 26.8				[132]
Spain	12 – 38		11 – 42		[123]
Germany *	6-16				[135]

The measured environmental concentrations (MECs) in surface waters are in the same range as the predicted environmental concentrations (PECs) [124, 130, 134]. These PECs are calculated based on consumption or prescription data, excretion of the substance, WWTP removal efficiency, and population data for defined areas, mostly countries, according to a European guideline [136]. Region independent data are the excretion value and the removal efficiency by conventional WWTPs. However, excretion values and removal efficiencies were not available or were neglected [124, 131, 134], except for the lowest reported PEC of 7 ng L^{-1} [130]. Here, an excretion factor of 0.3 was included for the fraction of the active compound, and no removal

during conventional wastewater treatment was applied. In this study the PEC was actually slightly underestimating the MEC [130]. As observed in this study, local or regional MECs can differ from the PEC, since PECs are usually based on country specific prescription/consumption data and not the actual local consumption.

It has to be noted, that although for TAM mainly glucuronidated and sulfonated phase II metabolites should be excreted [113, 137], detectable concentrations of tamoxifen can be observed in WWTP effluents and surface waters, as shown in Table 1-2. For other compounds it is reported that in activated sludge microbial cleavage of glucuronate conjugates can result in the release of the parent compound [138]. Also *N*-oxide metabolites can be reduced back to the parent compound under anaerobic conditions [139-141], although *N*-oxides are normally stable within biological treatment steps [20]. However, for TAM this would mainly implement a release of the activated metabolites 4-OH-TAM, endoxifen, or N-desmethyltamoxifen, and not TAM itself.

1.5.4 Ecotoxicological effects of tamoxifen

Several effects on aquatic organisms for tamoxifen have been reported so far. A comprehensive overview of all reported effects is given in Table 1-3 and Table 1-4. Shortly, TAM can be considered acute toxic for aquatic organisms at concentrations of more than 200 µg L^{-1} based on the EC_{50} [142], but especially influencing the reproduction or sex ratios of various species at concentrations above 0.15 µg L^{-1} – 0.81 µg L^{-1} [143, 144]. The latter effect can be attributed to the anti-estrogenic activity of tamoxifen. TAM affected in a 2-generation test the sex ratio of *Danio rerio* with an EC_{50} of 1.0 µg L^{-1} as 'population relevant endpoint' [143]. Hence, the endocrine effect of TAM onto aquatic organisms and populations is of high interest.

For the manufacturers' data presented in Table 3 [134], it has to be stated that experimental data were not accessible and hence, these ecotoxicological values have to be considered with care. For this reason the manufacturers' ecotoxicology data are discussed separately.

Table 1-3: Ecotoxicological data (NOEC and EC_{50}) for tamoxifen published and reported by the scientific community. # Tests performed with (a) tamoxifen or (b) tamoxifen citrate. Reported values for tamoxifen citrate were recalculated for TAM. * Algae growth inhibition tests with reported EC_{50} values are considered acute toxicity tests according to the European Technical Guidance Document on Risk Assessment [97]. ** NOEC obtained in standardized test, suitable for the calculation of the PNEC. *** NOEC based on trustworthy raw data.

trophic level	Species	Test type	endpoints	duration	guideline	NOEC / mg L^{-1}	EC_{50} / mg L^{-1}	Source	#
invertebrate	*Ceriodaphnia dubia*	chronic toxicity	reproduction	7 d	based on principles to ISO/CD 20665		0.00077	[144]	a
algae	*Pseudokirchinella subcapitata*	acute toxicity *	growth inhibition	72 h	OECD 201		0.98	[142]	a
algae	*Chlorella vulgaris*	acute toxicity *	growth inhibition	72 h	OECD 201		0.61	[4]	a
algae	*Chlamydomonas reinhardtii*	acute toxicity *	growth inhibition	72 h	OECD 201		0.47	[4]	a
invertebrate	*Daphnia magna*	acute toxicity	immobility	24 h	ISO 6341		1.53	[145]	a
invertebrate	*Daphnia magna*	acute toxicity	immobility	48 h	ISO 6341		0.21	[142]	a
invertebrate	*Daphnia magna*	chronic toxicity	reproduction, survival	21 d	OECD 211	0.00067 **		[142]	a
invertebrate	*Daphnia pulex*	chronic toxicity	reproduction	7 d	ISO CD 20665		0.0008	[145]	a
invertebrate	*Daphnia pulex*	multigeneration	reproduction, body length	56 d	non-standard	0.00081		[144]	a
invertebrate	*Brachionus calyciflorus*	acute toxicity	population growth	48 h	non-standard		0.25	[145]	a
fish	*Danio rerio* (zebrafish)	two generation fish full life cycle	sex ratio	155 d	non-standard	0.00051	0.00099	[143]	b
fish	*Danio rerio* (zebrafish)	two generation fish full life cycle	hatching success, growth, reproduction, fertilization rate	155 d	non-standard	0.0018		[143]	b
fish	*Danio rerio* (zebrafish)	two generation fish full life cycle	sex ratio	155 d	non-standard	0.00015 ***	0.00099	[143]	b
fish	*Danio rerio* (zebrafish)	biomarkers	vitellogenin	21 d	OECD 229		0.00198	[143]	b

Table 1-4: Ecotoxicological data (NOEC and EC_{50}) for tamoxifen reported by the manufacturer AstraZeneca [134]. (!) test data not accessible. # tests performed with (a) tamoxifen or (b) tamoxifen citrate. Reported values for tamoxifen citrate were recalculated for TAM. * Algae growth inhibition tests with reported NOEC values are considered chronic toxicity tests according to the European Technical Guidance Document on Risk Assessment [97]. ** NOEC obtained in standardized test, suitable for the calculation of the PNEC.

trophic level	*Species*	Test type	endpoints	duration	guideline	NOEC / mg L^{-1}	EC50 / mg L^{-1}	Source	#
algae	*Pseudokirchinella subcapitata*	chronic toxicity *	growth inhibition	14 d	FDA technical assistance document 4.01	0.065 **		[134] (!)	b
cyanobacteria	*Microcystis aeruginosa*	chronic toxicity *	growth inhibition	21 d	FDA technical assistance document 4.01	0.003 **		[134] (!)	b
invertebrate	*Daphnia magna*	chronic toxicity	reproduction, survival	21 d	FDA technical assistance document 4.09	0.03 **		[134] (!)	b
fish	*Lepomis macrochirus* (bluegill sunfish)	acute toxicity	not specified	96 h	FDA technical assistance document 4.11		0.15	[134] (!)	b
fish	*Oncorhynchus mykiss* (rainbow trout)	acute toxicity	not specified	96 h	OECD 203		0.21	[134] (!)	b
fish	*Oncorhynchus mykiss* (rainbow trout)	acute toxicity	not specified	96 h	FDA technical assistance document 4.11		0.27	[134] (!)	b
fish	*Pimephales promelas* (fathead minnow)	Fish full life cycle test	vitellogenin	284 d	based on US EPA fish life-cycle toxicity tests EPA 540/9-86-137	0.00005		[134] (!)	b
fish	*Pimephales promelas* (fathead minnow)	Fish full life cycle test	reproduction, growth	284 d	based on US EPA fish life-cycle toxicity tests EPA 540/9-86-137	0.003		[134] (!)	b
fish	*Pimephales promelas* (fathead minnow)	pair breeding	fecundity, reproduction	42 d	based on US EPA fish life-cycle toxicity tests EPA 540/9-86-137	0.0037		[134] (!)	b
fish	*Pimephales promelas* (fathead minnow)	pair breeding	vitellogenin	42 d	based on US EPA fish life-cycle toxicity tests EPA 540/9-86-137	0.00012		[134] (!)	b

1.5.5 Ecotoxicological assessment of tamoxifen

Acute effects by TAM onto aquatic organisms can be ruled out because concentrations causing acute effects (Table 1-3) exceed predicted and measured environmental concentrations (Table 1-2) by 3 – 4 orders of magnitude. However, endocrine effects were observed at concentrations higher than 150 ng L^{-1} and up to 212 ng L^{-1} are reported for a surface water, though only a single sample in one study showed this concentration level [126]. Nevertheless, this shows that effects in the environment might actually be induced by TAM.

For TAM two PNECs are already reported. One PNEC is 77 ng L^{-1} for TAM citrate, corresponding to 51 ng L^{-1} of TAM [134] and was calculated according to the guidelines. The second reported PNEC of 200 ng L^{-1} is not based on toxicity data, but calculated using a quantitative structure-activity relationship approach [124]. Since further NOEC values are reported, the PNEC can be calculated according to Equation 2 using the lowest reported NOEC and the risk assessment factor.

Although only standardized tests should be taken into account, the stated NOEC for *Danio rerio* by Knacker et al. [143] can be included, after thorough review of the data, since adaption of trustworthy non-standardized tests is allowed if insufficient data obtained by standardized test is available. Here, two NOECs can be used (RAF = 50) with the lower NOEC at 150 ng L^{-1} [143] and a PNEC of 3 ng L^{-1} is obtained. For a more conservative PNEC, the NOEC reported by Knacker et al. is excluded, as it is not derived from a standardized test and a PNEC of 6.7 ng L^{-1} is obtained by using a risk assessment factor of 100 since the NOEC of only one trophic level (670 ng L^{-1} [142]) can be implemented [97]. The ecotoxicological assessment should be based on the conservative PNEC of 6.7 ng L^{-1}, though both PNEC values are in the same concentration range. For the PNEC reported by the manufacturer reported NOECs are mostly not based on standardized tests and should therefore be neglected if other data are available [97]. If only standardized tests would have been considered here, the manufacturer's PNEC would be 323 ng L^{-1}.

Until now, TAM was considered a 'low environmental risk' with reported MEC/PNEC ratios of > 0.5 [124, 134]. Using the PNEC of 6.7 ng L^{-1} and the measured environmental concentrations of 12 ng L^{-1} or higher [123, 126-130, 132], the MEC/PNEC ratio is ≥ 1.8 and TAM has therefore to be considered as actual environmental hazard [146]. Hence, further investigation concerning possible

ecotoxicological effects, the environmental fate, and exposure assessment, as well as inclusion into monitoring programs of TAM are required [97].

Additionally TAM might be classified as persistent, bio-accumulative and toxic (PBT) substance, as it is an endocrine active substance [114] with a bioaccumulation factor of > 5000 [147]. Persistency in aquatic environments can be assumed due to the ubiquitous presence in a river basin in Spain [132] but has not been thoroughly studied yet. In conclusion, the fate of TAM and mechanisms for the reduction of the emission into the environment are of high interest.

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2 Aims and Scope

The overall aim of this thesis is to investigate the formation of transformation products in the reaction of TAM and ozone and to assess their effects on aquatic organisms. The scope of this work focused on (i) the ozonation of tamoxifen as a function of the pH and the identification of thereby formed transformation products, determination of (ii) anti-estrogenic activity and (iii) acute toxicity to aquatic organisms, both prior to and after ozonation and identification of effect inducing TPs, and (iv) synthesis of the effect inducing TP 270, identified as 4-(Dimethylaminoethoxy)-benzophenone. In accordance with these aims, the work was divided into four chapters, stated below. The relation of the individual chapters is visualized in Figure 2-1.

First, it was necessary to gain knowledge on the reaction of ozone and TAM. Hence, **Chapter 3** aims for the determination of the second order reaction rate constants for TAM and its' derivate TOM as well as for the identification of the TPs formed in ozonation as a function of the pH.

Since TAM is a SERM, the endocrine activity of the TPs formed in ozonation is of high interest. To elucidate this issue, **Chapter 4** investigates the anti-estrogenic activity after ozonation and the correlation of the observed change of effects with the formation of TPs to identify effect inducing TPs.

TAM does not solely induce endocrine effects in aquatic organisms; acute toxicity and chronic effects on several organisms have also been reported for TAM. For this reason, **Chapter 5** focuses on the investigation of the acute toxicity to the model organisms *Daphnia magna* and the green algae *Desmodesmus subspicatus* after the ozonation of TAM, and the identification of effect inducing TPs formed in the reaction with ozone.

Formation of one specific TP in the ozonation of TAM can be related to an increase of the anti-estrogenic activity and green algae growth inhibition, both observed after ozonation of TAM. To allow a further investigation of the ecotoxicological relevance of this TP, **Chapter 6** presents a synthesis route and further purification of TP 270, i.e. 4-(Dimethylaminoethoxy)-benzophenone.

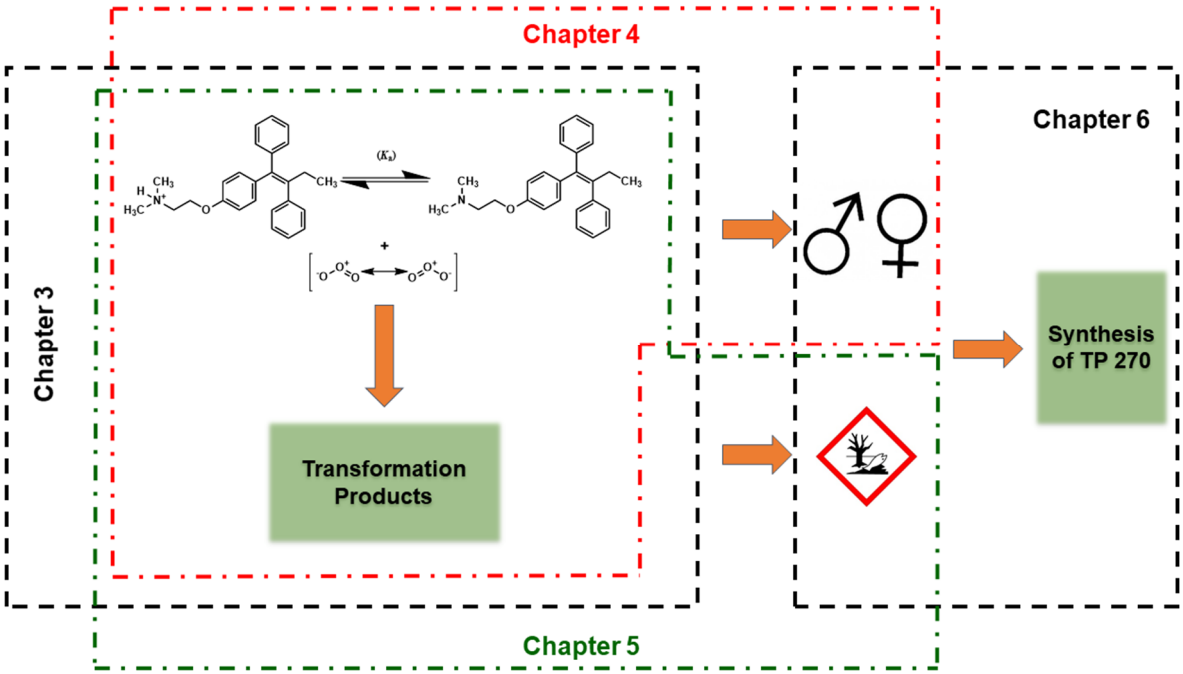


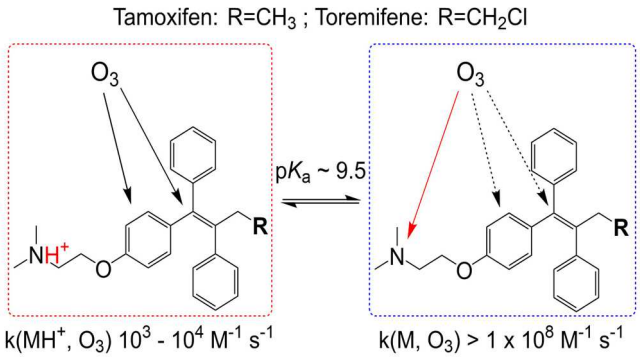
Figure 2-1: Illustration of the aims of this work and their connection. Source GIS pictogram: http://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/pictograms/Aquatic-pollut-red.gif

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

Adapted from: Knoop O., Hohrenk L.L., Lutze H.V., Schmidt T.C., Ozonation of Tamoxifen and Toremifene – Reaction Kinetics and Transformation Products. Submitted to Environmental Science and Technology (2018).

3.1 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 1.57×10^4 M^{-1} s^{-1} for tamoxifen and 4.37×10^3 M^{-1} s^{-1} for toremifene. The free tertiary amine (neutral species) has an unexpected high second order rate constant of 3.17×10^8 M^{-1} s^{-1} for tamoxifen and 1.46×10^8 M^{-1} s^{-1} for toremifene. Here 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 for the first time. Eight transformation products (TPs) were observed and identified based on MS/MS spectra or a reference standard. Primary products observed derived from Criegee reaction and hydroxylation as well as N-oxide formation. Secondary TPs showed to derive from N-oxide formation combined with either Criegee reaction or hydroxylation. Thus, reaction pathways can be derived and primary and secondary TPs distinguished for the first time.



3.2 Introduction

Endocrine disruptive compounds are an important group of micropollutants since they can have biological effects already at low concentrations [1, 2]. Indeed, endocrine effects have been observed in wastewater treatment plant effluents [3]. Therefore these are a major pathway for micropollutants to enter surface waters since most of the micropollutants are not fully degraded in the biological treatment step [4]. Oxidative processes offer an effective additional treatment to reduce micropollutant discharge into surface waters via wastewater treatment plant effluents. Here, ozone (O_3) is broadly discussed and tested in large scale [5-8]. Especially in wastewater treatment O_3 is largely consumed by organic matter. Hence, it is often dosed proportional to the dissolved organic carbon (DOC) content of the wastewater [9]. Second order rate constants are an important tool for assessing the degradation efficiency of micropollutants. Rate constants of $> 1 \times 10^3\ M^{-1}\ s^{-1}$ may result in a complete abatement of pollutants at an ozone dose of 1 g(O_3) per g(DOC) [10]. Another oxidant formed during ozonation is the hydroxyl radical ($^{\bullet}OH$), which also contributes to the degradation of micropollutants that react slowly with O_3 [11].

Even though ozonation does not result in a mineralization of pollutants, their biological effect can be removed (e.g., estrogenic activity [12]). However, some biological effects may persist such as anti-estrogenic activity, which was reported to remain partially, even after further biological treatment following ozonation [3]. Furthermore, ozonation can also result in formation of toxic transformation and by-products such as *N*-nitrosodimethylamin (NDMA) and bromate [13-17].

Tamoxifen (TAM) and its chlorinated derivate toremifene (TOM) are nonsteroidal selective anti-estrogenic estrogen receptor modulators, which are the most effective pharmaceuticals used in therapy to prevent reoccurrence of estrogen receptor-positive breast cancer [18]. These compounds are not removed by biological treatment during wastewater treatment and can therefore enter surface waters by wastewater discharge [19-22]. The highest reported concentration is 369 ng L^{-1} of TAM in a wastewater effluent and up to 212 ng L^{-1} reported in the river Tyne [23] exceeding the calculated PNEC value of 81 ng L^{-1}.

The possibility to degrade TAM in different (advanced) oxidative processes (e.g., ozonation or peroxone process) [24, 25] and by sunlight [26] were previously described in 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 earlier [27] and two further TPs were reported recently [28], but without information for validation of reported structures. Another study reported further TPs with proposed structures, reporting MS/MS spectra for one TP [25]. 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 life [6]. 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 toxicity [25], as well as for TPs from photo-transformation of TAM, showing also slightly reduced toxicity compared to TAM [26]. A recent study also showed that TPs formed during the ozonation of TAM preserve or amplify the anti-estrogenic effect caused by TAM in water [28]. Therefore investigations on formation of TPs as well as reaction mechanisms are of high 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 metoprolol [10]. Due to this pH dependency knowledge of the exact dissociation constant (pK_a) is important [29], 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.

3.3 Materials and Methods

3.3.1 Chemicals

TAM and TOM were purchased at Alfa Aesar (Karlsruhe). Available metabolites were 4-hydroxy-*N*-desmethyltamoxifen and 4-hydroxytamoxifen (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 ≥ 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 ≈ 0.5 – 0.7 mM). In case higher ozone concentrations were needed the solution was cooled using an ice bath (ozone concentration ≈ 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 (ε = 2950 M^{-1} cm^{-1}) [30]. Accuracy of spiking the ozone for batch experiments was demonstrated using the indigo method [10] with an accuracy of ± 5%. In all experiments added ozone reacted until complete ozone consumption.

3.3.2 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].

3.3.3 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 kinetics [33]. 100 mmol TBA was used to dissolve 10 µmol of TAM or TOM in 900 mL ultra-pure water. TBA was simultaneously used as scavenger for hydroxyl radicals formed during ozonation. 10 µmol of the competitor was added and phosphate buffer (preparation modified after Sörensen [34]) 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 µM for each analyte and competitor, and the pH was checked again. Ozone solution was added using glass syringes (Poulten&Graf, Wertheim) to 100 mL samples to obtain 0, 2, 4, 6, 8, 10, 12.5, 15, and 20 µM 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(cinnamic acid, O_3) = 5 x 10^4 M^{-1} s^{-1}, k(cinnamic acid anion, O_3) = 3.8 x 10^5 M^{-1} s^{-1}) [35]. All other second order rate constants were determined using phenol (pK_a 9.9) as competitor (k(phenol, O_3) = 1300 M^{-1} s^{-1} ± 200 M^{-1} s^{-1}, k(phenolate anion, O_3) = 1.4 x 10^9 M^{-1} s^{-1} ± 0.4 x 10^9 M^{-1} 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 5µm, 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 ± 1 nm and 278 ± 1 nm were used for quantification. For further information on the analytical method see SI 2.

3.3.4 Determination of TPs

The same experimental setup as for the reaction kinetics was used (10 µM 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 µM. Samples were analyzed with a Kinetex® C8 column (50 x 2.1 mm; 5 µm; 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/MS^n 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 µL syringe (Hamilton, Reno) at 7.5 µL 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.

3.4 Results and Discussion

3.4.1 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 values [32], 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 TOM [37].

3.4.2 Reaction kinetics

The determined second order rate constants are summarized in Table 3-1 and pH dependency is shown in Figure 3-1. 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 with hydroxyl radicals at diffusion controlled rates the excess of TBA will be sufficient to

scavenge > 95% of hydroxyl radicals (k (OH-radical + TBA) = 4.2-7.6 $\times$ 10^8 M^{-1} s^{-1} [38]). Hence, interferences by hydroxyl radicals can be ruled out.

As expected, the observed second order rate constants increase with pH due to an increasing fraction of the neutral deprotonated amine. For both compounds the second order rate constants of the neutral species are in the order of 10^8 M^{-1} s^{-1} which corresponds to the reaction of ozone at the free amine, although second order rate constants for amines are usually < 10^7 M^{-1} s^{-1} [39, 40]. The species specific second order rate constants of TAM and TOM were calculated using a data fitting analysis based on Equation A3-3. The same procedure was performed using the minima/maxima of experimental second order constants to estimate the accuracy of the regression, stated as error for TAM and TOM in Table 3-1. Second order reaction rates for TAM were also experimentally determined using 2-chlorophenol and phenol at pH 11 (see SI 2.1, Table A3-3). However, due to incomplete recovery in some experiments (data not shown), rate constants determined at pH 11 have to be considered with care and were therefore not implemented in the determination of the species specific rate constant.

The cation (protonated amine) probably reacts with ozone at the olefin moiety. The TAM cation reacts by a factor of three faster than the TOM cation. This can be explained by a reduced electron density of the olefin moiety due to the chlorine substituent in TOM, which is a major factor influencing ozone reaction with olefins [41]. The reactivity pK_a is defined as the pH where the reactions of ozone with each of the species present contribute equally to the apparent second order rate constant [10]. For TAM and TOM this is the case at pH 5.18 and pH 5.05, respectively. The connectivity of apparent and species specific second order rate constants and the pH is given in SI 2, Equation A3-3-S5.

A good correlation of determined kinetic data with the new determined pK_a values and consistency with a former reported second order rate constant at pH 7.1 [24] can be observed. Based on the structural similarity the species specific second order rate constants for the neutral species of TAM and TOM can be expected to be similar, which is confirmed in this study. However, the second rate constants deviate from the reported species specific second order rate constants of several other tertiary amines such as trimethylamine (4.1 – 5.1 x 10^6 M^{-1} s^{-1}) [36, 40], triethylamine (2.1 – 4.1 x 10^6 M^{-1} s^{-1}) [40, 42], and the pharmaceutical tramadol (1.0 x 10^6 M^{-1} 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 ($k(O_3)$: 1.1 x 10^7 M^{-1} s^{-1}, pKa: 9.2) [44]. 3-(dimethyl-aminomethyl)-indole ($k(O_3)$: 3.6 x 10^9 M^{-1} s^{-1}, pK_a: 10.0) is reported with an even higher species specific second order rate constant than TAM and TOM [44]. 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 ozone [44]. 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 3-1 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 tramadol (see SI, Chapter S5). This corroborates the high second order rate constant for TAM (k tramadol (1.0 x 10^6 M^{-1} s^{-1}) [43].

Table 3-1: Dissociation constants, species specific, and apparent second order rate constants for the reaction of ozone with of TAM and TOM; (neutral species: $k(M, O_3)$), cationic species: $k(MH^+, O_3)$, apparent rate constant at pH 7: $k(O_3$, pH 7), ratio of $k(MH^+, O_3)$ / $k(M, O_3)$, and reactivity pK_a. *Estimated values.

	Tamoxifen	**Toremifen**
pK_a	**9.49 ± 0.22**	**9.57 ± 0.22**
k(M, O3) / M^{-1} s^{-1}	**3.17 x 10^8 ± 1.19 x 10^8**	**1.46 x 10^8 ± 2.71 x 10^7**
k(MH+, O3) / M^{-1} s^{-1}	**1.57 x 10^4 ± 1.63 x 10^3**	**4.37 x 10^3 ± 1.62 x 10^3**
k(O3, pH 7) / M^{-1} s^{-1}	**1.04 x 10^6 ± 3.83 x 10^5**	**3.75 x 10^5 ± 2.13 x 10^4**
$k(MH, O_3)$ / $k(M, O_3)$	**4.94 x 10^5**	**2.99 x 10^5**
reactivity pK_a	**5.18**	**5.05**

For the reaction of ozone at the olefinic moiety of TAM and TOM (cationic species) second order rate constants in the same order of magnitude as other micropollutants containing olefinic groups (10^3 - 10^5 M^{-1} s^{-1}) such as carbamazepine, cephalexin, and anatoxin-a [6, 45-47] were found. For real water treatment (pH 7-8) [10] TAM and TOM have apparent second order rate constants > 10^3 M^{-1} s^{-1} and can thus be considered to be readily transformed during wastewater ozonation; (k(TAM, app.,O_3): 1.04 x 10^6 M^{-1} s^{-1}, k(TOM, app.,O_3): 3.75 x 10^5 M^{-1} s^{-1}, at pH 7).

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

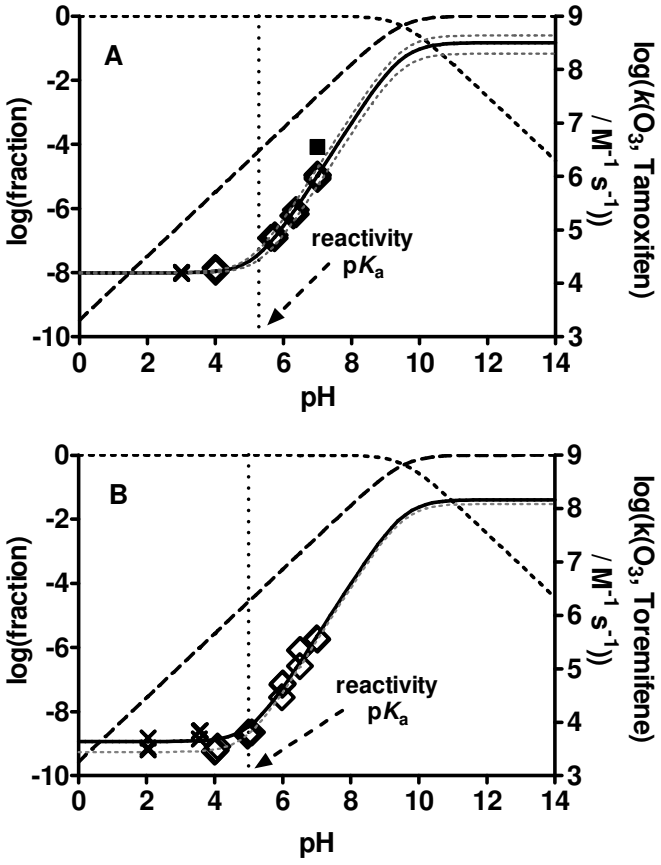


Figure 3-1: Individual Determined and calculated second order rate constants for ozonation of tamoxifen (A) and toremifene (B). - - [MH^+], ▬ ▬ [M], ▬▬ k(O_3, calculated) with error as dotted line in grey, k(O_3, determined) with different competitors: crosses: cinnamic acid, diamonds: phenol, closed square: second order rate constants of TAM from [24].

3.4.3 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 A3-5). Based on the m/z values one TP for the ozonation of TAM was reported in a previous study, but without information on structural identity [28]. 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 TPs [25].

Reaction pathways

Based on the observed transformation products the same reaction pathways can be assumed for TAM and TOM as shown in Figure 3-2. 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 peroxide [41]. TP 270 and TP A are also formed during photo-transformation of TAM, although propiophen-1-one is unstable under oxidative conditions [26] 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(O3): 290 M-1 s-1) or 1-phenoxy-2-propanol (k(O3): 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 conceivable [50], 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 formation [40] 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 enzymes [53]. 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 3-2). 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 3-2), 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].

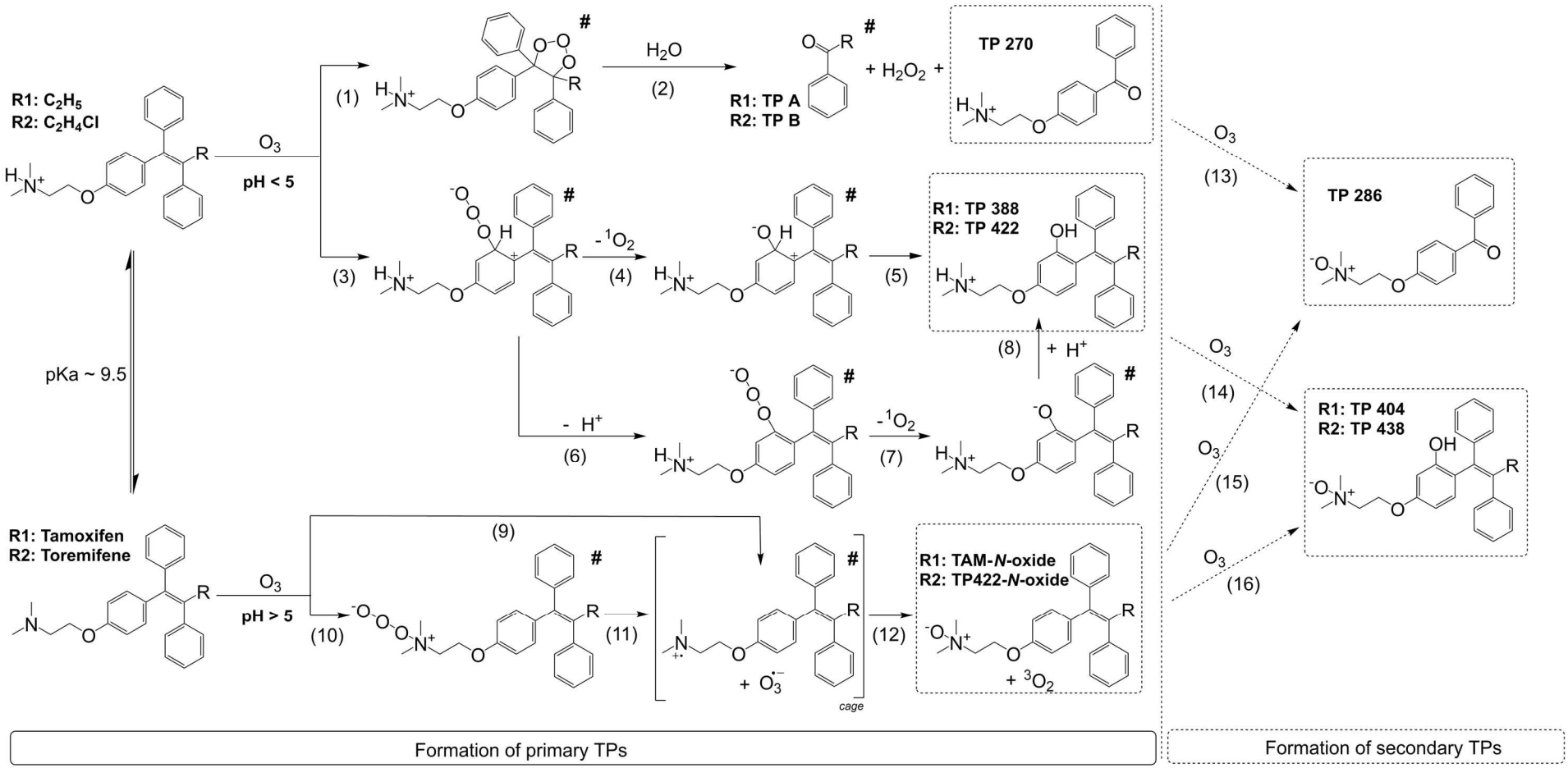


Figure 3-2: Reaction pathways for formation of primary TPs of tamoxifen (R1: CH_3) and toremifene (R2: CH_2Cl) by ozonation and formation of secondary TPs. Reactivity pK_a at pH 5. Measured TPs highlighted in dotted boxes, intermediates and TPs which have been hypothesized are marked with #.

