Ozone reactivity in wastewater treatment plant effluent and reverse osmosis concentrate

Ozonation of beta blockers: kinetic studies, identification of oxidation products and pathways

Dissertation

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Summary

Many pharmaceuticals (e.g. antibiotics, contrast media, beta blockers) are excreted unmetabolized and enter wastewater treatment plants (WWTPs) through the domestic sewage system. Research has shown that many of them are not effectively removed by conventional wastewater treatment and therefore are detected in surface waters.

Reverse osmosis (RO) is one of the most effective means for removing a wide range of micropollutants in water recycling. However, one significant disadvantage is the need to dispose the resultant RO concentrate. Due to the fact that there are elevated concentrations of micropollutants in the concentrate, a direct disposal to surface water could be hazardous to aquatic organisms. As a consequence, further treatment of the concentrate is necessary. In this study, ozonation was investigated as a possible treatment option for RO concentrates. Concentrate samples were obtained from a RO-membrane system which uses municipal WWTP effluents as feeding water to produce infiltration water for artificial groundwater recharge. In this study it could be shown that ozonation is efficient in the attenuation of selected pharmaceuticals, even in samples with high TOC levels (46 mg C/L).

Tests with chlorinated and non-chlorinated WWTP effluent showed an increase of ozone stability, but a decrease of hydroxyl radical exposure in the samples after chlorination. This may shift the oxidation processes towards direct ozone reactions and favors the degradation of compounds with high apparent second order rate constants. Additionally it might inhibit an oxidation of compound predominantly reacting with OH radicals.

Ozone reaction kinetics were investigated for beta blockers (acebutolol, atenolol, metoprolol and propranolol) which are permanently present in WWTP effluents. For beta blockers two moieties are common which are reactive towards ozone, a secondary amine group and an activated aromatic ring. The secondary amine is responsible for a pH dependence of the direct ozone reaction rate, since only the deprotonated amine reacts very quickly. At pH 7 acebutolol, atenolol and metoprolol reacted with ozone with an apparent

second order rate constant of about 2000 $M^{-1} s^{-1}$, whereas propranolol reacted at ~1.0 10^5 $M^{-1} s^{-1}$. The rate constants for the reaction of the selected compounds with $^{\bullet}OH$ radicals were determined to be 0.5-1.0 × 10^{10} $M^{-1} s^{-1}$.

Oxidation products (OPs) formed during ozonation of metoprolol and propranolol were identified via liquid chromatography (LC) tandem mass spectrometry. Ozonation led to a high number of OPs being formed. Experiments were carried out in MilliQ-water at pH 3 and pH 8 as well as with and without the radical scavenger tertiary butanol (*t*-BuOH). This revealed the influence of pH and the OH radical exposure on OP formation. The OH radical exposure was determined by adding the probe compound *para*-chlorobenzoic acid (pCBA).

Metoprolol: To define the impacts of the protonated and non protonated metoprolol species on OH radical formation, the measured pCBA attenuation was compared to modeled values obtained by a simplified kinetic model (Acuchem). A better agreement with the measured results was obtained, when the model was based on a stoichiometric formation of OH radical precursors ($^{\bullet}O_2^-$) during the primary ozone reaction of metoprolol. However, for reaction of a deprotonated molecule (attack of the aromatic ring) a formation of $^{\bullet}O_2^-$ could be confirmed, but an assumed stoichiometric $^{\bullet}O_2^-$ formation over-estimated the formation of OH radicals in the system.

Analysis of ozonated raw wastewater and municipal WWTP effluent spiked with 10 µM metoprolol exhibited a similar OP formation pattern as detected in the reaction system at pH 8 without adding radical scavenger. This indicated a significant impact of OH radical exposure on the formation of OPs in real wastewater matrices.

Propranolol: The primary ozonation product of propranolol (OP-291) was formed by an ozone attack of the naphthalene ring, which resulted in a ring opening and two aldehyde moieties being formed. OP-291 was further oxidized to OP-307, presumably by an OH radical attack, which was then further oxidized to OP-281. Reaction pathways via ozone as well as

OH radicals were proposed and confirmed by the chemical structures identified with MS^2 and MS^3 data.

It can be concluded that ozonation of WWTP effluent results in the formation of a high number of OPs with an elevated toxic potential (i.e. formation of aldehydes).

Zusammenfassung

Bei einigen Arzneimittel (z.B. Antibiotika, Kontrastmittel, Betablocker) wird ein Teil des Wirkstoffs unmetabolisiert ausgeschieden und gelangt so über das Abwasser in kommunale Kläranlagen. Studien haben gezeigt, dass viele dieser Wirkstoffe durch eine konventionelle Abwasserbehandlung nicht effektiv abgebaut werden und somit in Oberflächengewässern nachweisbar sind.

Eines der effektivsten Verfahren zur Entfernung organischer Mikroverunreinigungen und Mikroorganismen ist die Umkehrosmose. Ein bedeutender Nachteil dieses Verfahrens ist die Entsorgung der anfallenden Konzentrate, die erhöhte Konzentrationen von Mikroverunreinigungen und Mikroorganismen enthalten können. Dabei ist nicht auszuschließen, dass eine direkte Einleitung dieser Konzentrate das Ökosystem eines Gewässers schädigt. Um dieses Risiko zu minimieren, wäre eine gesonderte Behandlung des Konzentrats vor der Einleitung sinnvoll. In der hier vorliegenden Arbeit wurde die Ozonung als mögliches Oxidationsverfahren untersucht. Die untersuchten Konzentratproben stammen aus einer Kläranlage, in der der Ablauf nach Umkehrosmosebehandlung in das Grundwasser infiltriert wird. Durch die Untersuchungen konnte gezeigt werden, dass durch die angewendeten Ozondosen die ausgewählten Arzneistoffe weitgehend oxidiert wurden, obwohl das Umkehrosmosekonzentrat einen sehr hohen TOC –Gehaltes von bis zu 46 mg/L aufwies.

Zur Vorbeugung von Membranfouling wird Membrananlagenzuläufen, in diesem Fall dem Kläranlagenablauf, häufig Chlor zugesetzt. Eine Vergleichsstudie mit vorchlorierten und nicht vorchlorierten Kläranlagenabläufen zeigte einen Anstieg der Ozonstabilität nach der Chlorierung. Daraus resultierte aber auch eine Abnahme an OH-Radikalen. Die höhere Ozonstabilität könnte dazu führen, dass über eine direkte Ozonreaktion die Oxidation von Stoffen mit einer höheren Geschwindigkeitskonstante zweiter Ordnung bevorzugt würde. Der

Abbau der Stoffe, die hauptsächlich über OH-Radikale oxidiert werden, würde dann gleichzeitig herabgesetzt.

Für die Wirkstoffgruppe der Betablocker, die permanent in Kläranlagenabläufen nachweisbar ist, wurden die Geschwindigkeitskonstanten mit Ozon- sowie OH-Radikalen ermittelt. Untersucht wurden Acebutolol, Atenolol, Metoprolol und Propranolol. Betablocker enthalten zwei funktionelle Gruppen, die reaktiv gegenüber Ozon sind, zum einen ein sekundäres Amin und zum anderen einen aktivierten aromatischen Ring. Die Amingruppe ist dafür verantwortlich, dass die Geschwindigkeit der Ozonreaktion pH-Wert abhängig ist, da nur das deprotonierte Amin schnell mit Ozon reagieren kann. Die Geschwindigkeitskonstante für die Reaktion von Acebutolol, Atenolol and Metoprolol mit Ozon liegt bei etwa 2000 $M^{-1} s^{-1}$, wo hingegen Propranolol mit ~1.0 10^5 M^{-1} s^{-1} etwa zwei Größenordnungen schneller reagiert. Die Konstanten für die Reaktionen mit OH-Radikalen liegen bei 0.5-1.0 × 10^{10} $M^{-1} s^{-1}$.

Die während der Ozonreaktion von Metoprolol und Propranolol gebildeten Oxidationsprodukte (OPs) wurden über HPLC-Tandem-MS identifiziert. Die Ozonreaktionen führten zur Bildung 23 OPs im Fall des Metoprolols und etwa 30 OPs beim Propranolol. Um die Auswirkung des pH-Wertes sowie der OH-Radikalreaktion auf die OP-Bildung zu untersuchen, wurden die Experimente bei pH 3 und 8, sowie mit und ohne Zugabe des Radikalfängers *tert*-Butanol durchgeführt. Die Menge der gebildeten OH-Radikale wurde durch den Zusatz von *para*-Chlorbenzoesäure ermittelt.

Metoprolol: Der Einfluss des protonierten und des nicht protonierten Metoprolols auf die Menge der gebildeten OH-Radikale wurde über einen Vergleich der gemessenen *para*-Chlorbenzoesäureabnahme mit modellierten Werten ermittelt (Model: Acuchem). Es lassen sich dabei bessere Übereinstimmungen erzielen, wenn die Modelannahmen bei der primären Ozonreaktion des nicht protonierten Moleküls auf einer stöichiometrischen Bildung eines OH-Radikalvorproduktes ($^{\bullet}O_2^-$) basieren. Die Modellierung der Reaktion der

protonierten Spezies unterstützt ebenfalls die These der Bildung des Vorproduktes $^{\bullet}O_2^-$, allerdings in unterstöichiometrischer Menge.

Die Untersuchung eines Abwassers und eines Kläranlagenablaufs, die mit 10 µmol/L Metoprolol versetzt wurden, zeigte eine OP-Bildung ähnlich der bei pH 8 ohne Radikalfängerzugabe. Dies deutet auf einen signifikanten Einfluss der OH-Radikalreaktion für die Bildung der OP in realer Abwassermatrix hin.

Propranolol: Das primäre OP der Ozonreaktion des Propranolols (OP-291) wird über einen Angriff am Naphthalenring gebildet, der zu einer Ringöffnung und der Bildung zweier Aldehydfunktionen führt. OP-291 wird vermutlich über eine OH-Radikalreaktion weiter zu OP-307 oxidiert, welches anschließend zu OP-281 weitereagiert. Durch die mittels Massenspektrometrie identifizierten chemischen Strukturen der OPs, können die vorgeschlagenen Bildungsreaktionen sowohl über direkte Ozonreaktion als auch über OH-Radikalreaktion bestätigt werden.

Als Fazit kann festgestellt werden, dass eine Ozonung eines Kläranlagenablaufes zu der Bildung einer Vielzahl von OPs führt, die alle ein unbekanntes toxikologisches Potential haben (z.B. Bildung von Aldehydfunktionen).

1 General Introduction

1.1 Beta blockers

1.1.1 General information on beta blockers

Beta-adrenergic blocking agents (also known as beta blockers or beta-adrenergic antagonists) are pharmaceuticals used for various therapeutic purposes mainly for the treatment of cardiac malfunctions such as arrhythmia, hypertension, as well as post treatment after a myocardial infarction. They bind to beta-adrenergic receptors in the sympathetic nervous system preventing further binding of neurotransmitters (e.g. adrenaline). Beta blockers can be divided into selective and non-selective agents, depending on their selectivity of different types of beta receptors. The beta blockers selected for the research conducted in this thesis are shown in Table 1-1.

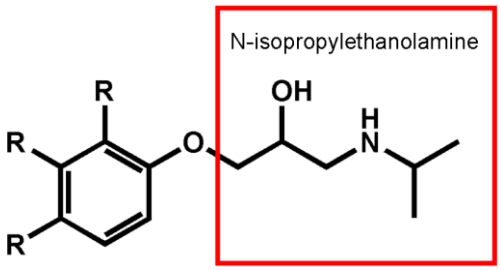
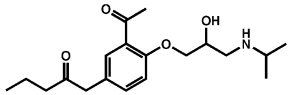
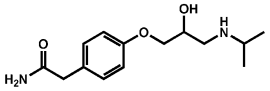
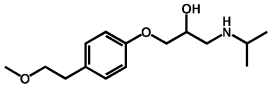
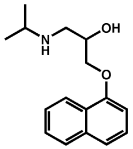


Figure 1-1: General chemical structure of beta blockers

The chemical structures of the selected beta blockers have two common functional groups or moieties, the aromatic ring and an ethanolamine (most cases N-isopropyl ethanolamine, Figure 1-1) side chain which binds to the aromatic ring with an ether bond. The oxygen in the ether bond acts as an electron donor and causes an increase in electron density in the π-system of the aryl group. This results in a higher reactivity towards the electrophile attack e. g. of ozone. The same goes for a deprotonated amine with alkyl substitutes acts as an electron donor with an elevated electron density. As the amino moieties of the selected beta blockers have pK_a values of ~ 9, the protonated species will be dominant at environmental as well as

wastewater relevant pH of 6-8. However, the deprotonated amine is up to 50 times more reactive than the activated aromatic ring, so that the apparent rate constant of the reaction with ozone will also be influenced at this pH range.

Table 1-1: Selected properties of the investigated beta blockers

Name	IUPAC name	MW [g/mol]	CAS	Structure	Select-ivity	Prescrip-tions Germany, 2004 [t]*	Human excretion rate [%] **	pK_a	LogP (*o/w*)
Acebutolol	N-[3-Acetyl–4-[2–hydroxy–3-[(1–methylethyl)amino] propoxy]-butanamide	336.4	3751–730–9		β_1	-	9-12 (60)	9.4	-
Atenolol	4-[2-Hydroxy–3-[(1–methylethyl) amino]propoxy] benzeneacetamide	266.3	29122–68–7		β_1	7.1	35-50	9.6	0.23
Metoprolol	1-[4-(2-Methoxyethyl) phenoxy] -3-[(1–methylethyl) amino]-2–propanol	267.4	37350–58–6		β_1	98.1	~ 10	9.7	1.9
Propranolol	1-[(1-Methylethyl) amino]-3-(1–naphthalenyloxy) -2–propanol	259.3	525–66–6		non	3.36	< 4	9.5	1.2

*(Lemmer 2006), **(Moffat 2004)

1.1.2 Occurrence of beta blockers in the aquatic environment

Pharmaceuticals, excreted either metabolized or unmetabolized, are entering wastewater treatment plants (WWTPs) through the sewer systems. In addition, unused or expired medications are known to be disposed directly in the sewage waters, elevating the concentrations of the pharmaceuticals. In most cases, these polar compounds are not completely removed from the water phase by the conventional wastewater treatment processes.

One therapeutic class of pharmaceuticals that is not effectively removed during wastewater treatment is beta blockers. Beta blockers have been detected in the high ng/L up to µg/L range in WWTP effluents (Bendz et al. 2005, Gros et al. 2006, Huggett et al. 2003, Lee et al. 2007, Ternes et al. 2003, Vieno, N. et al. 2007, Vieno, N. M. et al. 2006). As they are permanently discharged into surface waters, they have frequently been detected in several surface waters. Table 1-2 shows a summary of some concentrations found in European rivers.

Table 1-2: Concentrations of selected beta blockers from several European studies on surface waters [µg/L].

	Höje river[a] (Sweden)	Ebro river[b] (Spain)	German rivers[c]
Acebutolol	n. i.	n. i.	n. i.
Atenolol	0.06	0.072	n. i.
Metoprolol	0.06	<LOD	1.2
Propranolol	0.01	<LOD	0.44
Reference :	(Bendz et al. 2005)	(Gros et al. 2006)	(Ternes et al. 1998)
	Po river[c] (Italy)	Vantaa river[c] (Sweden) 2006	Vantaa river[c] (Sweden) 2007
Acebutolol	n. i.	0.008	0.012
Atenolol	n. i.	0.022	0.047
Metoprolol	0.039	0.077	0.101
Propranolol	n. i.	n. i.	n. i.
Reference :	(Calamari et al. 2003)	(Vieno, N. M. et al. 2006)	(Vieno, N. M. et al. 2007)

n. i.: not included in study; <LOD: below limit of detection. [a] grab sample; 7543 m downstream of WWTP; [b]given in average; [c] german rivers:Lahn, Kinzig, Fulda, Werra, Main, Rhine, Nidda, Schwarzbach; given as 90 percentile

1.1.3 Ecotoxicology of beta blockers

Pharmaceuticals are designed to have a pharmacological and biochemical response to target receptors in the human body, but could potentially be hazardous to the receiving ecosystems (i.e. non-target organisms). Additionally they might have an impact on the quality drinking if they reach the ground water.

Several studies focusing on the toxicological potential of beta blockers revealed their environmental relevance. Cleuvers (2005) measured EC_{50} values in algae tests (*desmodesmus subspicatus*) of < 1 mg/L for propranolol and 7.9 mg/L for metoprolol. According to the EU-Directive (Commission of the European Communities, 1996) metoprolol would then be classified as potentially toxic and propranolol as very toxic to aquatic organisms. A chlorophyll fluorescence test showed a positive correlation between fluorescence inhibition effects and hydrophobicity of the compound, with EC_{50} values ranging from EC_{50} 1335 mg/L for atenolol, EC_{50} 40 mg/L for metoprolol, and EC_{50} 4.1 mg/L for propranolol (Escher et al. 2006). Dzialowski et al. (2006) measured lowest observed effect concentrations (LOEC) of metoprolol and propranolol for *daphnia magna*. In addition to the typical ecotoxicological endpoints, growth ($LOEC_{propranolol}$ = 0.44 mg/L; $LOEC_{metoprolol}$ =12 mg/L), they also observed an impact on the heart rate of the test organisms ($LOEC_{propranolol}$ = 0.055 mg/L; $LOEC_{metoprolol}$ = 13.1 mg/L). A comparison of the LOECs confirmed a higher toxicological potential of propranolol compared to metoprolol. All these bioassays showed effects at concentrations at least one order of magnitude higher than the environmental relevant values. However, a 4-week exposure experiment by Huggett et al. exhibited that a concentration of only 500 ng/L propranolol had an effect on the reproduction and steroid levels in Japanese medaka (*Oryzias latipes*) (Huggett et al. 2002).

In addition, bioassays of beta blocker mixtures showed elevated effects, in comparison to the sum of single compound effects. The sum of EC_{50} values (*daphnia magna*) of propranolol, metoprolol and atenolol would theoretically only result in an inhibition of 21.3 %, but the

measured inhibition of the mixture was 65 %. This suggests that the mixture of beta blockers supports even a synergistic effect in these ecotoxicological tests, meaning the effect of the mixture is greater than the effect observed for each individual beta blocker or the sum of the individual effects.

1.2 Water Reclamation/ Reverse Osmosis

The global demand for high quality water is constantly increasing, with a number of regions facing water shortages. In order to produce enough clean water to meet the increasing water demands, several techniques have been developed to reuse municipal waste water for irrigation and even drinking water purposes. Especially for ground water recharge and drinking water production not only the removal of nutrients and microbial pollutants (e.g. pathogens) or dissolved organic carbon (DOC) are crucial, but also the removal of organic micropollutants need to be considered. Tight membrane processes, such as nanofiltration (NF) and reverse osmosis (RO), are molecular sieving techniques exhibiting very good performances also for removal of organic micropollutants. Pressure-driven membrane processes separate a feed stream (e.g. surface water, seawater, treated waste water) into a purified permeate fraction and concentrated retentate fraction (also called concentrate or brine).

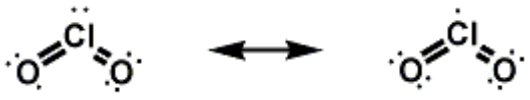
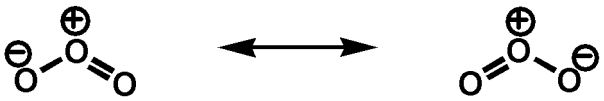
Studies on different types of NF and RO membranes demonstrate that some of the RO membranes rejected from 60 to over 90 % of micropollutants (e.g. pharmaceuticals). Rejection depends mostly on the charge of a compound, molecular size, steric hindrance and polarity. (Kimura et al. 2003, Kimura et al. 2004). However, the two major drawbacks of these processes are membrane fouling as well as the disposal of the resulting concentrate. (Van der Bruggen et al. 2003). The average concentration factor from the feed of a RO-processor to the retentate is about 3-4, including the DOC, salt content as well as micropollutants. Possible techniques for processing concentrates are recycling (e.g. into

WWTP), incineration and direct or indirect discharge into surface water. Recycling is only useful, if the concentrate contains a high amount of biodegradable substances, and most of the time the feed of a RO coming from a WWTP does not have biodegradable substances because they have already been eliminated. In the current study, reclamation of WWTP effluent via RO treatment was investigated. For incineration of the RO concentrate, high energy consumption and high costs are needed (Van der Bruggen et al. 2003). Direct discharge of a solution with elevated concentration of DOC, microorganisms and micropollutants into the environment might need further treatment to minimize the potential hazard to the receiving water. One possible treatment of the RO concentrate could be ozonation. Ozonation does not only lead to disinfection but also to an oxidation of organic molecule of the DOC as well as micropollutants, possibly increasing their biodegradability. This might justify a recycling of the concentrate into a WWTP.

1.3 Ozonation

With an oxidation potential of 2.1 eV ozone (Table 1-3) is one of the strongest oxidants of commonly known disinfectants.

Table 1-3: Comparison of oxidation potential of different common oxidants.

oxidant	chemical structure	oxidation potential [eV]
chlorine	$Cl_2 + H_2O \longrightarrow HOCl + HCl$	1.36
chlorine dioxide		1.15
hydroxyl radicals	$^{\cdot}OH$	2.8
ozone		2.1

Ozonation is a widely used and well known technique for disinfection of water (e.g. drinking water treatment) (Camel and Bermond 1998, von Gunten 2003) and very effective in the

removal of color, odor and taste. Several studies (Andreozzi et al. 2005, Huber et al. 2003, Huber et al. 2005, Ikehata et al. 2006, McDowell et al. 2005, Ternes et al. 2003) have confirmed that ozone treatment can be very efficient in the oxidation of a wide range of micropollutants (e.g. beta blockers, antibiotics, estrogens). In addition, ozonation has been found to reduce or to eliminate the pharmacological and biological effects of micropollutants. Estrogenic activity of 17α-ethinylestradiol (Huber et al. 2004) was reduced by a factor of 200-500 and the pharmaceutical effects of antibiotics (Dodd et al. 2006) were significantly reduced by ozonation. This could lower the risk of the formation of resistant strains against the common antibiotics in bacteria.