TP 286 was reported as the major formed TP by Ferrando-Climent et al. 2017 [25]. 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 acid [35]. 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 here [25].

(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 3-3. 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 A3-9 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 group [10] (σ_p (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 3-2. 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 dose [25]. Low solubility (c(TAM) < 1 µM) 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 A3-9 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 3-1). 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 compounds [55], low solubility has to be considered here as well. Hence, the formation of TP 422-*N* might be underestimated.

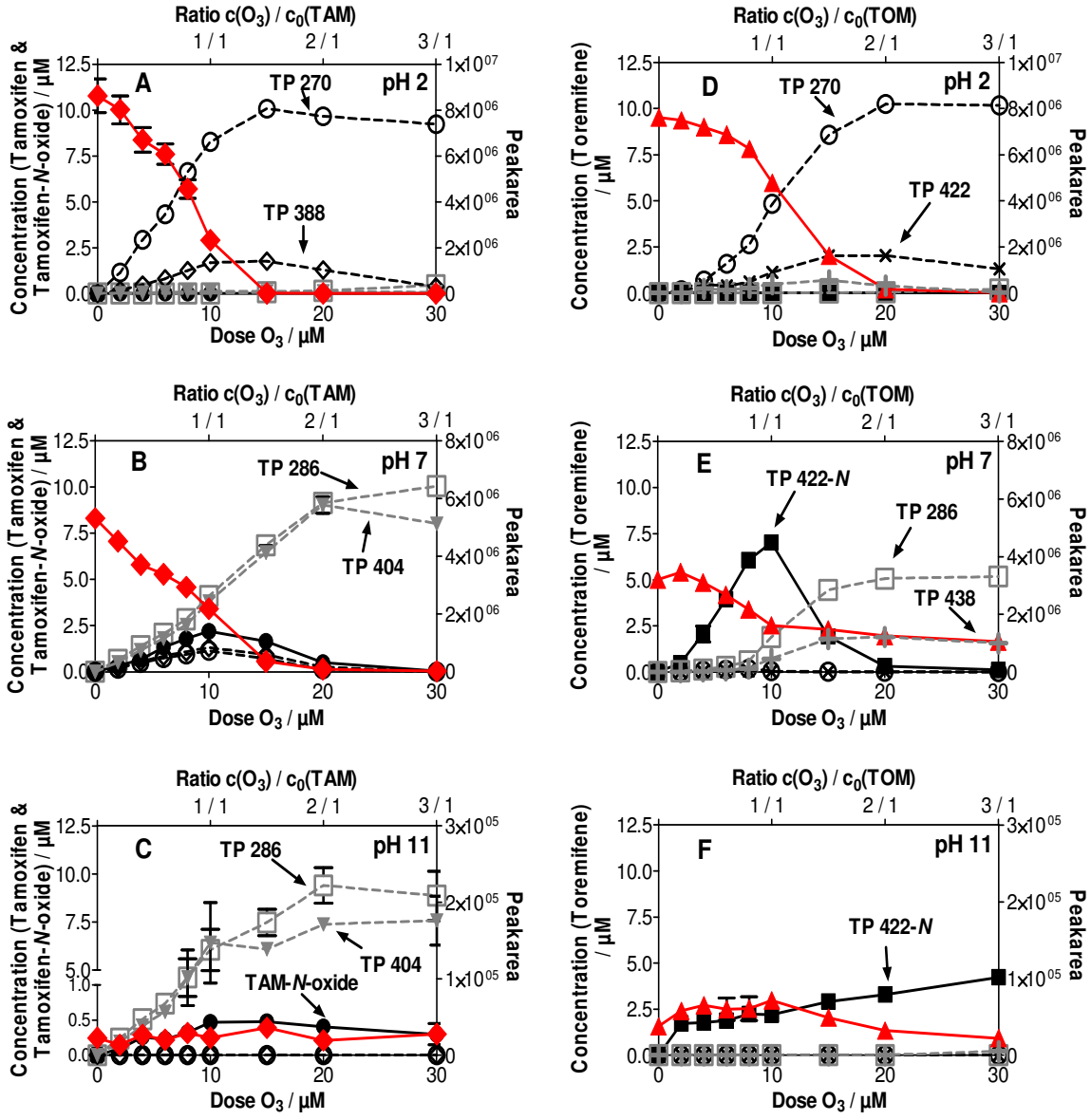


Figure 3-3: Degradation of TAM at pH 2 (A), pH 7 (B), and pH 11 (C) and TOM at pH 2 (D), pH 7 (E), and pH 11 (F) during ozonation and formation of TPs, semiquantititive. ○ - TP 270, □ - TP 286, ◊ - TP 388, ▼ - TP 404, ♦ - TAM, ● - TAM-N-oxide, x - TP 422, + - TP 438, ▲ - TOM, and ■ - TP 422-*N*. Initial nominal concentration c_0 of TAM/TOM: 10 µM.

3.4.4 Estimation of risk reduction

Based on pH during ozonation of wastewater (~pH 7-8) mainly *N*-oxides are formed from TAM and TOM in wastewater and in general, *N*-oxides showed to be persistent in biological post treatments but can be partially removed by sorption to activated carbon [57]. Only under anaerobic conditions in the environment *N*-oxides can be transformed back to their parent compounds [6, 55, 58, 59]. After ozonation though, oxic conditions are usually maintained during furhter treatment steps. Hence, ozonation may not be a final barrier for removing TAM and TOM. This emphasizes the importance to further study the environmental behavior of TAM and TOM and their TPs.

Concerning toxicity of the TPs, only for TP270 data are available with an EC_{50} of 0.89 µg L^{-1} for *Ceriodaphnia dubia* [26]. Nevertheless Ferrando-Climent et al. showed that the effect on *vibrio fisheri* can still be observed after full degradation of TAM and simultaneous formation of TP 286 [25]. The anti-estrogenic activity of TAM was monitored after ozonation in another study and showed that the anti-estrogenic effect can be preserved or even be amplified by roughly the factor 2 due to the formation of TP 270 [28]. This demonstrates the significance of monitoring toxicity of TPs formed during oxidative processes, but also the necessity of testing the toxicity with multiple organism species to allow a comprehensive environmental risk assessment concerning the success of toxicity removal of micropollutants by ozonation.

3.5 References

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3.7 Appendix A3: Supplementary Material

3.7.1 A 3-1 - Dissociation constants

Set up

100 mL beakers were filled with 30 mL of analyte solution and covered with paraffin. Nitrogen was permanently gently streamed into the solution to remove carbon dioxide, avoiding bubble formation, starting 5 min prior to titration. 0.01 M NaOH was added at a speed of 1 mL min^{-1} and the solution was constantly stirred, avoiding the formation of a vortex.

Cosolvent method

Most often the pK_a values for in water hardly soluble compounds are only calculated. Since these calculated pK_a values are often not precise, Völgyi et al. [1] developed a method to allow the experimental determination of the pK_a values of hardly soluble compounds. They showed a high correlation for their method of the pK_a and the p_sK_a in a predefined medium for 50 bases and acids in total. The 20%(V/V) MDM solution medium consists of a mixture of Methanol, Dioxane and Acetonitrile, each 1/3 (V/V), in water to allow all compounds of interest to be completely dissolved. Since the composition of their medium is well described, their method can be easily adapted.

Titration curves

Phosphoric acid

All replicates of the titration curves for phosphoric acid are given in Figure A3-1. Equilibration points (EQs), used for p*K*a determination, were located using the first derivative. The first $pK_{a,1}$ could not be determined since titration started at higher pH. Due to volume limitation $pK_{a,3}$ could also not be determined.

Determined equilibration points are given in Table A3-1 Uncertainty was calculated using error propagation, giving the overall accuracy of the set up.

Table A3-1: Equilibrium points (EQ) determined using first derivative.

EQ 1		EQ 2		$pK_{a,2}$
pH	mean pH	pH	mean pH	
4.81		9.19		
4.84	4.88 ± 0.09	9.42	9.28 ± 0.13	7.08 ± 0.22
4.98		9.22		

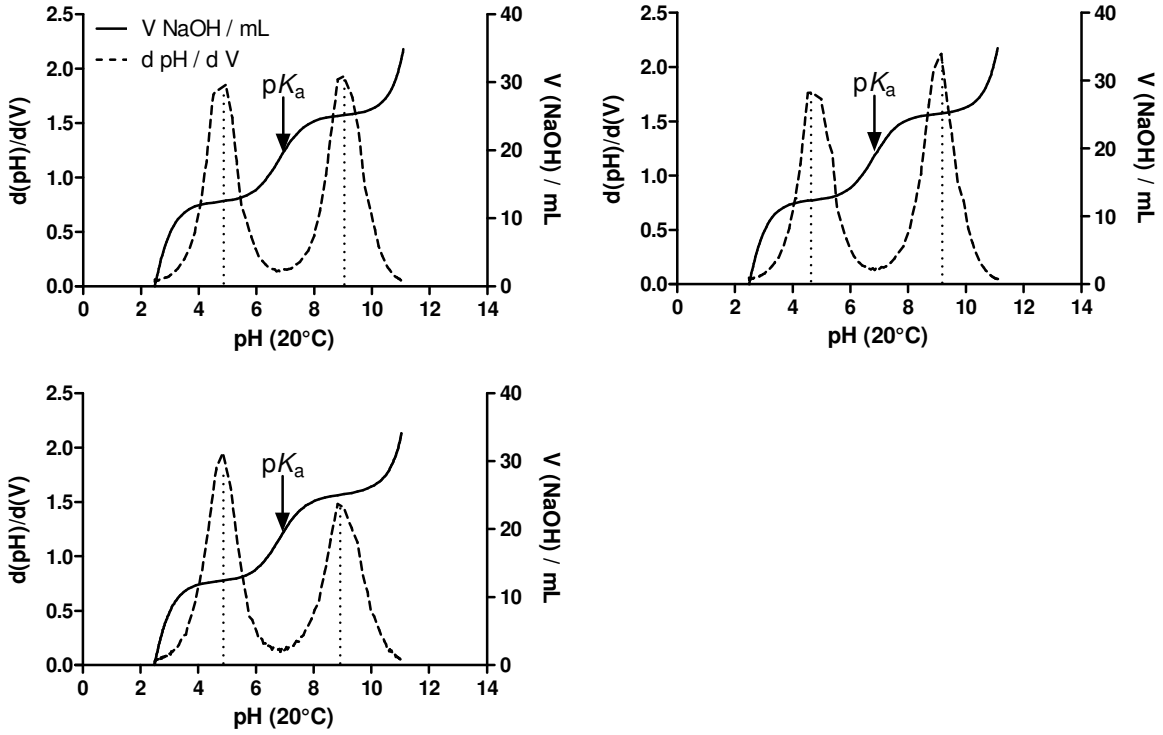

Figure A3-1: Individual titration curves for phosphoric acid and first derivative with marked equilibration points for pKa determination. n=3.

Tamoxifen & Toremifene

Recorded titration curves for tamoxifen (A – C) and toremifene (D – F) in 20%(v/v) MDM solution are given in Figure A3-2. Equilibration points, used for p_sK_a determination, were located using the second derivative. The calibration for acids in 20%(v/v) MDM solution developed by Völgyi et al.[1], see Equation A3-1 (r^2 = 0.9975, n = 25)**,** was used to calculate the pK_a value, given in Table A3-2.

Equation A3-1:

$$\mathrm{p}K_\mathrm{a} = 1.016\ \mathrm{p}_sK_{\mathrm{a\ (20\%/v\ MDM)}} - 0.382$$

Table A3-2: Co-solvent dissociation constants (p_sK_a) and calculted pK_a values for tamoxifen and toremifene.

	p_sK_a			pK_a		
Tamoxifen	8.99 8.94 8.91	8.96	± 0.11	9.49	±	0.11
Toremifene	8.92 9.08 9.12	9.04	± 0.10	9.57	±	0.11

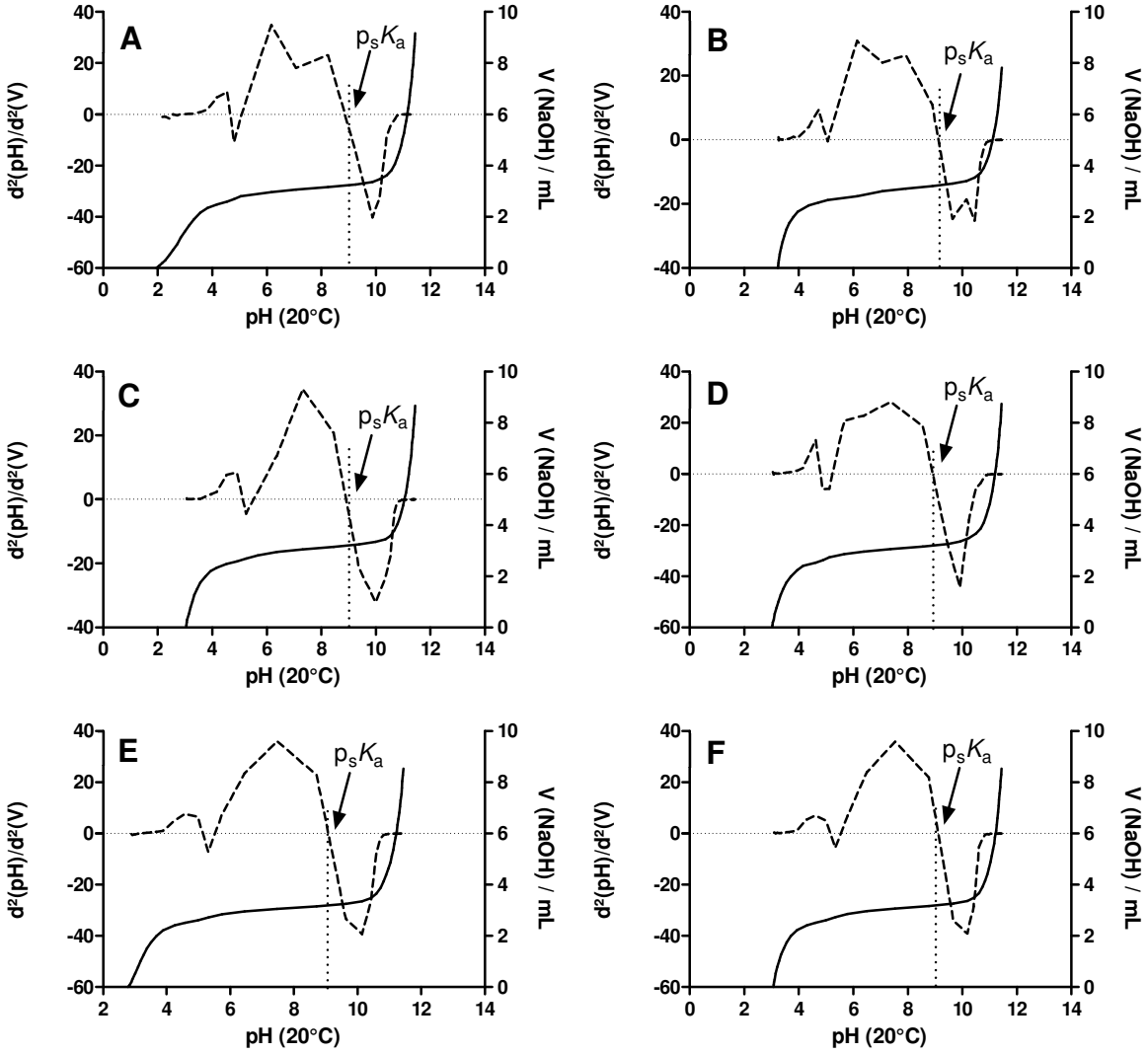

Figure A3-2: Individual titration curves and second derivative for (A – C) tamoxifen and (D – F) toremifene with marked equilibration points for p_sK_a determination.

3.7.2 A3-2 - Reaction kinetics

Instrumentation

Two different LC instruments were implemented. The Shimadzu liquid chromatograph consists of an LC-10A VP pump, a FCV-10A VP solvent mixer, a DGU-20A 5R degasser, a SCL-10A VP control unit, a SIL-10AD VP auto injector, a CTO-10AS VP column oven, and a SPD-M10A VP diode array detector. Data were evaluated using LC Solution version 1.25 SP4 (Shimadzu, Kyoto). For measuring the samples from the ozonation of toremifene at pH 2, 4, 5, 6, and 6.5 an Agilent (Waldbronn) liquid chromatograph 1100 series consisting of a G1379A degasser, a G1311A quarternary Pump, a G1313A automatic liquid sampler, a G1316A column oven, and a G1314A variable wavelength detector was used. ChemStation for LC version A10.02 [1757] was used for data evaluation. For all measurements a Kinetex® EVO C18 column (150 x 3.0 mm 5µm, 100 Å, Phenomenex, Aschaffenburg) with a C18 security guard column was utilized. An eluent gradient consisting of methanol and ultra-pure water, acidified to pH 2 with HCl, was applied.

For tamoxifen ozonation experiments at pH 3, 5, 6, 7, and 11 a flow rate of 0.8 mL min^{-1} was applied. The eluent started at 25 % methanol for 3 min, was increased to 70 % over 3 min, and hold for 8 min. For reconditioning 25 % methanol was hold for another 3 min. For experiments at pH 4 a flow rate of 0.6 mL min^{-1} was applied starting with 25 % methanol for 2 min, increasing to 45 % in 1 min and further to 75 % in another 3 min. 75 % methanol was then hold for 11 min and subsequent reconditioning was performed for 5 min at 25 % methanol.

For toremifene ozonation experiments at pH 3.5 and 7 a flow rate of 0.6 mL min^{-1} was applied. The eluent was started with 25 % methanol for 3 min, increasing to 90 % in 3 min and hold for 8 min. Reconditioning was performed for 9 min at 25 % methanol. For pH 2, 4, 5, 6, and 6.5 the eluent flow rate was 0.6 mL min^{-1} and started for 3 min at 30 % methanol. Methanol content was increased to 40 % in 0.4 min and further to 72 % in 17.6 min, finally increased to 80 % in 1 min and hold for 5.5 min. For reconditioning the eluent was set to 30 % for 7.5 min.

pH measurements were performed using a 6.0228.010 pH electrode (pH0…14/0…80 °C/NTC/3 M KCl, 827 pH lab, Metrohm, Herisau, Switzerland) before and after ozonation experiments. The electrode was calibrated on a daily base using pH 4 and pH 7 buffers.

Kinetic constants

Apparent and species specific second order rate constants were determined according to the following formula [2]:

Equation A3-2:

$$\ln\left(\frac{C}{C_0}\right) = \frac{k_{O_3.C}}{k_{O_3.R}} \ln\left(\frac{R}{R_0}\right)$$

C: concentration of tamoxifen/toremifene; C_0: initial concentration of tamoxifen/ toremifene; R: concentration of the competitor; R_0: initial concentration of the competitor; $k_{O_3.C}$: second order rate constant for the reaction of ozone and tamoxifen/toremifene; $k_{O_3.R}$: second order rate constant for the reaction of ozone and the competitor.

The apparent second order rate constant $k_{\mathrm{O_3+}MH_{\mathrm{total}}}$ of a monovalent acid MH can be calculated according to Equation A3-3, with the degree of dissociation α and the species specific second order rate constants $k_{\mathrm{O_3+MH}}$ and $k_{\mathrm{O_3+}M^-}$, given that MH is in equilibrium with its anion M^- (Equation A3-4).

Equation A3-3

$$k_{\mathrm{O_3+}MH_{\mathrm{total}}} = \left(\alpha * k_{\mathrm{O_3+MH}}\right) + \left((1-\alpha) * k_{\mathrm{O_3+}M^-}\right)$$

Equation A3-4

$$MH \leftrightarrows M^- + H^+$$

The corresponding dissociation constant K and the pH can be used to calculate the degree of dissociation α according to Equation A3-5.

Equation A3-5

$$\alpha = \frac{1}{1 + \frac{K}{10^{-pH}}}$$

At pH 11, the following competitors were used: 2-chlorophenol (pK_a 8.3) (n = 2, k(2-chlorophenol, O_3) = 1100 M^{-1} s^{-1} ± 300 M^{-1} s^{-1}, k(2-chlorophenolate anion, O_3) = 2 x 10^8 M^{-1} s^{-1} ± 1 x 10^8 M^{-1} s^{-1}) [2] and phenol (single experiments).

Table A3-3: Determined apparent second order rate constants for tamoxifen with the corresponding competitor and pH.

Competitor	pH	k (O_3, TAM) / M^{-1} s^{-1}	Mean k(O_3, TAM) / M^{-1} s^{-1}	S	S / %
Cinnamic Acid	3.00	1.60×10^4			
	3.01	1.53×10^4	1.57×10^4	3.40×10^2	2.17
	3.01	1.57×10^4			
Phenol	4.00	1.93×10^4			
	4.01	1.66×10^4	1.81×10^4	1.40×10^3	7.74
	4.03	1.85×10^4			
	5.64	7.06×10^4			
	5.74	7.03×10^4	7.34×10^4	5.05×10^3	6.88
	5.75	7.92×10^4			
	6.29	1.85×10^5			
	6.36	2.35×10^5	2.06×10^5	2.62×10^4	12.72
	6.37	1.96×10^5			
	7.00	1.05×10^6			
	7.00	9.53×10^5			
	7.00	1.08×10^6	1.03×10^6	6.80×10^4	6.61
	7.04	8.95×10^5			
	7.04	1.05×10^6			
	11.01	1.95×10^8			
2-Chlorophenol	11.10	6.69×10^8	6.32×10^8	5.25×10^7	8.31
	10.70	5.94×10^8			
Chen et al. [3]	7.10	3.56×10^6			
Calculated	13	2.54×10^8		1.46×10^8	57.46

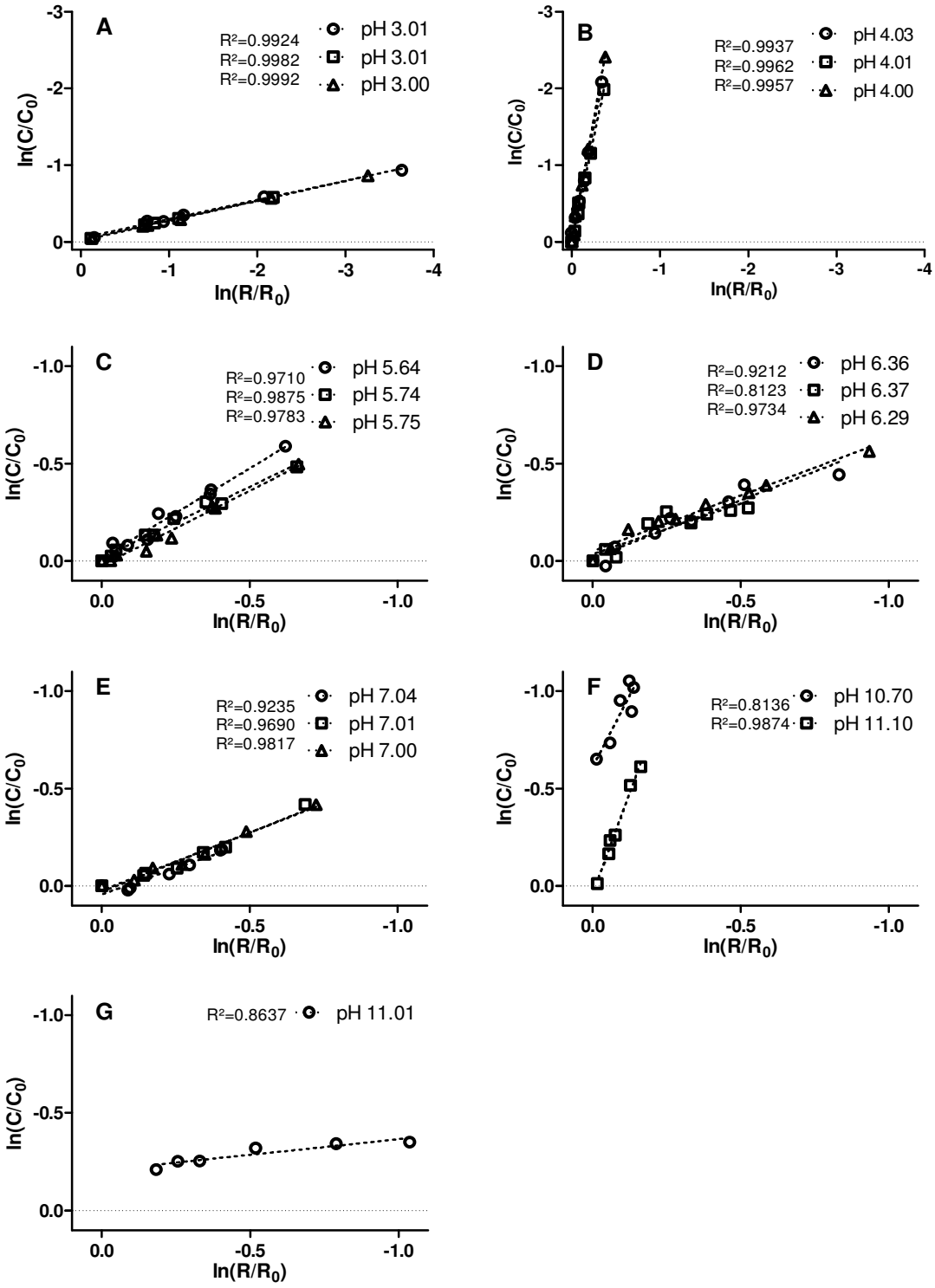

Figure A3-3: Competition kinetics for the determination of the second order rate constants for tamoxifen with cinnamic acid (A), Phenol (B-E, G), and 2-chlorophenol (F) as competitors.

Table A3-4: Determined apparent second order rate constants for toremifene with the corresponding competitor and pH.

Competitor	pH	k (O_3, TOM) / M^{-1} s^{-1}	Mean k(O_3, TOM) / M^{-1} s^{-1}	S	S / %
Cinnamic Acid	2.04	3.03×10^3	3.79×10^3	1.11×10^3	29.13
	2.04	5.06×10^3			
	2.03	3.29×10^3			
	3.58	6.96×10^3	6.20×10^3	1.15×10^3	18.51
	3.55	6.77×10^3			
	3.55	4.88×10^3			
Phenol	4.08	3.54×10^3	3.12×10^3	3.70×10^2	11.84
	4.04	2.99×10^3			
	4.00	2.84×10^3			
	5.00	6.38×10^3	6.16×10^3	4.70×10^2	7.63
	5.09	6.48×10^3			
	4.97	5.62×10^3			
	5.98	2.94×10^4	4.45×10^4	1.31×10^4	29.52
	5.99	5.06×10^4			
	6.00	5.34×10^4			
	6.50	2.22×10^5	1.86×10^5	6.33×10^4	34.05
	6.50	1.13×10^5			
	6.52	2.23×10^5			
	7.02	3.56×10^5	3.63×10^5	1.04×10^4	2.86
	7.00	3.70×10^5			
Calculated	13	1.46×10^8		2.72×10^7	18.55

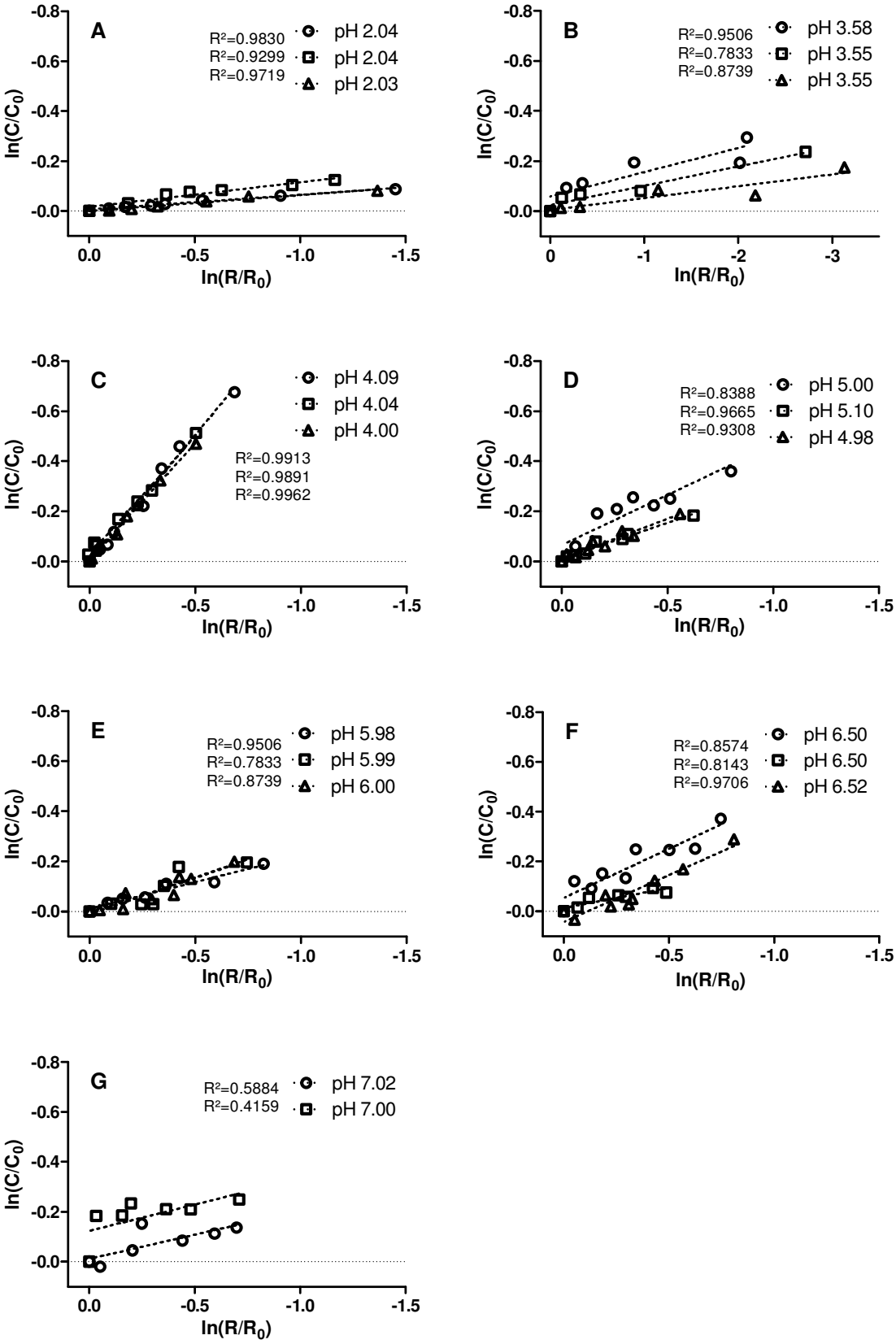

Figure A3-4: Competition kinetics for the determination of the second order rate constants for toremifene with cinnamic acid (A, B), and Phenol (C-G) as competitors.

3.7.3 A3-3 - Transformation Products

LC-MS Instrumentation

The Agilent (Waldbronn) LC-MS consists of a G1379A degasser, a G1312A binary Pump, a G1313A automatic liquid sampler, a G1316A column oven, and a 6120 quadrupole LC/MS. Data were evaluated using Open Lab ChemStation version C.01.07 [27].

Method

Methanol and water, both with 0.1 %/v formic acid were used. An eluent flow rate of 0.5 mL min^{-1} was applied. The eluent started at 45 % methanol for 0.5 min, was increased to 60 % in 1.5 min, further to 70 % in 3 min and hold for 6.5 min. For reconditioning eluent was set to 45 % methanol for another 3.5 min.

MS/MS Spectra

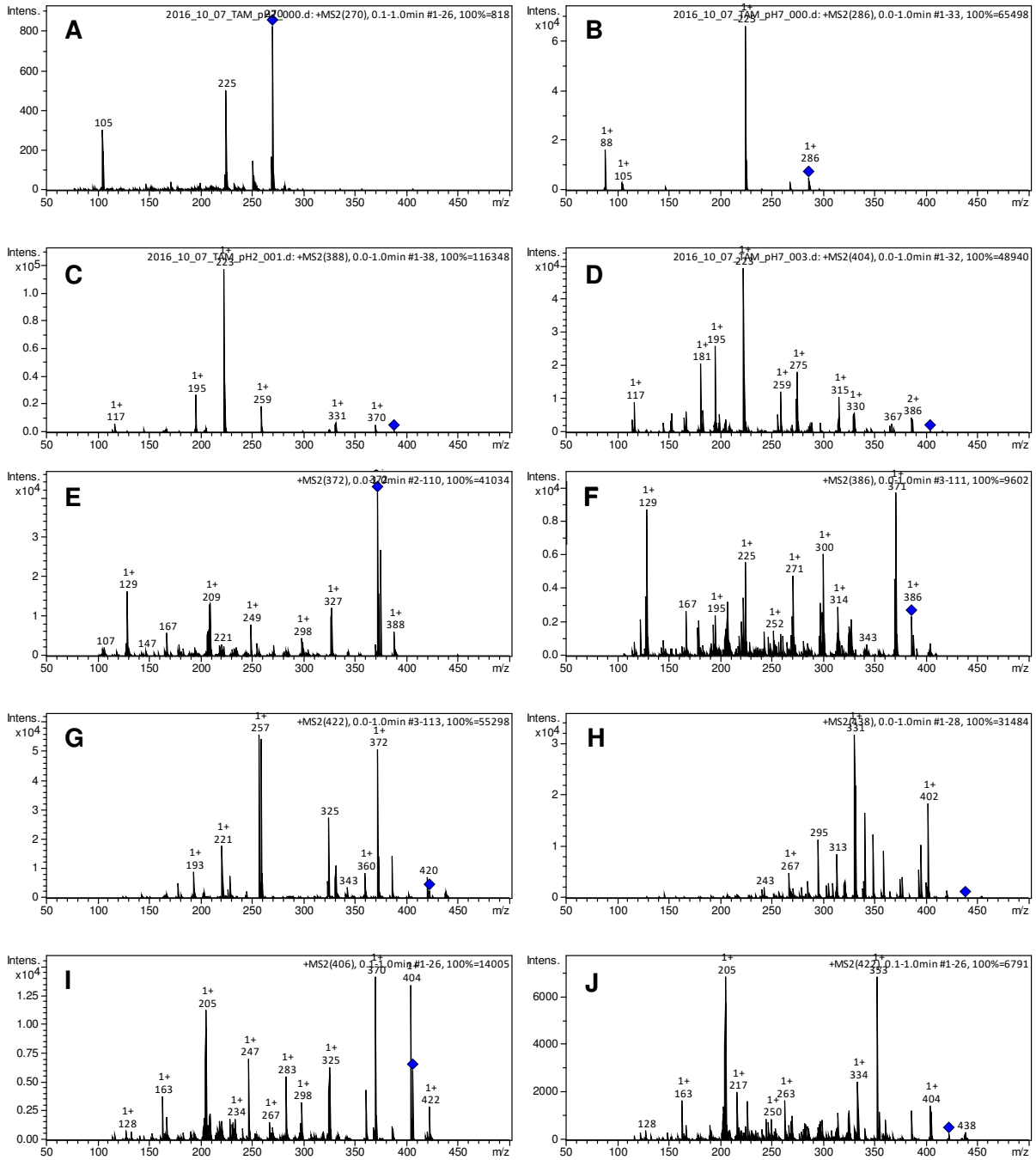

Figure A3-5: MS/MS spectra for (A) TP 270, (B) TP 286, (C) TP 388, (D) TP 404, (E) tamoxifen, (F) tamoxifen-N-oxide, (G) TP 422 (H) TP 438, (I) toremifene, and (J) TP 422-*N*.

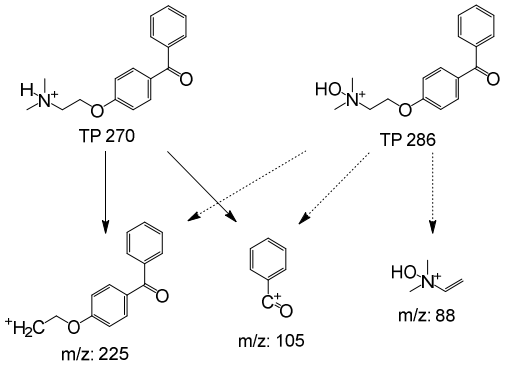

Figure A3-6: Fragments for TP 270 and 286

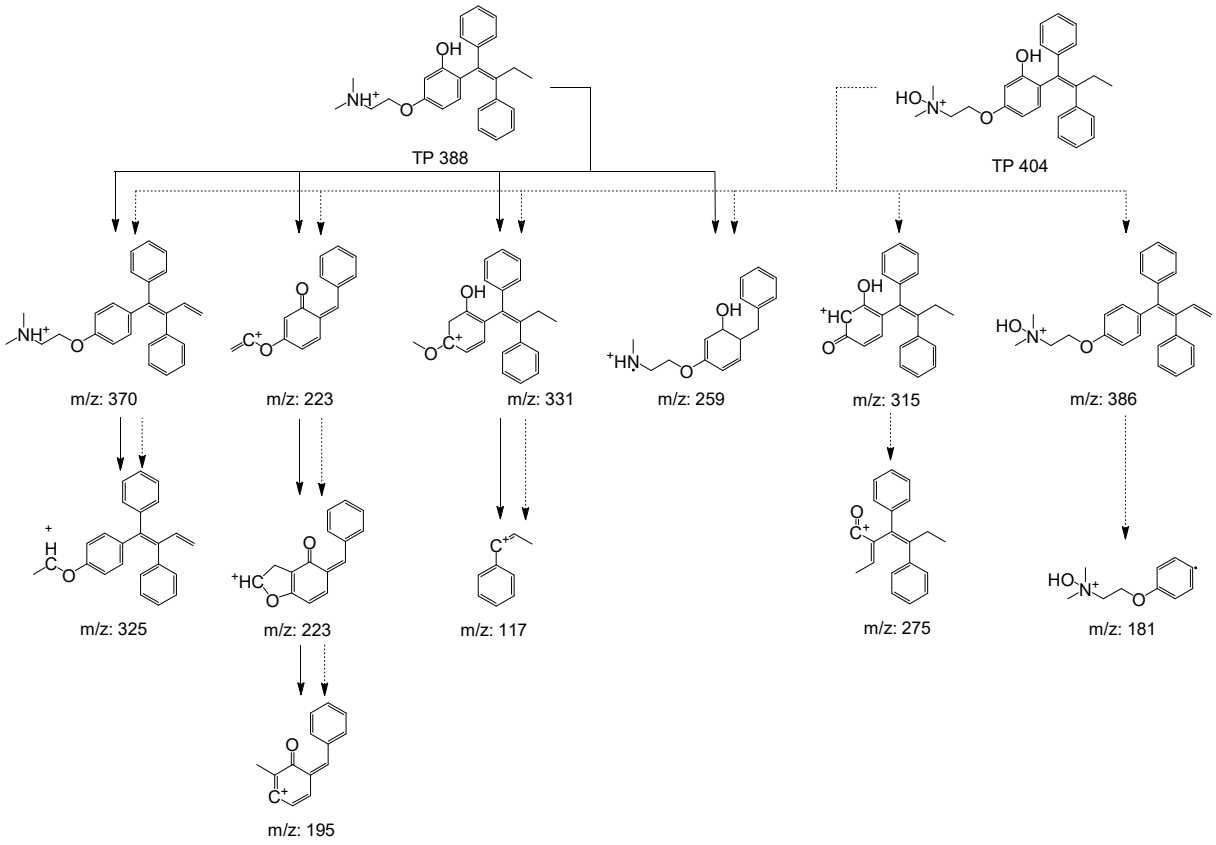

Figure A3-7: Fragments for TP 388 and TP 404.

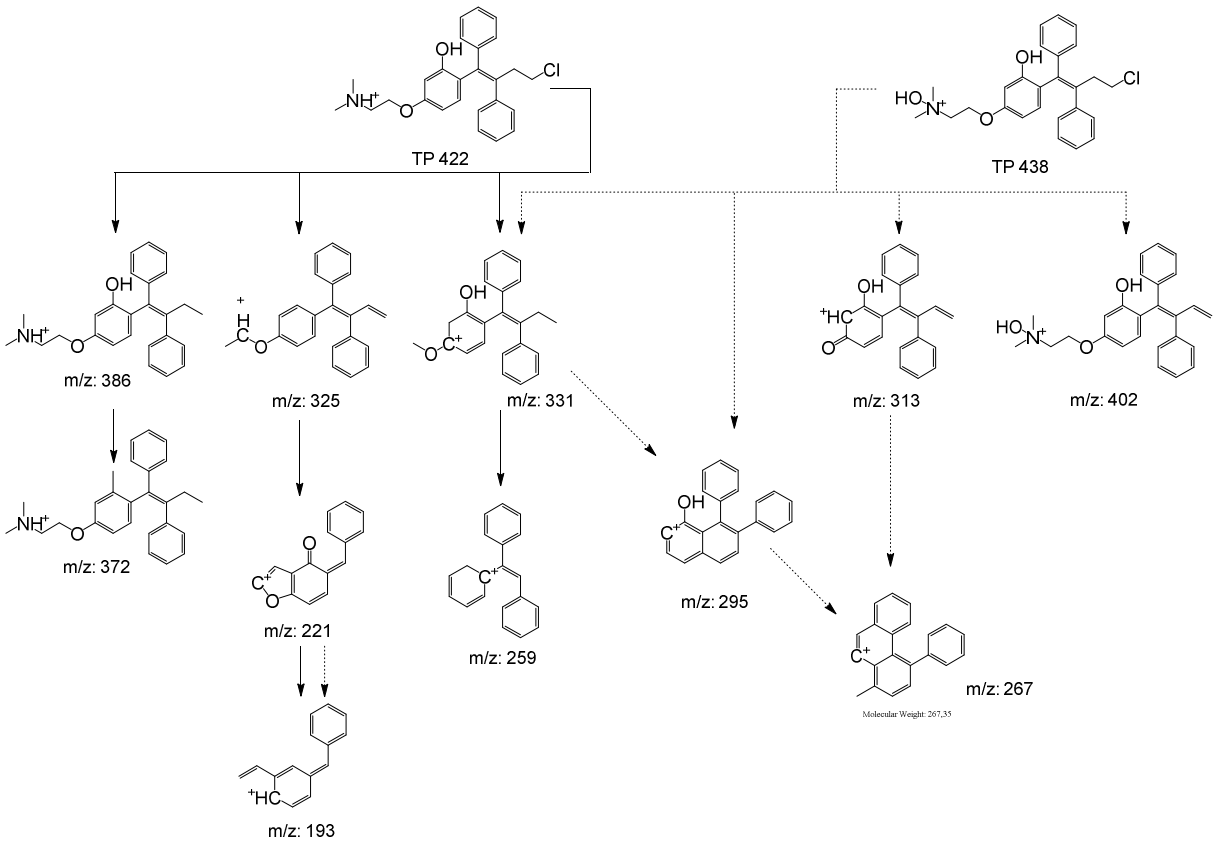

Figure A3-8: Fragments for TP 422 and TP 438.

Exact masses

Instrumentation

For the determination of the exact masses of the TPs a QExactive Orbirtap (Thermo, Michigan, USA) via direct injection with a 500 µL syringe (Hamilton, Reno) at 15 µL min^{-1} was used. Full MS scans (100-700 m/z) were performed at a resolution of 70,000, automatic gain control of 1e6 ions, and a maximum injection time of 50 ms. Positive electrospray ionization was performed at 3 kV with a sheath gas flow rate of 10 [ ]. No aux gas or sweep gas was applied. The aux gas heater was set to 50 °C and the inlet capillary to 250 °C. Mass calibration was performed prior to the experiments using the Pierce ESI Positive Ion Calibration Solution (Thermo, Michigan, USA). TAM and TOM were prepared as described in the manuscript, section 2.4. At pH 2, 7, and 11, an ozone dose of 10 µM was applied and left overnight. Prior to direct injection, samples were diluted 1:1 with methanol, containing 0.1 % (v/v) formic acid. For structure elucidation Top5-$ddMS^2$ mode was applied at a resolution of 35,000, automatic gain control of 1e5 ions, and a maximum injection time of 50 ms. The scan range was set to 50-500 m/z. Different collision energies (20, 30, 40, 50, 60, and 70 NCE) were tested, but nearly no specific fragments were observed.

Results

The exact masses of the TAM, TOM, and the TPs formed during ozonation are given in Table A3-5. No essential variation was observed in fragmentation using different collision energies. Main observed fragments are also given in Table A3-5.

Table A3-5: m/z ratios measured of TPs observed after ozonation of TAM & TOM in FullScan at a resolution of 70,000 and the deviation from the exact mass calculated on the chemical formula. m/z ratios and suggested chemical formulas for the fragments observed in Top5 ddMS² experiments at a resolution of 35,000. TPs formed during the ozonation of TAM or TOM are given below the component.

Compound	**FullScan** @ 70,000				**Top5 ddMS²** @ 35,000 HCD Fragments @ 30 NCE		
	Formula	exact mass	m/z	Delta ppm	m/z	Formula	Delta ppm
TAM	$C_{26}H_{30}NO$	372.2322	372.2323	0.29	72.0816	$C_4H_{10}N$	11.57
TP 270	$C_{17}H_{20}O_2N$	270.1489	270.1489	0.17	72.0816	$C_4H_{10}N$	11.57
TP 286	$C_{17}H_{20}O_3N$	286.1438	286.1439	0.49	72.0816	$C_4H_{10}N$	11.85
					88.0764	$C_4H_{10}ON$	8.06
					225.0912	$C_{15}H_{13}O_2$	0.64
TP 388	$C_{26}H_{30}O_2N$	388.2271	388.2272	0.11	72.0816	$C_4H_{10}N$	11.43
TP 404	$C_{26}H_{30}O_3N$	404.2220	404.2223	0.59	72.0816	$C_4H_{10}N$	11.43
TAM-*N*-oxide	$C_{26}H_{30}O_2N$	388.2271	388.2269	-0.30	72.0816	$C_4H_{10}N$	11.16
TOM	$C_{26}H_{29}ClNO$	406.1932	406.1934	0.40	72.0816	$C_4H_{10}N$	11.57
TP 270	$C_{17}H_{20}O_2N$	270.1489	270.1491	1.05	72.0817	$C_4H_{10}N$	12.27
TP 286	$C_{17}H_{20}O_3N$	286.1438	286.1440	0.80	72.0817	$C_4H_{10}N$	12.40
					88.0765	$C_4H_{10}ON$	8.62
					225.0912	$C_{15}H_{13}O_2$	0.95
TP 422	$C_{26}H_{29}O_2NCl$	422.1881	422.1882	0.21	72.0816	$C_4H_{10}N$	11.43
TP 438	$C_{26}H_{29}O_3NCl$	438.183	438.1836	1.33	72.0816	$C_4H_{10}N$	12.13
TP 422-*N*	$C_{26}H_{29}O_2NCl$	422.1881	422.1881	-0.15	72.0816	$C_4H_{10}N$	11.57

3.7.4 A3-4 - Ozone Consumption

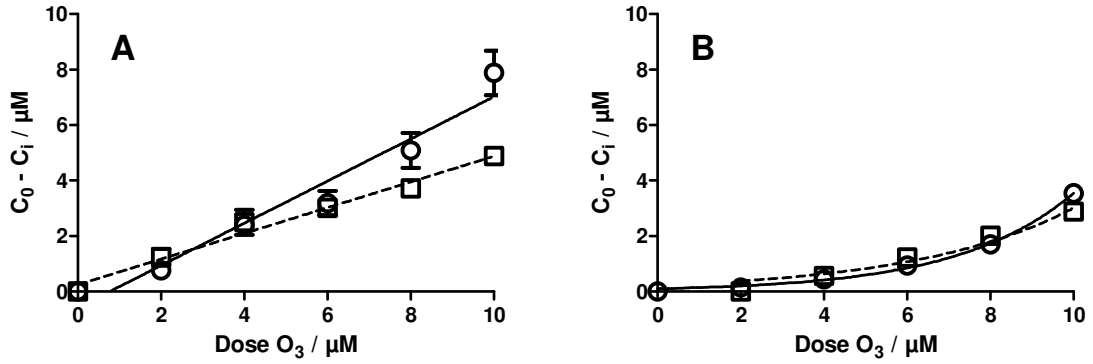

Figure A3-9: Abatement of TAM (A) and TOM (B) at pH 2 (circle, solid line) and pH 7 (square, dotted line). C_0: initial concentration, C_i: concentration after ozone dose *I* in µM.

3.7.5 A3-5 – Comparison of the degradation of TAM and tramadol by ozone:

Tramadol is a compound with a similar structure compared to TAM and TOM, with a substantially lower reaction kinetics [4]. For corroborating the high reaction rate of tramadol determined in our study we performed an additional competition experiment for TAM using tramandol as competitor. The results indeed show, that Tamoxifen reacts much faster with ozone than Tramadol (see Figure A3-10).

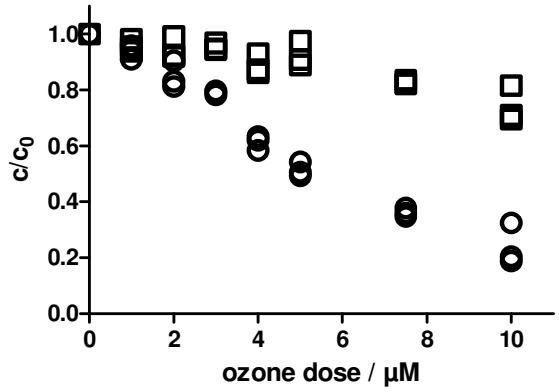

Figure A3-10: Degradation of tramadol (square) and TAM (circle) at different ozone dosages, tert-Butanol: 10 mM, pH 7.0 ± 0.05. Method: HPLC-UV (Shimadzu), The eluent was composed of methanol and water. The water was adjusted using HCl to pH 2. Gradient program: 0-17 min ramp 5-75 % methanol, 17-30 min isocratic 75 % methanol, used wavelength for UV detection: 278 for TAM and 271 for Tramadol,

separation column: Phenomenex Kinetex ® EVO C 18 100 × 3.0 mm, pore size 100 Å.

Tramadol hydrochloride was purchased from Sigma Aldrich, purity: ≥ 99.0 %.

3.7.6 A3-6 References

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4 Endocrine Effects after Ozonation of Tamoxifen

Adapted from O. Knoop, F. Itzel, J. Tuerk, H.V. Lutze, T.C. Schmidt, Endocrine effects after ozonation of tamoxifen, Sci. Total Environ., 622-623 (2018) 71-78. https://doi.org/10.1016/j.scitotenv.2017.11.286

4.1 Abstract

Ozonation is used as additional wastewater treatment option to remove recalcitrant micropollutants. It also removes the estrogenic activity found in wastewater but not always the anti-estrogenic activity. This can be explained by an incomplete removal of anti-estrogenic micropollutants or by formation of transformation products (TPs) which retain the activity. The present study investigates the degradation of the anti-estrogenic pharmaceutical tamoxifen in pure water, regarding TP formation and related anti-estrogenic effect using *Arxula adeninivorans* yeast estrogen screen (A-YES). In total, five transformation products were detected: three *N*-oxides and two further products (TP 270 and TP 388). For the transformation product TP 270 a correlation of the extent of formation with an increase of the anti-estrogenic activity was determined, demonstrating that transformation products from ozonation can be more active in a bioassay than the parent compounds. Our study shows also that the transformation of tamoxifen to *N*-oxides reduces the anti-estrogenic activity. The reactivity of amines towards ozone typically increases with pH, since only deprotonated amines react with ozone. Hence, removal of the endocrine activity by *N*-oxide formation may be disfavored at low pH.

4.2 Introduction

The determination of ecotoxicological effects, such as endocrine effects, is an important aspect for the evaluation of advanced wastewater treatment processes [1]. Endocrine effects can be caused by endocrine disruptive compounds (EDCs), a group of micropollutants affecting the hormonal system of organisms which can induce biological effects already at trace concentrations [2]. Estrogenic and androgenic EDCs, as well as their antagonists, are introduced into surface waters *via* wastewater treatment plant effluents since they are not completely removed during biological treatment [3].

The implementation of ozone as additional treatment option for wastewater is broadly discussed and tested in large scale facilities to reduce the discharge of micropollutants into the environment [4, 5]. Estrogenic activity monitored by *in vitro* bioassays is considered an important evaluation parameter to assess the impact of micropollutants as well as their metabolites and transformation products (TPs) [6, 7]. However, after ozonation of wastewater residual estrogenic effects [8] as well as their complete removal are reported [9, 10]. Anti-estrogenic activity on the other hand is reduced during biological treatment, but is not further reduced by ozonation [1, 3].

Remaining agonistic effects can be caused by three different factors or a combination thereof: (I) the micropollutants are ozone resistant, (II) active TPs are formed that maintain the biological effect of the precursor [4] or (III) compounds acting as antagonists are more efficiently removed, thus reducing a masking effect [11]. For anti-estrogenic or anti-androgenic micropollutants the same principle applies. This could for example be the case if 17α-ethinylestradiol (EE2) is present because it is well degraded by ozonation and no estrogenic TPs are formed [12].

To the authors' knowledge no investigations of (anti-) estrogenic effects of TPs after ozonation of tamoxifen (TAM) are reported yet. TAM is considered to be the prototype of nonsteroidal selective estrogen receptor modulators (SERMs) that show either an activating or deactivating estrogenic effect on different types of tissues [13]. It is used in therapy to inhibit reoccurrence of estrogen receptor-positive breast cancer [14].

Only few studies reported environmental concentrations of TAM with usually 25 to 200 ng L^{-1} in wastewater treatment plant (WWTP) effluents and 25 to 50 ng L^{-1} in surface waters [15-18]. In one study though, higher concentrations up to 369 ng L^{-1} TAM were reported in WWTP effluents and up to 212 ng L^{-1} in river Tyne [19]. Hence,

the predicted no effect concentration (PNEC) value of 200 ng L^{-1} [15] can be exceeded in surface waters. TAM reacts at sufficient rates with ozone to be completely removed in wastewater matrices forming several TPs depending on the pH [20, 21]. Some of these TPs showed a residual acute toxicity for aquatic organisms [22, 23].

This work focuses on (I) the removal of the anti-estrogenic activity of tamoxifen by ozonation at pH 3, 7, and 11, and (II) determination of the anti-estrogenic activity of formed TPs. A detailed study of kinetics and product formation at different pH values is out of the scope of this study but will be reported elsewhere.

4.3 Theory

4.3.1 Reaction of ozone with tamoxifen

In brief, five TPs can be formed by three pathways depending on the protonation of the tertiary amine (pK_a of corresponding acid = 9.5) (see Figure 4-1). A reaction at the olefin group causes bond cleavage resulting in formation of TP 270. TP 388 forms upon hydroxylation of the aromatic ring. Both reactions are favored at acidic conditions (pH < 5) which suppresses the attack at the amine (cationic species largely prevails). At higher pH values ozone reacts faster with the tertiary amine than with the double bond or the aromatic ring resulting in *N*-oxide formation. [21]

4.3.2 Effect induction at the estrogen receptor

The estrogen receptors alpha and beta (ERα and ERβ) have three major functional domains, the amino-terminal activation function-1 (AF-1) domain, the central DNA-binding domain (DBD) and the carboxyl-terminal ligand binding domain (LBD). Estrogenic compounds are bound to the hydrophobic cavity in the interior of the LBD. The binding of an estrogenic compound induces a conformational change of helix H12 that enables the activation function 2 (AF-2) and closes the entrance channel of the cavity thereby creating an interacting surface for coregulators to bind at the ER [24, 25]. These coregulators essentially carry out the DNA-transcription activity of the ER [13]. Antagonists have a basic moiety at a side chain, which on the one hand sterically blocks helix H12 and thus prevents to close the cavity. On the other hand the antagonist forms an ionic bond to aspartate 351 (ASP 351) of the ER protein, since the basic moiety is present as cationic species at pH 7. The ASP 351 otherwise stabilizes helix H12 in the closed position in presence of an agonist. Therefore, helix H12 blocks the AF-2 in presence of an antagonist [26, 27] thus inhibiting the DNA transcription. A

3D model of the ERα-agonist/antagonist complex is shown in Figure A4-4 adapted from Shiau et al. (1998) [28].

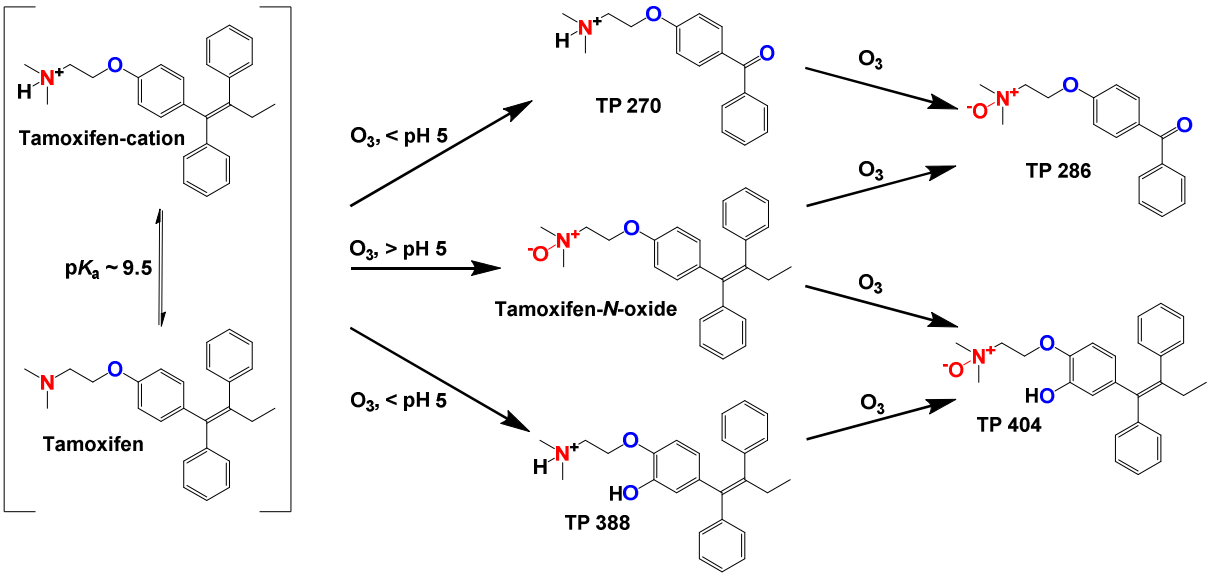

Figure 4-1: Proposed TPs for the ozonation of tamoxifen according to Knoop et al. (2016) [21].

Since agonists and antagonists show a different binding within the ER and therefore cause different conformational changes [24], coregulators will or will not bind to the ER, thus inducing either activation or suppression of gene expression [13]. SERMs have both agonistic and antagonistic estrogenic effects depending on the target tissue, structural variation of the ligand-ER-complex structure and different binding of coregulators and gene expression [29, 30].

Essential for the interaction of hormones or EDCs with the ER is a combination of hydrogen bonds and a non-polar structure, which is complementary to the binding cavity of the LBD [24]. Tamoxifen binds to the ER with a lower affinity than estradiol. The tertiary amine group of tamoxifen as terminal basic moiety at the side chain acts as the inhibitor of the AF-2, resulting in an anti-estrogenic activity. A partial agonistic activity remains since the AF-1 complex remains active [14]. Metabolic hydroxylation to 4-hydroxytamoxifen (4-OH-TAM) yields a higher structural similarity in the binding moiety to steroidal estrogens such as 17β-estradiol (E2) and a higher antagonistic activity [31]. Hydroxylation in the 4-position also gives an extreme increase in the relative binding affinity (RBA, compared to E2), similar to the RBA of ethinylestradiol (EE2) [32]. Endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen) as another phase-I-metabolite shows also an increased anti-estrogenic activity, whereas glucuronidation

during phase-II-metabolism removes the anti-estrogenic activity [31, 33]. However, TAM itself has a low but similar affinity to the ER (RBA = 1.6%) like the natural steroid hormone estrone (RBA = 7.3%), whereas only 4-OH-TAM (RBA = 175.2 %) can compete with the most potent estrogens E2 (RBA = 100.0%) and EE2 (RBA = 190.1%) [32]. The planar benzene ring is crucial for the affinity to the ER. A non-planar ring, as present in androgenic compounds, does not sterically fit into the ER-LBD cavity.