1.3.1 Ozone decay and OH radical formation in water

The decomposition of ozone in water is initiated by hydroxyl ions as shown in eq. (R1) (Staehelin and Hoigne 1982). The hydrogen peroxyl ion can react with another ozone molecule to form an OH radical, a dioxygen molecule and a superoxide radical (R2).

$O_3 + OH^- \rightarrow HO_2^- + O_2$, $k = 70\ M^{-1} s^{-1}$ (Staehelin and Hoigne 1982) R1

$O_3 + HO_2^- \rightarrow {}^\bullet OH + O_2 + {}^\bullet O_2^-$, $k = 2.8 \cdot 10^6\ M^{-1} s^{-1}$ (Staehelin and Hoigne 1982) R2

$O_3 + {}^\bullet O_2^- \rightarrow {}^\bullet O_3^- + O_2$, $k = 1.6 \cdot 10^9\ M^{-1} s^{-1}$ (Buhler et al. 1984) R3

The radical reaction chain is propagated with the reaction of superoxide radical with ozone, causing the formation of an ozonide ion (R3). If it is protonated it decomposes to form another OH radical and dioxygen molecule (R4 and R5).

${}^\bullet O_3^- + H^+ \rightarrow HO^\bullet_3$, $k = 5.2 \cdot 10^{10}\ M^{-1} s^{-1}$ (Buhler et al. 1984) R4

$HO^\bullet_3 \rightarrow {}^\bullet OH + O_2$, $k = 1.1 \cdot 10^5\ M^{-1} s^{-1}$ (Buhler et al. 1984) R5

If the ozonide ion decomposes directly an oxygen radical is formed (R6), which reacts with water to form an OH radical and a hydroxyl ion (R7).

${}^\bullet O_3^- \rightarrow {}^\bullet O^- + O_2$, $k = 2.8 \cdot 10^3\ M^{-1} s^{-1}$ (Elliot and McCracken 1989) R6

${}^\bullet O^- + H_2O \rightarrow {}^\bullet OH + {}^\bullet OH^-$, $k = 9.2 \cdot 10^7\ M^{-1} s^{-1}$ (Buxton 1970) R7

$^{\bullet}OH + O_3 \rightarrow HO^{\bullet}_2 + O_2$, $k = 2 \cdot 10^9\ M^{-1} s^{-1}$ (Staehelin et al. 1984) R8

The reaction of ozone and OH radicals leads to the formation of a HO_2 radical, which is the deprotonated form of an OH radical precursor (R8). Apart from these main pathways, reaction chain propagation is promoted by several more side reactions not mentioned here. Termination of the radical reaction chain occurs, whenever two radicals combine. For example, two OH radicals form hydrogen peroxide (R9) or a superoxide radical and an OH radical react resulting in a dioxygen molecule and hydroxyl ions (R10).

$^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2$, $k = 5 \cdot 10^9\ M^{-1} s^{-1}$ (Staehelin et al. 1984) R9

$^{\bullet}OH + {}^{\bullet}O_2^- \rightarrow O_2 + OH^-$, $k = 1 \cdot 10^{10}\ M^{-1} s^{-1}$ (Staehelin et al. 1984) R10

All radicals formed during the reaction chain, except OH radicals, do not oxidize any organic solutes. OH radicals are very strong oxidants (see Table 1), reacting in a non-selective way. During ozonation of natural water and wastewater matrices, OH radicals are formed but can be scavenged effectively by DOC. However, they can have a huge impact on the oxidation of organic compounds, especially those which are not reactive towards ozone.

1.3.2 Ozonation kinetics

Direct ozonation reactions (without involvement of OH radicals; in presence of a radical scavenger) can be described as followed (Hoigne and Bader 1983):

$$M + \eta\, O_3 \rightarrow M_{oxid}$$

Where M represents the compound being oxidize, η the stoichiometric factor of the number of ozone molecules consumed for an oxidation of M, and $M_{oxid,}$ the oxidation product of the target compound. If one is only looking at the primary attack of ozone, η becomes 1 and the 2nd order rate law for this reaction can be formulated as:

$$\frac{-d[M]}{dt} = k_{O_3}[O_3][M] \qquad \text{eq 1}$$

If M is a dissociating compound and the protonated and deprotonated species have different reactivities towards ozone, k_{O3} becomes pH dependent. Therefore, M can be described as

$$M + H^+ \rightleftharpoons MH^+$$

and k_{O3} can be formulated as

$$k_{O_3} = \left(\alpha \cdot k_{ox.MH^+} + (1-\alpha) \cdot k_{ox.M}\right) \qquad \text{eq 2}$$

with α defined as the degree of dissociation which is dependent on K_a, the equilibrium constant, of the protonation reaction:

$$\alpha = \frac{1}{1 + \frac{K_a}{\left[H^+\right]}} \qquad \text{eq 3}$$

A typical example are amines, in which the protonated species does not react with ozone, while the deprotonated form reacts quite fast. All beta blockers have a secondary amine moiety, causing the pH dependence of their rate constants. Since the pK_a values are known, the apparent rate constants for the deprotonated beta blockers can be extrapolated. Rate constants cannot be measured at high pH values (i.e. pH 12), because ozone is not sufficient stable during these experimental conditions.

Depending on the reactivity of the target compound, the determinations of rate constants are based on different theoretical considerations.

Pseudo-first-order condition (slow reacting compounds) (Hoigne and Bader 1983)*:*

To measure the apparent rate constants of slow reacting compounds in batch systems, the concept of pseudo-first-order kinetics is used. If ozone is added in high excess compared to the concentration of M, the change of ozone concentration due to reaction with M is negligible and can be considered stable. If this is applied to eq 1 it results in a pseudo-first-order reaction rate.

$$\frac{-d[M]}{dt} = k_{O_3,obs}[M] \qquad \text{eq 4}$$

$$\text{integrated form: } \ln\left(\frac{[M]}{[M]_0}\right) = -k_{o_3,obs} \cdot t \qquad \text{eq 5}$$

$$\text{with } k_{o_3,obs} = k_{O_3,app} \cdot [O_3]_0 \qquad \text{eq 6}$$

Rate constants for slowly reacting compounds were determined in batch systems by measuring the time depending attenuation of the target compound via HPLC with UV detection. Plotting the $\ln\left(\frac{[M]}{[M]_0}\right)$ versus the reaction time, resulted in a straight line with $-k_{o_3,obs}$ as slope.

Ozone concentrations in the samples were determined with an indigo-blue method and extrapolated to the time point 0. Dividing $-k_{o_3,obs}$ by the ozone concentration at the starting point resulted in the apparent second order rate constant $-k_{o_3,app}$ of the system.

The higher the pH in the batch system, the more elevated the ozone decay due to the decomposition initiated by the hydroxyl ions resulting in a non-linear correlation. If the decay of excess ozone was not caused by the reaction with the target compound, the measured values could be plotted against the ozone exposure (ct_{O3}) which is the intergraded ozone dose $\int [O_3] \cdot dt$. The slope of the resulting straight line represented the second order rate constant.

$$ct_{O_3} = \int [O_3] \cdot dt \qquad \text{eq 7}$$

Competition kinetics (fast reacting compounds):

For higher rate constants (> 2000 $M^{-1}s^{-1}$), a competition kinetic method with a competitor substrate of a known ozone rate constant was used. For this research, cinnamic acid (R, reference) ($k_{neutral,O3} = 5 \cdot 10^4$ $M^{-1}s^{-1}$ and $k_{anion,O3} = 3.8 \cdot 10^5$ $M^{-1}s^{-1}$ (Leitzke et al. 2001), pK_a =

4.4 (Lide 2001)) was used, which was mixed with the target compound (M) in a 1:1 ratio and several stoichiometric ozone doses were added.

$$\ln\left(\frac{[M]}{[M(0)]}\right) = \ln\left(\frac{[R]}{[R(0)]}\right)\frac{k_{O_3}(M)}{k_{O_3}(R)} \qquad \text{eq 8}$$

Plotting $\ln\left(\frac{[R]}{[R(0)]}\right)$ versus $\ln\left(\frac{[M]}{[M(0)]}\right)$ of the different ozone doses resulted in a straight line. By dividing the slope of $k_{O3}(R)$, $k_{O3}(M)$ could be elucidated (Hoigne and Bader 1983).

The same principle can be applied for $^{\bullet}OH$ radical rate constants. For a pure hydroxyl radical reaction, radicals were generated by *in situ* UV photolysis (low pressure mercury lamp) of H_2O_2 in solutions containing the competitor substrate para-chlorobenzoic acid (pCBA, with $k_{app}{}^{\bullet}{}_{OH} = 5.0 \cdot 10^9\ M^{-1}s^{-1}$ (Buxton et al. 1988)) with a known $k_{app}{}^{\bullet}{}_{OH}$ and the target compound. The experimental set-up with UV photolysis makes it necessary to account for direct photolysis of the compounds, and if this direct photolysis was $k_{app}{}^{\bullet}{}_{OH}$ could be determined.

Ozone and OH radical exposures in real water systems depend on different parameters, such as pH, DOC and the scavenging potential. If ozone and OH radical exposures can be measured for a certain matrix, the degree of oxidation of compounds (M) with known $k_{app,O3}$ and $k_{app}{}^{\bullet}{}_{OH}$ can be predicted according to eq. 4. (Elovitz and von Gunten 1999).

$$[M] = e^{-k_{app,O_3}\cdot\int[O_3]dt - k_{app,^{\bullet}OH}\cdot\int[^{\bullet}OH]dt}\cdot[M]_0 \qquad \text{eq 9}$$

Figure 1-2 shows an example comparing the measured and predicted attenuation of propranolol in a matrix sample (RO-concentrate).

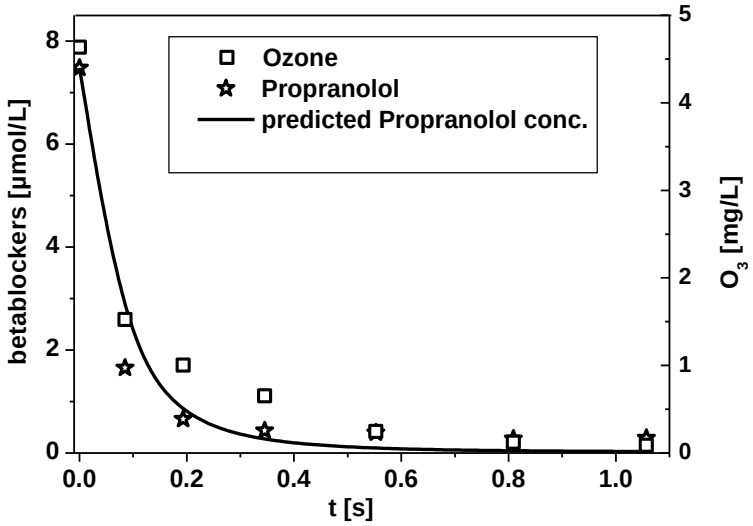


Figure 1-2: Continuous quench flow experiment with matrix (RO-concentrate, pH = 8) containing propranolol. Ozone dose: 5 mg/L; room temperature.

1.3.3 Formation of oxidation products

A major part of this thesis was the structural elucidation of ozonation products. As economic relevant ozone doses are insufficient to mineralize micropollutants, potential toxic oxidation products (OPs) could be formed. The two reactive sites of the beta blocker molecule do not only influence the kinetics of the oxidation reaction, but also the structures of the formed OPs. The attack of ozone at the double bond of an activated aromatic ring leads to the aromatic ring opening and formation of ketones, aldehydes and/or carboxylic moieties (Criegee 1975). During the reaction causing the formation of two aldehydes (Figure 1-3), a hydrogen peroxide ion (HO_2^-) is cleaved (Mvula and von Sonntag 2003), and HO_2^- can be a precursor for OH radical formation (Staehelin and Hoigne 1982) (see R2).

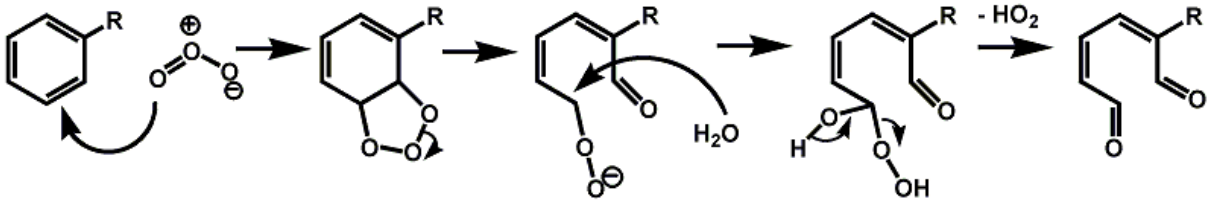


Figure 1-3: Ozonation of an activated aromatic ring following the Criegee mechanism (formation of two aldehyde moieties)

Ozone can also hydroxylate an aromatic ring activated by electron donating subtituents via electrophilic substitution (Figure 1-4). The ortho- and para-position of the electron donor are usually the preferred sites for hydroxylation (Mvula and von Sonntag 2003).

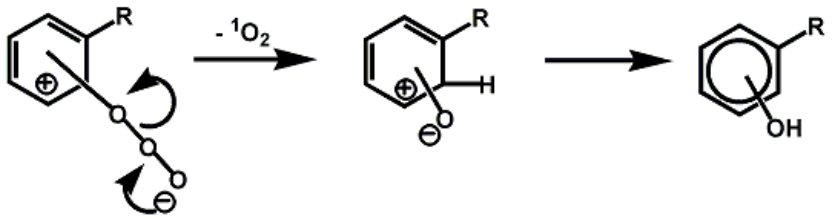
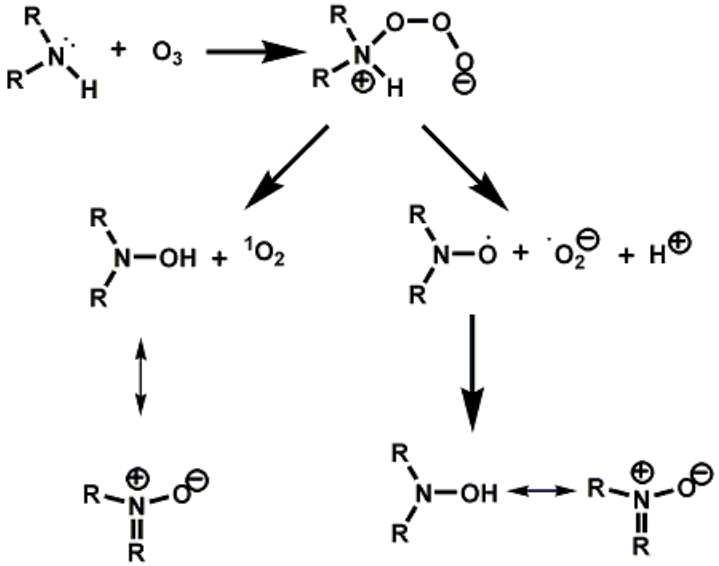


Figure 1-4: Electrophilic substitution of an activated aromatic ring (hydroxylation) by ozone.

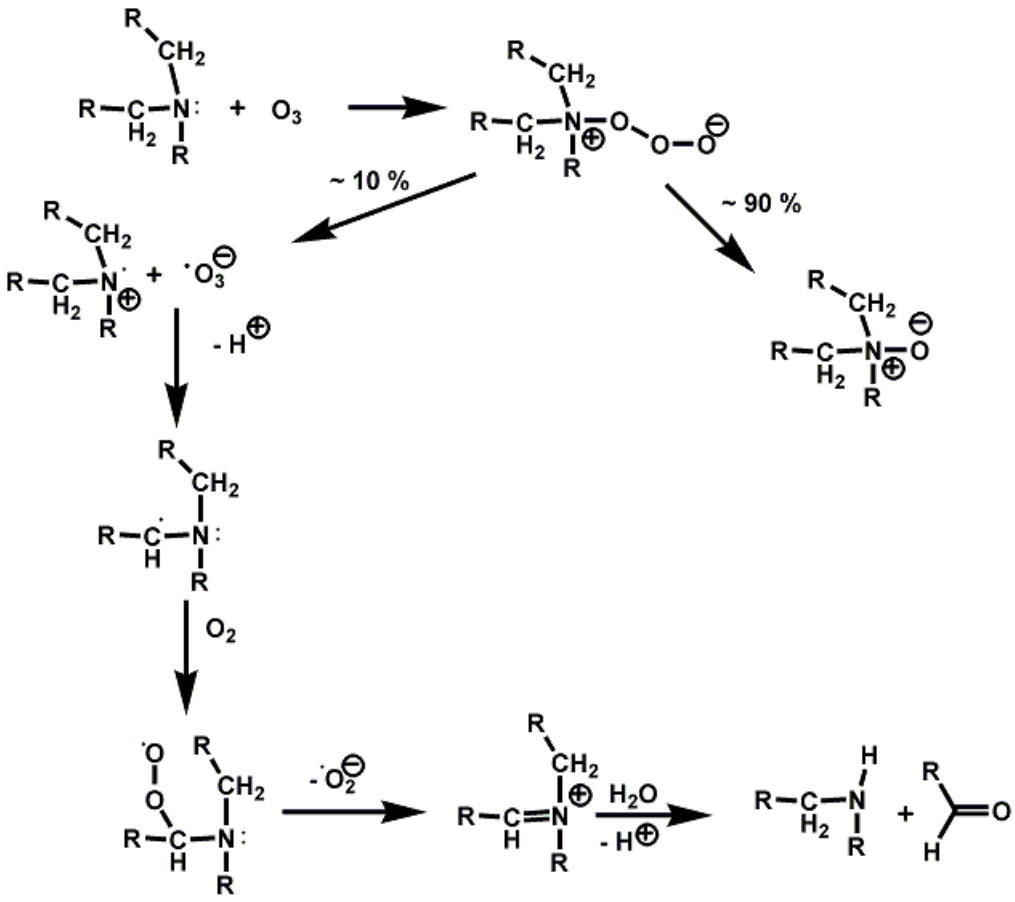
The reactivity of deprotonated amines depend on the degree of substitution. Comparing alkylated amines the following correlation can be stated: the higher the number of substituents, the stronger the electron density and the higher the reactivity (i.e. NH_3 < RNH_2 (primary amines) < R_2NH (secondary amines) < R_3N (tertiary amines)).

In general, ozonation of secondary amines results in the formation of hydroxyamines, either via formation of an amine oxyl radical leading to a superoxide radical or direct formation with a heterolytic cleavage of singulet oxygen (Scheme 1-1).



Scheme 1-1: Ozone reaction of secondary amines via production of singulet oxygen or superoxide radical formation (von Gunten 2003).

In case of tertiary amines about 90 % is transformed in an aminoxide as primary product while 10 % form an amine radical cation (Scheme 1-2). Further reactions with oxygen cause a cleavage of a substituent leading to formation of secondary amines (von Gunten 2003). In this reaction an ozonide radical as well as a superoxide is formed, both precursors for OH radical formation (Chapter 1.3.1).



Scheme 1-2: Ozone reaction of tertiary amines leading to production of a aminoxide (90%) or formation of a secondary amine via superoxide production (von Gunten 2003).

OH radicals react in a more non-selective manner, which makes it difficult to predict the oxidation products. Hydrogen abstraction as primary reaction leads to different kinds of radicals (von Gunten 2003). Further reactions of these radicals caused formation of a variety of OPs.

1.4 Objectives

One important aspect of this study was to investigate whether ozonation is an appropriate process to treat membrane concentrates. The behavior of ozone and the formation of OH radicals in such a concentrate should be determined. As many membrane processes use prechlorination to prevent biofouling of membranes, another task was the monitoring of the impact of chlorination on ozone stability and OH radical an exposure.

Another objective of this study was to investigate the ozonation of beta blockers on a molecular base. Ozone reactions of four selected beta blockers (acebutolol, atenolol, metoprolol and propranolol) were foreseen to be investigated. They can be used as a kind of model substance for other micropollutants with similar reactivity and chemical structures. Determination of the reaction rate constants of ozone and OH radicals was performed and applied to predict the extent of oxidation extent in RO concentrate and WWTP effluent samples. Another objective was the formation of oxidation products of propranolol and metoprolol. With the elucidation of chemical structures it is foreseen to propose oxidation pathways for most of the OPs. These pathways can help to understand the formation of OPs of structurally similar compounds. A comparison with modeled and measured OH radical exposures can indicate the influence of the different active moieties (aromatic ring, amine) on formation of OH radical precursors. An observation of a formation of specific functional groups which have known toxicological potential might indicate a toxicological relevance of the OPs.

1.5 Outline

The outline of this thesis is as followed:

Chapter 2 reports about the investigation of ozone reaction in reverse osmosis concentrate. Additionally kinetic rate constants of acebutolol, atenolol, metoprolol and propranolol were determined and tested for predicting their behavior in the RO concentrate matrix. Ozone stability and the formation of OH radical were monitored as well as the influence of chlorination.

Chapter 3 describes the formation of oxidation products of metoprolol. In addition to the elucidation of chemical structure of the OPs, the influence of metoprolol on OH radical formation in pure water was measured and then compared to the results from a model.

Chapter 4 deals with the identification of oxidation products of propranolol. Chemical structures of main oxidation products were elucidated via LC tandem MS and MS^3 spectra. Oxidation pathways and OP formation in wastewater matrix were investigated.

Chapter 5 discusses the results obtained and gives some general conclusions. In addition, an outlook on the on-going studies as well as suggestions on future research in this area is presented.