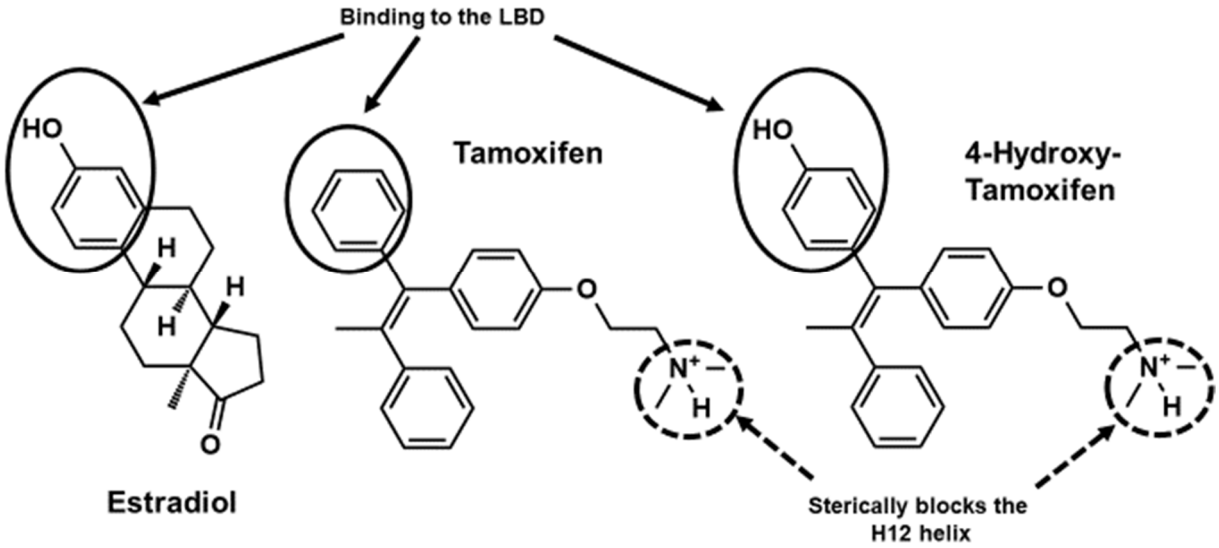

Figure 4-2: Structure of 17β-Estradiol (E2), TAM and 4-OH-TAM. Moieties binding to the LBD (full circles) and side chains blocking H12 helix (dashed circles) are marked.

4.4 Materials and Methods

4.4.1 Chemicals

Tamoxifen was purchased from Alfa Aesar (Karlsruhe, Germany), tamoxifen-*N*-oxide from LGC (Ann Arbor, Michigan, USA), tertiary butanol (TBA) from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate (Merck) were used for the preparation of a pH 7 buffer (100 mM). Sodium hydroxide (VWR, Darmstadt, Germany) and phosphoric acid (Fisher Scientific, Bremen, Germany) were used for pH adjustment. Purity of all chemicals was ≥ 98%. Ultra-pure water was produced onsite (Purelab Ultra, Elga LabWater, Celle, Germany). For liquid chromatography-mass spectrometry (LC-MS) measurements, LC-MS grade methanol (HiPerSolv CHROMANORM, VWR), water (LiChroSolv), and formic acid (Suprapur, Merck) were used as eluents.

Ozone-containing gas, produced onsite with an ozone generator (BMT 802 X, BMT Messtechnik, Berlin, Germany; feed gas: O_2 6.0, Linde, Düsseldorf, Germany), was

bubbled through ice-cooled ultra-pure water to produce an aqueous ozone stock solution. The concentration of the ozone solution was determined by UV absorption at 258 nm (ε = 2950 M^{-1} cm^{-1}) (Gilbert and Hoigne, 1983) using a UV-1650PC UV-visible spectrophotometer (Shimadzu, Kyoto, Japan).

4.4.2 Choice of pH

The pH values used for the experiments were chosen due to the pH dependency of the reaction of ozone with the tertiary amine. At pH 3 the reaction at the amine can be ruled out since protonated tertiary amines usually have low reaction rate constants, such as for the protonated dimethyl ethanolamine (DMEA, $k_{O3} < 0.2$ M^{-1} s^{-1}, pK_a = 9.2), which resembles the amine substructure of tamoxifen. The deprotonated DMEA shows a higher reaction rate constant ($k_{O3} = 1.1 \times 10^7$ M^{-1} s^{-1}) [34]. Reaction rate constants for the reaction of ozone and double bond moieties is mostly in the range of 10^2-10^5 [35]. Therefore at pH 11, other reactions than that of the tertiary amine are negligible. At pH 7, all three reactions can be observed. Hence, the three pH values chosen were necessary to allow the investigation of all TPs formed in more detail.

4.4.3 Sample preparation

10 µmol (3.715 mg) tamoxifen were dissolved in 100 mmol (9.38 mL) TBA and added to 900 mL ultra-pure water. The pH of the water was adjusted to pH 3, 7, and 11 using phosphoric acid and sodium hydroxide and subsequently volume was adjusted to 1000 mL by ultrapure water, resulting in a final tamoxifen concentration of 10 µM. 10 mL samples were aliquoted for ozonation. Ozone from the ozone stock solution were added using gastight syringes (Hamilton, Reno, USA) to gain ozone concentrations of 0, 2.5, 5, 7.5, 10, 15, 20, and 30 µM. Samples were stored in the dark for 2 h. For the yeast assay 2 mL of phosphate buffer (100 mM) were added to adjust all samples to pH 7 as required for the A-YES assay. All experiments were performed in duplicates. Blanks were produced accordingly without tamoxifen and spiked with either an ozone dose of 0 or 10 µM. No significant pH change (≤ ±0.1) was observed after ozone dosing. Due to the high amount of the radical scavenger TBA (100 mM) in the samples >95% of hydroxyl radicals eventually formed were scavenged. Finally, samples were stored at 4 °C and analyzed by LC/MS and A-YES assay without further treatment, considering the dilution due to addition of the ozone stock solution.

To ensure if changes of the effect are solely based on the TPs formed in the reaction of ozone and TAM, a wastewater matrix was not considered.

4.4.4 LC/MS measurements

An Agilent 1100 Series LC and a 6120 quadrupole LC/MS (Agilent, Waldbronn, Germany) were used. Samples were analyzed with a Kinetex® C8 column (50 x 2.1 mm; 5 µm; 100 Å; Phenomenex, Aschaffenburg) and a methanol (+0.1%/v formic acid) / water (+0.1%/v formic acid) gradient starting at 45 % methanol at a flow rate of 0.5 mL min^{-1}. 45% methanol was kept constant for 0.5 min, increased to 60% within 1.5 min, then increasing to 70% in 3 min and kept constant at 70% for 5 min. Reconditioning to 45% methanol was performed for 4 min. An injection volume of 20 µL was applied.

Electrospray ionization was operated at 3 kV and a nebulizer pressure of 30 psi. Dry gas flow rate was set to 10 L min^{-1} and heated to 300 °C. For quantitative (TAM and TAM-*N*-oxide) or semi-quantitative (TP 270, TP 286, TP 388, and TP 404) evaluation selected ion mode was used to measure the ions *m/z* 270.1, 286.1, 372.1, 388.2, and 404.2.

4.4.5 Yeast assay for anti-estrogenic activity

The samples were analyzed as described in the committee draft of ISO/CD 19040-2 (2016) using the validated *Arxula adeninivorans* yeast estrogen screen kit (A-YES, new_diagnostics, Freising, Germany) in quadruplicate with a variability of residuals of 5.1% (A-YES) indicating a high robustness of the assay. 400 µL of sample as well as blanks and standards were pipetted into 96-deep-well plates (2.2 mL, Ratiolab, Germany) and 100 µL of supplied yeast cells, suspended in yeast minimal maltose medium (YMM) were added. The 96-deep-well plates were incubated for 22 h at 30 °C on an orbital shaker at 750 rpm (Virbramax 100, Heidolph Instruments, Schwabach, Germany). After incubation, plates were centrifuged (900 g, GS-15R, Beckman Coulter, Krefeld, Germany) for 20 min to separate the cells from the supernatant. Fifty µL of supernatant were transferred into a 96-well microtiter plate, 50 µL 3.5 mM p-nitrophenyl phosphate in 0.1 M sodium citrate buffer (pH 3.9) were added and plates incubated for 60 min at 37 °C. Subsequently, 100 µL 3 M sodium hydroxide were added to stop the reaction and optical density was measured immediately at 405 nm (OD_{405}) (TECAN Infinite® M200, Tecan Group Ltd., Maennedorf, Switzerland). Cell growth was measured by re-suspending the centrifuged cells and diluting 30 µL of cell suspension

with 270 µL water. The optical density was measured at 620 nm (OD_{620}). The optical absorbance was measured with a multiwell plate reader (TECAN Infinite® M200, Tecan Group Ltd., Maennedorf, Switzerland). Data evaluation was conducted using the software BioVal® (QuoData GmbH, Dresden, Germany). Investigating antagonistic estrogenic effects (Anti-A-YES), the same test procedure was done by spiking the respective sample (380 µL) with 20 µL 17β-estradiol (1000 ng L^{-1}) inside the 96-well plate resulting in a 50 ng L^{-1} E2 concentration. As a reference sample, water was treated the same way for the Anti-A-YES. Water as blank without spiking was used to determine the OD_{405} background that was subtracted from samples and E2 standards. The OD was also determined for solvent blanks containing TBA and buffer.

The anti-estrogenic activity was defined as inhibition of 50 ng L^{-1} E2 in the A-YES essay and was calculated according to (1)

(1) $$\textit{Anti-estrogenic activity} = \left(\frac{e-x}{e}\right) * 100\%$$

with (1.1) $$e = OD_{405}(50\ ng\ L^{-1}\ E2) - OD_{405}(water\ blank)$$

and (1.2) $$x = OD_{405}(sample) - OD_{405}(water\ blank)$$

4.5 Results and Discussion

4.5.1 Chemical analysis and anti-estrogenic activity

A comprehensive overview of the concentrations of tamoxifen and TAM-*N*-oxide as well as the detected peak areas of the other TPs (A, B), and the anti-estrogenic activity are shown in Figure 4-3 (C, D). The tamoxifen concentration and formation of TPs of both individual sets of experiments are given in Figure A4-1 (A-F) and the antagonistic activity in Figure A4-2 (A-C).

pH 3

At pH 3 the concentration of tamoxifen decreased with increasing ozone dose and it was completely removed at an ozone dose of 20 µM. TP 270 and TP 388 were the main observed TPs. Only in excess of ozone the secondary TPs, TP 286 and TP 404, were formed, giving low signal intensities. The anti-estrogenic activity (Figure 4-3 C) increased twofold at an ozone dose of 20 µM indicating the formation of effect inducing TPs. This correlates with the formation of TP 270 and TP 388, see Figure 4-4 (B, D).

pH 7

Tamoxifen was completely removed at an ozone dose of 20 µM while primary TPs peak at 10 µM ozone dose. Further oxidation of TP 270, TP 388, and TAM-*N*-oxide to TP 286 and TP 404 was observed in excess of ozone to tamoxifen. Anti-estrogenic activity (Figure 4-3 D) did not decrease initially and was also not reduced after addition of 20 µM ozone which resulted in complete tamoxifen removal. This suggests that the remaining effect must be caused by one or more TPs. All TPs, except the *N*-oxide, were observed in the samples spiked with 20 µM ozone. With further increasing ozone dose, the activity was reduced below 10%. Here, the signals of the secondary TPs, TP 286 and TP 404, were most intense whereas TP 270 and 388 were further oxidized and not present anymore. This indicates that secondary TPs with *N*-oxide moieties, do not induce an effect to the receptor and the remaining anti-estrogenic activity was caused by TP 270 and/or TP 388. Higher standard deviations are due to variations of the individual sets of experiments, which are given in Figure A4-1 and Figure A4-2.

pH 11

Experiments at pH 11 were largely hampered by the low solubility of TAM at this pH. Neither a degradation of TAM nor a change of the effect was observed during ozonation. Hence, these results will not be discussed, but are shown in Figure A4-1 C, F, and Figure A4-2 C.

4.5.2 Correlation of anti-estrogenic activity and chemical analysis

To determine which TPs cause the endocrine effects, their signals or concentration where correlated with the anti-estrogenic activity (see Figure 4-4).

Anti-estrogenic activity and tamoxifen

In case no effect inducing products are formed the anti-estrogenic activity should decrease with the tamoxifen removal, giving a negative slope in the correlation. At pH 7 no correlation ($R^2 = 0.08$) was observed (Figure 4-4 A). However, pH 3 ($R^2 = 0.77$) revealed a positive slope indicating that TPs had an anti-estrogenic effect.

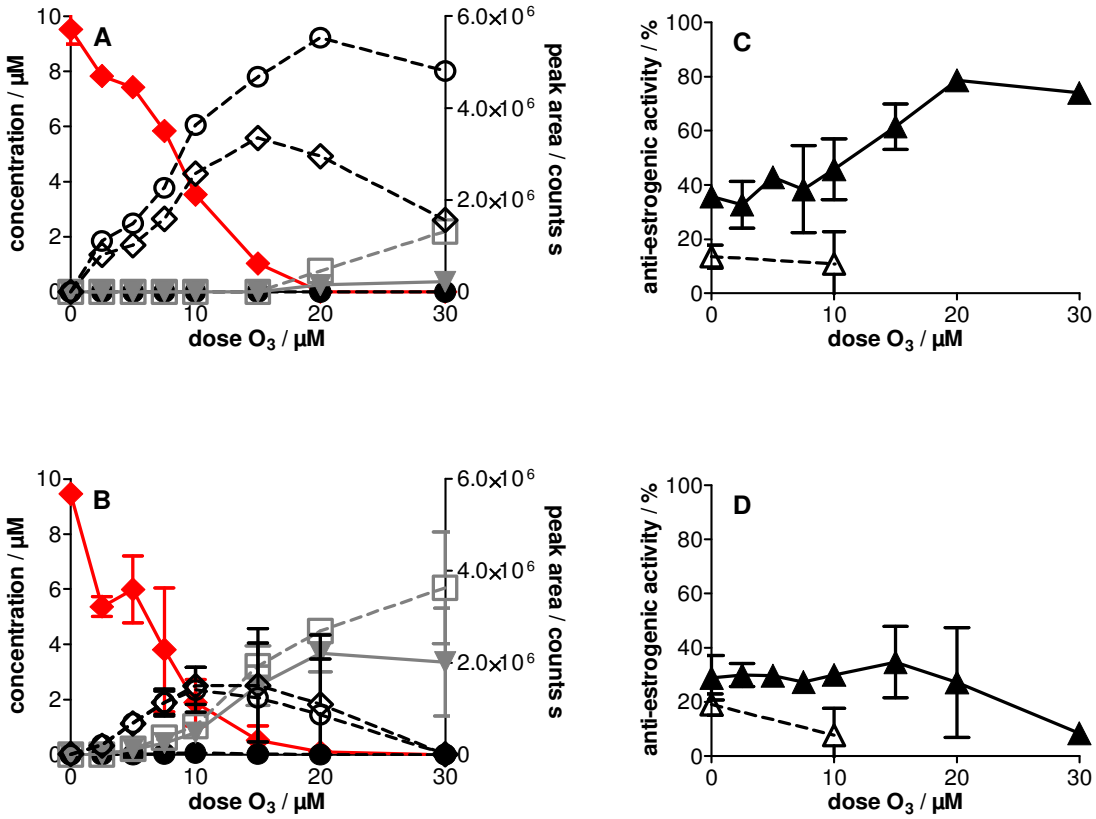

Figure 4-3: Degradation of TAM at pH 3 (A) and pH 7 (B) during ozonation and formation of TPs. ○ - TP 270, □ - TP 286, ◊ - TP 388, ▼ - TP 404, ♦ - TAM, and ● - TAM-N-oxide. Anti-estrogenic activity as inhibition of 50 ng L^{-1} E2 in % after ozonation of TAM (▲) and in solvent blanks (Δ) at pH 3 (C) and pH 7 (D). Deviation of the mean is given as error bars. If no error bar is visible, standard deviation is smaller than symbol size. Both individual data sets are presented in the supporting material in Figure A4-1 and Figure A4-2. Here standard deviations of replicate measurements are given as error bars.

Anti-estrogenic activity and TPs

Peak area of TP 270 (Figure 4-4 B) increases complying with the anti-estrogenic activity at pH 3 (R^2 = 0.72) but not at pH 7 ($R^2 \leq 0.15$). For TP 388 (Figure 4-4 D) poor correlations were determined at pH 3 (R^2 = 0.36) and at pH 7 ($R^2 \leq 0.18$). Nevertheless, for TP 270 and TP 388 a positive albeit weak correlation was determined. Since both TPs are always present simultaneously, at least one of these TPs has an anti-estrogenic effect. Due to the rather good correlation with the peak area for TP 270 we assume that this TP is responsible for the anti-estrogenic effect observed in this study. TP 388 might also induce an anti-estrogenic effect and therefore contribute to the

observed effect. Based on our results, this is not distinguishable. Indeed, both TPs have the essential moieties of TAM required for binding to the ER, i.e., the benzene ring and the amine (Figure 4-5).

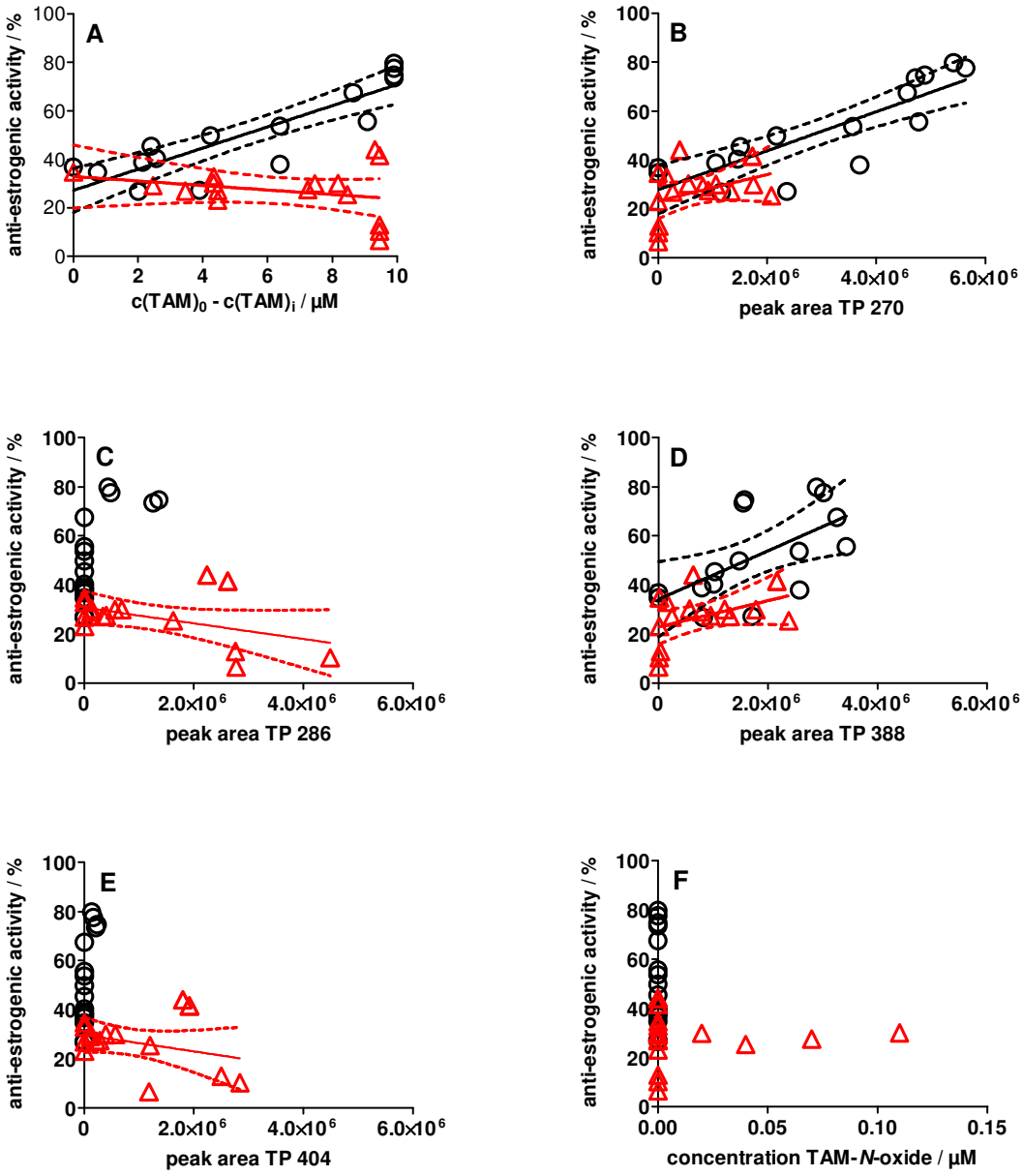

Figure 4-4: Correlation of observed anti-estrogenic activity and (A) removal of tamoxifen (initial concentration of tamoxifen ($c(TAM)_0$) – concentration of tamoxifen at ozone dose *i* ($c(TAM)_i$)), (B) peak area of TP 270, (C) peak area of TP 286, (D) peak area of TP 388, (E) peak area of TP 404, and (F) concentration of TAM-*N*-oxide. Data from Figure 4-3. ○ - experiments at pH 3 shown in black, **Δ** - experiments at pH 7 shown in red. (Confidence interval: 95%).

A correlation for TP 286 and TP 404 could not be established due to their formation only at pH 7 and insufficient correlation of peak area and anti-estrogenic effect (R^2 = 0.19; R^2 = 0.10, respectively). TAM-*N*-oxide was only determined at pH 7 in two samples hence, no correlation with the anti-estrogenic effect could be established. These three TPs, TAM-*N*-oxide, TP 286 and TP 404, are *N*-oxides (Figure 4-5). Due to their zwitterionic nature of the *N*-oxide, compared to the amine cation at pH 7, the stabilizing ionic bond to ASP 351 in the ER [25] is inhibited. Thus, the ligand-ER-complex might be destabilized indicating that these TPs have a lower affinity to the ER than TAM. In contrast, the cleavage of the double bond according to the Criegee reaction [36] yields a smaller molecule which still has all required moieties for binding to ER, i.e., a benzene ring for binding to the LBD cavity and an amine acting as a terminal basic moiety in the side chain. The reduced size could cause a higher affinity towards the ER compared to tamoxifen and hence give an increased anti-estrogenic effect as observed for TP 270 formed at pH 3.

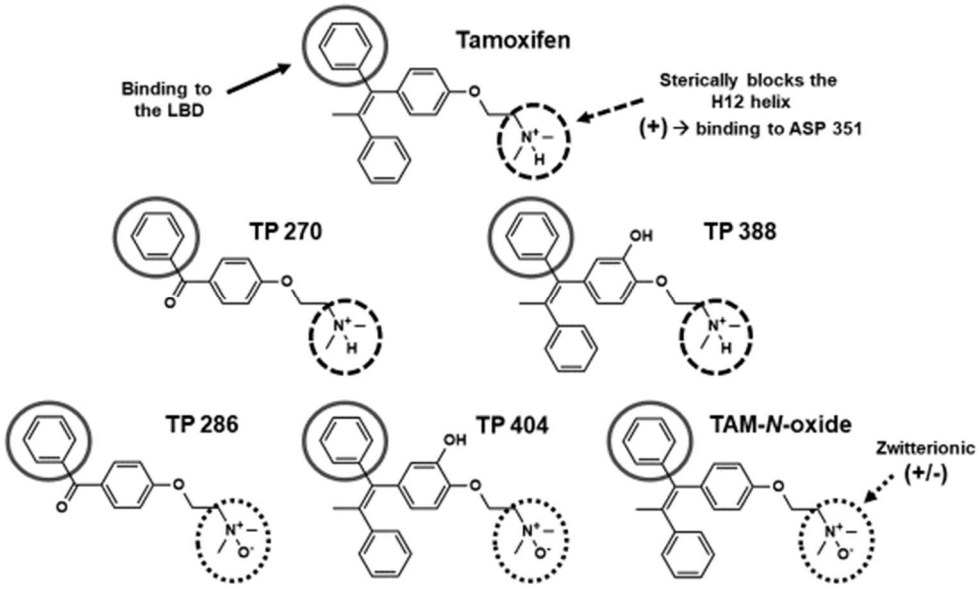

Figure 4-5: Structures of tamoxifen and the observed TPs. Moieties binding to the LBD cavity: full circle, tertiary amine (basic, at pH 7 positive charge): dashed circle, *N*-oxide (zwitter-ionic): dotted circle.

The present study has shown that TAM may be a precursor of transformation products formed during ozonation which are more harmful than TAM itself. Even though ozonation can remove undesired effects of pollutants such as ciprofloxacin, metoprolol and sulfamethoxazole [37], E2, EE2, and bisphenol A [2], formation of products having a toxicological relevance was already reported as well (e.g., TPs from diclofenac,

acyclovir and ibuprofen [38-41], *N*-nitrosodimethylamine (NDMA) and bromate [42, 43]). The observation of recalcitrant mutagenic effects and fish toxicity after wastewater ozonation, which could not be explained with the pollutants monitored [44], indicates that even more TPs or by-products may have a toxic potential.

The mechanistic considerations of the present study can be transferred to other SERMs such as toremifene (TOM), clomifene, and ospemifene (for structures see Figure A4-5). For TOM and clomifene the formation of TP 270 or a chlorinated derivative of TP 388 is possible. In ospemifene the tertiary amine is substituted by a hydroxyl group, which cannot be attacked by ozone. Hence, no *N*-oxide formation is possible and the olefin moiety will be the major target for ozone reactions at all pH values relevant for wastewater and water treatment. However, the TPs formed in these reactions have to be investigated for their (anti-) estrogenic activity. For other SERMs, such as raloxifene or lasoxifene, which vary strongly in the structure to tamoxifen, no estimation based on our results is possible.

The phase-I-metabolites of these TAM-like SERMs results in hydroxylation of their benzene moieties. This strongly activates the benzene rings which largely increases the reactivity towards ozone [35]. Hence, other TPs will likely be formed during ozonation of the TAM-like SERM metabolites. For the case of the phase-I-metabolites the benzene ring, which binds to the LBD is affected, alterations in the effect due to ozonation will show a significant change.

This study indicated that the TAM-(/TP-) *N*-oxides may reveal a lower anti-estrogenic potential than the parent compounds. However, these *N*-oxides can be biologically transformed to amines [45] resulting in a reappearance of TAM in a biological treatment step or in the natural aquatic environment. Hence, the result of the oxidative water treatment can be influenced by all treatment options in a water purification chain.

Based on the second order rate constant of TAM at pH 7 (3.56×10^6 $M^{-1}s^{-1}$) reported by Chen et al. (2008) [20], it can be assumed that during real water treatment at pH 7-8, tamoxifen is fully removed since compounds with second order rate constants $>10^5$ $M^{-1}s^{-1}$ are typically almost completely (>90%) degraded during wastewater treatment [46]. However, the anti-estrogenic effect in full scale ozonation of wastewater remains after ozonation [1, 3]. Our study shows for the first time that TPs formed during ozonation (here TP 270, TP 388) could explain the retained anti-estrogenic effect in treated wastewater. In future investigations, a step-wise increase in complexity of

water matrix towards a real wastewater is necessary in order to validate if and how these effects change.

4.6 Conclusions

- Based on the correlation of the formation of TP 270 and the anti-estrogenic activity, it can be concluded that TP 270 induces a strong anti-estrogenic effect.
- For TP 388, the correlation is not sufficient to allow a precise statement, but an anti-estrogenic activity of TP 388 cannot be excluded.
- At pH 7 (water treatment conditions), the endocrine effect of tamoxifen can be removed by ozone.
- Especially at pH 3 ozonation of TAM can form TPs with an increased anti-estrogenic effect.
- Formation of TPs needs to be considered for the evaluation of oxidative processes in water treatment.

4.7 References

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4.8 Appendix A4: Supporting Material

4.8.1 A4-1 Analysis and anti-estrogenic activity

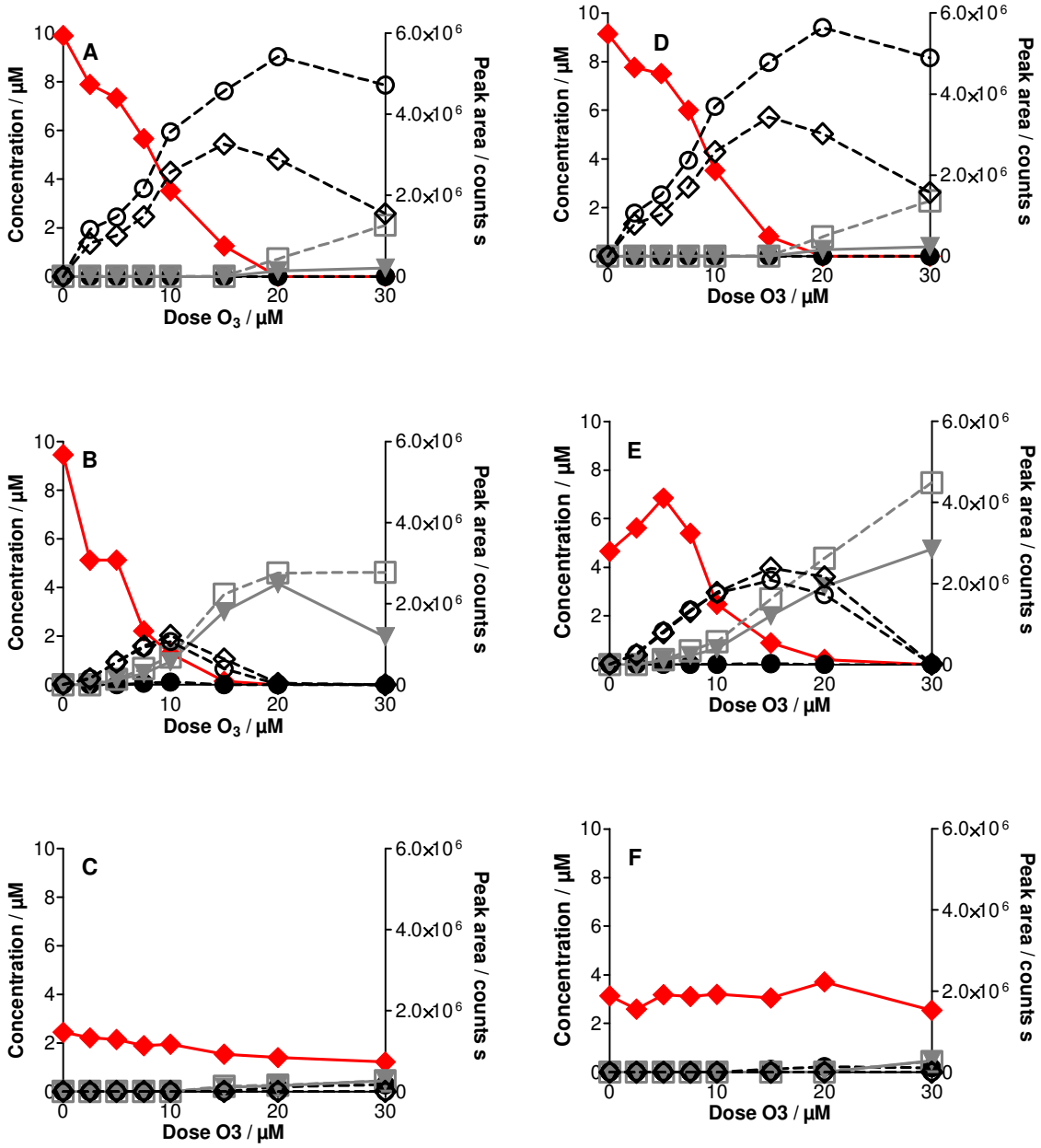

Figure A4-1: Degradation of TAM (experiment 1 (A, B, C) & experiment 2 (D, E, F)) at pH 3 (A & D), pH 7 (B & E), and pH 11 (C & F) during ozonation and formation of TPs. Peak areas for ○ - TP 270, □ - TP 286, ◊ - TP 388, and ▼ - TP 404, concentration for ♦ - TAM and ● - TAM-N-oxide. Standard deviations are shown as error bars.