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2 Ozonation of reverse osmosis concentrate: kinetics and efficiency of beta blocker oxidation

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*Water Research **2008**, 42, 3003-3012.*

Abstract

Reverse osmosis (RO) concentrate samples were obtained from a RO-membrane system which uses effluents of wastewater treatment plants (WWTP) as a feed water for the production of drinking water. A number of different pharmaceuticals (e.g. antibiotics, contrast media, beta blockers) were found in the WWTP effluent as well as in the RO-concentrate. Overall a concentration factor (feed:concentrate) of approximately 3-4 was measured. Beta blockers (acebutolol, atenolol, bisoprolol, celiprolol, metoprolol, propranolol, timolol) were found in the range of low ng/L to low µg/L. Because metoprolol and propranolol are classified as potentially toxic to aquatic organisms and all beta blocker molecules have moieties, which are reactive towards ozone (amine groups, activated aromatic rings), it was tested whether ozonation can be applied for their mitigation. Rate constants for the reaction of acebutolol, atenolol, metoprolol and propranolol with ozone and $^{\bullet}OH$ radicals were determined. At pH 7 acebutolol, atenolol and metoprolol react with ozone with an apparent second order rate constant (k_{O3}) of about 2000 M^{-1} s^{-1} whereas propranolol reacts with ~1.0 10^5 M^{-1} s^{-1}. The rate constants for the reaction of the selected compounds with $^{\bullet}OH$ radicals were determined to be 0.5-1.0 × 10^{10} M^{-1} s^{-1}. Experiments of RO concentrate showed that an ozone dose of only 5 mg/L resulted in a quantitative removal of propranolol in 0.8 s and 10 mg O_3/L oxidized 70 % of metoprolol in only 1.2 s.

Tests with chlorinated and non-chlorinated WWTP effluent showed an increase of ozone stability but a decrease of hydroxyl radical exposure in the samples after chlorination. This

may shift the oxidation processes towards direct ozone reactions and favors the degradation of compounds with high k_{O3}.

2.1 Introduction

Reverse osmosis (RO) is one of the most effective means for removing a wide range of micropollutants during water recycling. However, one significant disadvantage is the need to dispose the resultant RO-concentrate (brine) (Van der Bruggen et al. 2003). Due to the fact that there are elevated concentrations of micropollutants in the concentrate, a direct disposal to the environment could be hazardous to aquatic organisms. As a consequence, further treatment of the concentrate could be necessary. The site investigated in this study is producing infiltration water for recharge in a dune water catchment. The source for this infiltration water is an effluent from a municipal wastewater treatment plant (WWTP) with 80,000 population equivalent. Prior to infiltration, the WWTP effluent is treated in an advanced treatment step by ultrafiltration and reverse osmosis (Figure 1). The concentrate (brine) resulting from the reverse osmosis treatment is mixed with the water from the backwash-cycles of the ultrafiltration and then discharged into coastal surface water. In the past the WWTP effluent was directly discharged in the receiving water. Even though today the load is essentially the same as what was discharged without water re-use, the concentrations at the effluent stream are significantly higher and might, be ecotoxicologically harmful. As a consequence, further treatment of the concentrate could be necessary.

In this study, ozonation is being investigated as a potential treatment option for RO-concentrates. Several studies (Andreozzi et al. 2005, Huber et al. 2003, Huber et al. 2005, Ikehata et al. 2006, McDowell et al. 2005, Ternes et al. 2003, Vieno, N. M. et al. 2007) have shown that ozone treatment can be very efficient in the oxidation of a wide range of micropollutants (e.g. beta blockers, antibiotics, estrogens). The estrogenicity (Huber et al.

2004) as well as the antibiotic effects (Dodd et al. 2006) are significantly reduced during ozonation. Another crucial class of pharmaceuticals are beta blocker, which are permanently discharged by municipal WWTP (Bendz et al. 2005, Calamari et al. 2003, Gros et al. 2006, Ternes et al. 2003, Vieno, N. M. et al. 2006). Several studies focusing on the toxicological potential of beta blockers show, that they could be of environmental relevance. A 4-weeks exposure experiment by Huggett et al. (2002) showed that a concentration of 500 ng/L propranolol has an effect on the reproduction and steroid levels in medaka (*Oryzias latipes*) Cleuvers et al. (2005) measured EC_{50} values in algae tests (*desmodesmus subspicatus*) of < 1 mg/L for propranolol and 7.9 mg/L for metoprolol. Tests with mixtures of beta blockers indicated additive effects, which means that even a low concentration of propranolol might contribute to the overall toxic potential of the sum of compounds in an aquatic environment. This is of even more concern if the concentration levels of beta blockers in the aquatic environment might increase, for instance due to demographic reasons with an increasing percentage of the older people as expected for Germany in the next decades (Cleuvers 2005, Dzialowski et al. 2006, Escher et al. 2006, Fraysse and Garric 2005, Hernando et al. 2006, Owen et al. 2007).

Ozonation of effluent containing beta blockers could be an efficient tool to decrease the input of these compounds to the environment. One of the main objectives of this study was to investigate whether ozonation can be applied to treat brine. Since second order rate constants for the reaction of ozone and $^{\bullet}OH$ radicals with beta blockers are not known, they were determined in this study.

Furthermore, the efficiency of beta blocker oxidation in the brine was investigated. In addition, the stability of ozone and its exposure in the WWTP effluent and the concentrate were compared and used to predict beta blocker elimination.

2.2 Experimental Methods

2.2.1 Reverse osmosis concentrate: Analysis of Pharmaceuticals

RO-concentrate (brine) samples were obtained from a RO-membrane system which uses effluent of a WWTP as feed water (Figure 2-1). All samples were filtered (0.45-µm cellulose nitrate) upon arrival and stored at 4 °C until use. Each sample was analysed for selected antibiotics, antiphlogistic drugs, antiepileptic drugs, iodinated contrast media and beta blockers. The analytical methods are based on solid phase extraction (SPE) prior to liquid chromatography tandem mass spectrometry (LC tandem MS) detection as described elsewhere (Ternes 2001).

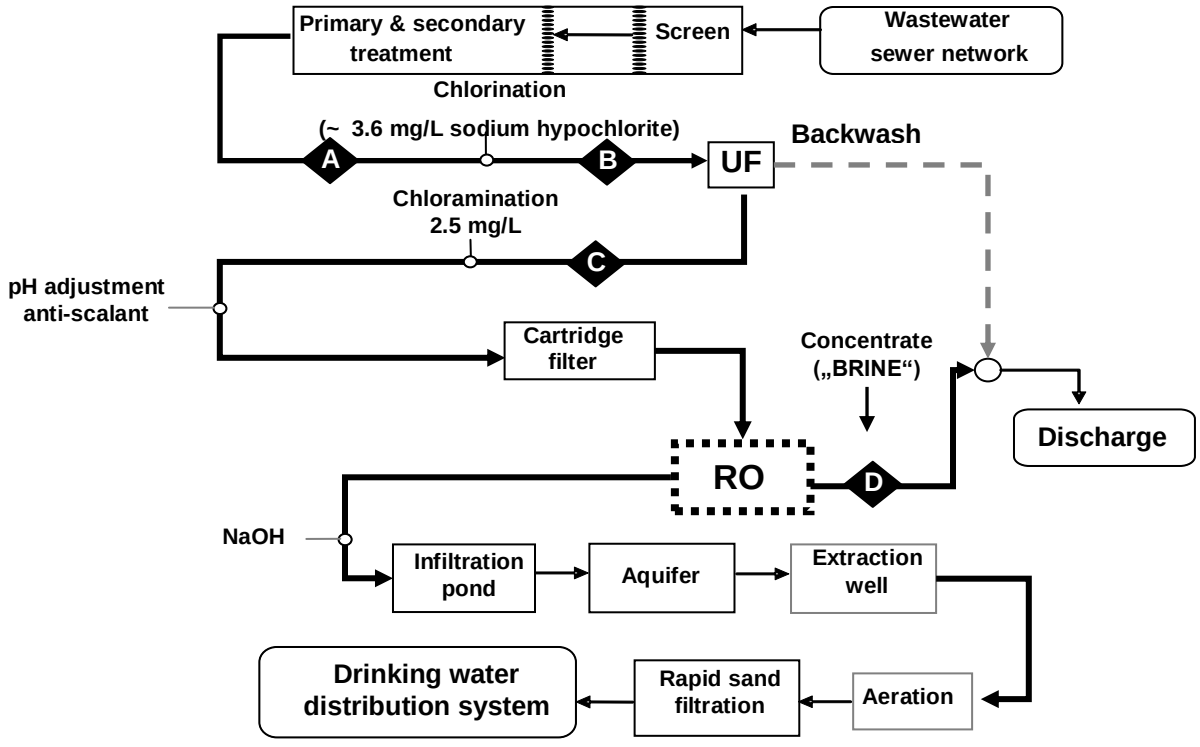


Figure 2-1: Scheme of indirect wastewater reuse for drinking water production; A-D: sampling points.

2.2.2 Determination of Rate Constants

The aqueous ozone stock solution (0.7-1.6 mM) was prepared by sparging ozone containing oxygen through an ice-bath cooled Milli-Q water (Bader and Hoigne 1981). The concentration of the ozone stock solution was measured directly by a UV spectrometer at 258 nm using $\varepsilon_{(O3)}$=3000 $M^{-1}cm^{-1}$. Rate constants for the reaction of ozone with the selected beta blockers were measured with different methods based on their reactivity. A detailed description of the kinetic methods was given previously (Dodd et al. 2006).

Briefly, rate constants for slowly-reacting compounds were determined at room temperature (20-22 ^{o}C) under pseudo-first-order conditions with an excess of O_3 ($[O_3]_0$:[substrate]$_0 \geq 10$) by measuring the reduction of the target concentration via HPLC with UV-detection. Glass bottles (250-mL) with a dispenser system were used as reaction vessels (Hoigne and Bader 1994). By adding ozone stock solution (end concentration ~10 µM) to the solution containing the compound to be investigated (~1 µM) and *tert*-butanol (~20 mM) (*t*-BuOH) as radical scavenger, the kinetic runs were started. After ~15 s (exact time recorded), the first sample (2 mL) was withdrawn with the dispenser system. Subsequently, sampling was performed in 5 to 15 s intervals, depending on the range of the rate constants being measured. The residual ozone was quenched immediately by dispensing into a vial containing an indigo solution. This enables to monitor the ozone decay during the experiment. The data were evaluated by plotting beta blocker concentrations versus ozone exposure ($\int [O_3] \cdot dt$). The slope of the resulting straight line represented the second order rate constant.

For higher rate constants (> 2000 $M^{-1}s^{-1}$) a competition kinetics method was applied using cinnamic acid ($k_{neutral,O3}$ = 5 x 10^4 $M^{-1}s^{-1}$ and $k_{anion,O3}$ = 3.8 x 10^5 $M^{-1}s^{-1}$ (Leitzke et al. 2001), pK_a = 4.4 (Lide 2001)) as competitor substrate. In general, the experiments were carried out at room temperature (20-22 ^{o}C) in 50-mL vials at a pH ranging from 7.5-8.5 (for propranolol pH

3-8.5, 10-50 mM phosphate buffer) with solutions containing equal concentrations of beta blocker and reference compound as well as *t*-BuOH (~20 mM). Different understoichiometric concentration levels of ozone were added with a glass syringe to a series of vials. During ozone injection the solutions were vigorously stirred. Remaining concentrations of target and reference compound were then analyzed by HPLC/UV. Based on eq 1 the data for each ozone dose was evaluated where $k_{O3}(R)$ and $k_{O3}(M)$ are the rate constants for the reference (R) and target compound (M), respectively.

$$\ln\left(\frac{[M]}{[M(0)]}\right) = \ln\left(\frac{[R]}{[R(0)]}\right)\frac{k_{O_3}(M)}{k_{O_3}(R)} \qquad (1)$$

$^{\bullet}$OH radical rate constants were determined by competition kinetics as well. Hydroxyl radicals were generated by in situ UV-photolysis (low pressure mercury lamp) of H_2O_2 (~2 µM) in solutions containing the competitor substrate para-chlorobenzoic acid (~ 1 µM pCBA, with $k_{app}{}^{\bullet}{}_{OH} = 5.0 \times 10^9\ M^{-1}s^{-1}$ (Buxton et al. 1988)) and the target compounds (~1µM). The rate constants were determined by accounting for direct photolysis of 4% for acebutolol, 3 % for atenolol, 0 % for metoprolol and 8 % for propranolol after 30 min irradiation. Further details about the irradiation equipment are given elsewhere (Canonica et al. 1995).

2.2.3 HPLC/UV and LC-tandem MS detection

All HPLC/UV analyses were performed using an Agilent 1100 HPLC system equipped with a variable wavelength UV diode-array detector. Separations were performed on a C 18 column (Macherey-Nagel CC125/4 Nucleosil 100-5) with acetonitrile and 0.05 M H_3PO_4 (adjusted with NaOH to pH 2.2) as mobile phase using gradient methods as required. UV detection was performed at a wavelength of 220 nm for the beta blockers, 256 nm for cinnamic acid and 243 nm for pCBA (LOQ in matrix: beta blockers =0.4 µM; pCBA= 0.2 µM; cinnamic acid was

not determined). The analysis of the samples from the competition kinetic measurements of metoprolol at pH 8.5 and 9 were done via LC- tandem MS using ammoniumacetate buffer (20mM) and acetonitrile as eluents. Metoprolol was detected in a positive ESI-mode (MRM transition 268.1/116.0) and cinnamic acid in a negative mode (MRM transition 146.8/102.9).

2.2.4 Ozonation experiments of matrix samples with a Continuous Quench Flow system (CQFS)

In all samples, ozone stability, $^{\bullet}$OH radical and ozone exposures ($\int [Ox] \cdot dt$) as well as the depletion of two beta blockers (metoprolol and propranolol) were investigated at room temperature (20-22 ^{o}C).

The CQFS-setup was previously described (Buffle et al. 2006). The quenching reagent was indigo blue. Residual ozone was continuously measured by the decrease of the blue colour in a flow-trough cell (1 cm pathlength) in a UV spectrometer at 600 nm (ε=20000 M^{-1} cm^{-1}) based on the indigo method (Bader and Hoigne 1981).

Table 2-1: CQFS-parameters

Loop	Loop size[µL]	Flow rate: 360 mL/h Reaction time [s]	Flow rate: 660 mL/h Reaction time [s]
1	15.6 ± 0.4	0.156 ± 0.004	0.085 ± 0.002
2	36± 1	0.36 ± 0.01	0.194 ± 0.007
3	45.9 ± 0.1	0.459 ± 0.004	0.250 ± 0.002
4	63.3 ± 0.1	0.633 ± 0.004	0.345 ± 0.005
5	101.3 ± 0.1	1.013 ± 0.009	0.553 ± 0.003
6	148.3 ± 0.5	1.483 ± 0.005	0.809 ± 0.002
7	193.8 ± 0.4	1.938 ± 0.004	1.057 ± 0.001
8	242 ± 2	2.416 ± 0.002	1.318 ± 0.002

The CQFS-parameters, such as loop sizes, flow rates and the resulting time resolution of the system are shown in Table 2-1. For the ozonation of the samples, an ozone stock solution was directly withdrawn into a piston pump from the ice-cooled vessel where the ozone containing oxygen was constantly bubbled through. To conserve the ozone in the solution for the time period of the experiment the reservoirs of the pump were cooled with ice packs and the solution was acidified with HCl to pH 3. This resulted in pH change of the mixture of ≤ 0.5, which could not be avoided as addition of any kind of buffer led to precipitations in the RO concentrate. Because the concentration of the ozone solution did not exceed 1.6 mM and the dilution of the sample had to be minimized, ozone doses of only 5(10) mg/L were used. This resulted in a ratio of sample:ozone-solution of 10:1 (5:1).

In contrast to the procedure applied by Buffle et al. (2006) single runs were done per loop. The samples for the HPLC analysis were taken after the flow-trough UV-measurement remained constant for at least 0.5 min (dead volume of cell). The $^{\bullet}OH$ radical exposure was back-calculated using the oxidation of pCBA analyzed with HPLC/UV (Elovitz and von Gunten 1999).

To monitor their depletion, metoprolol and propranolol were spiked into the samples (1-8 µM). The spiked concentrations were low enough to avoid significant changes in ozone half-lives or $^{\bullet}OH$ radical scavenging- capacities of the investigated waters. The ozone decrease did not vary significantly between the spiked and non-spiked samples.

2.2.5 Analysis of bromate formation

Bromate was determined according to a slightly modified method developed by Salhi and von Gunten (1999). Due to the complex matrix present in the concentrate, samples had to be diluted and the injection volumes had to be decreased, which led to a higher quantification limit of 5 µg/L.

2.3 Results and Discussion

2.3.1 Analysis of RO-concentrate

Several pharmaceuticals could be detected in the effluent of the conventional wastewater treatment plant, which indicates, that for the production of drinking water a more advanced treatment such as e.g. the reverse osmosis is necessary (Table 2-2). As all investigated pharmaceuticals were found to be below the limit of quantification (LOQ = 2-25 ng/L) in the reverse osmosis permeate (data not shown here), this technology proved to be a very efficient tool to remove micropollutants from the water phase.

The reverse osmosis concentrate is diluted with the backwash water from the ultrafiltration prior to disposal. As this results in higher volumes to be treated, the ozonation of the pure concentrate is more economic. Therefore, this study focuses on the pure concentrate which was analysed for different micropollutants (Table 2-2).

Table 2-2: Concentrations (in µg/L) of selected pharmaceuticals in WWTP effluent and the reverse osmosis concentrate

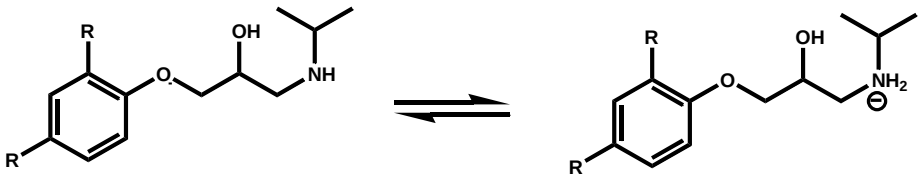
compound	WWTP effluent	RO-concentrate	concentration-factor
antiepileptic drug			
Carbamazepine	1.2 ± 0.1	3.4 ± 0.2	2.8
antibiotics			
Sulfamethoxazole	0.4 ± 0.2	1.19 ± 0.050	3.0
Trimethoprim	0.22 ± 0.02	0.6 ± 0.1	2.7
Clarithromycin	0.32 ± 0.02	0.8 ± 0.2	2.5
contrast media			
Iomeprol	0.9 ± 0.2	3.9 ± 0.5	4.3
Iopromide	1.38 ± 0.07	7 ± 1	5.1
antiphlogitics			
Ibuprofen	0.25 ± 0.01	1.33 ± 0.07	5.3
Diclofenac	0.53 ± 0.09	1.5 ± 0.1	2.8
lipid regulator			
Naproxen	0.36 ± 0.04	0.98 ± 0.06	2.7
beta blockers			
Acebutolol	0.23 ± 0.03	0.76 ± 0.03	3.3
Atenolol	1.7 ± 0.2	2.9 ± 0.3	1.7
Bisoprolol	0.24 ± 0.01	0.94 ± 0.06	3.9
Celiprolol	1.2 ± 0.1	1.8 ± 0.6	1.5
Metoprolol	0.25 ± 0.02	0.88 ± 0.03	3.5
Propranolol	0.36 ± 0.01	1.05 ± 0.02	2.9
Timolol	0.0083 ±0.0003	0.018 ± 0.001	2.1
DOC	12.000	46.000	3.8

The comparison of the concentration of the different pharmaceuticals showed an average concentration factor of 3-4, which is also represented in the DOC values of the measured WWTP effluent and RO concentrate. However, there are significant deviations for some pharmaceuticals, which can be explained with variations occurring in the real samples. The samples were taken on the same day, but not corrected for the residence times of the water in the different treatment steps.

2.3.2 Determination of rate constants for the reaction of 4 beta blockers with ozone and •OH radicals.

To predict the behaviour of beta blockers in the investigated water matrixes during ozonation the rate constants for the oxidation of acebutolol, atenolol, metoprolol and propranolol by O_3 (k_{app,O_3}) and •OH radicals ($k_{app,\bullet OH}$) were determined.

Ozone is a highly selective electrophile. All the investigated beta blockers contain two reactive sites for this reaction: an activated aromatic ring and a secondary amine-moiety (Table 3). The reaction of the aromatic structure is independent of the solution pH. In contrast, the protonated amino group does not react with ozone (Hoigne and Bader 1983). Therefore, the reactivity of amines depends strongly on the p*K*a of the amines and the pH of the solution, with the corresponding speciation:



To determine the pH-dependence of second order rate constants, kinetic measurements were performed over a pH range (3-8.5). The observed k_{app,O_3} were used to extrapolate the k_{deprot,O_3}, of the deprotonated species according to eq. 2 and 3 which is illustrated in Figure 2-2.

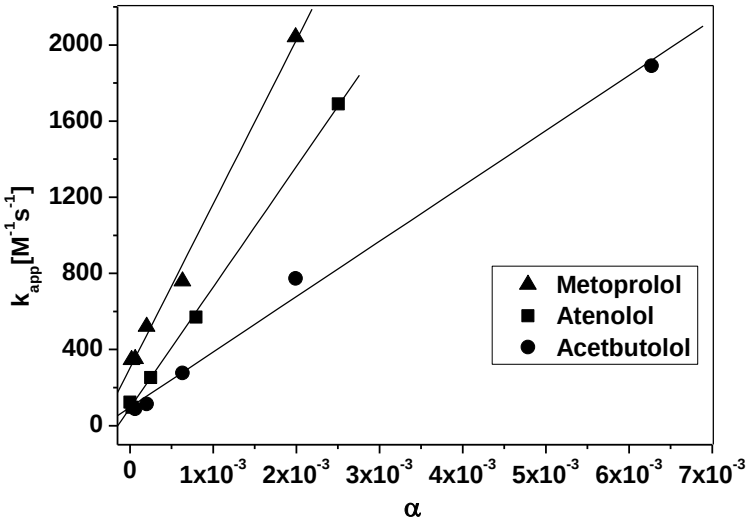


Figure 2-2: Plot of degree of dissociation $k_{app,O3}$ versus (α). Regression used for the calculation of k_{deprot}.

$$k_{app} = \alpha \cdot k_{O_3,deprot} + (1-\alpha) \cdot k_{O_3,prot} \qquad \text{degree of dissociation, } \alpha = \frac{1}{1+\frac{[H^+]}{K}} \tag{2}$$

$$\Rightarrow k_{app} = \alpha \cdot (k_{O_3,deprot} - k_{O_3,prot}) + k_{O_3,prot} \cdot \tag{3}$$

The lines in Figure 2-3 a-c represent the modeled $k_{app, O3}$ as a function of pH for acebutolol, atenolol and metoprolol according to eq. 2. The triangles show the values for experiments with pH > 7.5, which could only be determined by competition kinetics. These values agree quite well with the prediction from the batch experiments, even though the errors for competition kinetics are larger and more difficult to estimate, partly due to the errors induced by the use of reference compounds (Huber et al. 2003).

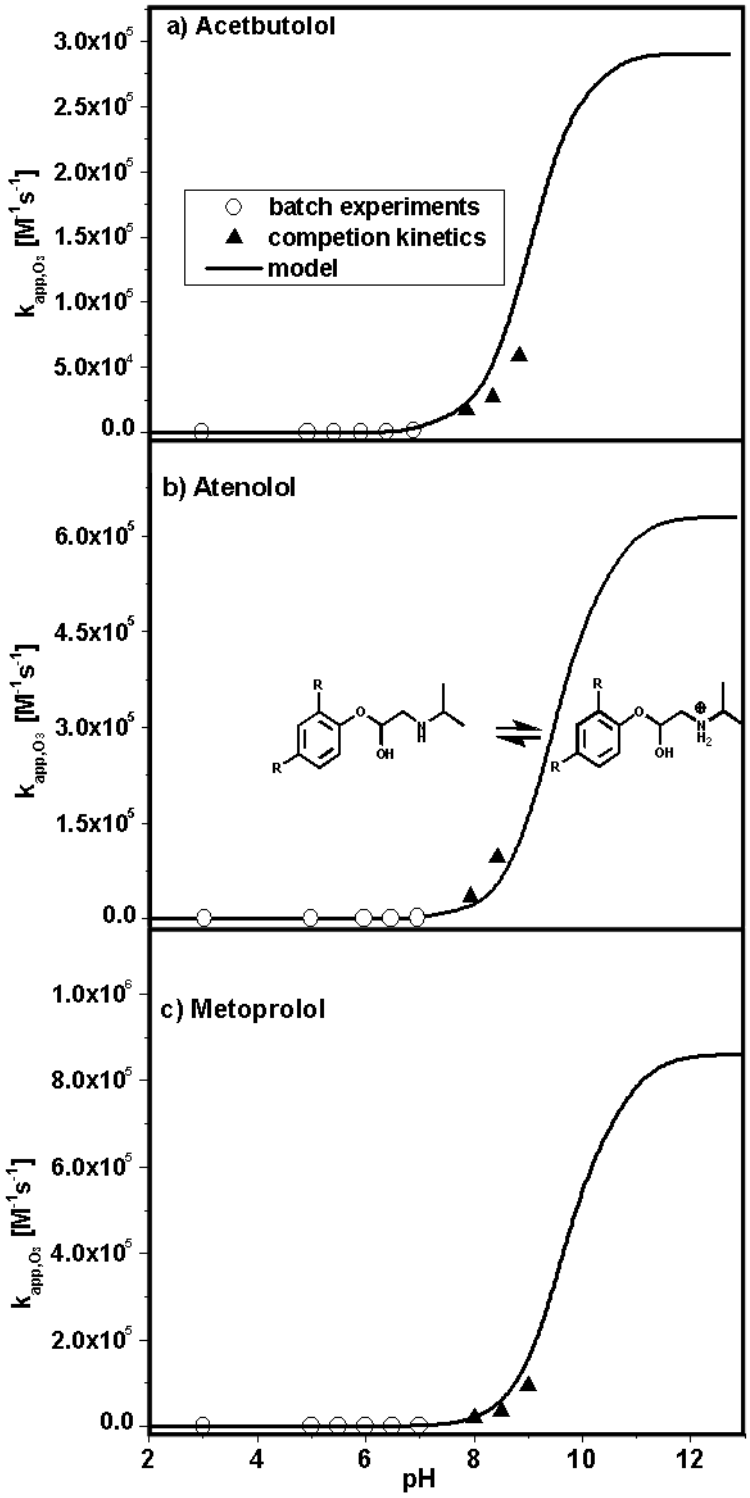


Figure 2-3: pH dependence of k_{app,O_3} of acebutolol (a), atenolol (b) and metoprolol (c). Circles: batch experiment, triangles: competition experiment, lines: model calculations.

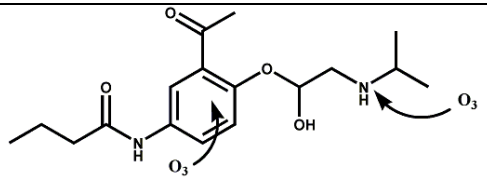
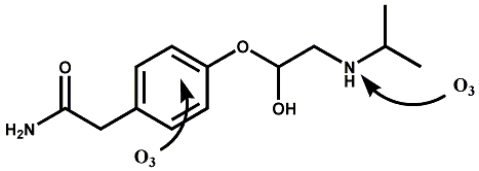
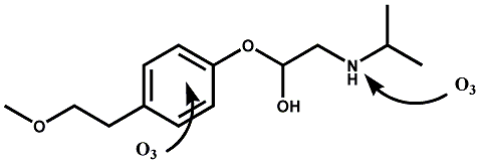
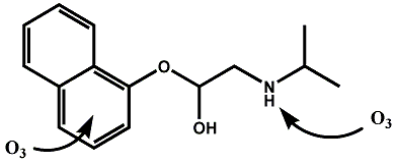
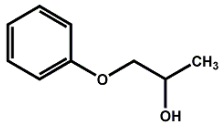
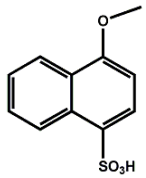
Consequently only the values from the batch experiments were used to calculate the deprotonated rate constant even though the extrapolation of values several orders of

magnitude lower than the calculated values will magnify even these very small errors. These extrapolated values are also shown in Table 2-3.

All kinetic experiments with propranolol could only be performed by competition kinetics and an average rate constant for propranolol of $(1.0 \pm 0.2) \cdot 10^5$ M^{-1} s^{-1} was found. In the measured range of pH 3- 7.5 no significant increase in the apparent rate constants could be detected. It seems, that the baseline-reactivity of the protonated propranolol species is already so high, that the accuracy of the method was not sufficient to observe this effect.

Table 3 lists the obtained ozone and hydroxyl radical rate constants for the protonated and deprotonated species and the rate constants at pH 7, respectively. For the ozonation reaction the values for metoprolol, atenolol and acebutolol at pH 7 are all in the same range. Only propranolol is reacting about 2 orders of magnitude faster than the other compounds. The major difference of its structure lies in the naphthalene moiety while the other three beta blockers contain a phenyl group (Table 2-3). In addition, the rate constants for 1-phenoxy-2-propanol and 4-methoxy-1-naphthalene sulfonic acid were measured, modelling the aromatic rings of the different structures. Even though the apparent rate constant for the substance with the naphthalene-moiety is one order of magnitude higher, it is not as prominent as observed for the beta blockers (2 orders of magnitude). The sulfonic acid group attached to the methoxy-naphthalene might lower the electron-density in the model substrate, but it is unclear whether this effect is strong enough to explain this discrepancy.

Table 2-3: Chemical structures and second-order rate constants for the reaction of ozone or ˙OH radicals with selected beta blockers

compound	Chemical structure	pK_a ($R_2NH_2^+ \rightarrow R_2NH$)	$k_{O_3,prot}$ [M^{-1} s^{-1}]	$k_{O_3,deprot}$ [M^{-1} s^{-1}]*	k_{O_3} [M^{-1} s^{-1}] at pH 7	$k_{\cdot OH}$ [M^{-1} s^{-1}]
Acebutolol		9.2	60	$2.9 \cdot 10^5$	$(1.9 \pm 0.6) \cdot 10^3$	$(4.6 \pm 0.7) \cdot 10^9$
Atenolol		9.6	110	$6.3 \cdot 10^5$	$(1.7 \pm 0.4) \cdot 10^3$	$(8.0 \pm 0.5) \cdot 10^9$
Metoprolol		9.7	330	$8.6 \cdot 10^5$	$(2.0 \pm 0.6) \cdot 10^3$	$(7.3 \pm 0.2) \cdot 10^9$
Propranolol		9.5	$\sim 1 \cdot 10^5$	-	$\sim 1 \cdot 10^5$	$(1.0 \pm 0.2) \cdot 10^{10}$
1-Phenoxy-2-propanol		-	-	-	320 ± 40	-
4-Methoxy-1-Naphthalene sulfonic acid		-	-	-	3600 ± 300	-

* extrapolated values (see Figure 2-2)

The apparent rate constants for the reaction with hydroxyl radicals with the compounds are all in the same order of magnitude. Song et al. (Song et al. 2008) found the same values for the reaction constants for atenolol ($7.05 \pm 0.27 \cdot 10^9$ M^{-1} s^{-1}), metoprolol ($8.4 \pm 0.06 \cdot 10^9$ M^{-1} s^{-1}) and propranolol ($1.07 \pm 0.02 \cdot 10^{10}$ M^{-1} s^{-1}). For acebutolol, atenolol and metoprolol this oxidation pathway will play a more important role than in the case of propranolol, because their k_{O3} are lower.

2.3.3 Ozonation of beta blockers and stability of ozone in the brine matrix

To understand and to predict the behaviour of ozone in the brine matrix, the stability of ozone as well as the $^{\bullet}OH$ radical formation in the RO-concentrate were measured. A continuous quench flow system (CQFS) allowed to monitor the instantaneous loss of ozone in the complex matrix (Buffle et al. 2006). Samples were spiked with propranolol and metoprolol (4-8 µM) and their dissipation was measured by HPLC/UV. As shown in Figure 2-4, propranolol is attenuated in the same time interval as ozone (1.2 s). The very fast dissipation of ozone indicates that for a significant oxidation of compounds reacting much slower than propranolol, higher ozone doses will be required.

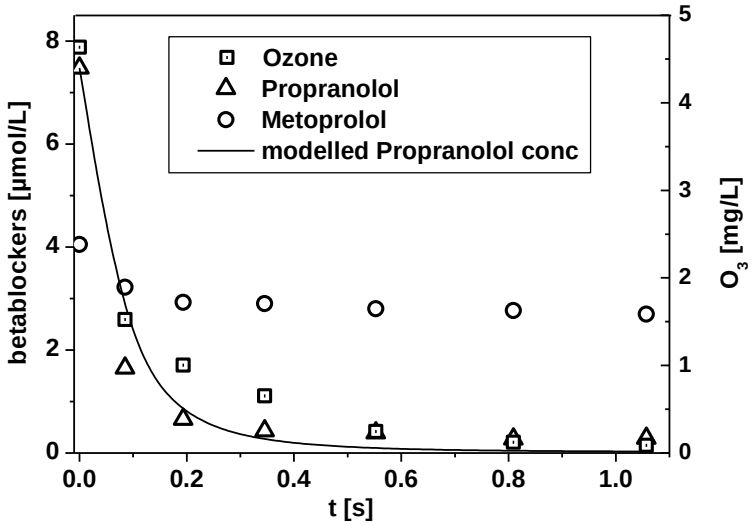


Figure 2-4: CQFS-experiment with RO-concentrate (pH =8) containing propranolol and metoprolol. Ozone dose: 5 mg/L; room temperature. Symbols: measured data, line: model calculations (see eq. 4 modeled for pH 8)

This assumption can be confirmed by the metoprolol behaviour. Its attenuation was incomplete, since it has a lower rate constant for its reaction with ozone. With 10 mg/L ozone metoprolol was oxidized to a much higher extent (Figure 2-5). A batch experiment using the same ozone dose showed that residual ozone was present until 50 s after ozone addition, which can continuously oxidize residual metoprolol.

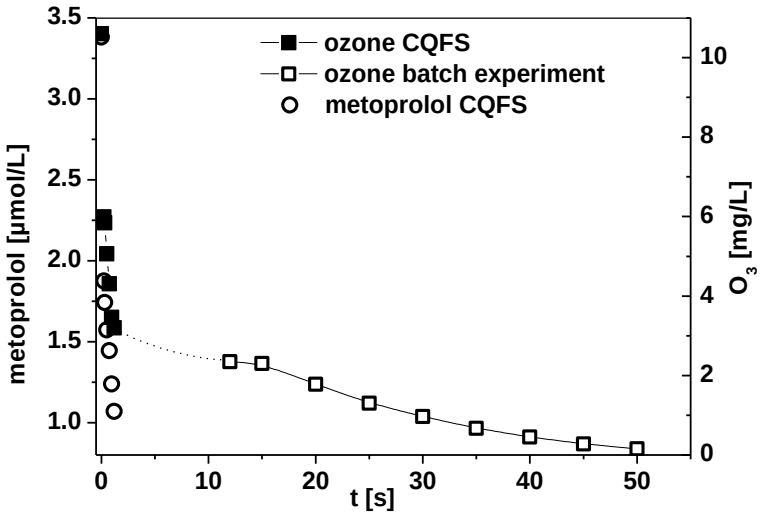


Figure 2-5: Ozonation of RO-concentrate (pH =8) containing metoprolol. Ozone dose: 10 mg/L, room temperature. Residual ozone measured with CQFS (up to 1.2 s) and batch system (up to 50 s).

Due to the higher ozone dose the reaction of propranolol was faster than the time resolution of the CQFS. Therefore only the loss of metoprolol, the beta blocker with the lower reactivity could be monitored for the 10 mg/L ozone dose.

The extent of oxidation of the investigated beta blockers [X] can be predicted according to eq. 4. and the measured values for the ozone exposure, the $^{\bullet}OH$ radical exposure as well as the apparent rate constants k_{O3} and $k_{\bullet OH}$ (Elovitz and von Gunten 1999).

$$[X] = e^{-k_{app,O_3} \cdot \int [O_3] dt - k_{app,^{\bullet}OH} \cdot \int [^{\bullet}OH] dt} \cdot [X]_0 \qquad (4)$$

Figure 2-4 shows the comparison of the calculated and measured values for propranolol. It can be seen that there is a good agreement between the two data sets (data: triangles, model: line).

With the used ozone doses a mineralisation of the compounds is unlikely. There will be a formation of unknown oxidation products which will most probably have lost the former biological activity but their toxicological potential is still not known. Currently an on-going study is dealing with the identification of the structures and toxicity of these products.

2.3.4 Bromate formation during ozonation of brine matrix

Bromate, a potential human carcinogen, is formed during ozonation of samples containing bromide (von Gunten 2003). With a bromide level in the reverse osmosis concentrate sample of about 1200 µg/L there is a potential of elevated bromate concentrations after ozonation. To investigate bromate formation, different ozone doses were applied to a sample spiked with propranolol and metoprolol (1 µM). The resulting bromate concentration and the dissipation of the beta blockers were measured (Figure 2-6.).

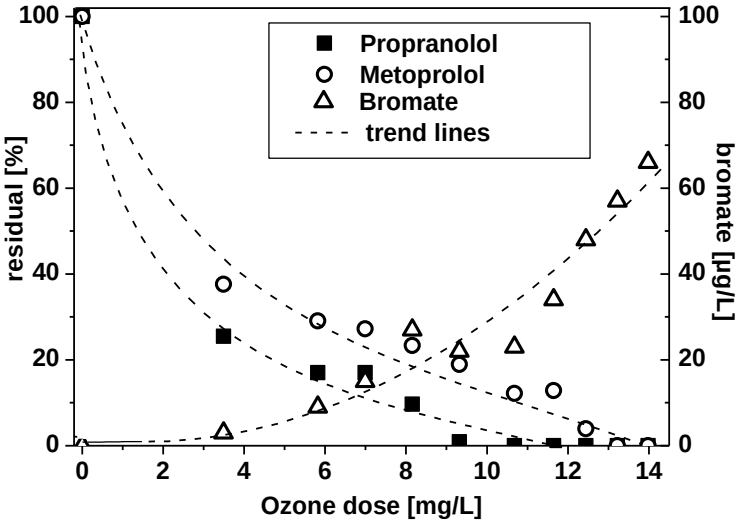


Figure 2-6: Bromate formation and depletion of metoprolol and propranolol in the RO-concentrate (pH=8) as a function of the ozone dose. Room temperature.

The dose needed for a 90 % elimination of propranolol of ~ 8 mg O_3/L (metoprolol ~ 11 mg O_3/L) caused a bromate concentration of ca. 24 µg/L (35 µg/L). These are relatively low bromate concentration for an effluent discharged. Hutchinson et al. (1997) propose not to exceed the bromate concentration beyond 3.0 mg/L for the protection of aquatic organisms based on long term adverse effects. However, data on long term and chronic influence of bromate to the aquatic ecosystems is still very limited and further studies are needed.

2.3.5 Comparison of ozone stability from different sampling points

The stability of ozone in different sampling points in the reuse process (see Figure 2-1) was investigated to evaluate potential alternative points of ozone application. Samples were taken prior to (A) and after (B) the addition of chlorine, as well as after the ultrafiltration (C) (for sampling points see Figure 2-1). To be able to compare the ozone stability in the effluent and the RO-concentrate, it was diluted down to a comparable DOC level. As the carbonate content also plays an important role for the ozone stability, Na_2CO_3 was added to obtain a similar alkalinity while maintaining the pH at 8 (von Gunten 2003). Figure 7 shows the residual ozone (ozone dose: 2 mg/L) in the different samples, measured with the CQFS. A significant increase of the ozone stability after chlorine addition (B) was found (Figure 2-7).

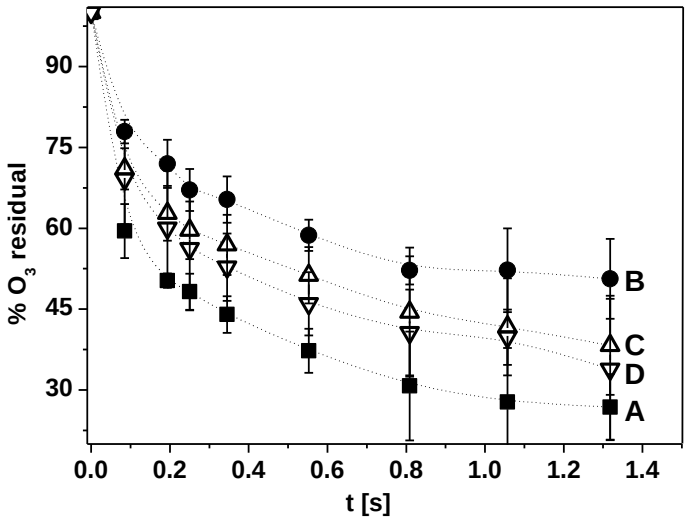


Figure 2-7: Ozone stability in water from different sampling points according to Figure 1 (pH 8, room temperature, measured with CQFS). A: WWTP effluent before chlorination; B: WWTP effluent after chlorination; C: Ultrafiltrate; D: diluted concentrate; transferred ozone dose: 2 mg/L.

A reason for the increase of the ozone stability could be the reaction of chlorine with amine moieties of the organic matrix. This would lead to a formation of chloramines, which are no longer susceptible for a direct reaction with ozone (Hoigne and Bader 1983). The ultrafiltration (C) and the diluted concentrate sample (D) should both still contain the formed chloramines, as they are obtained from later sampling points in the process and their DOC

level does not change. However, the measured ozone stability in these samples exhibited that this effect seems to be partially reversible, because their ozone stability lies between the chlorinated and the non-chlorinated sample. This is probably due to a partial back reaction of chloramines to amines with a recovery of ozone reactivity.

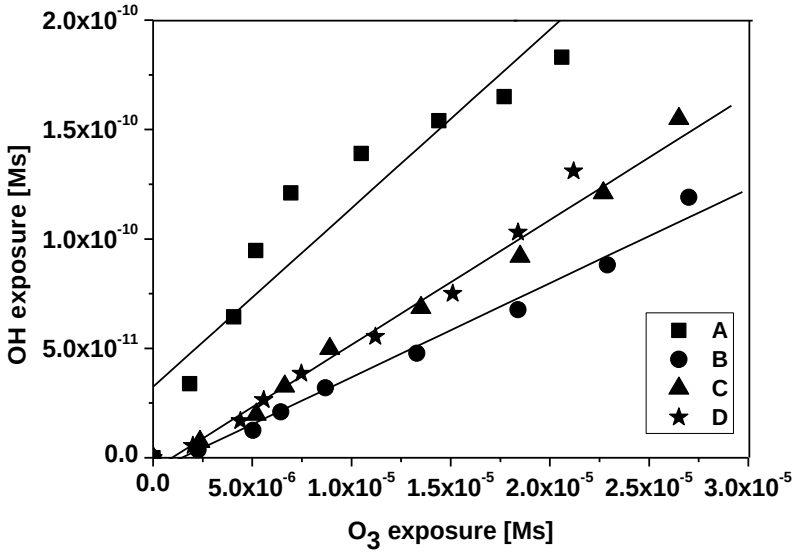


Figure 2-8: OH exposure vs. O_3 exposure of ozonated water collected at different sampling points according to Figure 2-7. A: WWTP effluent before chlorination; B: WWTP effluent after chlorination; C: Ultrafiltrate; D: diluted RO concentrate; transferred ozone dose: 2 mg/L.