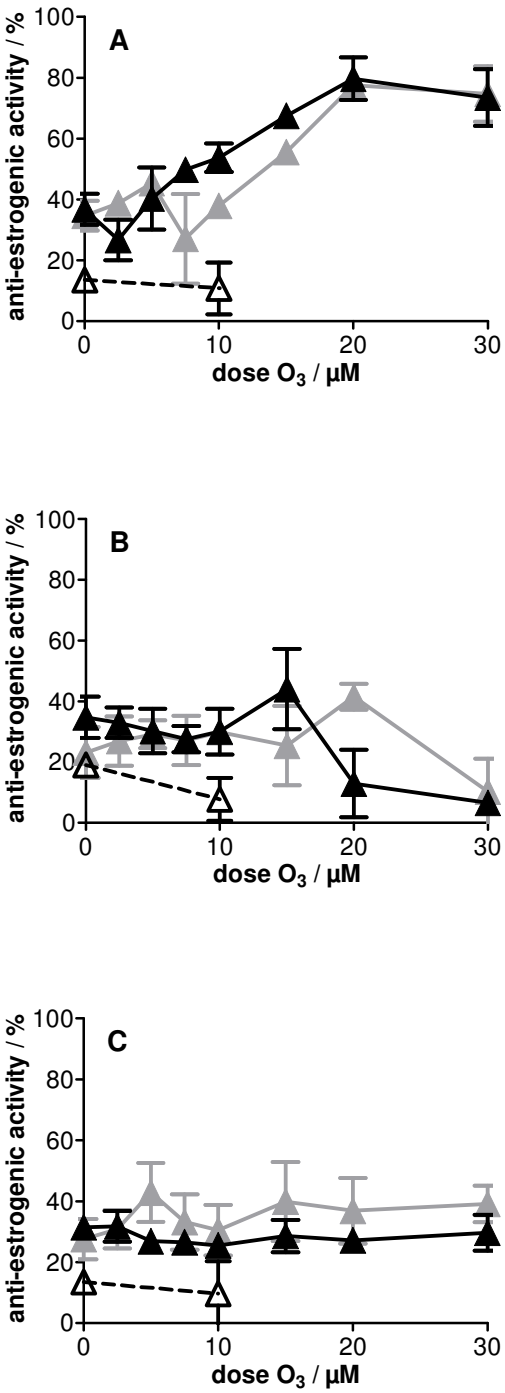

Figure A4-2: Anti-estrogenic activity as inhibition of 50 ng L^{-1} E2 in % after ozonation (TAM ▲ - experiment 1, ▲ - experiment 2, and Δ – solvent blank) at pH 3 (A), pH 7 (B), and pH 11 (C). Standard deviations are shown as error bars.

4.8.2 A4-2 Experiments at pH 11

At pH 11 only lower concentrations of tamoxifen were detected (> 2.5 µM), which decreased only slightly with the increasing ozone dose. In these experiments tamoxifen showed to be hardly soluble at pH 11 even in 100 mM TBA and a slight turbidity indicated precipitation of tamoxifen. However, since pH was adjusted to pH 7 subsequent to the ozonation in all samples, the turbidity disappeared. The quantitative evaluation showed low recovery. Additionally, TP formation was only observed with peak areas an order of magnitude below those at pH 3 and pH 7. This indicates that only a small fraction of ozone reacted with tamoxifen. Formed hydroxyl radicals were scavenged by the radical scavenger TBA (100 mM). Therefore degradation of tamoxifen by $^{\bullet}OH$ can be ruled out. Only *N*-oxide containing TPs were observed. For pH 11 no correlation of activity and formed TPs can be assumed.

4.8.3 A4-3 Quantification Limits

Limit of detection (LOD) and limit of quantification (LOQ) were determined according to the DIN 32645:2008-11 and are given in Table A4-1. Quantification of TAM was performed using nonlinear regression.

Table A4-1: Correlation coefficient, LOD, and LOQ calculated according to the DIN 32645 using linear regression in the concentration range from 0.1-1.0 µM.

	TAM	TAM-N-oxide
R^2	0.9930	0.9976
LOD / µM	0.05	0.03
LOQ / µM	0.15	0.09

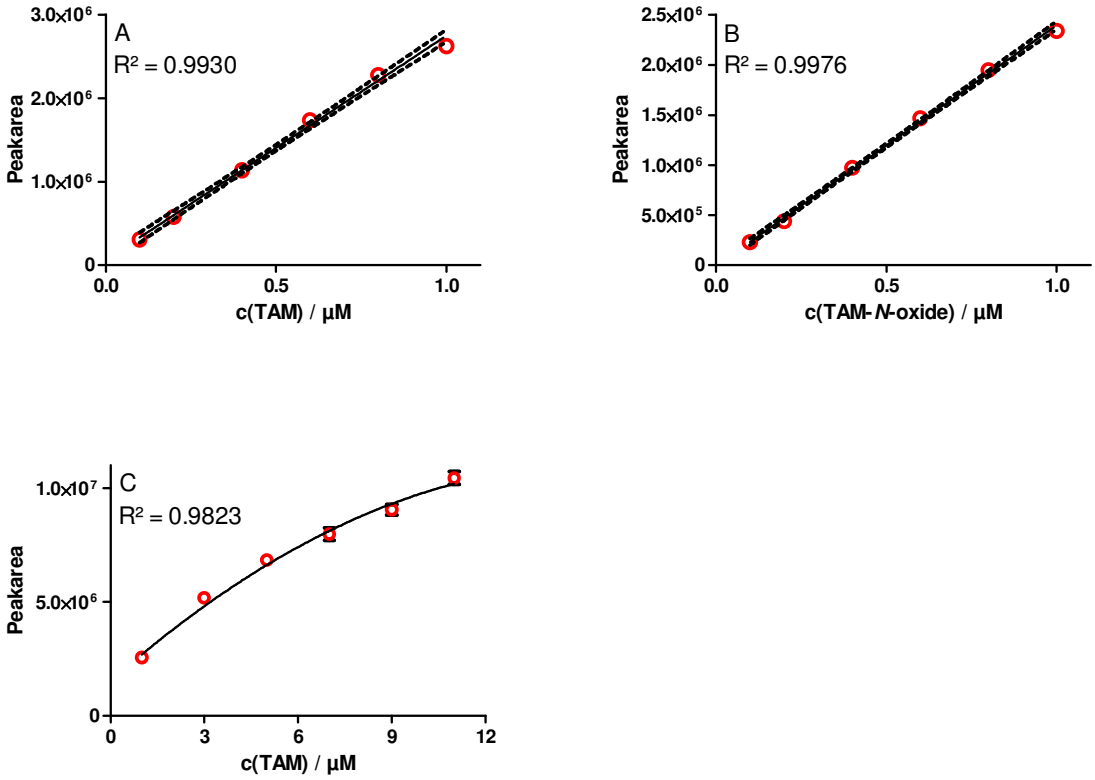

Figure A4-3. Linear calibration used for the determination of the LOD and LOQ for TAM (0.1-1.0 µM, A), LOD, LOQ and quantification for TAM-*N*-oxide (0.1-1.0 µM, B), and nonlinear calibration (second order polynomial) for quantification of TAM (1.0-11.0 µM, C). Calibration standards were measured in triplicates. Standard deviation is given as error bar. 95% confidence intervals are shown for linear calibration.

4.8.4 A4-4 Visualization of the Estrogen Receptor

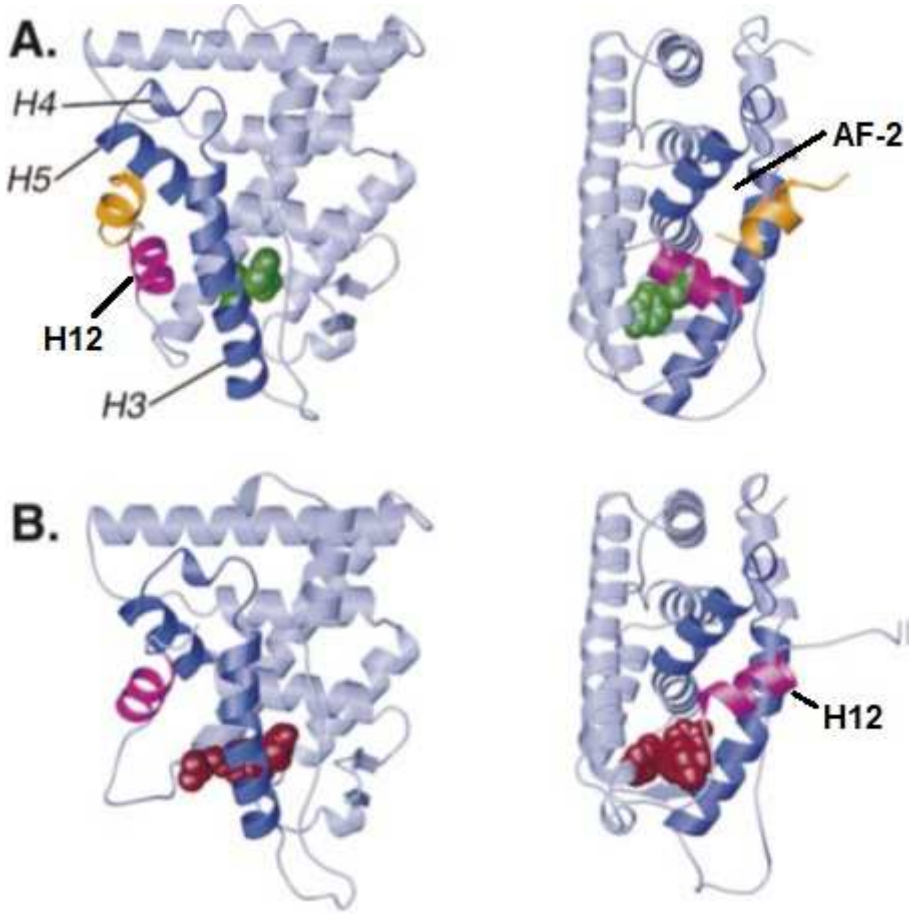

Figure A4-4. Overall Structures of the diethylstilbestrol-ERα LBD-coactivator complex and of the 4-OH-TAM-ERα LBD complex. Two orthogonal views of A) the diethylstilbestrol (DES)-ERα LBD-coactivator complex and (B) the 4-OH-TAM-ERα LBD. The coactivator peptide (gold) and the LBD are shown as ribbon drawings. Helix 12 (residues 538–546) is colored magenta and helices 3, 4, and 5 (H3, H4, and H5, respectively) are colored blue. DES (green) and 4-OH-TAM (red) are shown in space-filling representation. Source: Shiau et al. (1998) [1])*

* Reprinted from Cell, 95, Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, et al., The Structural Basis of Estrogen Receptor/Coactivator Recognition and the Antagonism of This Interaction by Tamoxifen, 927-937, 1998, with permission from Elsevier.

A vivid video showing the 3D structure of the ER is available online http://pc1664.pharmazie.uni-marburg.de/book/html/msv/Abb28_6.html (access date 28.08.2017; Klebe (2003) [2]).

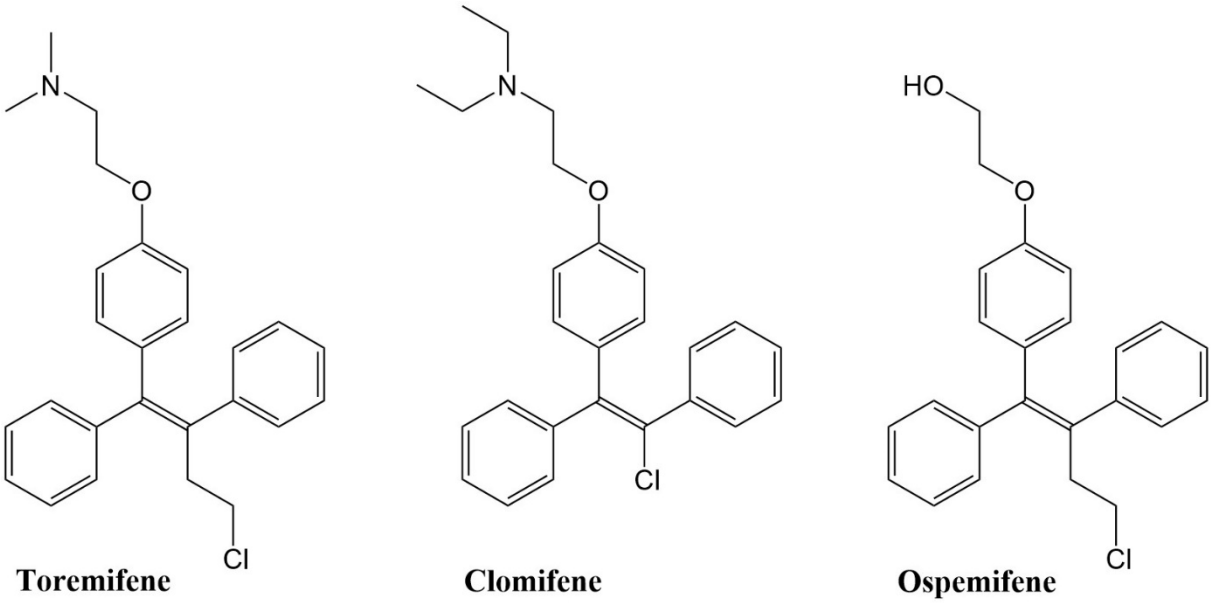

Figure A4-5: Tamoxifen analogical SERMs toremifene, clomifene, and ospemifene.

4.8.5 A4-5 Refferences

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5 Ecotoxicological effects prior to and after the ozonation of Tamoxifen

Adapted from O. Knoop, M. Woermann, H.V. Lutze, B. Sures, T.C. Schmidt, Ecotoxicological effects prior to and after the ozonation of Tamoxifen, Journal of hazardous materials, 358 (2018) 286-293.
https://doi.org/10.1016/j.jhazmat.2018.07.002

5.1 Abstract

The endocrine disrupting micropollutant tamoxifen can induce several effects on aquatic organisms. It is introduced into the environment mainly by wastewater treatment plant effluents. To reduce the discharge of micropollutants into surface waters, ozonation can be used as additional wastewater treatment option. For only few transformation products (TPs) formed by ozonation ecotoxicological data are available. To enable an initial estimation of ecotoxicological potentials of the TPs formed after the ozonation of tamoxifen, acute toxicity (immobilization) to *Daphnia magna* and green algae growth inhibition using *Desmodesmus subspicatus* were determined for several ozone doses spiked at pH 3 and pH 7. The initial immobilization of *D. magna* by tamoxifen was not further observed after ozonation. In contrast, the green algae growth inhibition increased due to ozonation of tamoxifen. Overall, five transformation products were observed. For three TPs, positive correlations of green algae growth inhibition and peak area were determined, whereas two TPs do not induce the residual effects. Based on our observations, TP 270 can be assumed as most potent of the formed TPs concerning green algae growth inhibition. Since the effect is not induced by formed *N*-oxides, green algae growth inhibition could be reduced by sufficient ozone exposure during wastewater treatment.

5.2 Introduction

Micropollutants such as pharmaceuticals, personal care products, and other anthropogenic chemicals, can be found in nearly all surface waters and can induce biological effects already in low concentrations [1, 2]. One major source of micropollutants are the effluents of wastewater treatment plants since most of them cannot be removed sufficiently by conventional wastewater treatment [2-4]. Here, advanced treatment processes can be used to reduce the amount of micropollutants emitted into receiving surface waters [5, 6]. Ozonation was shown to be an effective treatment to reduce the discharge of micropollutants and has successfully been tested in large scale [7-10]. Along with a reduction of micropollutant discharge most toxicological effects (e.g. estrogenic activity) can also be reduced by ozonation [11, 12]. However, the reaction of micropollutants with ozone does not result in mineralization but rather in the formation of transformation products (TPs) [13]. Some of these TPs, e.g. *N*-nitrosodimethylamine (NDMA) and bromate (from oxidation of bromide), can also induce toxicological effects [14-17]. but only few TPs are identified yet, compared to the broad range of original micropollutants and for even less of the known TPs toxicological information is available [8, 16, 18]. Accordingly, a combination of analytical and effect monitoring is necessary for the evaluation of a risk reduction by advanced wastewater treatment [19, 20].

Endocrine disruptive compounds (EDCs) affect the hormonal system of organisms and can thus affect aquatic organisms already at very low concentrations [21]. The antineoplastic pharmaceutical tamoxifen (TAM) is used for breast cancer therapy due to its anti-estrogenic activity [22]. TAM can be found in wastewater treatment plant effluents in typical concentrations of 25 to 200 ng L^{-1} [23-25], with 369 ng L^{-1} being the highest reported concentration [26]. Thereby, concentrations in surface waters were found to range between 25 to 50 ng L^{-1} [23, 25, 27] with a maximum of 212 ng L^{-1} in the river Tyne [26]. These TAM concentrations are in the same range as predicted environmental concentrations (PECs) of TAM in Sweden and England (36 to 63 ng L^{-1}) [23, 28].

Effect concentrations with an effect of 50 % (EC_{50}) for acute toxicity of TAM are only available for the immobilization of the invertebrate *Daphnia magna* (EC_{50} = 1530 µg L^{-1} (24 h) [29] and 210 µg L^{-1} (48 h))[30]. Since TAM was found continuously in a river basin in concentrations 3 orders of magnitude below available acute toxicity values [27], no acute effects are expected. Therefore, chronic toxicity with observable effects

at lower concentration levels is of higher interest. For *Daphnia* species, the $EC_{50,\ reproduction}$ has a concentration of 810 ng L^{-1} [29]. For the inhibition of the growth of green algae similar effect levels are reported for the inhibition concentration (IC_{50}) for different green algae species (470 to 980 ng L^{-1}) [30]. However, TAM has an $EC_{50,\ sex\ ratio}$ on zebra fish (*Danio rerio*) populations at concentrations of 150 ng L^{-1} due to its anti-estrogenic activity [31], but no acute toxicity was found during zebrafish embryo tests (≤ 1850 µg L^{-1}) [30]. Based on reported no observable effect concentrations (NOEC) available for *Daphnia magna* [30] the predicted no effect concentration for TAM is 6.7 ng L^{-1}, calculated using a risk assessment factor of 100 [32]. This NOEC is below all reported concentrations [23, 25-27] and therefore underlines the need to further study TAM behavior in advanced wastewater treatment. Here, especially TP formation and their biological effects are of high interest [18].

TAM reacts fast with ozone and is readily transformed during ozonation of wastewater effluents [33]. Thereby several TPs are formed, of which two can enhance the anti-estrogenic effect of TAM [34]. Two TPs formed during ozonation were also observed during advanced oxidation processes and photolysis, for which a residual acute toxicity for *Aliivibrio fischeri* is reported [29, 35]. Structures of conceivable TPs formed in ozonation of TAM were proposed previously (see Figure 5-1) [36]. Contribution of hydroxyl radicals for TAM abatement was calculated using the approach of Lee & von Gunten (2016) [18], arriving at ≤ 1 % contribution of hydroxyl radicals to TAM transformation. Hence, the contribution of radical reactions to TP formation can be neglected.

In order to gain first information about possible adverse effects of TPs, standardized tests such as the OECD 201 [38] and OECD 202 [39] guidelines can be applied as they are easy to handle. Nevertheless, precursor specific effects, such as anti-estrogenic activity are of high interest and should also be monitored. The anti-estrogenic effect after the ozonation of TAM has already been reported elsewhere [34].

The current study therefore focuses on (I) effect on acute toxicity of tamoxifen on *Daphnia magna* and (II) effect on growth inhibition of the algae *Desmodesmus subspicatus* following ozonation of tamoxifen, both at pH 3 and pH 7.

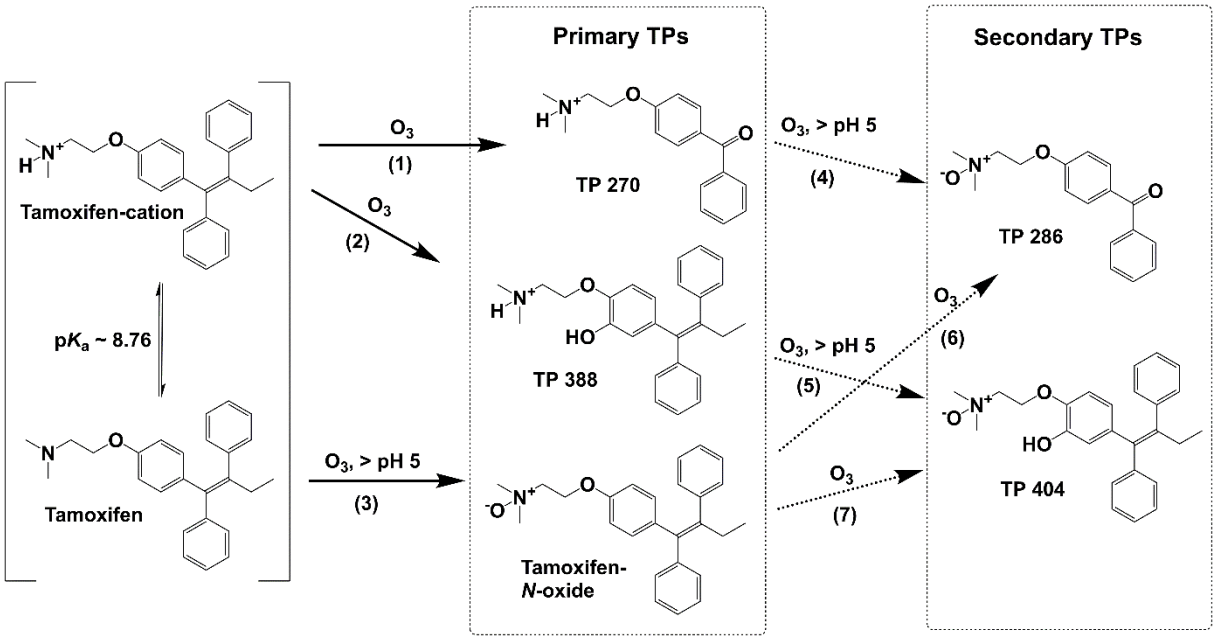

Figure 5-1: Proposed transformation products and major reaction pathways for the ozonation of TAM at stated pH ranges. TP 270, TP 388, and TAM-*N*-oxide as primary TPs, and TP 286 and TP 404 as secondary TPs[36]. Note that reaction 3 will dominate at pH > 5 [36], due to the high second order rate constants for the reaction of tertiary amines and ozone [37]. Formation of *N*-oxides (reactions 3, 4 & 5) require presence of the corresponding deprotonated species and are most important at a pH > 5, whereas Criegee reaction (1 & 6) and hydroxylation of the benzene ring (2 & 7) are most important at pH < 5. Reaction of the phenolic moieties of TP 388 and TP 404 with ozone have yet not been reported.

5.3 Materials and Methods

5.3.1 Chemicals

Tamoxifen (CAS: 10540-29-1) was purchased from Alfa Aesar (Karlsruhe, Germany), tamoxifen-*N*-oxide (analytical standard) from LGC (Ann Arbor, Michigan, USA), dimethyl sulfoxide (analytical reagent grade) from VWR (Darmstadt, Germany). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate (Merck) were used for the preparation of a pH 7 buffer (100 mM). Sodium hydroxide (VWR, Darmstadt, Germany) and phosphoric acid (Fisher Scientific, Bremen, Germany) were used for pH adjustment. Purity of all chemicals was ≥ 98 % if not stated otherwise. Ultra-pure water was produced onsite (Purelab Ultra, Elga LabWater, Celle, Germany). For liquid chromatography-mass spectrometry (LC-MS) measurements, LC-MS grade methanol (HiPerSolv CHROMANORM, VWR), triple distilled water, and formic acid (Suprapur, Merck) were used as eluents.

To obtain an aqueous ozone stock solution ozone-containing gas was produced onsite with an ozone generator (BMT 802 X, BMT Messtechnik, Berlin, Germany; feed gas: O_2 6.0, Linde, Düsseldorf, Germany) and bubbled through ice-cooled ultra-pure water. Ozone concentration in the stock solution was determined by UV absorption at 258 nm (ε = 2950 M^{-1} cm^{-1}) [40] using a UV-1650PC UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). Accuracy of spiking ozone was ± 5 % and determined using the indigo method [37].

Reconstituted freshwater, or Aachener Daphnien Medium (ADaM) modified after Klüttgen et al. [41], was prepared and aerated continuously until use. A description of the composition is given in Table A5-2.

A modified algae growth medium (AAM) was used for algae culture maintenance. For growth inhibition tests, twice the concentration of AAM was prepared as double AAM (DAAM) stock solution to gain same concentrations in the test vessels due to 1:1 (v/v) dilution. Compositions of both media are given in Table A5-5.

5.3.2 Experimental set up

2 L stock solutions, containing 10 µM TAM (3.71 mg L^{-1}), were prepared in volumetric flasks using a 5 mM TAM solution in DMSO, yielding a 5 mM DMSO concentration. Further, dimethyl sulfoxide (DMSO) was added to gain a final concentration of 10 mM DMSO. The pH was adjusted using phosphoric acid and sodium hydroxide to pH 3 and pH 7 before adjusting the volume finally with ultrapure water. As samples, aliquots of 150 mL were spiked with the ozone stock solution to gain final concentrations of 0, 5, 10, 15, 20, 30, and 40 µM ozone. Samples were stored overnight and pH was checked the next day. Subsequently, the pH was adjusted to pH 6.5 ± 0.1 by spiking 1.5 mL of a 100 mM phosphate buffer (pH 6.5) to each sample, and sodium hydroxide if required, to gain suitable conditions for the toxicity test. Blanks containing DMSO and phosphate buffer were produced accordingly and spiked with the ozone stock solution to gain final ozone concentrations of 0, 20, and 40 µM. 1 mL of each sample was used for LC/MS analysis. Samples were then stored until toxicity testing in the dark at 4 °C for a maximum of 10 days. The concentration of added radical scavenger DMSO (10 mM) in the samples allowed scavenging of > 95 % of hydroxyl radicals eventually formed. Dilution due to addition of the ozone stock solution was ≤3 %. Ozonation experiments at basic pH were not performed, since previous experiments showed a limited reaction at pH 11 and minor formation of TPs due to a lowered solubility of TAM under basic

conditions. Furthermore, the reaction of tamoxifen at pH > 5 is largely controlled by the tertiary amine. Hence, the primary point of attack of ozone can be assumed to be the same at pH 7 and pH > 7 (i.e., tertiary amine) [34].

Experiments in wastewater matrix were not performed to ensure that any changes of the effect were solely based on the TPs formed in the reaction of ozone and TAM.

5.3.3 LC/MS measurements

Measurements were performed as described previously [34] and are described here only in brief. The LC/MS system used consisted of an Agilent 1100 Series LC and a 6120 quadrupole LC/MS (Agilent, Waldbronn, Germany), using a Kinetex® C8 column (50 x 2.1 mm; 5 µm; 100 Å; Phenomenex, Aschaffenburg) for separation. The applied gradient used methanol (+0.1 %/v formic acid) / water (+0.1 %/v formic acid) started at 45 % methanol at a flow rate of 0.5 mL min^{-1} and was kept constant for 0.5 min. The gradient was then increased to 60 % within 1.5 min and further increased to 70 % within 3 min and kept constant at 70 % for 5 min, before reconditioning at 45 % methanol for 4 min. The injection volume was set to 10 µL and all samples were analyzed in triplicates.

Positive electrospray ionization was set to 3 kV and the nebulizer pressure to 30 psi. Dry gas was heated to 300 °C and the flow rate was set to 10 L min^{-1}. The ions of *m/z* 270.1, 286.1, 372.1, 388.2, and 404.2 were monitored using selected ion mode. 20 % of the signal time were operated in scan mode. TAM and TAM-N-oxide (*m/z* 388.2) were quantified using an external calibration. For the other TPs no reference standards were available, hence a semi-quantitative evaluation was performed for these, namely TP 270, TP 286, TP 388, and TP 404. Names are based on the corresponding *m/z*.

5.3.4 Daphnia magna immobilization tests

D. magna acute toxicity test was performed following the OECD guideline 202 [39] to determine the $EC_{50,\ immobility}$ and effect change after ozonation at ozone doses of 0, 20 and 40 µM. For toxicity testing, daphnids younger than 24 h were obtained from cultures with daphnids aged 3-12 weeks. Cultures of each ~20 daphnids are kept in 1 L beakers filled with ADaM at a light cycle of 8/16 h dark/light at (20 ± 1°C), fed three times a week with concentrated algae (*Desmodesmus subspicatus*) and water exchanged three times a week.

Test solutions were prepared by diluting pH 7 TAM or DMSO stock solutions as described in 2.2 for $EC_{50,\ immobility}$ determination with at least 50 mL ADaM in a 100-mL volumetric flask. Highest TAM concentration in the test was hence 5 µM (1.86 mg L^{-1}). Applied DMSO concentrations were tested in advance and did not show any effect on the daphnids or deviation from the ADaM as blank and the highest applied concentration of DMSO (5 mM) was hence used as negative control. For determining the effect after ozonation, aliquots of 34 mL of each sample were diluted accordingly with ADaM to a total volume of 100 mL, resulting in a theoretical TAM concentration of 3.4 µM (1.26 mg L^{-1}) in the test. Dilution was chosen based on results obtained during EC_{50} determination to allow observation of decrease and increase of the effect.

For each tested concentration/sample 4 aliquots of 20 mL were filled into separate 50 mL glass beakers. Subsequently, 20 healthy neonates were preselected and given into the remaining 20 mL of the corresponding concentration/sample before 5 daphnids were distributed into each beaker to avoid dilution in the test vessels (n=20). Physicochemical properties were checked before and after each experiment using a LE621 IP67 dissolved oxygen sensor (Mettler-Toledo, Greifensee, Switzerland) for oxygen saturation and temperature, and pH using test stripes (DOSATEST® pH 6.0 – 10.0, VWR). Daphnia tests were kept in darkness at constant temperature (21 ± 1°C). Mobility was monitored by visual inspection after 24 and 48 hours. pH was in the range of 6.4 – 7.0 and dissolved oxygen 4 – 6 mg L^{-1}. Potassium dichromate was used as positive control to ensure reliability of the test. As defined by OECD Guideline 202, Annex 1 [39], organisms unable to swim within 15 seconds after gentle agitation of the test vessels were considered immobile. $EC_{50,\ immobility}$ was calculated and dose-response curves were plotted using Graph Pad Prism 5.01.

5.3.5 Algae growth inhibition tests

Algal growth inhibition was tested using the freshwater green algae *Desmodesmus subspicatus f*ollowing the respective OECD 201 guideline [38]. Algae cultures were maintained and harvested according to ISO 8692 [42], using a modified cultivation medium (AAM). As test vessels, sterile 24-well microplates (Cat. #10062-896, VWR, USA) with a sample volume of 2 mL per well were used. Biomass was determined by measuring the fluorescence of the chlorophyll content with a multimode reader Infinite M200 (Tecan, Switzerland) based on a previously determined factor. For more details see A3.2.

For the determination of the IC_{50} value 5 concentrations were prepared by diluting the pH 7 TAM stock solution as described in 2.2 in the range from 0.5 to 3.7 mg L^{-1} with water in 10-mL volumetric flasks. The highest amount of DMSO (10 mM) gained in the test solutions was used as solvent control. Ultrapure water was used for negative control blanks. Each well was prefilled with 1 mL DAAM containing a predefined biomass of algae and subsequently 1 mL of the according test solutions were added to a well according to the experimental design (Table A5- A). The highest tested TAM concentration in the test solutions was 5 µM (1.86 mg L^{-1}).

Algae growth inhibition of TAM-*N*-oxide was tested in the concentration range of 0.15 – 3 µM (same concentrations were formed during ozonation experiments), using a TAM-*N*-oxide stock solution in DMSO. Thereby the same procedure as described for TAM was used, gaining 3 µM as highest concentration of TAM-*N*-oxide in the test (1.16 mg L^{-1}).