Figure 2-8 shows a plot of the OH radical exposure as a function of the ozone exposure for the 4 different waters discussed in Figure 2-7. It can be seen that the non-chlorinated sample exhibits a much higher OH exposure for a given ozone exposure. This is again an indication for the removal of the OH radical forming moieties such as amines by chlorination. Buffle et al. (2006) hypothesized that amine groups are responsible for a high $^{\bullet}$OH-radical generation during ozonation. The decrease of $^{\bullet}$OH formation in the chlorinated water could therefore confirm the hypothesis of a chloramine formation. For compounds being oxidized predominantly by $^{\bullet}$OH, this could lead to a decrease in their removal efficiency.

Table 4 shows extrapolated •OH radical exposures values for an ozone exposure of 10 µMs, taken from Figure 2-8. These values were used to calculate the expected residual concentration of propranolol with eq. 4. The measured and calculated relative elimination for the different sampling points are in the same range (Table 2-4). With eq. 5 the fraction of propranolol reacting with •OH radical and ozone can be calculated for the different matrixes:

$$f_{\bullet OH} = \frac{k_{\bullet OH} \cdot \int [OH]\, dt}{k_{O_3} \cdot \int [O_3]\, dt + k_{\bullet OH} \cdot \int [OH]\, dt} \quad ; \quad f_{O_3} = 1 - f_{\bullet OH} \tag{5}$$

$f_{\bullet OH}$ and f_{O_3} for propranolol are shown in Table 4 for water from sampling points A-D according to Figure 2-8. The comparison shows, that before chlorination a 2.5 times higher fraction will be oxidized by •OH radicals than in the chlorinated samples. This is again an indication for the reduced OH radical formation after pre-chlorination. However, the values of the residual propranolol (calculated and measured) show that the impact of this on propranolol is nearly negligible.

Table 2-4: Measured and calculated degradation of propranolol and fraction reacting with hydroxyl radicals and ozone for an ozone exposure of 1 x 10 $^{-5}$ [Ms] (Figure 2-8)

Sampling point	Residual propranolol [%] calculated (eq 4.)	Residual propranolol [%] measured	•OH exposure [Ms]	$f_{\bullet OH,propra}$	$f_{O_3,propra}$
WWTP effluent before chlorination (A)	3.1	11.8	$1.1 \cdot 10^{-10}$	0.40	0.60
WWTP effluent after chlorination (B)	4.3	8.9	$3.7 \cdot 10^{-11}$	0.15	0.85
Ultrafiltrate (C)	5.8	6.9	$5.2 \cdot 10^{-11}$	0.19	0.81
Diluted concentrate (D)	6.1	11.0	$5.2 \cdot 10^{-11}$	0.20	0.80

2.4 Conclusions

Second order rate constants for the reaction of four beta blockers (acebutolol, atenolol, metoprolol and propranolol) with ozone and OH radicals were measured. The rate constants for the reaction of the selected beta blockers with ozone showed, that the naphthalene-moiety of propranolol causes an increase of the reactivity of about two orders of magnitude. Although ozone has a lower stability in samples with elevated DOC concentration (46 mg/L) of the RO brine, the moderate ozone doses applied (5-10 mg/L) in our experiments were sufficient to remove beta blockers efficiently. Therefore, ozonation of WWTP effluents and brines is a very efficient tool to decrease the discharge of beta blockers in the aquatic environment.

Even though chlorination during the process does not attenuate the DOC, it increases the ozone stability in the selected samples. As this extends the life time of the ozone it leads to a more efficient oxidation of micropollutants with high rate constants for the reaction with ozone. Furthermore, also disinfection is enhanced, because mainly direct ozone reactions are responsible for the inactivation of microorganisms. However, it was shown that the $^{\bullet}OH$ radical exposure for a given ozone exposure is decreased, which could lead to a decrease of the oxidation of compounds with low rate constants for the direct reaction with ozone.

The comparison of effluent and diluted brine indicated, that ozone behaves similar apart from the influence of the pre-chlorination. Considering the high bromide concentration in the brine, bromate formation for a 90% elimination of the investigated beta blockers is only moderate (< 40 µg/L). This is far below the ecotoxicological threshold value of 3 mg/L which was previously suggested.

2.5 Acknowledgment

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3 Ozonation of Metoprolol: Elucidation of Oxidation Pathways and Major Oxidation Products

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submitted to Environmental Science and Technology

Abstract

Oxidation products (OPs) formed during ozonation of metoprolol were identified via liquid chromatography and hybride Qq-LIT-MS. Experiments carried out at pH 3 and 8 showed the formation of different OPs, depending on pH. The analysis of samples with and without tertiary butanol (*t*-BuOH) revealed the influence of OH radical reactions. The OH radical exposure was measured by adding a probe compound (*para*-chlorobenzoic acid, pCBA). Elucidation of chemical structures confirmed the formation of aldehyde moieties as well as the occurrence of hydroxylation reactions. Several reaction pathways for the formation of the oxidation products are proposed. Analysis of ozonated raw wastewater and the effluent of a municipal wastewater treatment plant spiked with 10 μM metoprolol exhibited a similar OP formation pattern as detected in the reaction system at pH8 without a radical scavenger. This indicates a significant impact of OH radical exposure on the formation of OPs in real wastewater matrices.

3.1 Introduction

Metoprolol, as a β_1-selective beta blocker, is administered to treat hypertension, tachycardia, and heart failure, and is a highly prescribed pharmaceutical. In 2004, approximately 98.1 t of metoprolol was prescribed in Germany (Lemmer 2006). About 10 % of the applied metoprolol dose is excreted unchanged, and hence it is present in wastewater treatment plant (WWTP) influents at concentrations of 0.6-1.4 µg/L (Bendz et al. 2005, Gros et al. 2006, Ternes et al. 2003, Vieno et al. 2007a, Vieno et al. 2007b)

Several studies focusing on the toxicological potential of metoprolol indicate its potential environmental relevance (Cleuvers 2005, Dzialowski et al. 2006, Escher et al. 2006, Fraysse and Garric 2005, Hernando et al. 2006, Owen et al. 2007). This might be even more of a concern if the concentrations of beta blockers increase in the aquatic environment, for instance due to demographic reasons with an increasing percentage of older people as expected for Germany in the next decades (Nicholas and Smith 2006).

A previous study has shown that ozone treatment is very efficient in the oxidation of metoprolol, and an effective wastewater treatment technology for reducing the discharge of metoprolol into the aquatic environment (Benner et al. 2008).

Direct Oxidation by Ozone. Two moieties of metoprolol (for chemical structures refer to Scheme 3-2) are predominantly reactive toward a direct ozone attack: the activated aromatic ring and the secondary amine. However, as shown before (Benner et al. 2008), the ozone reactivity at these two sites are very different. The amine moiety is only reactive in its deprotonated form (pKa = 9.7, $k_{O3,\ deprot} = 8.6 \cdot 10^5\ M^{-1}\ s^{-1}$) (Benner et al. 2008), and hence, the apparent rate constant is pH dependent (Munoz and von Sonntag 2000). The protonated metoprolol molecule is likely to be attacked by ozone at the aromatic ring with an apparent rate constant of 330 $M^{-1}\ s^{-1}$ (Benner et al. 2008). At pH 3 the amine moiety is completely protonated, and thus, the only reactive site for an ozone attack is the activated aromatic ring. Therefore, the resulting OPs are probably formed by a modification at the ring moiety. The concentration of the deprotonated amine species increases with higher pH, leading to OPs possibly formed by the amine group being attacked. Although with a pKa of 9.7, at pH 8 only 2 % of the metoprolol occurs in the neutral nonprotonated form, the amine reaction is still contributing to the reaction rate (uncharged amine ~ 250 times more reactive).

Indirect Oxidation by OH Radicals. Ozonation involves two different major oxidative species: ozone and OH radicals. One very important reaction leading to OH radicals is the decay of ozone into the superoxide radical ($^{\bullet}O_2^-$). The formation of $^{\bullet}O_2^-$ is catalyzed by

hydroxides (OH^-) resulting in higher OH exposure at higher pH (Buhler et al. 1984) but this reaction contributes only to a minor extent as has a second-order rate constant of 70 M^{-1} s^{-1} (Staehelin and Hoigne. 1982).

However, several researchers have reported a correlation between the ozone reaction of amines and elevated OH radical exposures, indicating that during the oxidation reaction of an amine a precursor for OH radicals is formed ($^{\bullet}O_2^-$ or $^{\bullet}O_3$ (Buffle and Von Gunten 2006, Buhler et al. 1984)). Additionally, several studies report the potential of OH radical formation during ozonation of phenolic compounds (Buffle and Von Gunten 2006, Mvula and von Sonntag 2003).

Economically relevant ozone doses might not mineralize metoprolol, causing the formation of oxidation products (OPs). Liquid chromatography coupled with hybride triple quadrupole with linear ion trap (LC-Qq LIT MS), was used for the elucidation of chemical structures of the OPs. A comparison of the LC-MS data and chemical structures of e-beam and gamma radiolysis products of metoprolol proposed by Slegers et al. (2006) resulted in the confirmation of some chemical structures of the OPs formed.

Ozonation reactions can lead to OPs with toxicological relevant functional groups, e.g., aldehydes or hydroxylated aromatic compounds. Several substances with aldehyde functions (e.g., the α,β-unsaturated carbonyl 4-hydroxynonenal) are known to interact with DNA and exhibit genotoxic and carcinogenic properties (Richardson et al. 2007, Kuchenmeister et al. 1998, Roberts et al. 2003, Eckl et al. 1993). Hydroxylated PCBs, PDBEs, and PAHs were found to have elevated endocrine potentials in comparison to the aromatic compound without hydroxyl substitution (Sumbayev et al. 2008, Hamers et al. 2008, Kester et. al 2002 Kester et al. 2000).

In this study the formation of metoprolol OPs was investigated during ozonation of spiked Milli-Q water at acidic and neutral pH as well as the ozonation of raw and conventionally treated wastewater at the ambient pH.

3.2 Materials and Methods

3.2.1 Sample preparation

An aqueous ozone stock solution (~ 0.7 mM) was prepared by sparging ozone-containing oxygen through ice-bath-cooled deionized water (Bader and Hoigne 1981). The concentration of the ozone stock solution was determined directly by a UV spectrometer at 258 nm using $\varepsilon_{(O3)} = 3000\ M^{-1}cm^{-1}$.

Ozone stock solution was added to the reaction solution containing metoprolol (100 µM), phosphate buffer (50 mM) at pH 3 or 8 and *tert*-butanol (100 mM) (*t*-BuOH) as a radical scavenger, resulting in ozone to compound ratios of 1:5, 1:3, 1:1, 2.5:1, 5:1, and 10:1. For a comparison of OP formation without radical scavenging, samples without *t*-BuOH addition were prepared in parallel as described previously. To determine the formation of OH radicals, *para*-chlorobenzoic acid (pCBA, 2 µM) was added. The samples were analyzed 24h after the addition of ozone to ensure that no more ozone was present.

3.2.2 Chromatography Development

Samples containing the mixture of OPs were separated with an Agilent 1100 HPLC system (Agilent Technologies, USA-Santa Clara) using a Synergi 4u Hydro-RP column, 3 mm i.d., 250 mm, 4 µm (Phenomenex®, Aschaffenburg, Germany) at room temperature. Deionized water (A) and acetonitrile (B) both consisting of 0.5 % aqueous formic acid were used as eluents (refer to table 3-1 for optimized gradient). The UV system was operated at 254 and 280 nm for detection of metoprolol and OPs, and at 240 nm for pCBA quantification (LOQ = 0.2 µM).

Table 3-1:Optimized gradient Used for LC-MS and LC-UV Experiments.

step	time [min]	flow rate [µl/min]	A [%]	B [%]
0	0.00	400	95.0	5.0
1	30.00	650	90.0	10.0
2	40.00	750	80.0	20.0
3	50.00	800	60.0	40.0
4	55.00	400	95.0	5.0

3.2.3 Determination of Molecular Weights and Fragmentation by Mass Spectrometry (MS)

Mass spectrometry was performed using an Applied Biosystems/MDS Sciex 4000 Q Trap® Qq-LIT-MS (Applied Biosystems, Langen, Germany). The system consisted of a hybrid triple quadrupole and linear ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. Nitrogen was used as drying, nebulizing, and collision gas. For the LC-system, the same gradient than describes above was used. To determine the molecular weights,we performed LC-Q1 scans in positive and negative ion mode. The OPs could only be detected with positive ionization, therefore, all following MS experiments were performed only in positive mode.

For structural elucidation, the MS fragmentation pathways of all OPs were studied by performing product ion scans (MS^2), using the linear ion trap of the LC tandem MS. The obtained product ions were incorporated into a LC tandem MS method, using multiple reaction monitoring (MRM) transitions (Table S3-2 and S3-3). For MS^3 spectra, the sample was directly injected into the mass spectrometer at a flow of 10 to 15 µL/min. The excitation energy, for these experiments was optimized for each single OP and varied between 20-100 V, with a collision energy spread of 5 V.

3.2.4 OP formation in Matrix Samples

Raw wastewater (DOC = 65 mg/L, pH 7.7) and conventionally treated municipal wastewater (DOC = 8.5 mg/L, pH 8.4) were spiked with metoprolol (10 µM), and ozone stock solution was added to obtain ozone to compound ratios of 3:1, 6:1, and 12:1. OH radical formation in the matrix was monitored by adding 1 µM *para*-chlorobenzoic acid (pCBA) to the samples. pCBA concentration was analyzed by HPLC-UV as described before.

3.3 Results and Discussion

3.3.1 Oxidation Pathways of Metoprolol

To monitor the influence of OH radical reactions in the oxidation process of metoprolol, we performed experiments in the presence and absence of *t*-BuOH as a radical scavenger. The addition of pCBA, a probe compound only reacting with OH radicals and not with ozone, allowed for OH radical formation to be determined in all experiments (pH 3 scavenged, pH 3 non scavenged, pH 8 scavenged, and pH 8 non scavenged).

Table 3-2 Attenuation of pCBA in Different Experimental Setups[a]

experiment	ozone:metoprolol ratio	attenuation pCBA [%]
pH 3 scavenged	1 : 5	0
	1 : 3	1
	1 : 1	0
	2.5 : 1	0
	5 : 1	1
	10 : 1	5
pH 3 non scavenged	1 : 5	0
	1 : 3	5
	1 : 1	0
	2.5 : 1	1
	5 : 1	24
	10 : 1	59
pH 8 scavenged	1 : 5	0
	1 : 3	1
	1 : 1	4
	2.5 : 1	4
	5 : 1	3
	10 : 1	5
pH 8 non scavenged	1 : 5	4
	1 : 3	5
	1 : 1	18
	2.5 : 1	60
	5 : 1	79
	10 : 1	100
raw waste water	3 : 1	9
	6 : 1	6
	12 : 1	15
WWTP effluent	3 : 1	6
	6 : 1	13
	12 : 1	31

[a] $k_{app,OH,\ pCBA} = 5 \cdot 10^9\ M^{-1}\ s^{-1}$; $k_{app,OH,\ t\text{-}BuOH} = 5.9 \cdot 10^8\ M^{-1}\ s^{-1}$ (Bader and Hoigne 1981

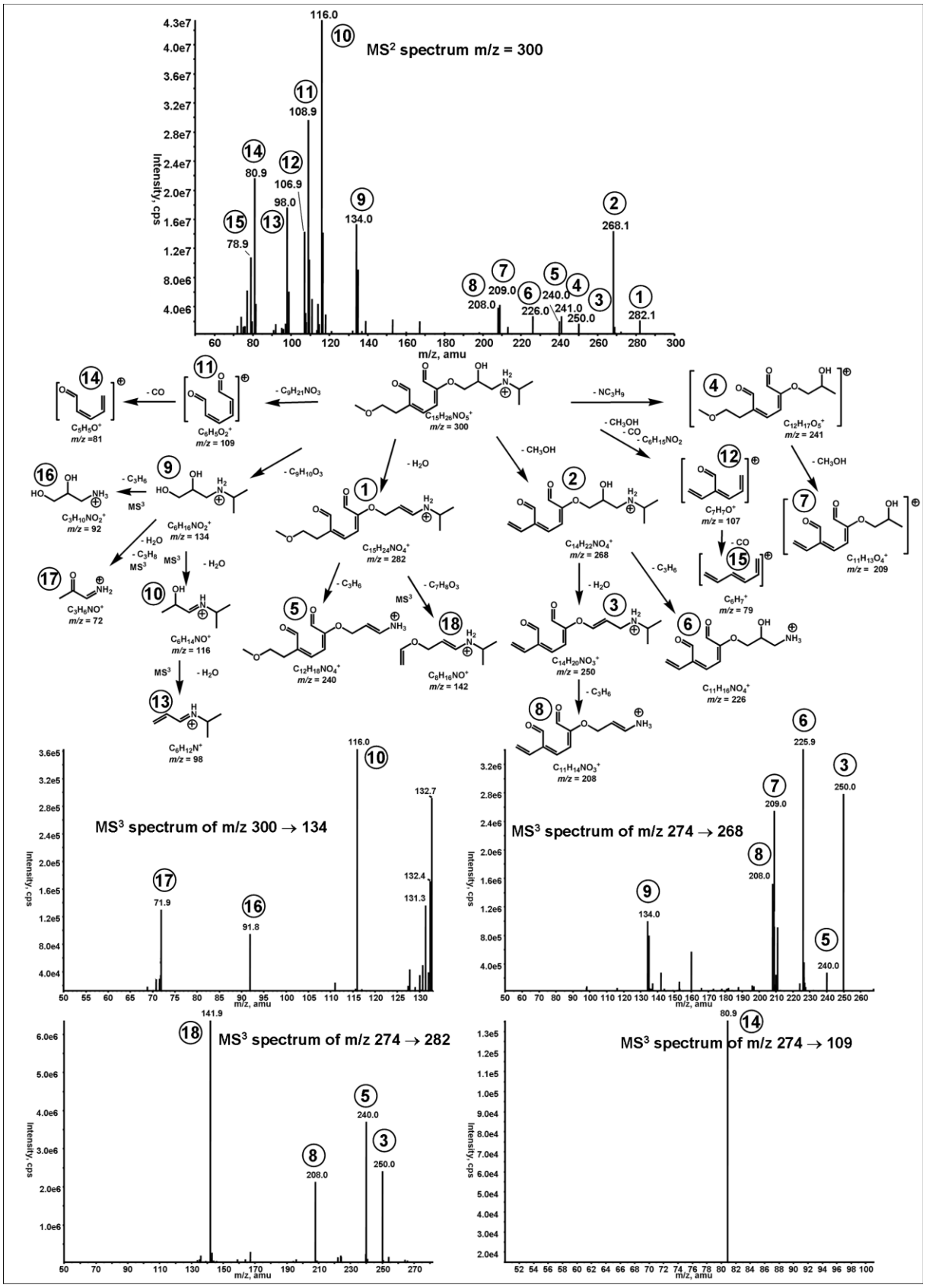
3.3.2 Oxidation Products

Because OH radicals and ozone molecules can be present, ozone and OH radical reactions as well as different combinations of both were taken into account. The information on product formation of ozonation and OH radical reactions for different functional groups in the literature (Beltran et al. 1993, Boncz et al. 1997, von Gunten 2003) allowed for possible reaction pathways for the oxidation of metoprolol and possible OP structures to be proposed. The nominal masses and the fragmentation pattern from product ion scans supported the proposed structures of the OPs (refer to Table 3-3 for chemical structures, and to SI Chapter

3.5.2 for missing MS^2 spectra and fragmentation pathways). Direct injection MS^3 experiments were performed for a limited number of OPs at higher concentrations. The fractions were obtained by fractionation via LC followed by freeze drying (refer to TextS 3-1 of the Supporting Information for the procedure). If the OP concentrations were sufficiently high, the chemical structures of OPs were confirmed by MS^3 spectra (section 3.5.2 of the Supporting Information).

In total, 23 different OP signals were detected in which 13 OP chemical structures could be proposed. Although MS^2 and MS^3 spectra (section 3.5.3 of the Supporting Information) were available, the chemical structure of 10 signals could not be identified.

Product formation at pH 3. The OP formation at pH 3 is very similar for the scavenged and non scavenged system. The same OPs could be detected to a similar extent. The main OP formed at pH 3 is M3/299/1-3. (M srefers to the OP of metoprolol; 3 or 8 indicates the pH where this OP was formed, 299 refers to the nominal mass of the OP and 1-3 refer to the isomer discussed), resulting from an attack at the aromatic ring following the Crigée mechanism (Scheme 3-2) (Dowideit and von Sonntag 1998). This leads to a ring opening and the formation of two aldehyde moieties.