Samples spiked with ozone concentrations of 0, 10, 20, 30, and 40 µM were prepared accordingly, including solvent controls spiked with ozone concentrations of 0, 20, and 40 µM. Here, either samples were used without dilution, gaining a dilution of 1:1 (v/v) in the well, or by reducing the sample amount to 0.5 mL and addition of 0.5 mL ultrapure water, gaining a dilution of 1:3 (v/v), resulting in theoretical TAM concentrations of 1.86 mg L^{-1} and 1.24 mg L^{-1} in the wells, respectively. Overall, 6 replicates per concentration were inserted into microplates using the pre-defined experimental design (Table A5-8 B & C). The initial biomass concentration of 0.48 mg L^{-1} in each well was confirmed via fluorescence measurements. Additionally to the standard lid, test plates were sealed with PARAFILM® (Brand, Wertheim, Germany) and incubated using a Celltron shaker (InforsHT, Bottmingen, Switzerland) at 100 rpm, 21 ±1°C and light intensity of 120 µeinstein m^{-2} s^{-1}, measured respectively by a thermometer HI 98128 (Hanna, USA) and a quantum-radiometer-photometer LI-185B (Li-Cor, USA) coupled to a LI-190SB quantum sensor. Prior to measurement of the biomass growth after 24, 48, and 60 h, each well was mixed thoroughly and uncovered microplates were pre-shaken automatically for 30 s again. For further details of the fluorescence measurements see S3.2. Growth rates and percent inhibition of the growth rate were calculated according to OECD 201 [38]. For IC values dose-response curves were plotted as described for acute toxicity and values estimated in accordance with the guideline [38].

5.4 Results and Discussion

5.4.1 Effect concentrations

Effect and inhibition concentrations of TAM for an effect of 50 % (EC_{50}/IC_{50}) were determined to allow for comparability with previously reported EC_{50} values. Effect-concentration curves are shown in Figure 5-2 for *D. magna* (A) and *D. subspicatus* (B) and thereby calculated EC_{50}/IC_{50} values are given in Table 5-1.

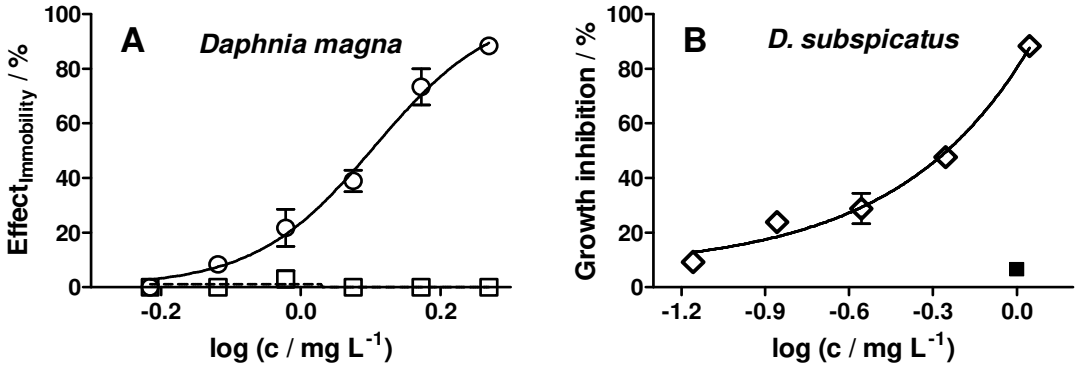

Figure 5-2: Determination of the $EC_{50,\ immobility}$ of *D. magna* after 24 h (open squares) and ○ 48 h (circles) at pH 7 (A) of TAM and the IC_{50} of the growth inhibition for *D. subspicatus* (diamonds) and DMSO blanks (filled square) after 60 h at pH 7 (B).

The $EC_{50,\ immobilization,\ 48\ h}$ for *D. magna* is in the same range as the values reported previously [29, 30] considering the known variation occurring in these tests [43]. For *D. subspicatus* the $IC_{50,\ growth}$ is also in a similar range to the reported value for *Pseudokirchneriella subcapitata* and two further green algae [30], albeit direct comparison is limited since effect concentrations are species specific. Nevertheless, effects at similar concentrations were observed and hence, changes of the effects after ozonation can be evaluated based on tests applied in this study. To the authors' best knowledge, no ecotoxicological data of *D. subspicatus* for TAM are available in the literature. Due to the test design, no higher concentrations were tested and IC values cannot be calculated, but estimated from the dose-response curve in accordance with the guideline [38]. Hence, calculation of the confidence interval was not possible. Higher stock solutions of TAM could not be used due to the limited solubility of TAM.

Table 5-1: Reported and determined EC_{50}/IC_{50} for TAM. Confidence interval (CI) of 95 % is stated if available. * Calculation of CI for *D. subspicatus* was not possible in this study.

Species	Source	Exposure time / h	Endpoint	EC_{50} / µg L^{-1}	CI (95 %) / µg L^{-1}
Daphnia magna	[30]	48	immobility	210	
Daphnia magna	[29]	24	immobility	1530	
Daphnia magna	**present study**	48	immobility	**1280**	**1080 - 1500**
Species	**Source**	**Exposure time / h**	**Endpoint**	**IC_{50} / µg L^{-1}**	**CI (95 %) / µg L^{-1}**
P. subcapitata	[30]	72	growth	980	830 - 1360
D. subspicatus	**present study**	60	growth	**580**	*

5.4.2 Degradation of TAM and formation of TPs

An overview of the concentration of TAM and TAM-*N*-oxide, as well as the semi-quantitative (peak area) formation of the TPs, vs. spiked ozone dose, is given in Figure 5-3 for pH 3 (A & B) and pH 7 (C & D). Similar results have been reported in a previous study using tertiary butanol as OH-radical scavenger [34]. The formation of propiophenone as possible low molecular weight TP formed by the Criegee reaction was not observed in this study.

pH 3

The concentration of TAM decrements with increasing ozone dose during ozonation at pH 3 (Figure 5-3 A). Full abatement was observed at an ozone dose of 30 µM, while 20 µM ozone already resulted in 98 % reduction of TAM. Here, only TP 270 and TP 388 were formed (B) and no *N*-oxide formation was observed. At pH 3 TAM is almost completely dissociated and present as corresponding acid. Due to the protonation at the tertiary amine ozone cannot attack at the amine (p*K*a = 8.76 [44]) and hence absence of *N*-oxide formation can be explained.

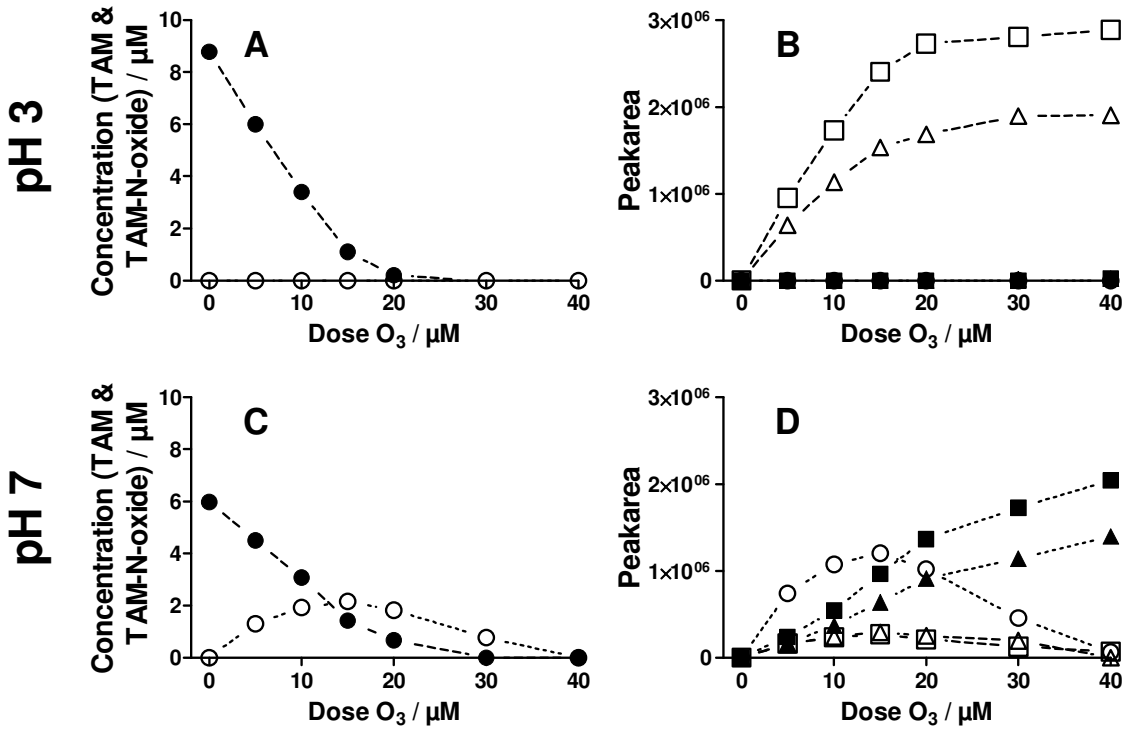

Figure 5-3: Degradation of ● - TAM at pH 3 (A) and pH 7 (C) during ozonation and formation of ○ - TAM-*N*-oxide. Semiquantititive formation of TPs at pH 3 (B) and 7 (D). □ - TP 270, ■ - TP 286, Δ - TP 388, ▲ - TP 404,. Initial applied concentration of TAM: 10 µM.

pH 7

At pH 7 the initial TAM concentration was reduced to 6 µM instead of 10 µM presumably due to working near the aqueous solubility and potentially subsequent sorpition or precipitation. Since initial concentrations were always measured, this should not affect results of TP formation and effect monitoring. Here, TAM was also completely transformed at an ozone dose of 30 µM. However, the reduction of the TAM concentration at lower ozone doses is somewhat less pronounced than at pH 3 (< 90 % of TAM transformation at an ozone dose of 20 µM) (Figure 5-3 C). All three primary TPs, TP 270, TP 388, and TAM-*N*-oxide, were formed simultaneously with TAM-*N*-oxide as main product (D). However, the secondary products TP 286 and TP 404 were also observed at the lowest tested ozone dose and are the main products at the highest ozone doses. TPs containing a phenolic moiety such as the suggested structure of TP 404 and TP 388 will be further oxidized at higher ozone doses, though TP 404 persisted at ozone dosages of 60 µM at pH 7 (see Figure A5-2 D) and no further TPs

were detected doses using reversed phase LC/MS, as tested in preliminary experiments. The reason of TP 404 being so persistent is yet unclear. One explanation is that other compounds which escaped detection might have competed for ozone with TP 404. Furthermore, the molecular structure, albeit conceivable, is just proposed. Further transformation of TP 286 due to ozone reactions is highly unlikely due to the deactivation of the aromatic moieties by the ketone [45].

5.4.3 Effects after Ozonation

Daphnia magna immobilization tests

D. magna immobilization of TAM samples ozonated at pH 3 and pH 7 is shown in Figure 5-4 A & B, respectively. Effect of the initial TAM concentrations was in both cases about 80 %. After ozonation of TAM no further immobilization was observed in the tested samples, spiked with either 20 µM or 40 µM as final ozone concentration at both, pH 3 and pH 7.

Green algae growth inhibition tests

Growth inhibition of the green algae *D. subspicatus* for the ozonation of TAM at pH 3 and pH 7 is shown in Figure 5-4 C & D, respectively. Two dilutions (1:1 and 1:3 (v/v)) of each sample were tested. At pH 3 (C) the 1:1 (v/v) dilution of the ozonated samples resulted in no significant change in the inhibition of the algae, indicating that the toxicity of the samples was way above the range covered by the test. Therefore, experiments were repeated using the 1:3 (v/v) dilution in the tests. Here, the initial effect increased by the factor of 2 at an ozone dose of 10 µM and remained constant at about 80 % growth inhibition, which is still at the upper limit of the test.

Samples ozonated at pH 7 showed an increase of the growth inhibition with increasing ozone dose of up to 20 µM, which decreased at higher ozone doses in the 1:1 (v/v) dilution. For the 1:3 (v/v) dilution the initial growth inhibition (30 %) increased by a factor of 2.6 at an ozone concentration of 10 µM (80 %) and subsequently decreased.

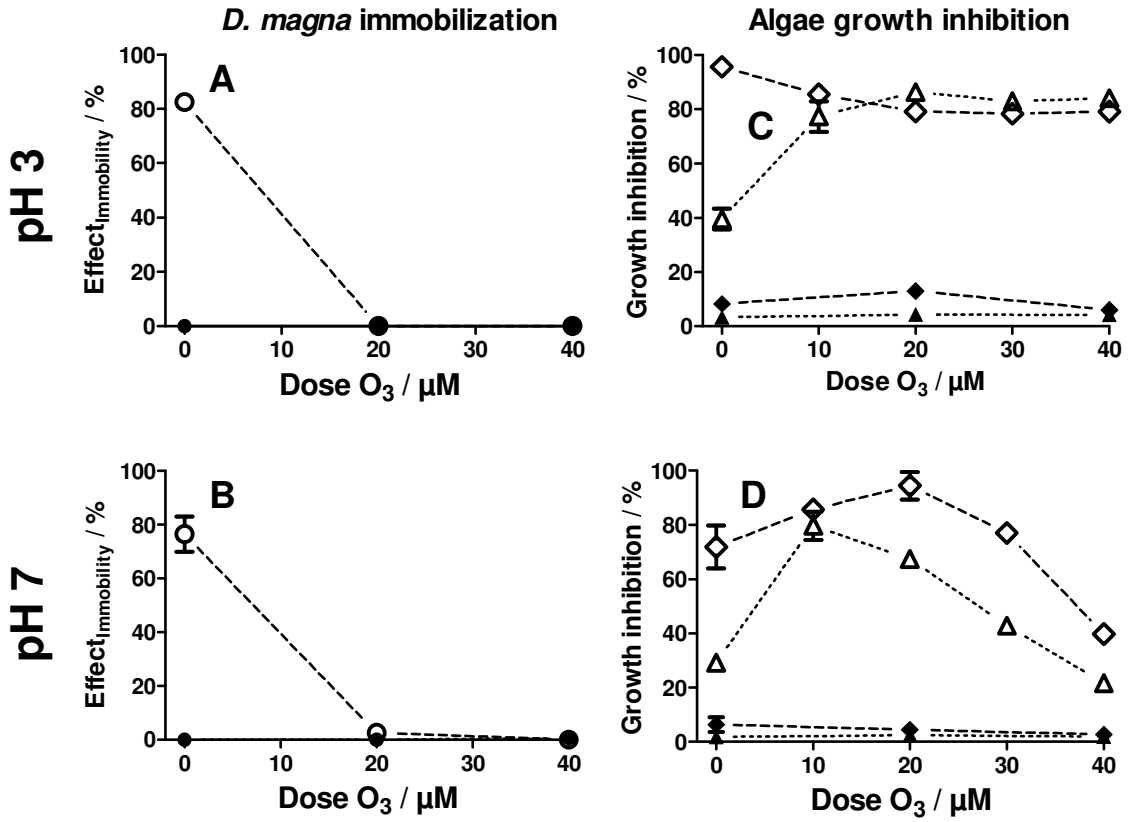

Figure 5-4: Immobility of *D. magna* after 48 hours at pH 3 (A) and pH 7 (B) of ○ - TAM and ● – DMSO controls over ozone dose in 1:2 (v/v) dilution. Growth inhibition (60 h) of TAM over final ozone dose for *D. subspicatus* at pH 3 (C) and pH 7 (D) with ◊ - in 1:1 and ∆ - 1:3 (v/v) dilution of samples in test media. DMSO controls ♦ - 1:1 (v/v) diluted and ▲ - 1:3 (v/v) diluted. Standard deviations of replicates are indicated by error bars that sometimes are smaller than the symbol size.

At pH 3 and 7 growth inhibition was observed although TAM was completely abated. This points to the formation of effect inducing TPs. Indeed, the effect vs. the abatement of TAM shows a positive slope for pH 3. At pH 7 the effect peaks at a TAM degradation of 2.5 µM (Figure 5-5 A). To further investigate this observation, the correlation of TP peak area and effect (1:3 (v/v) dilution) were analyzed (Figure 5-5 B – F).

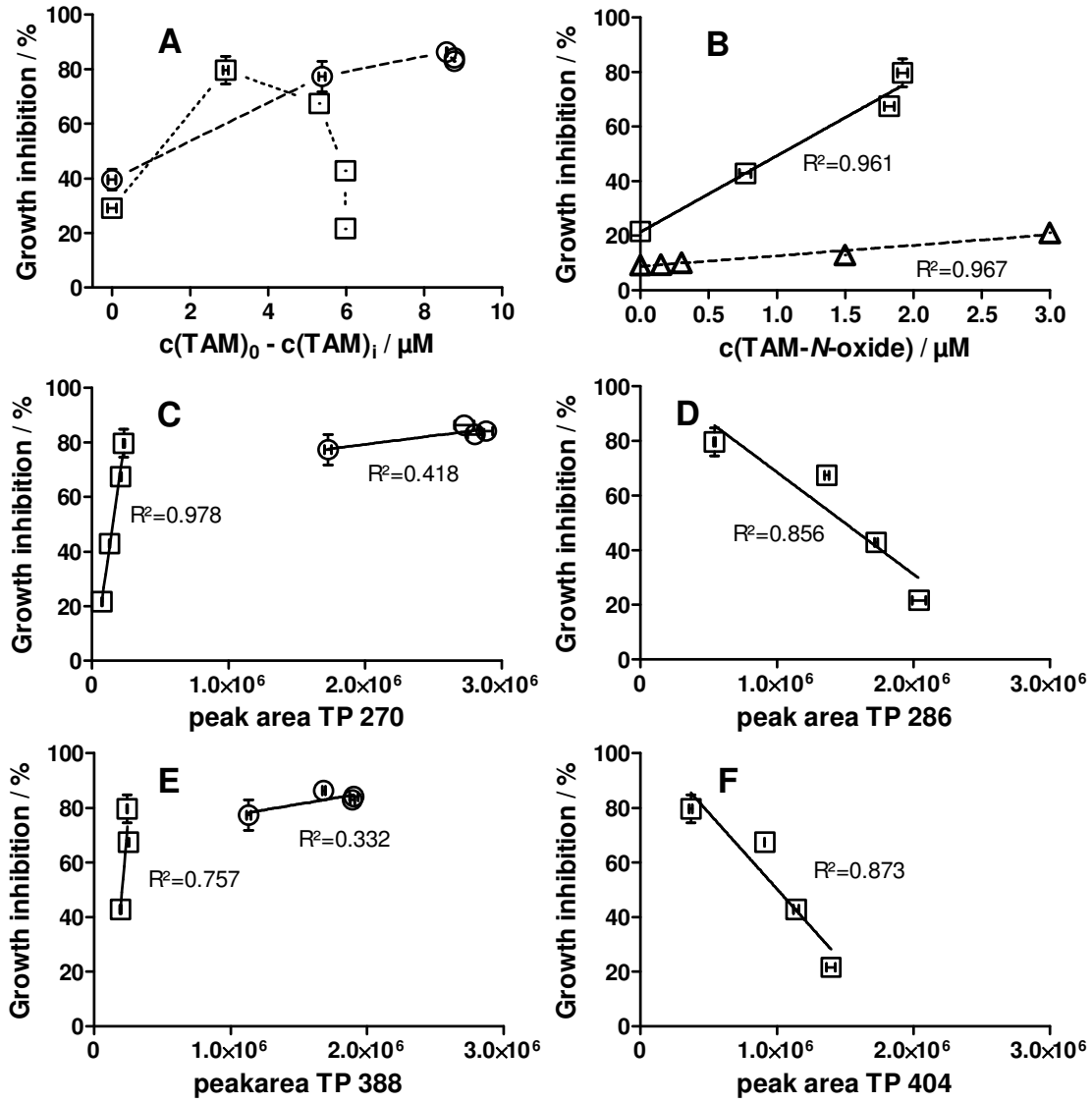

Figure 5-5: Effect over the abatement of TAM (initial concentration of TAM ($c(TAM)_0$) – concentration of TAM at ozone dose *i* ($c(TAM)_i$)) (A). Correlation of green algae growth inhibition (1:3 (v/v) dilution) and formation of TPs, as concentration or peak area for TAM-*N*-oxide (B), TP 270 (C), TP 286 (D), TP 388 (E), and TP 404 (F) at ○ – pH 3 and □ – pH 7; Δ - growth inhibition of TAM-*N*-oxide, tested individually. Standard deviations are indicated as error bars.

For TP 270 and TP 388, positive correlations were obtained at pH 3 and 7 (Figure 5-5 C & E). However, for both TPs the obtained slopes vary significantly. At pH 3, solely effects of > 80 % were observed, resulting in weak correlations for both TPs. Since the observed effects were at the upper limit of the test, the correlation is not valid. Yet, no other TPs were formed at pH 3 which could be responsible for the strong increase of

the effect (Figure 5-4 E). For both TPs also a positive correlation was obtained at pH 7. Here, a high correlation coefficient (R^2=0.978) for TP 270 was observed. TP 388 was present in only 3 of 4 ozonated samples, but also obtained a good correlation (R^2=0.757) at pH 7. Since TP 270 and TP 388 are always present simultaneously one cannot clearly determine the individual contribution of these TPs to the observed effect. Nevertheless, the correlated data for pH 3 and pH 7 are not coherent since effects observed at pH 3 are at the upper limit of the test and hence linearity gained at pH 7 is more meaningful. For TAM-*N*-oxide (Figure 5-5 B) also a high correlation (R^2 = 0.961) was obtained, indicating the induction of the growth inhibition by TAM-*N*-oxide. As previously mentioned, TAM-*N*-oxide was only observed simultaneously with TP 270 and the individual growth inhibition of 3 µM TAM-*N*-oxide was 21 %. Lower tested concentrations did not differ significantly from the DMSO control (9.1 ± 5.4 %). Therefore, the observed effect is probably not caused by TAM-*N*-oxide, although mixture effects cannot be ruled out [46]. For TP 286 and TP 404 (Figure 5-5 D & F) negative correlations were obtained and on that account a contribution by these to the effect can be ruled out. Hence, three TPs formed during the ozonation of TAM might proliferate the green algae growth inhibition of TAM. However, at pH 7 TP 388 was not found at the highest ozone dose, thus the remaining growth inhibition of 21 % has to be attributed to TP 270 and/or TAM-*N*-oxide, both remaining present with low intensities. Since TP 270 was always observed with a remaining effect, we assume that this is the TP inducing the strongest green algae growth inhibition, and TP 388 and TAM-*N*-oxide either do only weakly induce the effect and are observed as mixed toxicity effect or are solely incidentally observed. For TAM-*N*-oxide only a minor effect induction was observed. However, individual effects of each TP would be needed to assess if synergistic, additive or inhibitory effects might have influenced the observed effects in the mixture of the TPs as tested here.

5.5 Conclusion

The TPs formed by ozonation of TAM did not lead to a remaining immobilization effect of *D. magna*. In contrast, an increase of the growth inhibition of the green algae *D. subspicatus* was observed and correlated to the presence of two TPs, namely, TP 270 and TP 388. Hence, importance of different tests for the ecotoxicological evaluation of TPs and formation of toxicologically relevant TPs could be shown. For the secondary TPs, namely TP 286 and TP 404, no positive correlation to the residual effect was obtained and formation of TAM-*N*-oxide results in a reduced algae growth

inhibition. Principally, ozonation my lead to a reduction of the growth inhibition for green algae *D. subspicatus* of TAM at pH values typical for wastewater ozonation (pH 7-8), since mainly *N*-oxides will be formed. However, in practice a reduction of this effect is probably not required since algae growth inhibition of TAM is of minor importance at concentrations typically determined in the environment (ng L^{-1} range). Additionally, wastewater matrix effects are not considered in this study. Individual effects of single TPs could not be determined because beside TAM-*N*-oxide, TPs are not available as authentic standards. Furthermore, the systematic assessment of mixture effects was therefore not possible. For the determination of the ecotoxicological potential of individual TPs, isolation of the TPs will be necessary and will be addressed in future work.

5.6 References

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5.7 Appendix A5: Supplementary Material

5.7.1 A5-1 - LC-MS validation

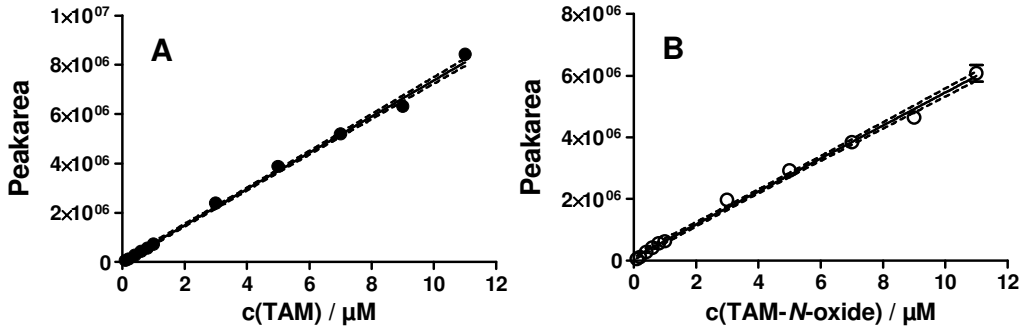

Figure A5-1: Calibration for TAM (A) and TAM-*N*-oxide (B). Dotted lines as confidence interval (95 %).

Table A5-1: Limit of detection (LOD) and limit of quantification (LOQ) determined according to DIN 32645.

	TAM	**TAM-*N*-oxide**
LOD / µM	0.03	0.03
LOQ / µM	0.08	0.08
R^2 (0.1-11 µM)	0.9962	0.9922

5.7.2 A5-2 – Ozonation and formation of TPs

Due to limitations of the toxicity tests, the highest ozone dose (60 µM) was not considered for toxicity testing. For a comprehensive data presentation Figure A5-2 shows the TAM abatement and formation of TPs over the whole range of ozone doses.

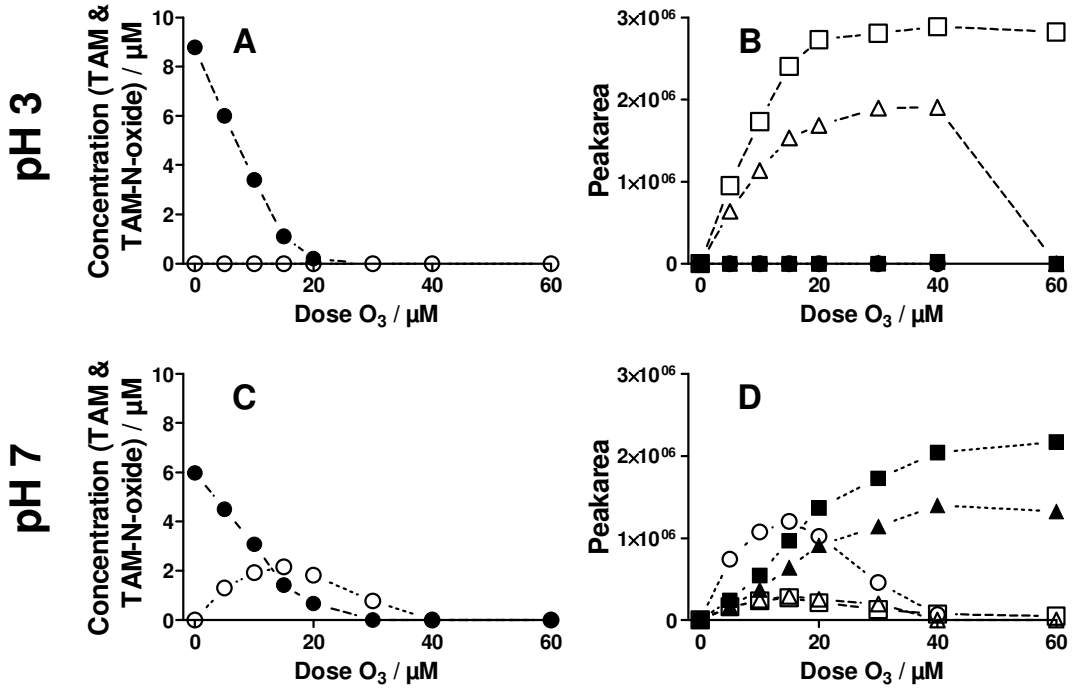

Figure A5-2: Degradation of ● - TAM at pH 3 (A) and pH 7 (C) during ozonation and formation of ○ - TAM-*N*-oxide. Semiquantititive formation of TPs at pH 3 (B) and 7 (D). □ - TP 270, ■ - TP 286, Δ - TP 388, ▲ - TP 404,. Initial applied concentration of TAM: 10 µM.

5.7.3 A5-3 - *Daphnia magna* immobilization tests

Aachener Daphnien Medium (ADaM) composition is given in Table A5-2. ADaM was aerated continuously before use to ensure a high oxygen saturation.

Table A5-2: Composition of ADaM modified after Klüttgen et al. (1994) [1].

Solution	Chemical	m / g	Solution preparation	V / L for 10 L ADaM
1	$CaCl_2$	117.6	Make up to 1 L with ultrapure water	0.023
2	$NaHCO_3$	25.2	Make up to 1 L with ultrapure water	0.022
3	SeO_2	0.7	Make up to 1 L with ultrapure water	0.001
3.33 g artificial sea salt* added directly to 10 L ADaM				

* hw-Marinemix professional (artificial sea salt) was purchased from Wiegandt GmbH & Co.KG (Krefeld, Germany).

Table A5-3: Concentrations applied and observations for determination of EC_{50} values for *Daphnia magna* immobility tests.

			Test solution			13.12.2017			29.01.2018 *		
Sample	V (total) / L	V (TAM stock) / L	c / mg L^{-1}	c / mM	log (c / mg L^{-1})	mobile 48 h	immobile 48 h	**%Effect 48 h**	mobile 48 h	immobile 48 h	**%Effect 48 h**
TAM C6	0.1	0.016	0.609	1.6	-0.215	20	0	**0**	x	x	**x**
TAM C5	0.1	0.020	0.761	2.0	-0.118	18	2	**10**	14	1	**7**
TAM C4	0.1	0.026	0.952	2.6	-0.022	17	3	**15**	10	5	**33**
TAM C3	0.1	0.032	1.189	3.2	0.075	13	7	**35**	7	8	**53**
TAM C2	0.1	0.040	1.487	4.0	0.172	4	16	**80**	5	10	**67**
TAM C1	0.1	0.050	1.858	5.0	0.269	2	18	**90**	2	13	**87**
DMSO	0.1	0.000	0.000	0.0		20	0	**0**	15	0	**0**
$K_2Cr_2O_7$	0.1	0.100							1	14	**93**

* 3 replicates with 5 daphnids each per concentration tested.

Table A5-4: Concentrations applied and observations for determination of *Daphnia magna* immobility after ozonation of tamoxifen at pH 3 and pH 7.

	Ozonation at pH 3					
	22.01.2018			31.01.2018		
Sample	mobile 48 h	immobile 48 h	**%Effect 48 h**	mobile 48 h	immobile 48 h	**%Effect 48 h**
T 0	4	16	**80**	3	17	**85**
T 20	20	0	**0**	20	0	**0**
T 40	20	0	**0**	20	0	**0**
B 0	20	0	**0**	20	0	**0**
B 20	20	0	**0**	20	0	**0**
B 40	20	0	**0**	20	0	**0**
	Ozonation at pH 7					
	23.01.2018			30.01.2018		
Sample	mobile 48 h	immobile 48 h	**%Effect 48 h**	mobile 48 h	immobile 48 h	**%Effect 48 h**
T 0	6	14	**70**	3	15	**83**
T 20	19	1	**5**	18	0	**0**
T 40	20	0	**0**	18	0	**0**
B 0	20	0	**0**	18	0	**0**
B 20	20	0	**0**	18	0	**0**
B 40	20	0	**0**	18	0	**0**

5.7.4 A5-4 - Green algae growth inhibition tests

Algae growth medium

Artificial algae medium (AAM) was used for *Desmodesmus subspicatus* cultivation and twice concentrated artificial algae medium (DAAM) for chronic tests.

Table A5-5: Preparation of stock solutions for AAM and corresponding volumes required for preparation of 1 L AAM/DAAM.

Solution	Chemical	m / g	Solution preparation	V / L for 1 L AAM	V / L for 1 L DAAM
1	$Ca(NO_3)_2 \cdot 4\ H_2O$ $NaNO_3$	5.9 46.7	Make up to 1 L with ultrapure water	2.5	5
2	$K_2HPO \cdot 3\ H_2O$	4.1	Make up to 1 L with ultrapure water	2.5	5
3	$MgSO_4 \cdot 7\ H_2O$	2.5	Make up to 1 L with ultrapure water	2.5	5
4	$NaHCO_3$	16.8	Make up to 1 L with ultrapure water	10	20
5	NaEDTA $FeSO_4 \cdot 7\ H_2O$	1.145 0.3	Complete to 60 mL with ultrapure water, autoclave and make up to 1 liter with ultrapure water	10	20
6	H_3BO_3 $MnSO_4 \cdot H_2O$ $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ $ZnSO_4 \cdot 7\ H_2O$ $Co(NO_3)_2 \cdot 6\ H_2O$ $CuSO_4 \cdot 5\ H_2O$	3.1 1.69 0.09 0.29 0.15 0.13	Make up to 1 L with ultrapure water	0.8	1.6

Algae measurement and biomass determination

For the determination of algal biomass a calibration of chlorophyll fluorescence and cell concentration was performed. Cell concentration was determined by visual counting (Neubauer's chamber method) and chlorophyll fluorescence was determined using a multimode reader Infinite M200 (Tecan, Switzerland) in the range from $1.86 \times 10^3 - 1.86 \times 10^6$. Parameters for fluorescence measurements are given in Table A5-6.

Table A5-6: Multimode reader Infinite M200 parameters used for the measurement of chlorophyll fluorescence using fluorescence top reading mode.

Exitation Wavelength / nm	450	± 9
Emmision Wavelength / nm	685	± 20
Gain	100	manual
Number of Reads	25	
Integration Time / µs	2000	
Lag Time / µs	20	
Settle Time / ms	0	

The calibration is shown in Figure A5-2. Limit of detection for cells was 3.49 x 10^4 cells mL $^{-1}$.

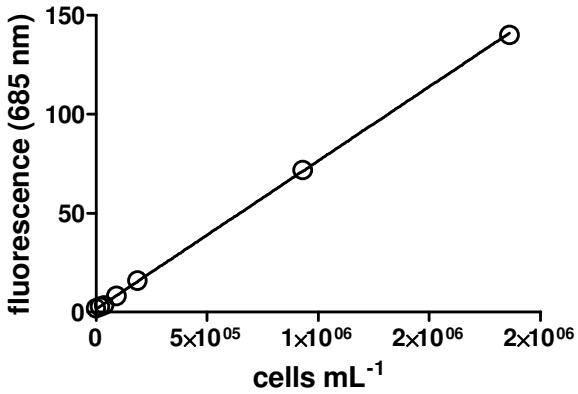

Figure A5-3: Calibration of chlorophyll fluorescence and algal cells per mL. R^2 = 0.9981.

Biomass was calculated using the cell dry weight (3-4 x 10^{-8} mg cell^{-1}) of *D. subspicatus*, stated in Annex 2 of the OECD guideline 201 [2]. In this study, 3.5 x 10^{-8} mg cell^{-1} was used.

Concentrations tested, replicates and plate design

Table A5-7: Measured concentrations of TAM in applied test solutions and green algae growth inhibition for EC determination.

	TAM in test solution*		**growth inhibition (I(r)) / %**			
Sample	c / mg L-1	c / µM	Plate 1	Plate 2	mean I (r) / %	S_{dev}
C5	0.069	0.19	8.8	9.7	9.26	0.64
C4	0.139	0.37	21.0	26.7	23.84	4.09
C3	0.277	0.75	23.3	34.4	28.87	7.86
C2	0.555	1.49	46.9	48.3	47.61	1.00
C1	1.110	2.99	87.2	89.4	88.31	1.51
DMSO	0.000	0.00	5.6	7.6	6.64	1.41

*measured concentration

Table A5-8: Experimental design for sample distribution in 24-well microplates. Design A was used for determination of the EC_{50}, Design B was used for the determination of the effect before (T 0) and after ozonation with 20 µM (T 20) and 40 µM (T 40) concentrations of ozone spiked and corresponding solvent controls (B 0, B 20, B 40). Both sets were performed on two plates with 3 wells of each tested concentration/sample per plate (n=6). Design C was used for the determination of the effect ozonation with 10 µM (T 10) and 30 µM (T 30) concentrations of ozone spiked with 6 samples on one plate (n=6). Per plate 6 negative controls were included.

A

	1	2	3	4	5	6
A	Blank	C4	Blank	C3	Blank	C4
B	C1	C5	DMSO	C2	C1	C5
C	C2	Blank	C5	C1	C2	Blank
D	C3	DMSO	C4	Blank	C3	DMSO

B

	1	2	3	4	5	6
A	Blank	B 0	Blank	T 0	Blank	B 0
B	T 0	B 20	B 0	T 20	T 0	B 20
C	T 20	B 40	B 20	T 40	T 20	B 40
D	T 40	Blank	B 40	Blank	T 40	Blank

C

	1	2	3	4	5	6
A	Blank	T 30	Blank	T 10	Blank	T 30
B	T 10	T 10	T 30	T 30	T 10	T 10
C	T 30	T 30	T 10	T 10	T 30	T 30
D	T 10	Blank	T 30	Blank	T 10	Blank

Biomass over time

Biomass over time for all experiments are given in Figure A5-3 and Figure A5-4 below. All validation criteria were met and are given in Table A5-9.

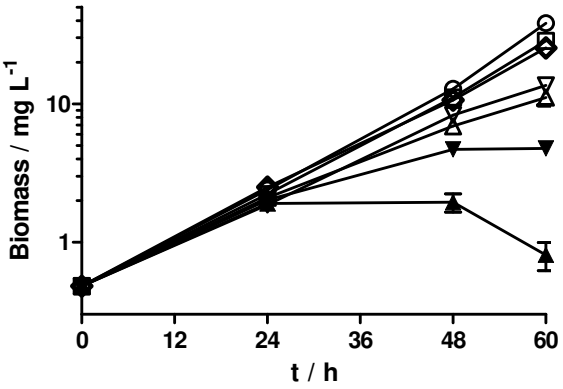

Figure A5-4: Biomass over time for the determination of algal growth inhibition of TAM and IC values. Negative controls: AAM (open circle), 10 µM DMSO (open square). TAM: C1 (closed triangle up), C2 (close triangle down), C3 (open triangle up), C4 (open triangle down), and C5 (diamond).

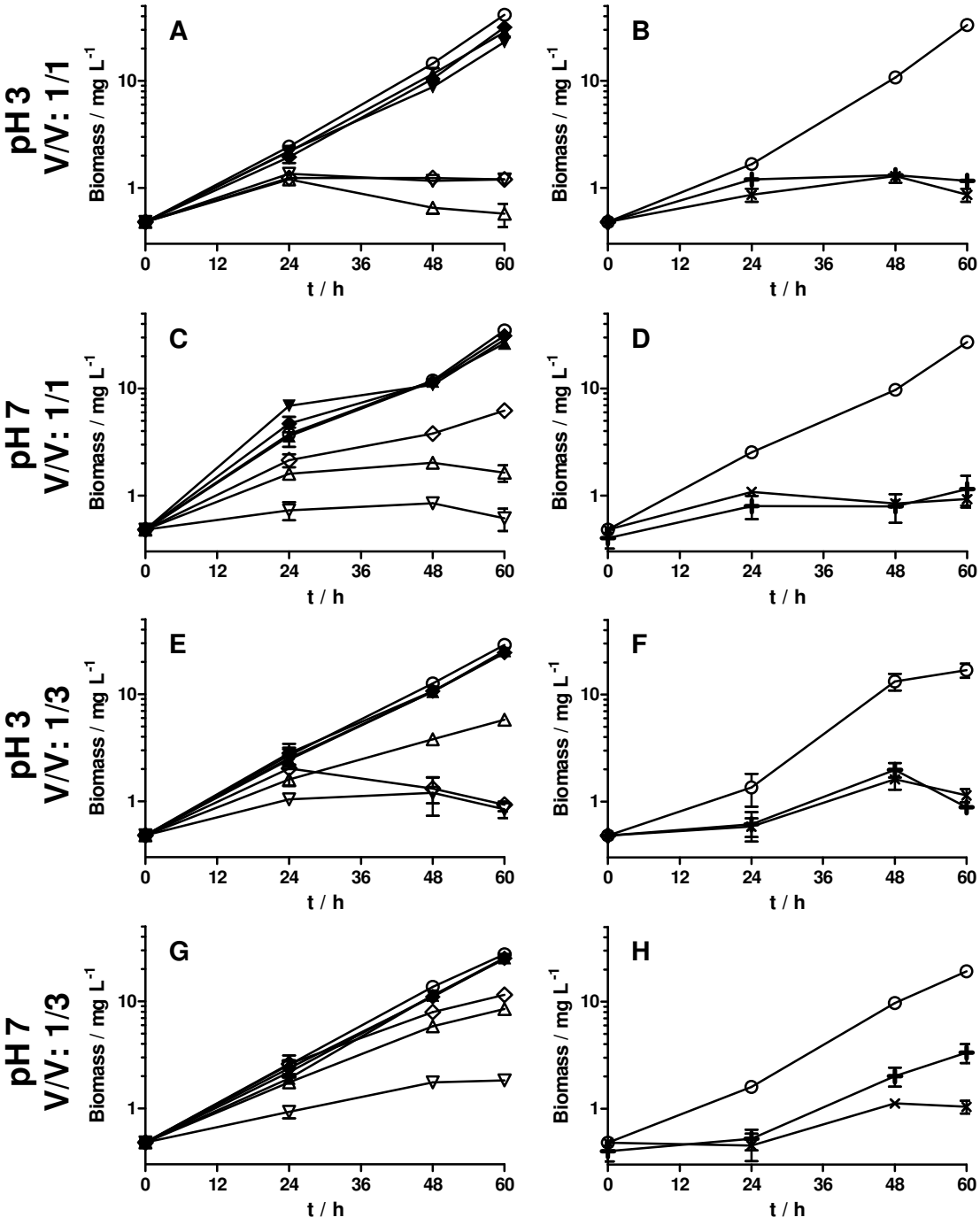

Figure A5-5: Biomass over time for the determination of algal growth inhibition of TAM after ozonation at pH 3 (A, B) and pH 7 (C, D) using a 1:1 (v/v) dilution and pH 3 (E, F) and (G, H) using a 1:3 (v/v) dilution. Negative controls: AAM (open circle). DMSO (10 mM) spiked with ozone concentrations of 0 µM (closed triangle up), 20 µM (closed triangle down), and 40 µM (closed diamond). TAM spiked with ozone concentrations of 0 µM (open triangle up), 10 µM (cross), 20 µM (open triangle down), 30 µM (x), and 40 µM (open diamond).

Validation of algae tests

Table A5-9: Validation criteria for the green algae growth inhibition test in AAM as negative controls. [a] for determination of IC values. [b] minimum factor: 16. [c] average specific growth rates during whole test in replicate controls. [d] coefficient of variation of average specific growth rates, must not exceed 7 % for *Pseudokirchnella subcapitata* and *D. subspicatus*.

dilution (v/v)	**Plate**	increasing factor (60 h) [b]	average specific growth rate / day^{-1} [c]	% CV [d]
	TAM [a]	79.4	**1.74**	**5.1**
		78.7	**1.74**	**5.6**
1:1	**pH 3-1**	87.2	**1.79**	**1.6**
		82.4	**1.76**	**1.9**
	pH 3-3	68.3	**1.68**	**5.5**
	pH 7-1	68.0	**1.68**	**4.7**
		68.2	**1.68**	**4.5**
	pH 7-3	55.8	**1.60**	**4.0**
1:3	**pH 3-1**	59.9	**1.64**	**2.1**
		59.0	**1.63**	**0.6**
	pH 3-3	39.8	**1.47**	**3.4**
	pH 7-1	55.6	**1.61**	**1.7**
		57.5	**1.62**	**4.7**
	pH 7-3	39.7	**1.47**	**1.9**

Note: section-by-section specific growth rates in controls did not exceed 35 %. Positive controls were tested and reported previously [3].

5.8.1 A5-5 References:

[1] B. Klüttgen, U. Dülmer, M. Engels, H. Ratte, ADaM, an artificial freshwater for the culture of zooplankton, Water research, 28 (1994) 743-746.
[2] OECD, Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Publishing, Paris, 2011.
[3] A.R. Ribeiro, B. Sures, T.C. Schmidt, Ecotoxicity of the two veterinarian antibiotics ceftiofur and cefapirin before and after photo-transformation, Sci. Total Environ., 619 (2018) 866-873.

6 Synthesis of 4-(Dimethylaminoethoxy)-benzophenone (TP 270)

6.1 Introduction

4-(Dimethylaminoethoxy)-benzophenone (4DAEB, TP 270) has been reported as transformation product of tamoxifen (TAM) and toremifen (TOM) in ozonation [1] and as indicator for TAM in sunlight-based photolysis in water [2]. For 4DAEB ecotoxicological effects, such as anti-estrogenic activity [3] and green algae growth inhibition [4] were correlated with the formation of 4DAEB during ozonation and were assumed to be higher than for the parent compound TAM [3-5]. Additionally, acute and chronic tests using cladocera species after phototransformation of TAM [2] are reported. However, 4DAEB is commercially not available as authentic standard, hence, synthesis of 4DAEB is required to assess its ecotoxicological potential and to validate potential ecotoxicological effects that are reported to remain after transformation processes [Kapitel 4 & 5][2].

Various synthesis routes are available for 4DAEB [6]. The synthesis of 4DAEB is reported as part of the synthesis of tamoxifen and its derivates [7, 8], but also an endocrine effect of the compound has been assumed and was hence patented [9, 10].

The synthesis route with least steps reported is a nucleophilic aromatic substitution reaction using 4-hydroxybenzophenone and 2-chloro-*N*,*N*-dimethylethylamin hydrochloride in acetone using potassium carbonate as catalyzer as shown in Figure 6-1 [7-10]. Synthesis can be performed in dried organic solvents. Reported products were either brown [8] or yellow viscous fluids [7, 9], a further purification is required. ^{1}H-and ^{13}C-NMR spectra of the synthesized product and photolysis TP are identical [2, 7, 8].

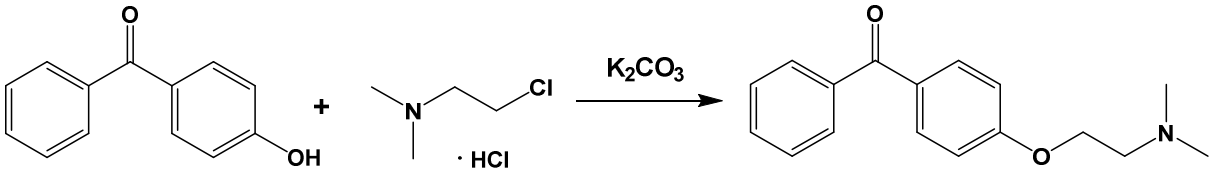

Figure 6-1: Synthesis route for 4-(dimethylaminoethoxy)-benzophenone using 4-hydroxybenzophenone and dimethylaminoethyl chloride hydrochloride under basic conditions.

Aim of this work was to evaluate a simple synthesis route, including a purification procedure to obtain purified 4DAEB, suitable for characterization and toxicity testing.

6.2 Materials and Methods

6.2.1 Chemicals

The educts 2-chloro-*N*,*N*-dimethylethylamin hydrochloride (ClDMEA) and 4-hydroxybenzophenone (4HBP) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide (VWR, Darmstadt, Germany) and hydrochloric acid (37 %) (Fisher Scientific, Bremen, Germany) were used for pH adjustment. Acetone and toluene were purchased from Merck (Darmstadt, Germany), diethyl ether (DEE) and potassium carbonate from Fisher Scientific, and dichloromethane (DCM) from VWR.

Ultra-pure water (UPW) was produced onsite (Purelab Ultra, Elga LabWater, Celle). Chloroform-d1 (99.8 %, Deutero GmbH, Kastellaun, Germany) was used for NMR measurements. For LC-MS measurements LC-MS grade methanol (HiPerSolv CHROMANORM, VWR), water (LiChroSolv), and formic acid (Suprapur; Merck) were used.

6.2.2 Synthesis routes

Synthesis was performed twice, using two different ratios of educts. Masses of the educts and reagents are presented for both synthesis routes in Table 6-1. Synthesis routes were based on reported synthesis pathways, but varied in the ratios of educts used. Route 1 is based on Chatterjee et al. [9] and route 2 on Guzi et al. [10].

Table 6-1: Educts and reagents used in both synthesis approaches.

compound	**Synthesis route 1**			**Synthesis route 2**		
	V / L	n / mol	m / g	V / L	n / mol	m / g
ClDMEA		0.038	5.46		0.038	5.45
4HBP		0.086	17.10		0.025	5.00
Acetone	0.1125			0.3		
K2CO3		0.217	30.00		0.126	17.40

4HBP and acetone were prefilled into a round bottomed flask and subsequently potassium carbonate and ClDMEA added. The mixture was thoroughly stirred using a magnetic stirrer and boiled under reflux using a Liebig condenser for 24 h. For heating an oil bath was used and set to 90 °C. Subsequently, acetone was removed under vacuum using a rotary evaporator (Büchi Labortechnik GmbH, Essen, Germany).

From this point onwards, for both synthesis routes individual purification procedures were tested. Purification procedure 1 and 2 were applied for products of synthesis routes 1 and 2, respectively.

6.2.3 Purification procedure1

The first steps of the purification procedure were adapted from Chatterjee et al. [9]. Each 120 mL of UPW and toluene were added to the dried product, mixed thoroughly and given into a separation funnel. After phase separation, the product was extracted in toluene. Extraction in toluene was repeated once with 120 mL of toluene. The combined extract was transferred into a clean separation funnel and washed successively with 5 % sodium hydroxide (50 mL) and UPW (80 mL). Toluene was removed under vacuum in a rotary evaporator at 40 °C and 200 mbar. The product was tested using high resolution mass spectrometry (HRMS) and ^{1}H-NMR.

Subsequently to the reported purification procedure, the product was dissolved in toluene and 2 % hydrochloric acid (50 mL). Toluene was removed using a separation funnel. Then 50 mL of 5 % sodium hydroxide were added and mixed with the remaining water phase, containing the product. Subsequently, the product was extracted twice with toluene (2 x 50 mL). The toluene extract was washed with de-ionized water. Finally, toluene was removed under vacuum and the remaining product tested using ^{1}H-NMR. The remaining product was stored in a desiccator under dry conditions.

6.2.4 Purification procedure 2

For purification of the product of synthesis 2 an enhanced washing procedure was developed based on the calculated partitioning of educts and the product depending on the pH. Partition coefficients for water/octanol (K_{OW}) systems of the individual species were calculated using ChemAxon [11], as well as the dissociation constant K_a. These were then used to calculate the distribution coefficients for all species of one compound (D) as a function of the pH. The K_{OW} can be used to estimate the distribution of a compound in between polar and nonpolar solvents, such as toluol which was applied in this study. All calculated data are given in Table 6-2 and visualized in Figure 6-2.

Table 6-2: Distribution coefficients as function of the pH, partition coefficients and dissociation constants for educts and 4DAEB, all calculated using Marvin (ChemAxon) [11].

pH	log*D*		
	4HBP	ClDMEA	4DAEB
1.7	3.13	-2.65	-0.21
4.6	3.13	-2.23	0.13
6.5	3.11	-0.56	1.76
7.4	3	0.24	2.57
8.0	2.75	0.6	2.98
	4HBP	**ClDMEA**	**4DAEB**
logK_{OW}	3.13	0.85	3.29
pK_a	7.85	7.9	8.03

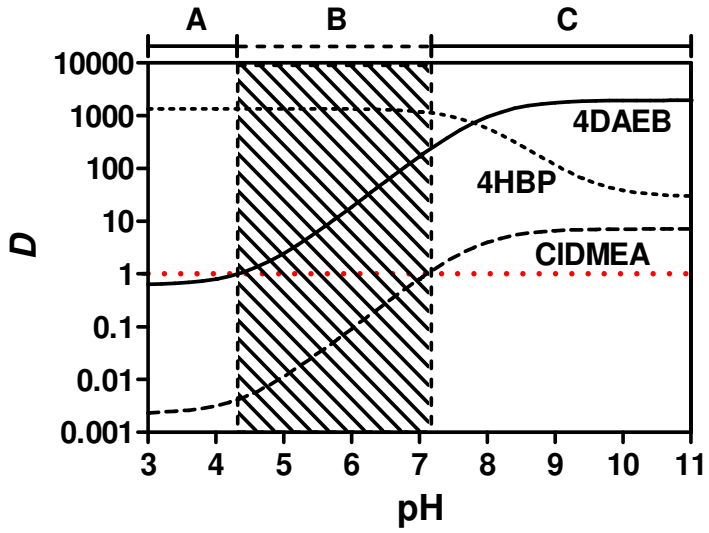

Figure 6-2: Calculated distribution coefficients between octanol and water (K_{OW}) as a function of the pH for the product 4DAEB, and the educts 4HBP and ClDMEA.

As depicted in Figure 6-2, solubility of product and educts can be divided into three pH ranges. Above a pH of 7.2 (C) all three compounds are extractable into a nonpolar organic solvent. In the pH range from 4.4 to 7.2 (B), ClDMEA will be extracted into the water phase, whereas below pH 4.4 (A) 4DAEB will also be extractable in water. Based on the different distribution constants, a washing procedure was developed. It has to be mentioned that calculated dissociation constants for amines often show an error [12]. To consider these errors, safety intervals were included for planning the pH values for the corresponding extractions.

The product was solubilized in 200 mL diethyl ether, added into a separation funnel and washed twice with 2 x 200 mL water (pH 6.5), to remove ClDMEA and remaining K_2CO_3. Then 2 x 100 mL of 1 M hydrochloric acid were used to extract the product. The extract was mixed with 250 mL of 1 M sodium hydroxide in a clean separation funnel to gain alkaline conditions and the combined water phase was extracted overnight in 2 x 200 mL DCM. DCM was subsequently removed under vacuum using a rotary evaporator. Subsequently, the product was stored under dry conditions. ^{1}H- and ^{13}C-NMR were recorded and the exact mass determined using HRMS.

Both purification procedures are visualized in Figure 6-3.

6.2.5 Analytical approaches

For the determination of the exact masses of the product a QExactive Orbitrap mass spectrometer (Thermo Fischer Scientific, Waltham (MA), USA) *via* direct injection with a 500 µL syringe (Hamilton, Reno) at 10 µL min^{-1} was used. Full MS scans (100-500 m/z) were performed at a resolution power of 140,000 and the automatic gain control was set to 3e6 ions. A maximum injection time of 100 ms was used. Positive electrospray ionization was performed at 4 kV with a sheath gas flow set to 40 [ ]. Aux gas was set to 5 [ ] and sweep gas to 10 [ ]. The aux gas heater was set to 100 °C and the inlet capillary to 350 °C. Mass calibration was performed prior to the experiments using the Pierce ESI Positive Ion Calibration Solution (Thermo Fischer Scientific, Waltham (MA), USA). Samples were dissolved in a 1:1 (v/v) mixture of water and methanol, containing 0.1 % (v/v) formic acid. For structure elucidation MS/MS experiments for the five most intensive ions (Top5-ddMS^2 mode) was applied at a resolution of 35,000, automatic gain control of 1e5 ions, and a maximum injection time of 50 ms. The scan range was set to 50-500 m/z. Collision energy was set to 35 eV.

^{1}H-NMR and ^{13}C-NMR measurements were performed using a Bruker DRX 500 by the NMR department of the faculty of chemistry at University of Duisburg-Essen.

Weighted aliquots of the purified products were solubilized in either 1:1 (v/v) methanol/water for HRMS-measurements or chloroform-d1 for NMR measurements.

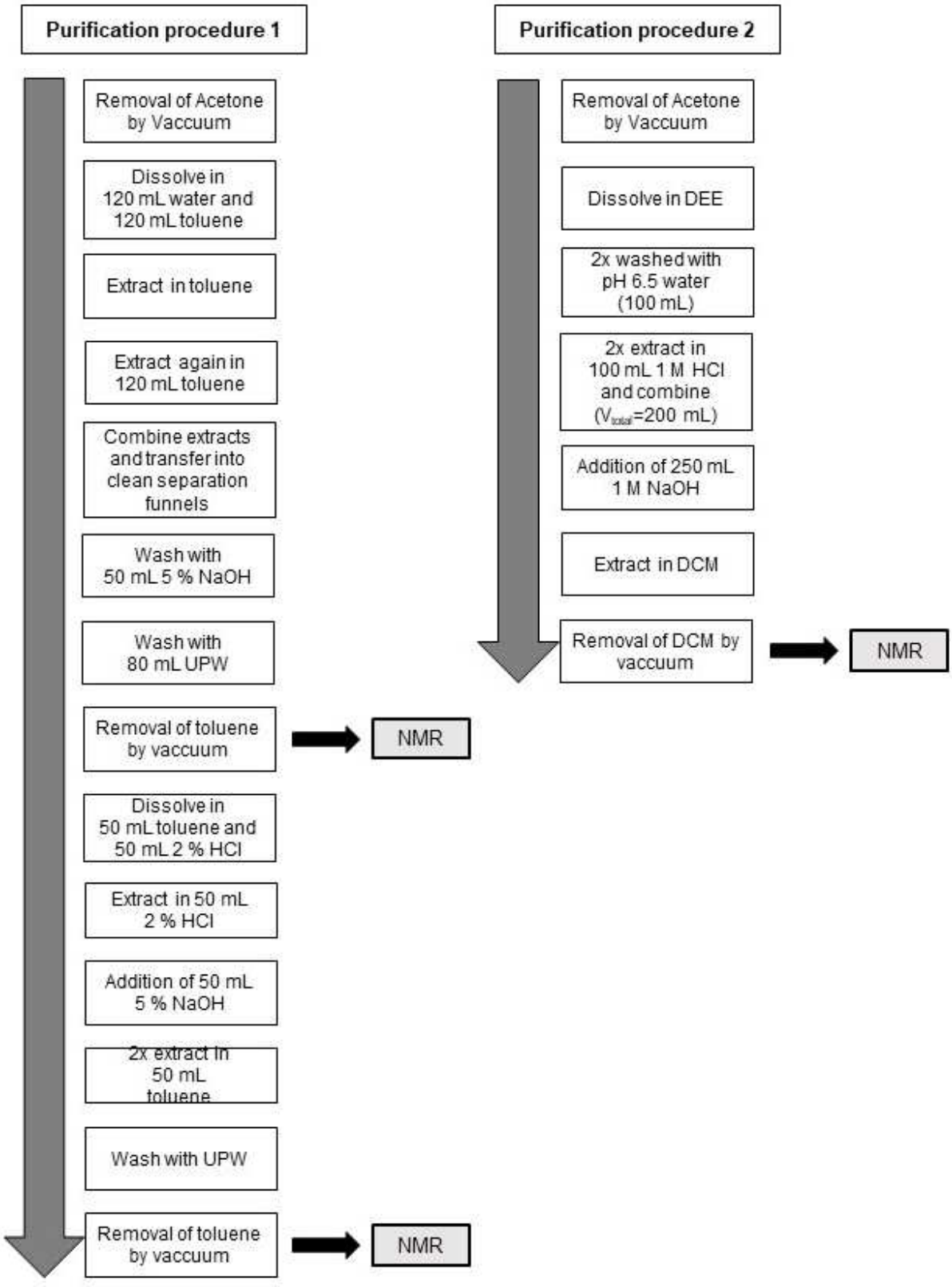

Figure 6-3: Purification procedures 1 and 2 for synthesis 1 and 2, respectively, including sampling points for NMR measurements.

6.3 Results and Discussion

6.3.1 Visual observations

A strong yellow color was observed as soon as 4HBP was mixed with potassium carbonate in acetone. The yellow color remained throughout both synthesis routes.

The alkaline aqueous washing phases showed a strong yellow color, due to the extraction of 4HBP into the aqueous phase. The product was extracted from organic phases into aqueous phases at low pH, but formed an emulsion after adjustment to a high pH. These emulsions were visible at the border of organic and aqueous phase. During extraction, the product dissolved slowly in the organic phase again.

After evaporation of the solvents, the purified product remained as yellow colored oily liquid. When stored at ambient temperature and dry conditions in a desiccator, the oil crystallized within a week. For synthesis route 1, an incomplete crystallization was observed and the yellow oil phase remained. For synthesis route 2, crystallization was complete. The crystalline phase was divided into two parts, i. e., white crystals, covered by a light yellowish top layer. Hence, a remaining yellow contamination can be assumed, which delays crystallization of 4DAEB. This also explains the incomplete crystallization observed for synthesis 1, where a strong yellow oily phase is observed. For NMR analysis of the product of synthesis route 2 the lower white crystal layer was tested. For storage, the combined product of synthesis 2 was transferred into a flask.

6.3.2 Mass spectrometry

Mass spectrometric measurements showed successful synthesis for both synthesis routes based on determined exact masses. Results are shown in Table 6-3.

Table 6-3: Exact masses (theoretical) of 4DAEB and measured mass to charge ratios (m/z) observed after synthesis 1 and 2.

Synthesis	**FullScan** @ 140,000				**Top5 ddMS²** @ 35,000 HCD Fragments @ 35 NCE		
	Formula	exact mass	m/z	Delta ppm	m/z	Formula	Delta ppm
1	$C_{17}H_{20}O_2N$	270.14885	270.14794	-0.915	72.08167	$C_4H_{10}N$	0.894
2	$C_{17}H_{20}O_2N$	270.14885	270.14783	-0.555	72.08165	$C_4H_{10}N$	0.874

The fragment observed in MS/MS experiments is probably a rather unspecific dimethylethylamine cation. No further signals were observed in the ESI-MS/MS measurements.

6.3.3 ^{1}H-NMR

Figure 6-4 shows the atom labeling of 4DAEB. Spectra for the products obtained by synthesis routes 1 and 2 are given in Figure 6-5. Predicted and reported ^{1}H-NMR signals as well as signals of products of synthesis 1 and synthesis 2 are given in Table 6-4.

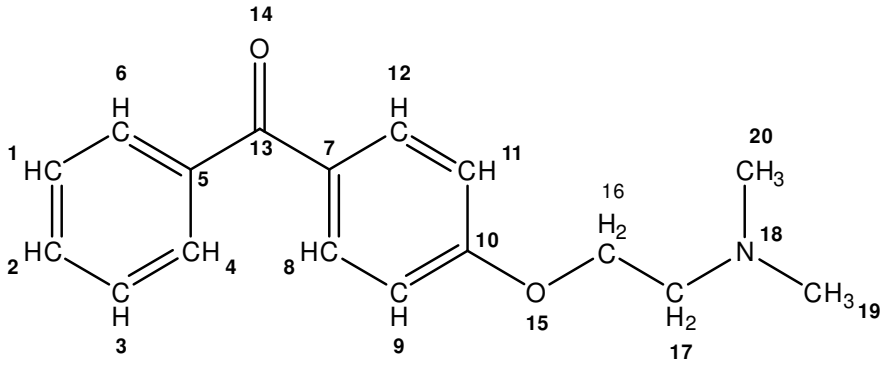

Figure 6-4: Structural labeling of 4DAEB.