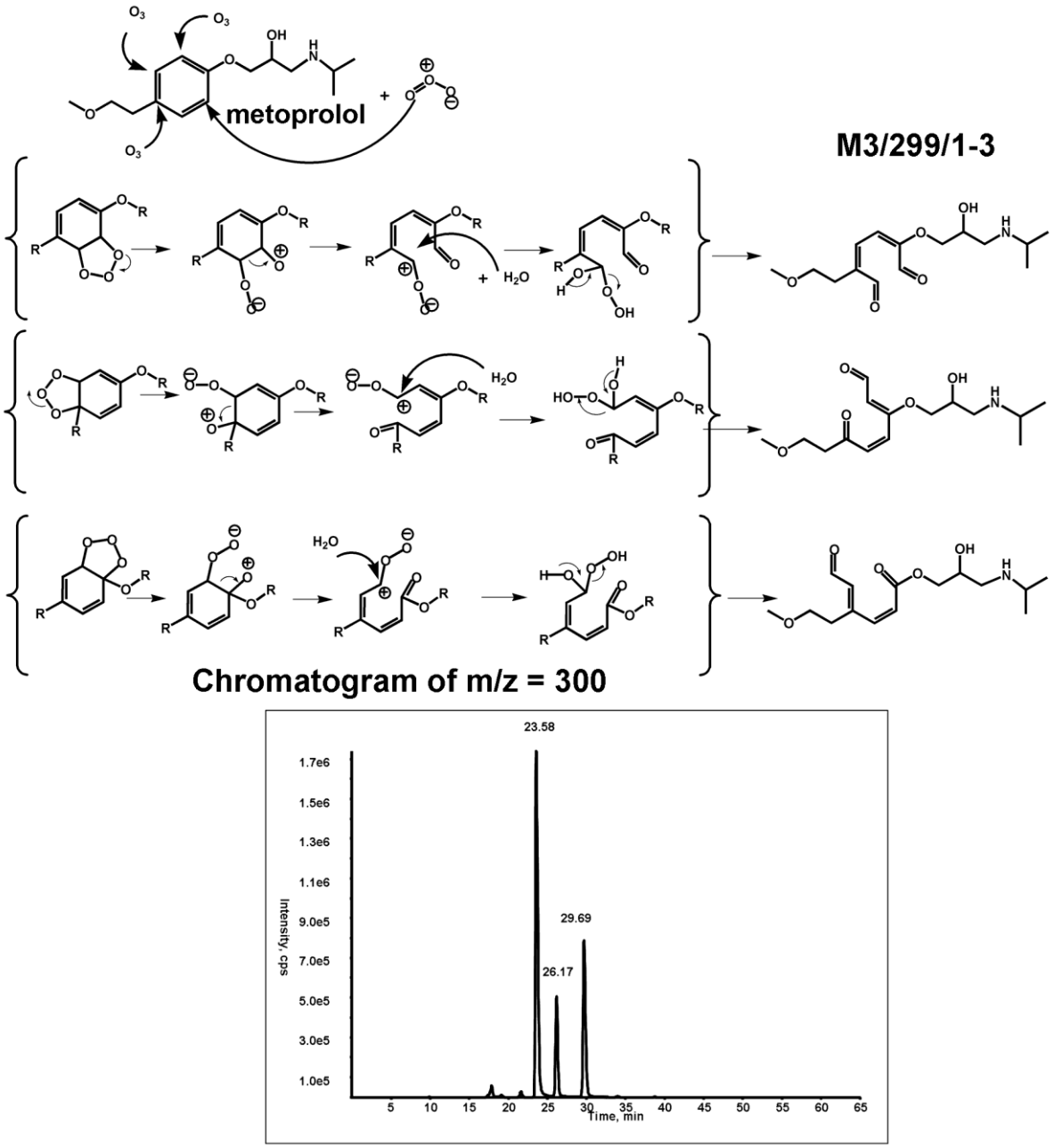


Scheme 3-1: Proposed Fragmentation Pathway for One Possible Structure of M3/299 with MS^2 Spectrum and MS^3 Spectra.

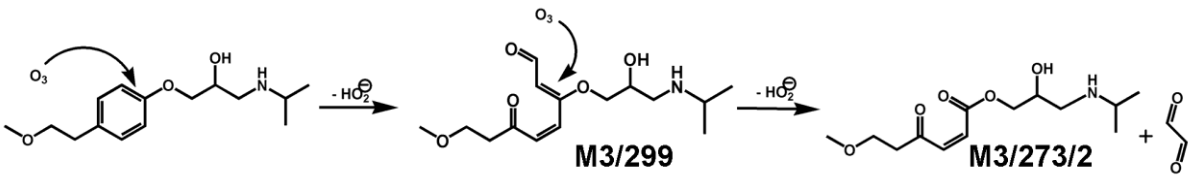
Several peaks with a nominal mass of 299 Da were found. Their MS^2 and MS^3 spectra exhibited equal fragmentation patterns. Scheme 3-1 illustrates the proposed fragmentation pathway for one possible isomer. A mass difference of 32 Da when compared to metoprolol corresponded to an addition of two oxygen atoms. In the MS^2 spectra the neutral loss of 59 Da (fragment 4) and the occurrence of fragments 9, 10 and 13 indicated that the isopropyl amine side chain remained unchanged (compare to metoprolol fragmentation Scheme S 3-1). The single loss of water (18 Da) confirmed that only one hydroxyl group next to an extractable proton is present. The loss of 32 Da, resulting in fragments 2 and 7, confirmed an unchanged ether side chain.

The *m/z* 109 fragment ion implied that oxidation took place at the aromatic moiety. The proposed formation of two aldehyde moieties instead of a double hydroxylation on the ring was supported by the fragment ion *m/z* 81 (109 Da -28 Da; loss of CO). The MS^3 spectra confirmed the proposed fragmentation pathway.

Scheme 3-2 shows the proposed reaction pathways for the three isomers of M3/299, during which a hydrogen peroxide ion (HO_2^-) is cleaved. The three major peaks detected correlated well with the three possible isomers formed by the ring opening. Staehelin et al. (1982) stated that HO_2^- can be a precursor for OH radical formation. However, at pH 3 HO_2^- will very quickly be protonated, so the probability of OH radical formation resulting from this reaction is low. Another reaction leading to the production of HO_2^- is the further oxidation of M3/299 via the same mechanism resulting in M3/273/2 (Scheme 3-3). Further oxidation reactions following the same mechanism such as an attack of the third double bond of M3/273/2 might be possible. A combination of all these reactions supports elevated OH radical formation in the non scavenged sample at pH 3.



Scheme 3-2: LC Chromatogram of OPs with *m/z* = 300 and Proposed Oxidation Reaction Pathways of the Formation of OP M3/299.



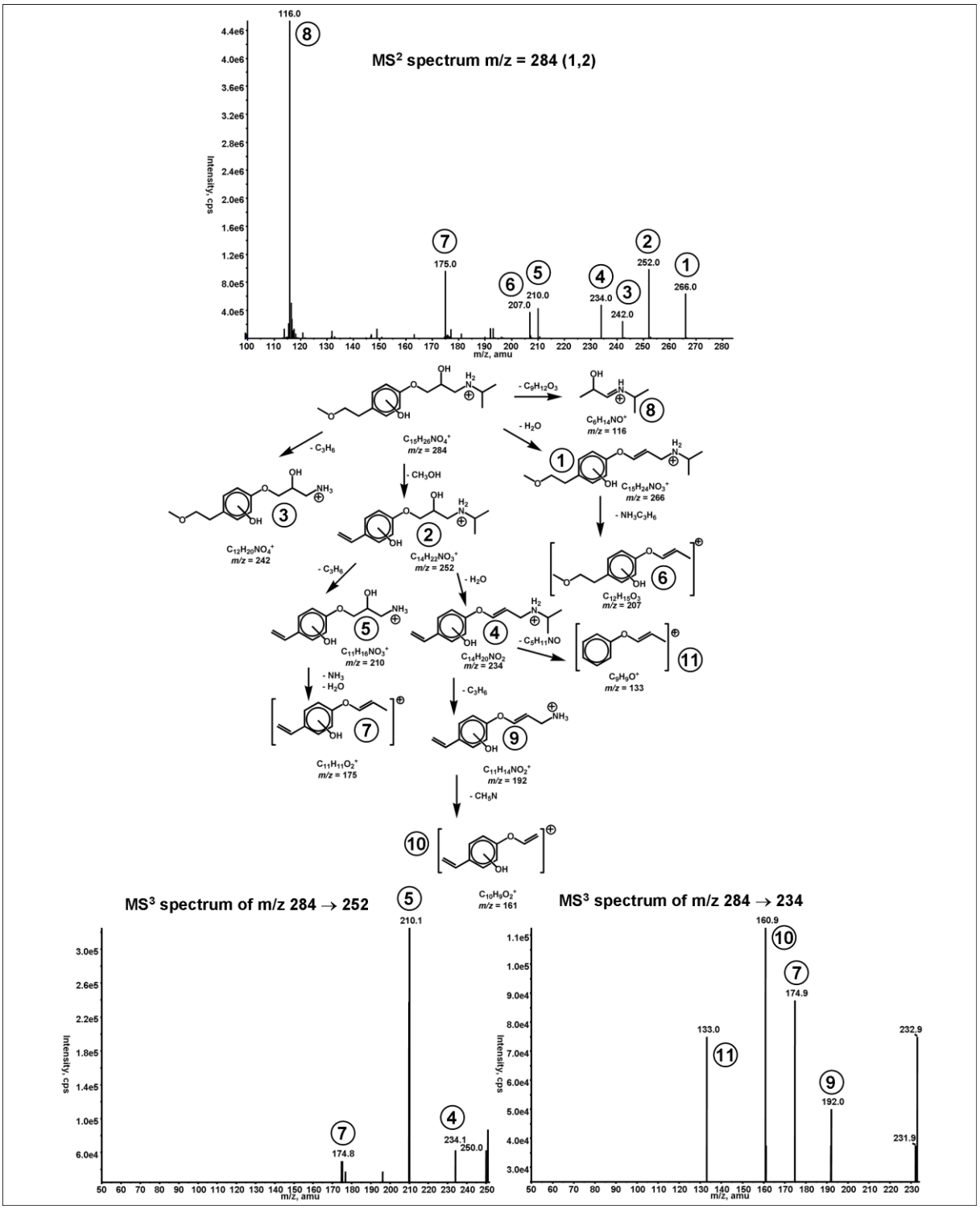
Scheme 3-3: Proposed Oxidation Reaction Pathway of M3/273/2 and F urther Oxidation of M3/299.

Formation of Constitutional Isomers. Several of the formed OPs had the same nominal mass but different retention times (e.g. chromatogram in Scheme 3-2). In most cases these OPs also exhibited the same MS^2 fragmentation pathways, indicating formation of constitutional isomers. Scheme 3-2 illustrates examples for possible reaction pathways of M3/299 formed at pH 3. The formation of constitutional isomers could be due to the ozone attack at different carbon atoms of the activated aromatic ring. Most of the MS^3 fragmentation of these OPs showed no differences, again confirming the presence of constitutional isomers. Unfortunately with the same fragmentation pattern it was impossible to allocate the isomers to the different retention times.

Product Formation at pH 8. Performing the experiments at pH 8 in the presence and absence of *t*-BuOH showed a higher diversity of OP formation. Some OPs detected in the scavenged system were not found in the non scavenged and *vice versa*. The elucidation of the structures of the main OPs indicated that this is likely due to a higher extent of OH radical oxidation in the non scavenged sample.

The OPs M8/283/1 and 2 resulted from hydroxylation at the aromatic ring, either at the *ortho*- or *meta*-position of the metoprolol molecule.

The proposed structure was confirmed by the MS^2 and MS^3 spectra (Scheme 3-4). The loss of water (fragments 1 and 7) as well as the loss of 42 Da (corresponding to the isopropyl moiety, fragments 3, 5 and 7) implied an unchanged isopropyl amine side chain. The loss of 32 Da, corresponding to the cleavage of methanol (fragment 2) confirmed the ether bond of the second side chain was not modified. Comparison of the fragments with a phenoxy moiety during fragmentation of metoprolol and the MS spectra of M8/283/1 and 2, revealed once more the mass difference of 16 Da, confirming the hydroxylation of the aromatic ring (fragments 4, 6 and 7).

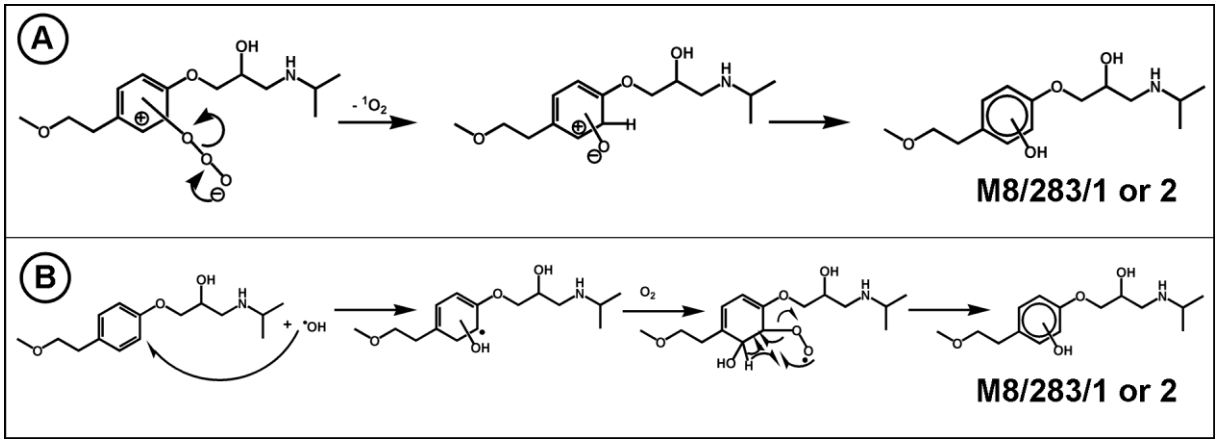


Scheme 3-4: Proposed Fragmentation Pathway of M8/283/1 and 2 with MS^2 Spectrum and MS^3 Spectra.

A hydroxylation can occur via direct ozone reaction (Scheme 3-5 A) (Boncz et al. 1997) or OH radical attack (Scheme 3-5 B) (Song et al. 2008). It is likely that both reactions occur simultaneously at pH 8, but the OH radical reaction would be more pronounced due to higher

OH radical exposure in the non scavenged system. At pH 8 most of the ozone will be consumed by the direct ozone decay, and therefore based on the OH radical attack is more likely to be the predominant reaction pathway. As a consequence, at pH 8, a higher production of M8/283/1 and 2 was found for the non scavenged system.

The occurrence of a third isomer (M8/283/3) indicated hydroxylation at another position of the molecule.The MS product ion spectrum revealed in contrast to the two other isomers, a second water elimination. Cleavage of an unchanged isopropyl moiety (3.5.2 of the Supporting Information) shows it was not altered. A hydroxylation in α- position of the amine or one of the ether bonds would lead to a formation of a hemiaminal or –acetal, respectively. Even if this reaction occurs, it would be impossible to detect these structures because they are known to be unstable, especially in aqueous solution. The only remaining hydroxylation possibilities would lead either to a formation of a hydroxylamine or to formation of a hydroxylation in β-position of the metoxy moiety. According to literature (von Gunten 2003), the formation of a hydroxylamine is much more likly, as the deprotonated amine has the higher electron density and, therefore, is prone to react with ozone as well as with OH radicals. Because M8/283/3 was found at pH 8 without *t*-BuOH addition, formation via a OH radical reaction is more plausibel. However, on the basis of the MS fragmentation, the identification of the exact position of the hydroxyl group was not feasible (refer to section 3.5.2 of the Supporting Information for possible fragmentation pathways for both possible products).

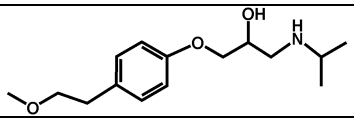
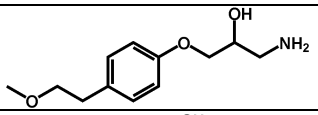
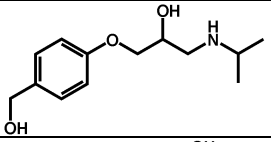
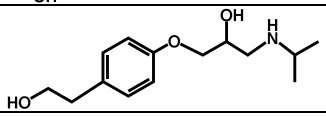
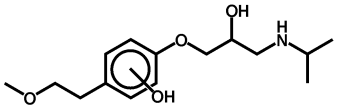
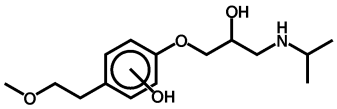
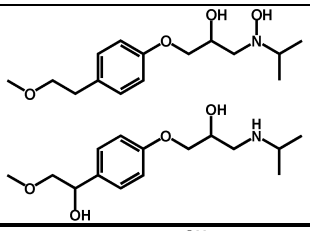
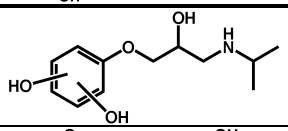
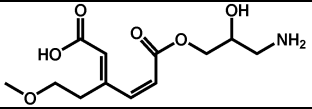
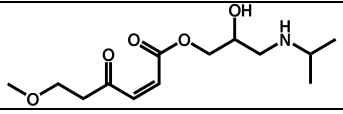
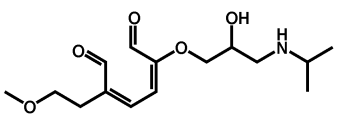


Scheme 3-5: Proposed Reaction Pathway For Oxidation of the Aromatic Ring (pH 8) Leading to M8/283/1 and 2 via Ozone (A) or OH Radical Reaction (B).

The formation of OP M8/253/1 (refer to Table 3-3 for the chemical structure and to Scheme S 2-4 of the Supporting Information for MS fragmentation) only occured in the nonscavenged setup at pH 8, indicating a OH radical reaction. The reaction took place at the ether bond of the side chain, supporting the attack by OH radical because ozone shows no reactivity with ethers (von Gunten 2003).

The OP M8/225 was mainly found in the scavenged system. Its structure (Table 3-3) was easy to identify, as the fragmentation pattern was very similar to the one found for metoprolol, apart from the cleavage of the isopropyl group (Chapter 3.5.2 in SI). There are two possible reaction pathways for the formation of M8/225, either via direct ozone reaction at the secondary amine or via OH radical oxidation. Currently, the dealkylation of an amine has only been presented in literature for tertiary amines (Munoz and von Sonntag 2000, Munoz et al. 2001), but a similar reaction pathway might be possible for the cleavage of the isopropyl moiety. This hypothesized reaction would also lead to formation of $^{\bullet}O_3^-$, another precursor for OH radical formation, supporting the high OH radical exposure detected in the nonscavenged samples (Buffle and von Gunten 2006, von Gunten 2003). In the literature, ozonation of secondary amines is usually described with a formation of $^{\bullet}O_2^-$ and a hydroxylated amine (Buffle and von Gunten 2006, von Gunten 2003).

Table 3-3: : Oxidation Products of Metoprolol at pH 3 and pH 8[a].

	m/z	RT [min]	Molecular formula	Proposed structure	Abbreviation
metoprolol	268	41	$C_{15}H_{25}NO_3$		
ozonationat pH 8					
mainly with *t*-BuOH	226	29.2	$C_{12}H_{19}NO_3$		M8/225
only without *t*-BuOH	240	16.7	$C_{12}H_{17}NO_4$		M8/239/1
only without *t*-BuOH	254	21.6	$C_{14}H_{23}NO_3$		M8/253/1
both conditions, more without *t*-BuOH		25.6	"	unknown	M8/253/2
both conditions, more without *t*-BuOH	284	13.8	$C_{14}H_{21}NO_5$		M8/283/1
both conditions, more without *t*-BuOH		18.8	"		M8/283/2
both conditions, more without *t*-BuOH		22.1	"		M8/283/3
ozonation pH 3	242	11.6	$C_{12}H_{19}NO_4$		M3/241
both conditions	274	13.7			M3/273/1
both conditions		18.9			M3/273/2
both conditions	300	8.8 22.5 25.6 28.1	$C_{15}H_{25}NO_5$		M3/299/1 M3/299/2 M3/299/3 M3/299/4

[a] structures shall not implicate any defined stereo or structural isomers of the OPs; Apart from M8/283/3 in case of possible constitutional isomers, only one of the possible structures is shown.

3.3.3 OP stability

A comparison of different ozone:metoprolol ratios indicated that many of the formed OPs react further with ozone. Benause no isolation of pure compounds was feasible, quantification of the OPs was impossible. By normalizing the peak areas of one specific OP to the highest peak found in the whole set of applied ozone doses, its potential for further oxidation could be correlated (Figure 3-1 a-c).

In panels a-c of Figure 3-1 the normalized areas for the main (most intense) OPs of the different experimental systems were plotted against the ozone:metoprolol ratio. Panels a and b of Figure 3-1 illustrate the development of the OPs formed at pH 8 with and without OH radical scavenging and the attenuation of metoprolol. The reaction with radical scavenging led to the main OPs M8/225 and M8/239/2, which were still present in the highest applied ozone dose of 18 mg O_3/L, indicating stability against further ozone attacks in the order of OP M8/239/2 > OP M8/225 > OP M8/253/2, as the latter reached the maximum already at an ozone dose of 4.3 mg O_3/L with a complete attenuation at 18 mg O_3/L.