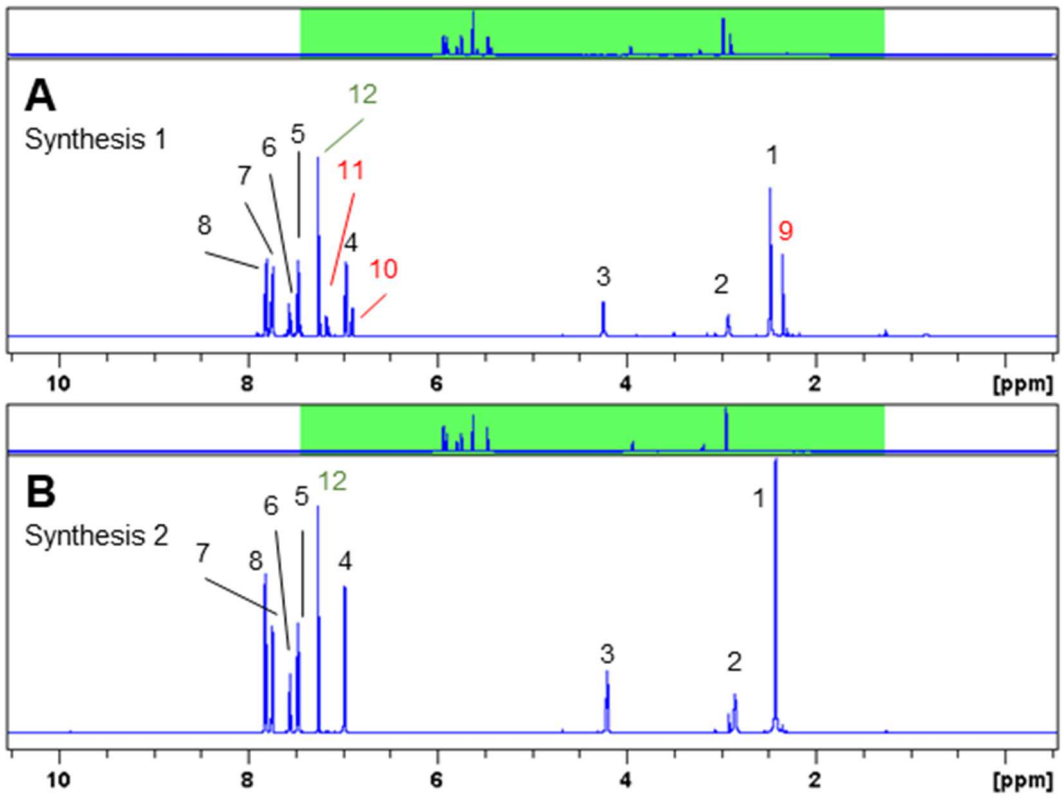

Figure 6-5: ^{1}H-NMR-Spectra for 4DAEB obtained by synthesis 1 (A) and synthesis 2 (B) and signal labels according to Table 6-4.

For both syntheses, the reported signals for 4DAEB were observed and structures could be assigned accordingly. However, for synthesis 1 signals 9, 10, and 11 indicate impurities. Based on the ^{1}H-NMR spectrum of the product from synthesis 2, no contamination was observed.

6.3.4 ^{13}C-NMR

Since for synthesis 2 a high similarity of the ^{1}H-NMR spectrum with the reported spectrum was obtained, a ^{13}C-NMR spectrum was also recorded and is shown in Figure 6-6. ^{13}C-NMR signals are stated in Table 6-5 as well as predicted and previously reported ^{13}C-NMR signals.

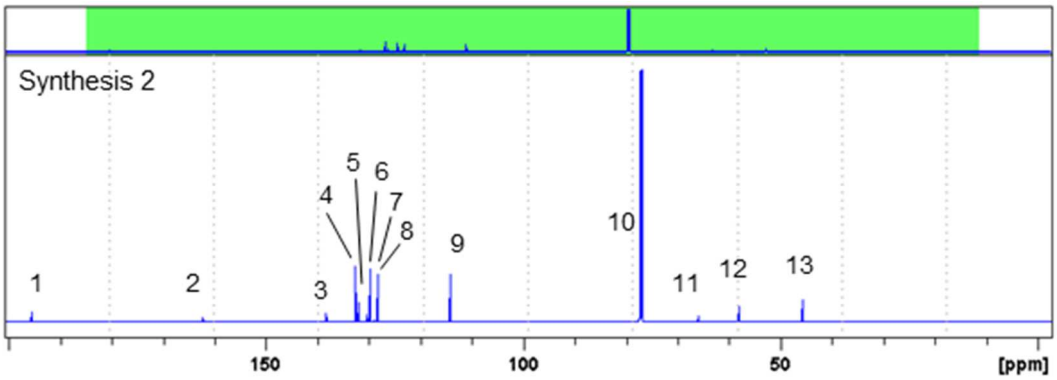

Figure 6-6: ^{13}C-NMR spectrum of 4DAEB derived by synthesis 2.

Table 6-4: ^{1}H-NMR-signals predicted [13], reported [2] and measured for 4DAEB derived from synthesis route 1 and synthesis route 2, assigned structures. Atom / signal labeling refers to Figure 6-4 / Figure 6-5. * impurities; ** solvent signal.

Predicted [13]		**DellaGreca [2]**		**Synthesis 1**		**Synthesis 2**		**Signal in Figure 6-5**	**Atom labeling in Figure 6-4**
δ / ppm	**structure assignment**	**δ / ppm**	**structure assignment**	**δ / ppm**	**structure assignment**	**δ / ppm**	**structure assignment**		
				2.36	* CH3 (NC_2H_6)			**9**	
2.29	6H, CH_3	2.70	2H, CH_2N	2.48	CH_3	2.43	CH_3	**1**	**19 & 20**
2.77	2H, CH_2N	2.98	6H, CH_3	2.94	CH_2N	2.86	CH_2N	**2**	**17**
4.24	2H, CH_2O	4.62	2H, CH_2O	4.25	CH_2O	4.22	CH_2O	**3**	**16**
				6.91	* (C_6H_5)OH			**10**	
6.98	2H, H-9 & H-11	7.00	2H; H-9 & H-11	6.98	H-9 & H-11'	6.99	H-9 & H-11	**4**	**9 &11**
				7.17	* C_6H_6			**11**	
				7.27	** CCl_3-d1	7.28	** CCl_3-d1	**12**	
7.497	2H, H-4 & H-6								**4 & 6**
7.502	2H, H-1 & H-3	7.49	2H, H-1 & H-3	7.48	H-1 & H-3	7.48	H-1 & H-3	**5**	**1 & 3**
7.53	1H, H-2	7.58	1H, H-2	7.56	H-2	7.55	H-2	**6**	**2**
7.59	2H, H-8 & H-12	7.75	2H, H-6 & H-4	7.75	H-6 & H-4	7.76	H-6 & H-4	**7**	**6 & 4**
		7.84	2H, H-8 & H-12	7.83	H-8 & H-12	7.82	H-8 & H-12	**8**	**8 & 12**

Table 6-5: ^{13}C-NMR-signals predicted, reported and from 4DAEB derived by synthesis 2, and assigned structures. Atom / signal labeling refer to Figure 6-4 / Figure 6-6. ** solvent signal

Predicted [13]		DellaGreca [2]		Synthesis 2		Signal in Figure 6-6	Atom labeling in Figure 6-4
δ / ppm	**structure assignment**	**δ / ppm**	**structure assignment**	**δ / ppm**	**structure assignment**		
194.7	CO	195.2	CO	195.7	CO	1	13
158.5	C-10	160.4	C-10	162.4	C-10	2	10
137.8	C-5	137.7	C-5	138.4	C-5	3	5
132.1	C-8 & C-12	132.5	C-8 & C-12	132.7	C-8 & C-12	4	8 & 12
129.6	C-2	132.1	C-4 & C-6	132.1	C-4 & C-6	5	4 & 6
128.9	C-4 & C-6	131.3	C-2	130.5	C-2	6	2
128.7	C-7	129.7	C-7	129.9	C-7	7	7
128.5	C-1 & C-3	128.2	C-1 & C-3	128.4	C-1 & C-3	8	1 & 3
114.6	C-9 & C-11	114.1	C-9 & C-11	114.2	C-9 & C-11	9	9 & 11
				77.0	** CCl_3-d1	10	
68.2	CH_2O	63.2	CH_2O	66.0	CH_2O	11	16
58.2	CH_2N	56.8	CH_2N	58.1	CH_2N	12	17
45.6	$(CH_3)_2N$	44.0	$(CH_3)_2N$	45.8	$(CH_3)_2N$	13	19 & 20

Based on the ^{13}C-NMR spectrum and the structural assignment of the signals, the successful synthesis of 4DAEB can be confirmed and no remaining contamination was visible in the spectrum of the product (synthesis 2).

6.4 Conclusion and Outlook

Identification of 4DAEB can be confirmed based on exact mass and fragmentation obtained by HRMS, ^{1}H-NMR and ^{13}C-NMR spectra for both synthesis routes. However, the newly developed purification procedure following synthesis route 2 gained a higher purity for the product compared to the suggested procedure [9], though a contamination still remained as evident, in form of the light yellow colored crystal layer above a completely white crystal layer. Therefore, the formation of 4DAEB by ozonation of TAM, as well as by photolytic transformation [2], can be confirmed. As the crystal layers of the product of synthesis 2 were combined for storage, a further ^{1}H-NMR was recorded (not shown) and showed a purity of > 95 % based on the signal areas. Hence, no further purification of the product is required for application in ecotoxicological tests. Further investigations will focus on the ecotoxicological potential of 4DAEB, as previous studies correlated an anti-estrogenic effect and an algae growth

inhibition to the presence of 4DAEB, as TP 270, in mixtures of transformation products yielded by ozonation of TAM.

6.5 References

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6.6 Appendix A6: Supplementary Material

6.6.1 A6-1 – Predicted ^{1}H-NMR Spectra

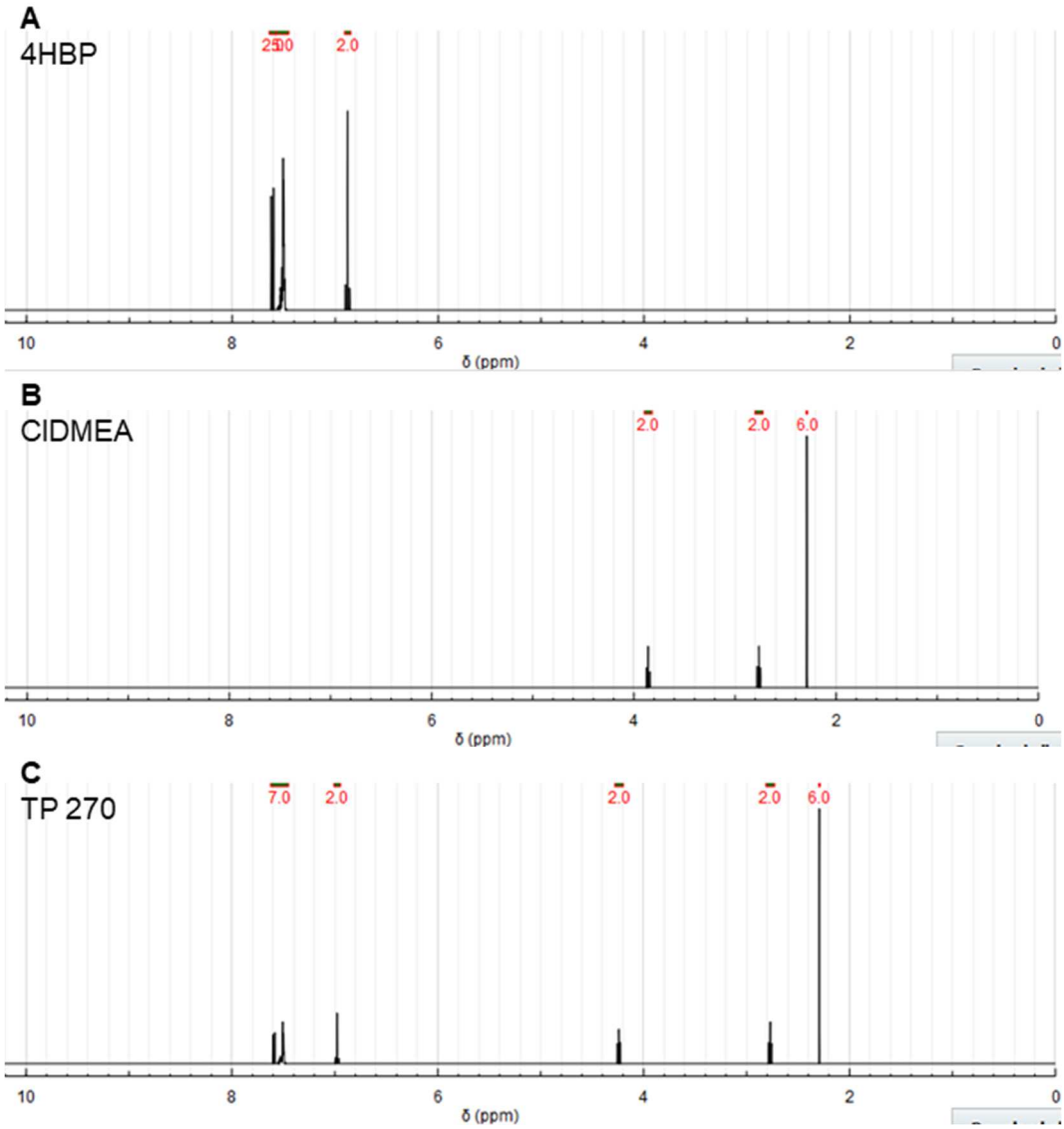

Figure A6-1: Predicted 1H-NMR spectra for (A) 4HBP, (B) ClDMEA, and (C) 4DAEB. [13]

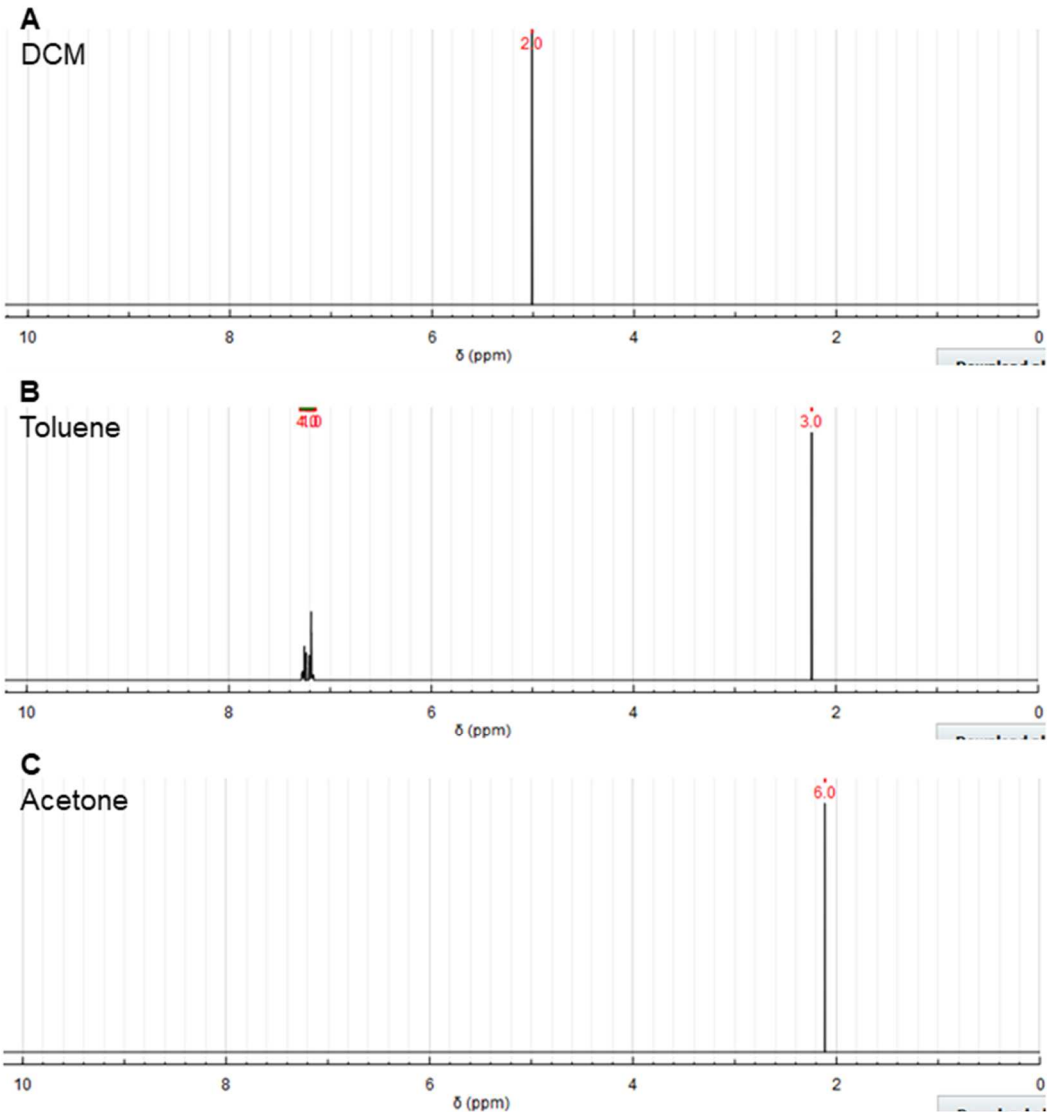

Figure A6-2: Predicted 1H-NMR spectra for (A) DCM, (B) toluene, and (C) acetone. [13]

6.6.2 A6-2 – Reported NMR, IR, and EIMS data

DellaGreca et al. reported a ketone as TP formed during photolysis. They isolated the TP using preparative chromatography and characterized it as follows [2]:

Ketone 5:
IR ($CHCl_3$): ν_{max} 1654 (C=O) cm^{-1}.
^{1}H-NMR: δ ($CDCl_3$) 7.84 (d, J = 8.5 Hz, 2H, H-7 and H-7'), 7.75 (d, J = 7.5 Hz, 2H, H-3 and H-3'), 7.58 (t, J = 8.4 Hz, 1H, H-9), 7.49 (t, J = 8.4 Hz, 2H, H-8 and H-8'), 7.00 (d, 2H, J = 8.5 Hz, H-2 and H-2'), 4.62 (m, 2H, CH2O), 2.70 (m, 2H, CH_2N), 2.98 (s, 6H, $(CH_3)_2N$).
^{13}C NMR: δ ($CDCl_3$) 195.2 (CO), 160.4 (C-1), 137.7 (C-6), 132.5 (C-3), 132.1 (C-9), 131.3 (C-4), 129.7 (C-7), 128.2 (C-8), 114.1 (C-2), 63.2 (CH_2O), 56.8 (CH_2N), 44.0 ($(CH_3)_2N$).
EIMS m/z 269 $[M]^+$, 198 [M_C4H10N]$^+$, 105 $[C_7H_5O]$+, 58 $[(CH_3)_2N{=}CH_2]^+$.

7 General Conclusions and Outlook

The present study elucidated the reaction of ozone and TAM as SERM surrogate. In contrast to the well investigated reactions of ozone and estrogenic active compounds, little information was available until now for the ozonation of anti-estrogenic compounds and the ecotoxicological potential of the formed transformation products.

Five TPs formed in the reaction of TAM and ozone were identified in this study. Primary and secondary TPs could be differentiated and reaction pathways could be assigned based on known ozone reactions [1]. The high variation in the species specific second order reaction rate constants for the neutral TAM species and the corresponding acid also strongly influence the formation of primary TPs. TPs reported previously were not observed [2]. Additionally, formation pathways of these TPs were not consistent with known ozone reactions.

Within the investigation of the reactions of ozone and TAM, a rather high second order rate constant was determined for the reaction of ozone with the tertiary amine moiety. The obtained second order rate constant of $> 10^8$ M^{-1} s^{-1} exceeds the usually reported second order rate constants for amines ($\leq 10^7$ M^{-1} s^{-1}) [1]. Only for one tertiary amine a higher second order rate constant was reported until now (2×10^9 M^{-1} s^{-1}) [3]. The extrapolated second order rate constant obtained in this study was validated using two different competitors. However, a feasible explanation for the high value of the second order rate constant cannot be given in this study, though they are matching the previously reported apparent second order rate constant of 3.56×10^7 M^{-1} s^{-1} at pH 7 [4].

Although three primary and two secondary TPs can be differentiated, not all possible reactions of primary TPs are investigated within this study. One primary TP, namely TP 388, is formed by hydroxylation of a benzene ring within TAM, resulting in the formation of a phenolic moiety. This phenol moiety is expected to be highly reactive towards ozone, but was outcompeted by the other reactive moieties within TAM (the olefin and the tertiary amine). Further transformation of the phenolic moiety was not elucidated in this study, as no further TPs were observed using reversed phase LC/MS.

The formation of TP 270, 4-(Dimethylaminoethoxy)-benzophenone, can be correlated with a proliferation of the anti-estrogenic effect after ozonation. Both effect inducing moieties, the tertiary amine and the benzophenone, are preserved in this TP. It is

formed by the Criegee reaction at the olefin. This product is also formed in the reaction of ozone and TOM and could be formed in the reaction of ozone with the SERMs, droloxifene and clomifene. Other triphenylethylene based SERMs such as ospemifene and idoxifene can also react with ozone according to the Criegee mechanism and therefore can also result in the formation of TPs preserving the effect inducing moieties. With the proliferated anti-estrogenic activity after ozonation assigned to TP 270, we identified the first reported anti-estrogenic TP formed in ozonation. Since the anti-estrogenic activity was shown to remain after ozonation in full scale WWTPs [5], but can be attributed to other fractions after the treatment [6], this study can contribute to understand the fate of anti-estrogenic activity in more complex matrices.

Formation of TP 270 as well as TP 388 showed to be coherent with an increase of the algae growth inhibition over an increasing ozone dose, though the mode of action here is unknown and could not be assessed within this study. The formation of *N*-oxides results in a reduction of the algae growth inhibition. However, ozonation of TAM can result in an increased ecotoxicological risk due to the observed residual anti-estrogenic activity and algae growth inhibition in presence of TP 270.

Since ozonation has become one of the main options for advanced water treatment for the removal of micropollutants [7] and metabolites and transformation products should be included in the risk assessment of chemicals [8], the formation of TPs during ozonation is of high interest. This study shows the formation of a TP that might need to be considered for the risk assessment of TAM according to the European risk assessment guidelines [8]. Nevertheless, quantification of TP 270 was not possible in this study and the formation of the TPs in wastewater matrix were also not included.

The environmental fate of the formed TPs, especially the fate during biological filtration subsequent to ozonation needs to be investigated in the future. Hence, matrix effects during ozonation and the fate of formed TPs in further treatment and the environment need to be determined concerning a consideration in the risk assessment for TAM and other triphenylethylene based SERMs. Especially for TP 270 long-term tests to determine the endocrine effect on the development of individual organisms and also populations should be performed.

Since further transformation reactions can occur during the ozonation of TAM further investigations are required to gather a more comprehensive overview of the reaction pathways for the ozonation of TAM. Here, the reaction of ozone and the phenol moiety

in TP 388 and TP 404 might be of high interest. However, more sensitive methods will be required for the detection of further TPs, since their yields might be too low for detection with our methods. This could be achieved by implementing of a more sensitive detector or an enrichment via SPE-methods specifically designed for the TPs [9]. Further, formation of propiophenone might be monitored using gas chromatography-mass spectrometry.

The ozonation of the active TAM metabolites 4-hydroxytamoxifen and endoxifen are also of high interest, since these show a significantly increased anti-estrogenic activity [10]. Desalkylation at the amine will influence product formation and reaction kinetics, as well as the phenolic moiety. Hence, the results obtained in this study cannot be extrapolated to predict toxicological effects of TPs formed in the ozonation of the TAM metabolites. However, the investigation of TP formation will be compounded by the fact that both, 4-OH-TAM and endoxifen, form enantiomers in aquatic solutions [11]. This will especially impede the elucidation of reaction pathways. As the enantiomers are also separated by RPLC, products will also be present as individual signals. Only TP 270 and TP 286 will not be affected as the stereo center will be removed by the Criegee mechanism.

As also shown in this study, the identification of the relevance of TPs formed in technical and biological processes is achieved using the combination of chemical analytical and toxicological techniques [12]. Only the combination of these complementary approaches will allow a comprehensive evaluation of treatment processes in water treatment. However, since micropollutants can induce a broad range of different effects, relevant effects need to be identified and monitored for each micropollutant. In addition, unspecific tests, such as algae growth inhibition, luminescence of *Aliivibrio fischeri*, and immobilization of *Daphnia magna* need also to be included to allow for an estimation of the ecotoxicological risk [13].

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8 Supplementary Material

8.1 List of Abbreviations

$^{\bullet}OH$	Hydroxyl radicals
4-DAEB	4-(Dimethylaminoethoxy)-benzophenone
4HBP	4-Hydroxybenzophenone
4-OH-TAM	4-Hydroxy tamoxifen
AAM	Modified algae growth medium
ADaM	Aachener Daphnien Medium
AF-1	Activation function 1
AF-2	Activation function 2
alpha-OH-TAM	Alpha-hydroxytamoxifen
AOP	Advanced oxidation processes
ASP 351	Aspartate 351
AWT	Advanced Wastewater Treatment
A-YES	*Arxula adeninivorans* yeast estrogen screen assay
BAC	Biological activated carbon
CI	Confidence interval
ClDMEA	2-chloro-*N*,*N*-dimethylethylamin hydrochloride
CYP	Cytochrome P450
DBD	DNA-binding domain
DCM	Dichloromethane
DEE	Diethyl ether
DES	Diethylstilbestrol
DMEA	Dimethyl ethanolamine
DNA	Deoxyribonucleic acid
DOC	Dissolved organic compound
DOM	Dissolved organic matter
E2	17β-estradiol
EC_{50}	Effect concentration 50 %
EDA	Effect directed analysis

EDC	Endocrine disruptive compounds
EE2	17α-ethinylestradiol
EQ	Equilibration point
ER	Estrogen receptor
HOMO	Highest occupied molecular orbital
HPLC	High Performance Liquid Chromatography
HRMS	high resolution mass spectrometry
IC_{50}	Inhibition Concentration x %
k	Second order rate constant
K_{OW}	Partition coefficients for water/octanol
LBD	Ligand binding domain
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MDM	Methanol, dioxane, and acetonitrile
MEC	Measured environmental concentration
MS	Mass Spectrometry
NDMA	*N*-nitrosodimethylamine
NMR	Nuclear magnetic resonance spectroscopy
NOEC	No observed effect concentration
O_3	Ozone
OD_λ	Optical density measured at λ
PBT	Persistent, bio-accumulative and toxic
PEC	Predicted no effect concentration
pK_a	Dissociation constant
PNEC	Predicted no effect concentration
p_sK_a	Cosolvent dissociation constant
RAF	Risk assessment factor
RBA	Relative binding affinity
SERM	Selective estrogen receptor modulator
SULT	Sulfotransferase

TAM	Tamoxifen
TAM-*N*-oxide	Tamoxifen-*N*-oxide
TBA	Tertiary butanol
TCEP	Tris(1,3-dichloro-2-probpyl)phosphate
TOM	Toremifen
TOrCs	Trace organic compounds
TP	Transformation Product
TP 270	[4-[2-(Dimethylamino)ethoxy]phenyl](phenyl)methanone
TP A	Propiophen-1-one
TP B	3-chloropropiophen-1-one
UGT	UDP-glucuronosyltransferase
UPW	Ultra-pure water
WWTP	Wastewater Treatment Plant
YMM	Yeast minimal maltose medium

8.2 List of Publications

Publications in peer-reviewed journal

- I. Ieropoulos, J. Greenman, D. Lewis, O. Knoop, Energy production and sanitation improvement using microbial fuel cells, Journal of Water Sanitation and Hygiene for Development, 3 (2013) 383-391. doi:10.2166/washdev.2013.117
- Knoop O., Hohrenk L.L., Lutze H.V., Schmidt T.C., Ozonation of Tamoxifen and Toremifene – Reaction Kinetics and Transformation Products (submitted to Environmental Science and Technology)
- O. Knoop, F. Itzel, J. Tuerk, H.V. Lutze, T.C. Schmidt, Endocrine effects after ozonation of tamoxifen, Sci. Total Environ., 622-623 (2018) 71-78. doi.org/10.1016/j.scitotenv.2017.11.286
- O. Knoop, M. Woermann, H.V. Lutze, B. Sures, T.C. Schmidt, Ecotoxicological effects prior to and after the ozonation of Tamoxifen, Journal of hazardous materials, 358 (2018) 286-293. doi.org/10.1016/j.jhazmat.2018.07.002

Oral presentations

- Ieropoulos I., Greenman J., Lewis D., Knoop O., 2012, Energy Production and Sanitation Improvement Using Microbial Fuel Cells Water Research Commission, International Faecal Sludge Management Conference 2 (FSM2), Durban, South Africa, 29-31 October 2012. Pretoria South Africa.
- Knoop O., Itzel F., Türk J., Schmidt T.C., 2017, Identifying the risk – Monitoring of Water Quality needs more than conventional chemical analysis, ASLO 2017, Honululu, United States of America. .
- Knoop O., Itzel F., Türk J., Schmidt T.C., Ozonation of Tamoxifen Generates Endocrine Active Transformation Products, MicroPol 2017, Wien, Östereich,

Poster presentations

- Knoop O., Lewis D., Greenman J., Ieropoulos I., 2013, 'Urine-tricity': Electricity from Urine and Sludge *Center for Research in Biosciences* Bristol (UK) (veröffentlicht)

- Knoop O., Bronja A., Schmtiz O.J., 2015, Establishment of a new gas chromatographic-atmospheric pressure photoionization ion source for Orbitrap-mass spectrometry Anakon2015, Graz, Austria, 23-27 March 2015.
- Knoop O., Lutze H.V., Schmidt T.C., 2015, Degradation of Tamoxifen During Ozonation: pH Dependency Micropol & Ecohazard Conference 2015, Singapore, Singapore, 22-26 November 2015.
- Knoop O., Kaziur W., Orschler L., Lutze H.V., Schmidt T.C., 2016, The Ozonation of Tamoxifen is pH dependent Wasser2016, Bamberg, Germany, 2-4 May 2016.
- Knoop O., Kaziur W., Orschler L., Lutze H.V., Schmidt T.C., 2016, The Ozonation of Tamoxifen is pH dependent 13th IWA Leading Edge on Water and Wastewater Technologies Conference 2016, Jerez de la Frontera, Spain, 13-18 June 2016.
- Knoop O., Hohrenk L., Lutze H.V., Schmidt T.C., 2017, *Degradation pathways of Tamoxifen during Ozonation* Wasser2017, Donau-Eschingen, Germany, 22-24 May 2017.

Other publications

- Kosse P., Knoop O., Lübken M., Schmidt T.C., Wichern M., 2018, Methane emissions from wastewater treatment plants, WaterSolutions (1), 3-6 (Journal, not peer reviewed)

8.3 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten

8.4 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Formation and Effects of Transformation Products during the Ozonation of Tamoxifen”

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im Juli 2018

Oliver Knoop

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