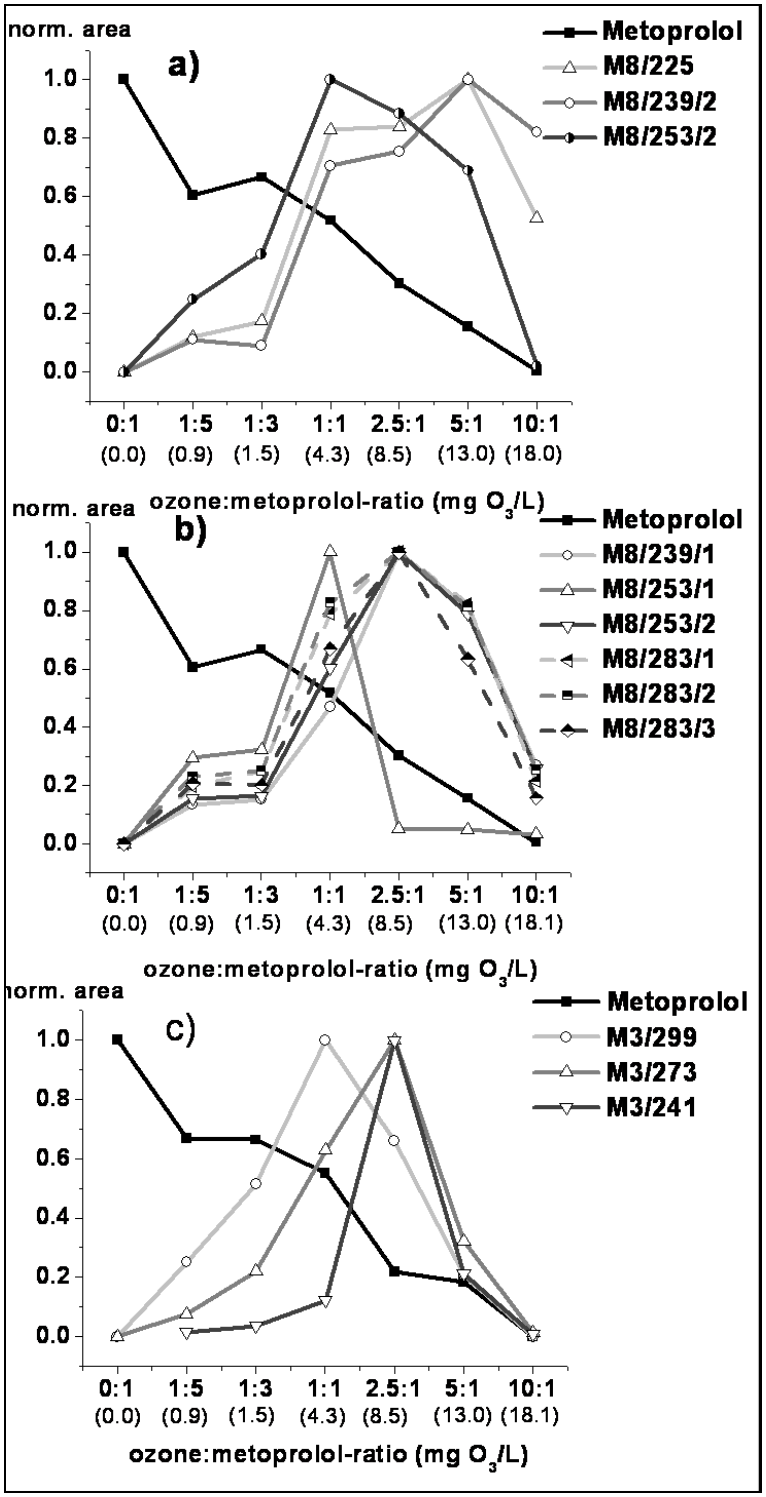


Figure 3-1: Comparison of the main OPs at different ozone:metoprolol ratios. a) Experiment at pH 8 with **addition of a radical scavenger (100 µM *t*-BuOH). b) Experiment at pH 8** without **addition of a radical scavenger. c) Experiment at pH 3, with addition of radical scavenger (100 µM *t*-BuOH).**

In the nonscavenged systems, most of the main OPs followed a similar pattern. They reached their maximum concentration at an ozone dose of 8.5 mg O_3/L and were further oxidized with

higher ozone doses. Their concentrations were strongly attenuated at an ozone dose of 18.1 mg O_3/L. OP M8/253/1 had a different reactivity. The maximum concentration was already reached at an ozone:metoprolol ratio of 1:1 (ozone dose 4.3 mg O_3/L). Ozone in excess led to further reactions and a complete attenuation at an ozone dose of 8.5 mg O_3/L. Although MS^2 and MS^3 fragmentation patterns indicated the same or a very similar structure for M8/253/1 and M8/253/2, this implies that there has to be a significant difference in their structures.

At pH 3, several peaks for the constitutional isomers of OP M3/299 and M3/273 were found. The formation and further reaction of these constitutional isomers were very similar. To simplify the analysis of Figure 1 c, the areas of all isomer peaks were summed and then normalized. OP M3/299 reached the maximum concentration at a stoichiometric ratio of 1:1 and the other two main products M3/273 and M3/241 at 2.5:1. An ozone dose of 18 mg O_3/L resulted in all three OPs being oxidized completely.

3.3.4 Product formation in Wastewater Matrix

One possible application of ozone is the ozonation of raw waste water as pretreatment or WWTP effluent as posttreatment to reduce micropollutant input to surface waters. However, limited information is available about the formation of stable oxidation products. As wastewater is a complex matrix and the predominant reactions in such a matrix are difficult to predict, raw wastewater (DOC = 65 mg C/L; pH = 7.7) and WWTP effluent (DOC = 8.5 mg C/L; pH = 8.4) were spiked with metoprolol (10 µM) and then ozonated. To follow possible OH radical formation in the samples, 1 µM pCBA was added and its consumption was analyzed by HPLC-UV (Table 3-2). Using the MRM method developed with the neat metoprolol solutions, different OPs could be analyzed semi-quantitatively. The pCBA analysis confirmed a significant OH radical formation in raw wastewater and WWTP

effluent samples (Table 3-2). In Figure 3-2, ozonated metoprolol samples with and without *t*-BuOH (ozone dose 18.1 mg O_3/L) and the spiked raw wastewater sample (ozone dose 4.8 mg O_3/L) were compared. It has been shown before that this metoprolol concentration should not influence the ozone and OH radical exposure in the matrix (Benner et al. 2008).

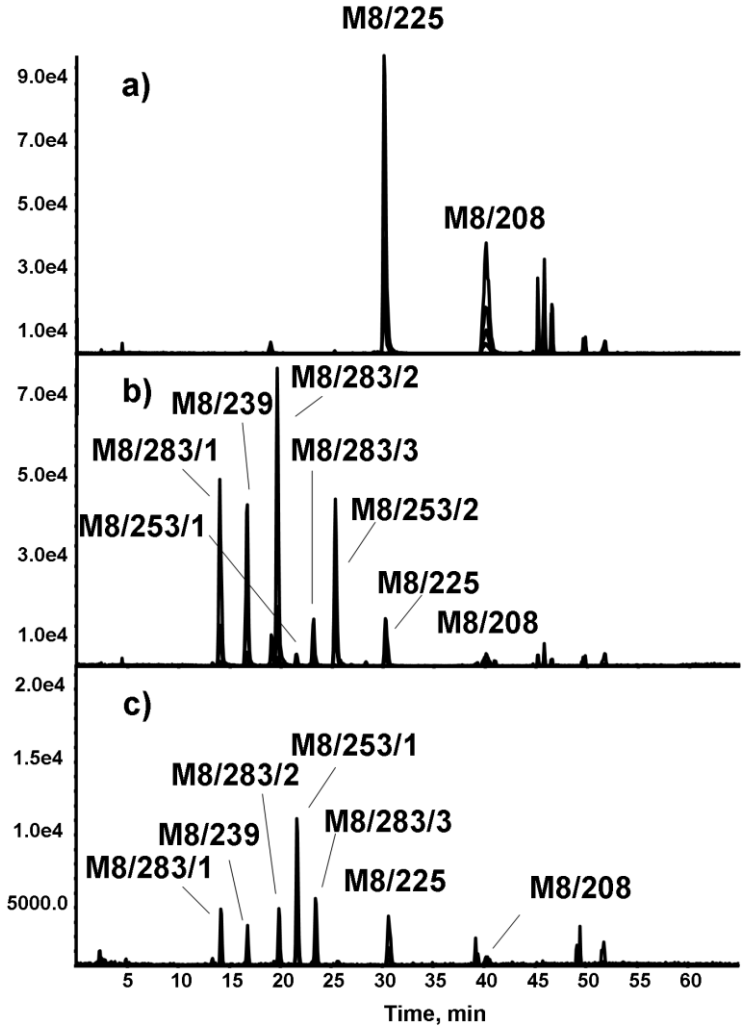


Figure 3-2: Comparison of ozonated samples. a) 100 µM metoprolol at pH 8 with 100mM *t*-BuOH (ozone:metoprolol ratio of 10:1). b) 100 µM metoprolol at pH 8 without *t*-BuOH (ozone:metoprolol ratio of 10:1). c) Raw wastewater (pH = 7.7) spiked with 10 µM metoprolol (ozone:metoprolol ratio of 12:1).

The high number of similarities between the nonscavenged system and the spiked wastewater as well as the attenuation of pCBA indicated that in a real matrix such as raw or conventional treated municipal wastewater, OH radical reactions are occurring in addition to ozone

reactions. For a molecule such as metoprolol reacting only in a moderate rate with ozone ($k_{app, O3, pH\ 7} = 2.0 \pm 0.6 \cdot 10^3\ M^{-1}\ s^{-1}$) but relatively fast with OH radicals ($k_{app, OH} = 7.3 \pm 0.2 \cdot 10^9\ M^{-1}\ s^{-1}$) (Benner et al. 2008), the latter process might dominate the OP formation. Although an extrapolation of the investigated reactivity and OP formation of metoprolol to structurally similar compounds will be rather difficult due to the low selectivity of OH radical reactions, a multitude of hydroxylated OPs can be expected.

3.4 Acknowledgments

The authors thank Michael C. Dodd for fruitful discussions. We thank Jennifer Lynne Kormos and Carmen Pies for reviewing the manuscript as well as Manoj Schulz for his support in MS data elucidation. For financial support, the European Commission for the EU-project RECLAIM WATER (Project No. 018309) is gratefully acknowledged.

3.5 Supporting information

3.5.1 LC-MS parameter and properties

Text S 3-1: HPLC/UV time wise fractionation.
The samples containing the mixture of OPs were separated with an Agilent 1100 HPLC system (Agilent Technologies, USA-Santa Clara) using a Synergi 4u Hydro-RP column, 3 mm i.d., 250 mm, 4 µm (Phenomenex®, Aschaffenburg, Germany) at room temperature. Milli-Q water and acetonitrile both consisting of 0.5 % aqueous formic acid were used as eluents. Injection volumes varied between 20-100 µL. The UV system was operated at 254 and 280 nm. Highly concentrated samples (metoprolol start concentration: 5 mM) were separated by a fraction collector in ten fractions with varying time intervals depending on the retention times of the formed peaks. The time wise fractions were then concentrated by freeze drying. To monitor any possible loss or degradation of OPs, samples were measured before and after the freeze drying process.

Table S 3-1: LC-MS setup properties.

LC Pump Properties	
Pump Model	Agilent 1200 Binary Pump SL
Columnoven Temperature	28 °C
Autosampler Properties	
Autosampler Model	Agilent 1200 High Performance Autosampler SL
Syringe Size (µl)	100
Injection Volume (µl)	50.00
Source Parameter	
Source and desolvation temperature	450.00 °C
collision activated dissociation gas	High
capillary voltage positive mode	5500.00
Scan Type	MRM (multiple reaction mode)
Polarity	Positive
Ion Source	Turbo Spray

Table S 3-2: Multiple reaction mode (MRM) transitions for metoprolol OPs formed at pH 8.

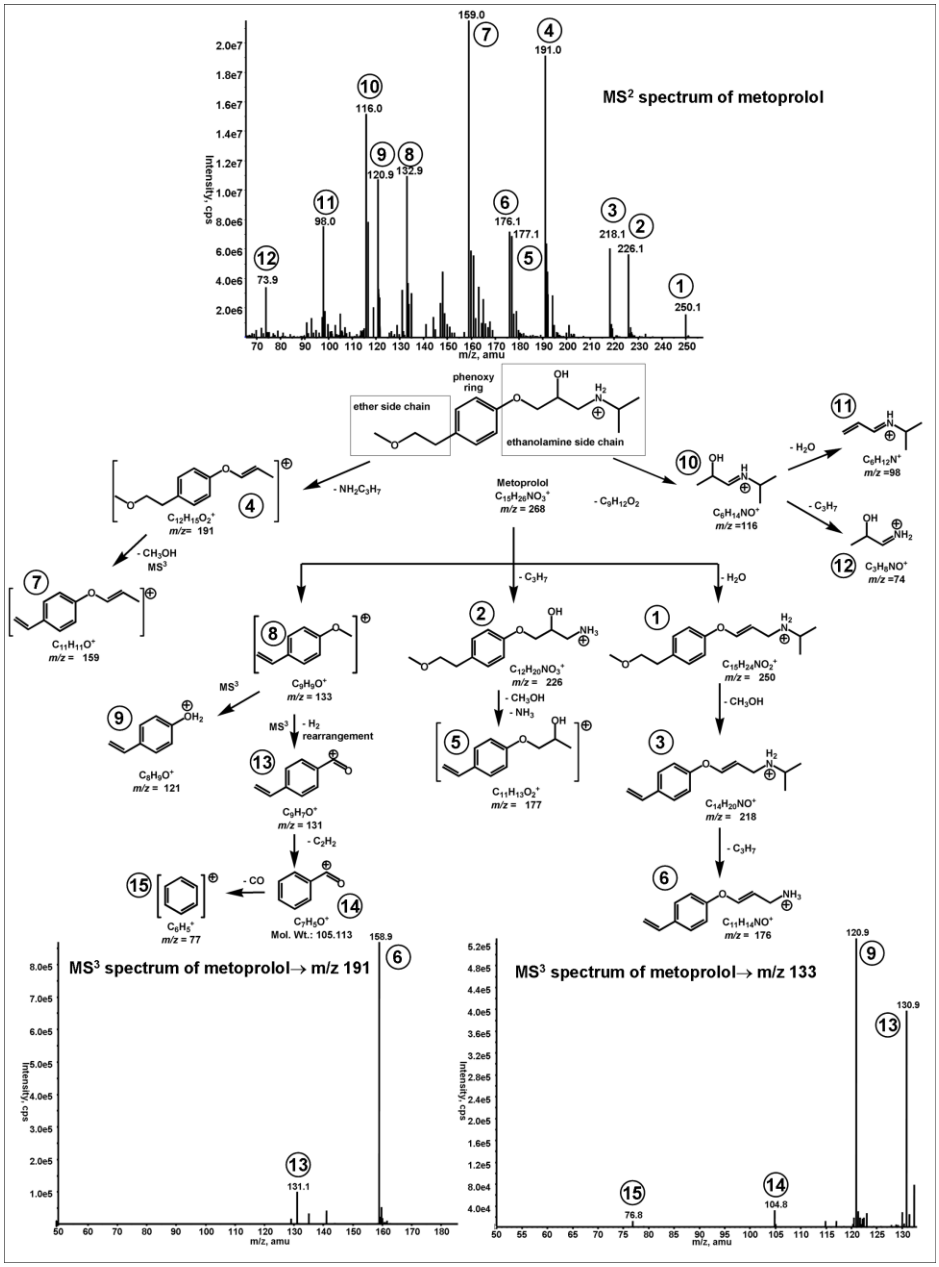
Q1 → Q3 *m/z* (amu)	dwell time [ms]	Collision energy [V]	collision cell exit potential [V]	Entrance potential [V]	Declustering potential [V]
268 → 116 (metoprolol)	75	27	50	10	10
268 → 74 (metoprolol)	75	35	50	10	10
226 → 121	100	30	50	10	10
226 → 123.9	100	30	50	10	10
226 → 132	100	30	50	10	10
226 → 147.9	100	30	50	10	10
226 → 151.9	100	30	50	10	10
226 → 159	100	30	50	10	10
240 →1 63	100	30	50	10	10
240 → 144.9	100	30	50	10	10
240 → 132.9	100	30	50	10	10
240 → 121	100	30	50	10	10
254 → 212	100	30	50	10	10
254 → 177	100	30	50	10	10
254 → 151	100	30	50	10	10
254 → 132.9	100	30	50	10	10
254 → 115.9	100	30	50	10	10
284 → 115.9	100	30	50	10	10
284 → 175	100	30	50	10	10
284 → 132.9	100	30	50	10	10
284 → 207	100	30	50	10	10
300 → 131.9	100	30	50	10	10
300 → 208	100	30	50	10	10
Unidentified OPs					
207 → 116.9	100	30	50	10	10
207 → 144.9	100	30	50	10	10
260 → 120.9	100	30	50	10	10
260 → 145	100	30	50	10	10

Table S 3-3: Multiple reaction mode (MRM) transitions for metoprolol OPs formed at pH 3.

Q1 → Q3 *m/z* (amu)	dwell time [ms]*	Collision energy [V]	collision cell exit potential [V]	Entrance potential [V]	Declustering potential [V]
242 → 115.9	150	30	50	10	10
242 → 108.9	150	30	50	10	10
274 → 115.9	150	30	50	10	10
274 → 110.9	150	30	50	10	10
300 → 115.9	150	30	50	10	10
300 → 282	150	30	50	10	10
Unidentified OPs					
208 → 115.9	150	30	50	10	10
232 → 116	150	30	50	10	10
254 → 159	150	30	50	10	10
254 → 133	150	30	50	10	10
254 → 191	150	30	50	10	10
260 → 145.9	150	30	50	10	10
260 → 115.9	150	30	50	10	10
282 → 250	150	30	50	10	10
282 → 196	150	30	50	10	10
282 → 154	150	30	50	10	10
290 → 272	150	30	50	10	10
290 → 134	150	30	50	10	10
290 → 115.9	150	30	50	10	10

* higher dwell time than pH 8 method possible due to less transitions

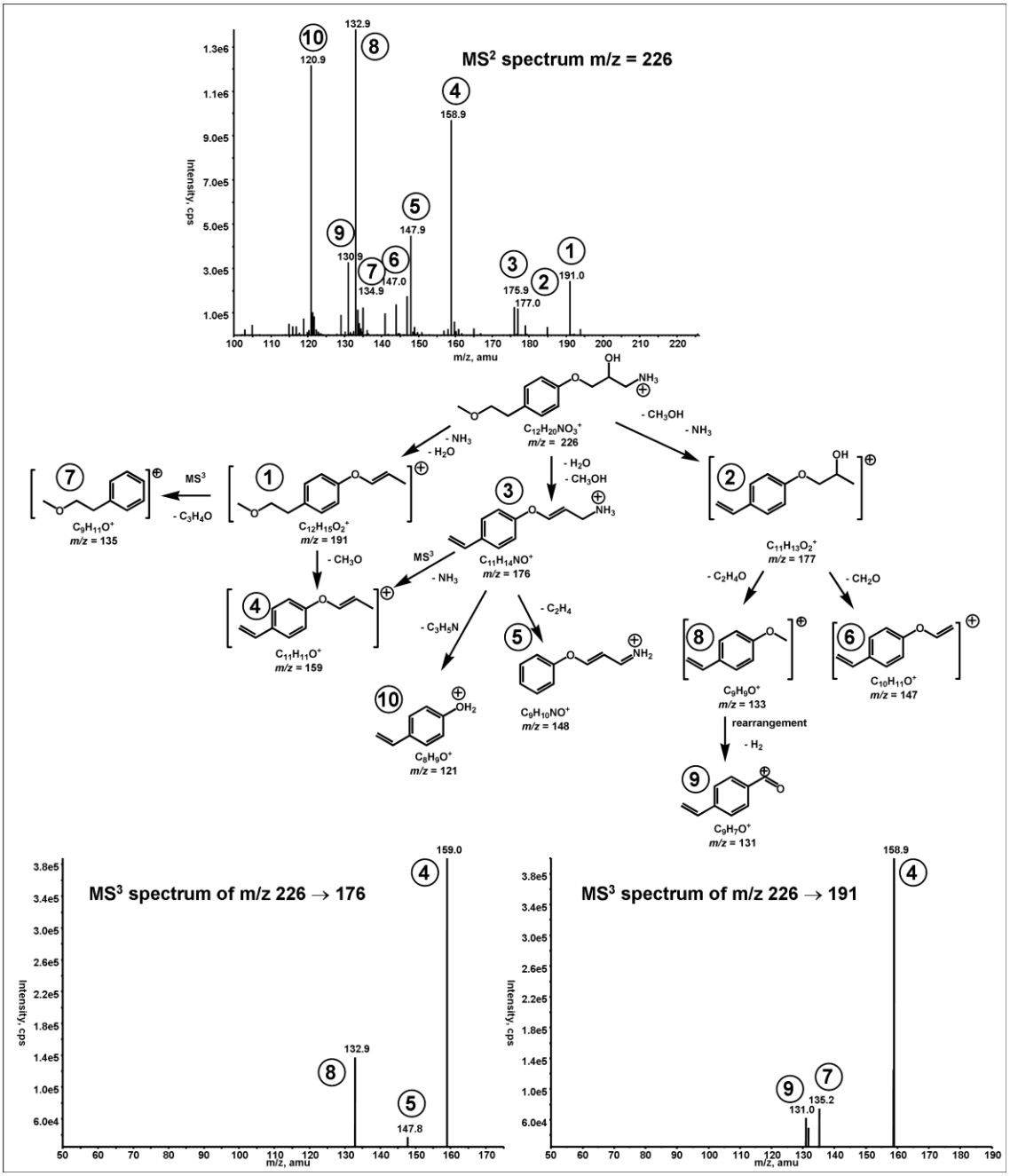
3.5.2 MS^2 and MS^3 spectra of identified OPs of metoprolol at pH 3 and pH 8



Scheme S 3-1: Proposed fragmentation pathway of metoprolol with MS^2 spectrum and MS^3 spectra.

Text S 3-2: Proposed fragmentation pathway of metoprolol.

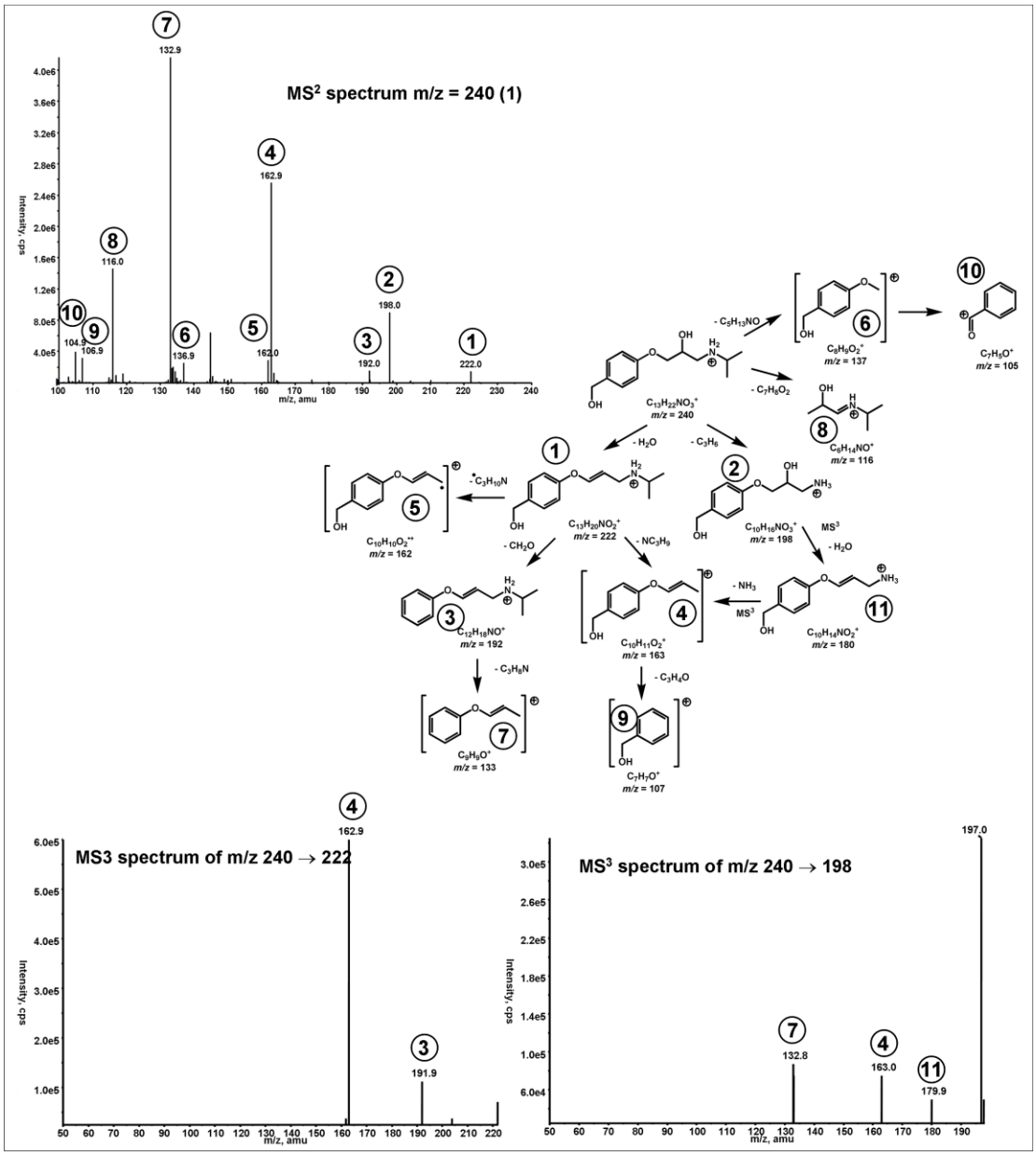
- Slegers et al. (2006) published LC-MS data for metoprolol and the e-beam and gamma radiolysis products of metoprolol.
- Same fragments as Slegers et al (2006): 1, 2, 3, 4, 6, 7, 9, 10, 11.
- Important fragments for identification of OPs:
    - Ethanolamine side chain: fragments 10, 11, 12, loss of 18 Da (H_2O) corresponding to the elimination of the hydroxyl group and the loss of 42 Da, corresponding to the loss of isopropyl moiety
    - Ether side chain: loss of 32 Da corresponding to the elimination of methanol
    - Phenoxy ring: fragments 8, 9, 13, 14, 15.



Scheme S 3-2: Proposed fragmentation pathway of M8/225 with MS^2 spectrum and MS^3 spectra.

Text S 3-3: Proposed fragmentation pathway of M8/225.

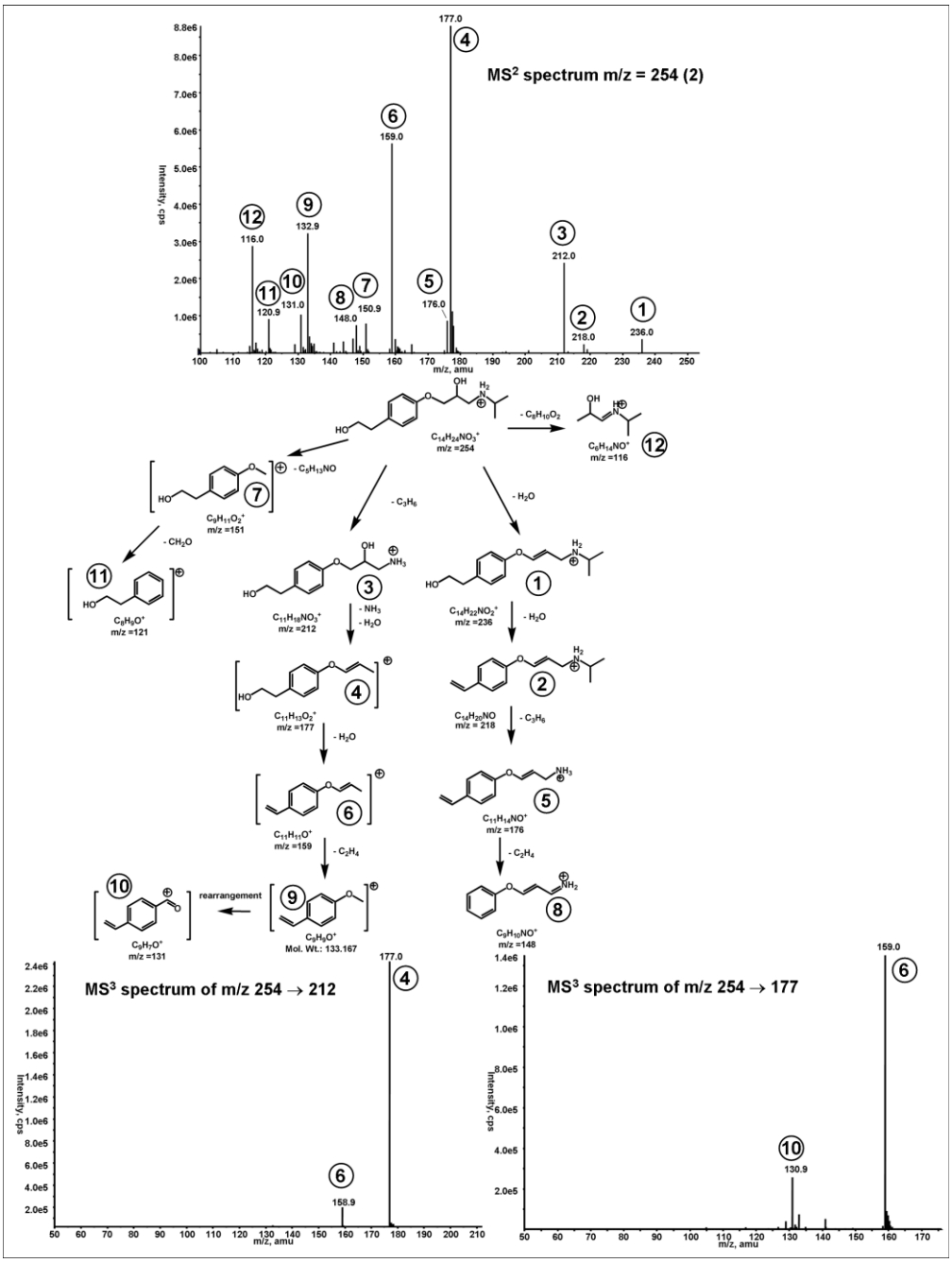
- Difference to metoprolol is -42 Da, corresponding to the cleavage of the isopropyl moiety.
- EPI spectrum of metoprolol contains a fragment of m/z = 226, fragments also occurring in metoprolol spectrum: 1, 2, 3, 4, 8, 10.
- Accordance with m/z of 226 (product 10).
    - Analog fragments with Slegers et al. (2006): 1, 3, 4, 10
    - Some fragments of Slegers et al. (2006) are similar, but a fragmentation pathway is not described.
- Ethanolamine side chain: No fragment with m/z of 116 and no loss of 42 Da (loss of isopropyl group).
- Ether side chain: loss of 32 + 18 (+17) Da corresponding to the elimination of methanol and water (ammonia) (fragment 3 (2)).
- Phenoxy ring: fragments 8, 9, 10.



Scheme S 3-3: Proposed fragmentation pathway of M8/239/1 with MS^2 spectrum and MS^3 spectra.

Text S 3-4: Proposed fragmentation pathway of M8/239/1.

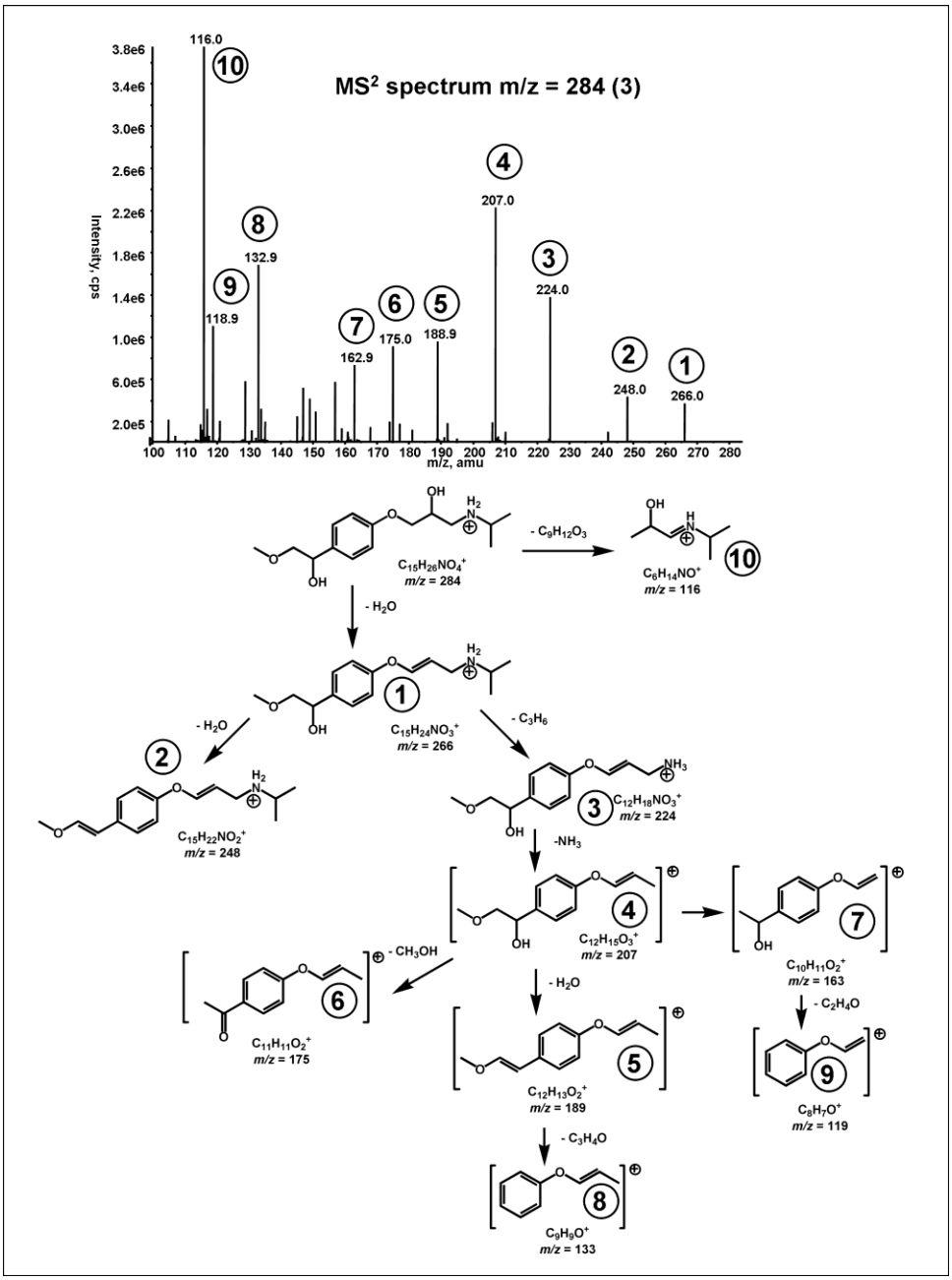
- Difference to metoprolol is -28 Da, corresponding to a loss of C_2H_4.
- Ethanolamine side chain intact due to:
  - Fragment 8
  - Loss of water (fragments 1 and 11)
  - Loss of 42 Da corresponding to isopropyl group (fragment 2).
- Ether side chain not intact as no loss of 32 occurs.
- Phenoxy ring: fragments 10.
- Slegers et al. (2006) published LC-MS data of e-beam and gamma radiolysis product with m/z of 238, similar structure than proposed here, but with an aldehyde instead of the hydroxyl group (product 2).
  - Slegers et al. (2006) show similar pattern than here only -2 Da.



Scheme S 3-4: Proposed fragmentation pathway of M8/253/1 with MS^2 spectrum and MS^3 spectra.

Text S 3-5: Proposed fragmentation pathway of M8/253/1.

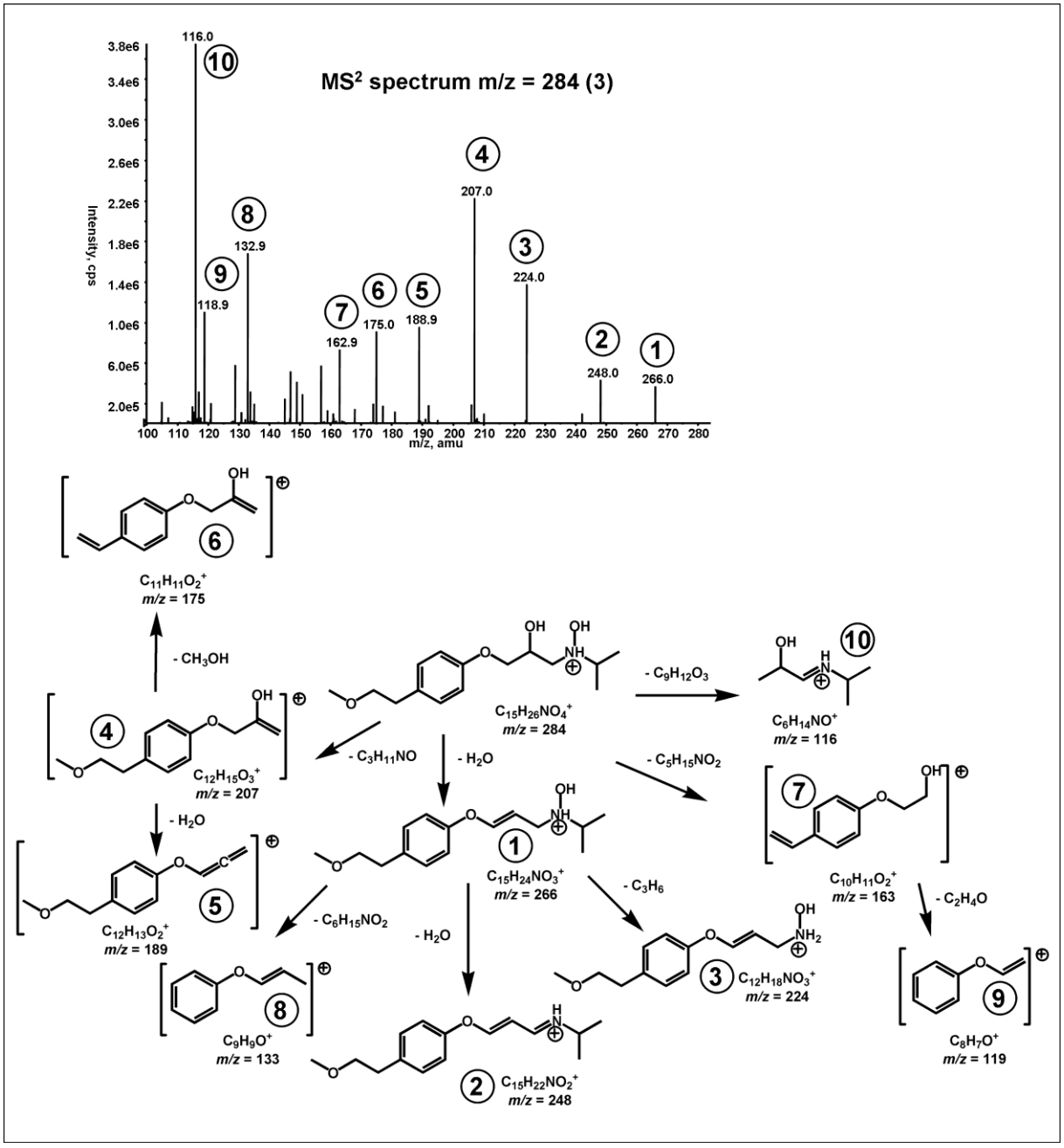
- Difference to metoprolol is -14 Da, corresponding to a loss of CH_2.
- Ethanolamine side chain intact due to :
  - Fragment 12
  - Loss of water (fragments 1 and 4)
  - Loss of 42 Da corresponding to isopropyl group (fragments 2, 3).
- Ether side chain not intact as no loss of 32 occurs.
- Phenoxy ring intact: fragments 9, 10.
- Second loss of water (fragments 2 and 6) proves the existence of a second hydroxyl group next to an extractable proton.
- Slegers et al. (2006) published LC-MS data of e-beam and gamma radiolysis product with m/z of 254 and proposed the same structure.
  - Analog fragments with Slegers et al. (2006): 1, 2, 3, 4, 6, 12.



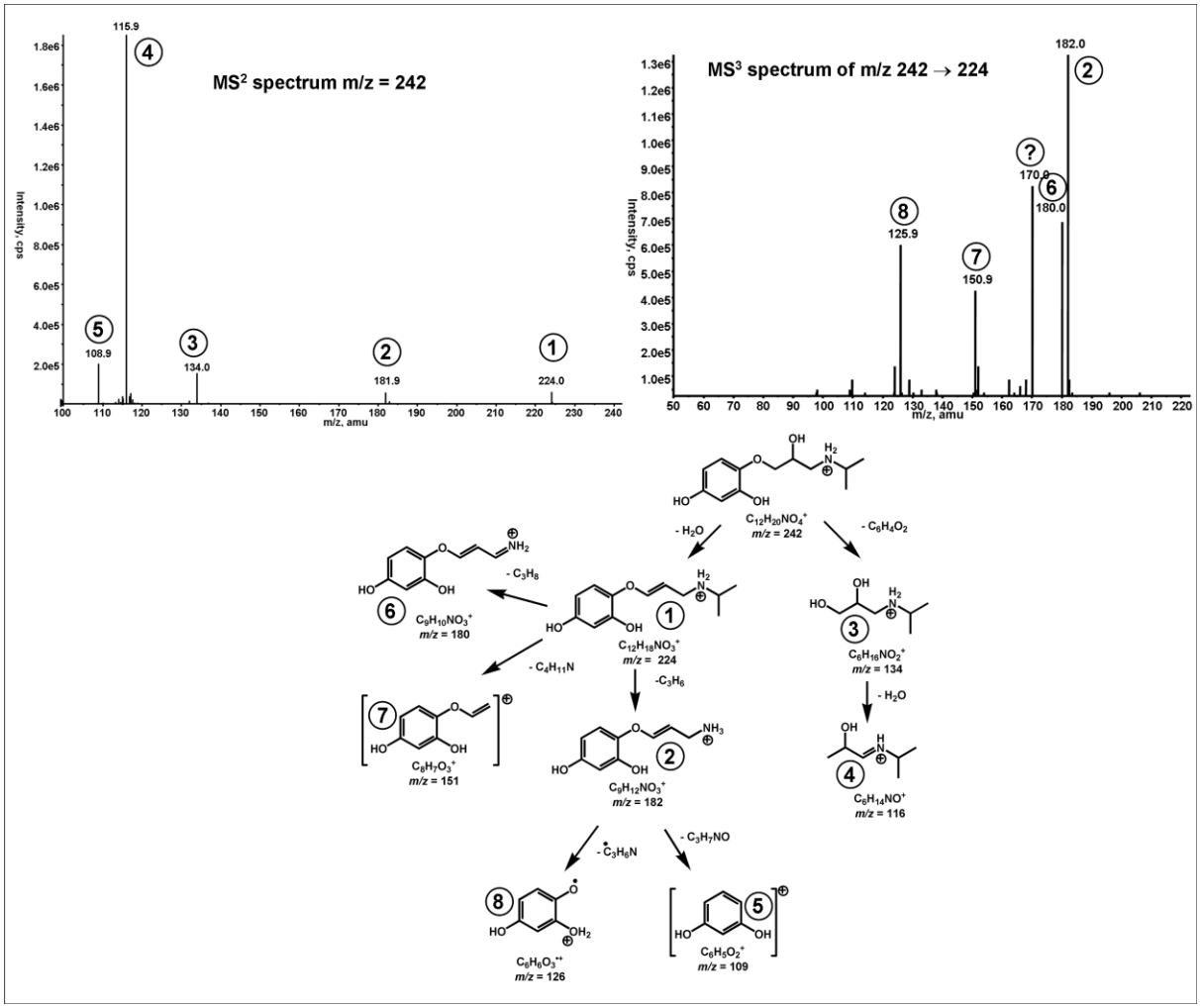
Scheme S 3-5: Proposed fragmentation pathway of one possible structure of M8/283/3 with MS^2 spectrum.

Text S 3-6: Proposed fragmentation pathway of M8/283/3.

- Difference to metoprolol is +16 Da, corresponding to an addition of an oxygen atom via a hydroxylation.
- Ethanolamine side chain intact due to:
  - Fragment 10
  - Loss of water (fragment 1)
  - Loss of 42 Da corresponding to isopropyl group (fragment 3).
- Different MS^2 spectrum pattern than M8/284/1 and 2.
- Ether side chain: ether bond intact as a loss of 32 occurs (fragment 6).
- Second loss of water (fragments 2 and 5) proves the existence of a second hydroxyl group next to an extractable proton.
- As ethanolamine side chain is proven to be unchanged, hydroxylation most probable on either of the secondary carbon atoms of the ether side chain.



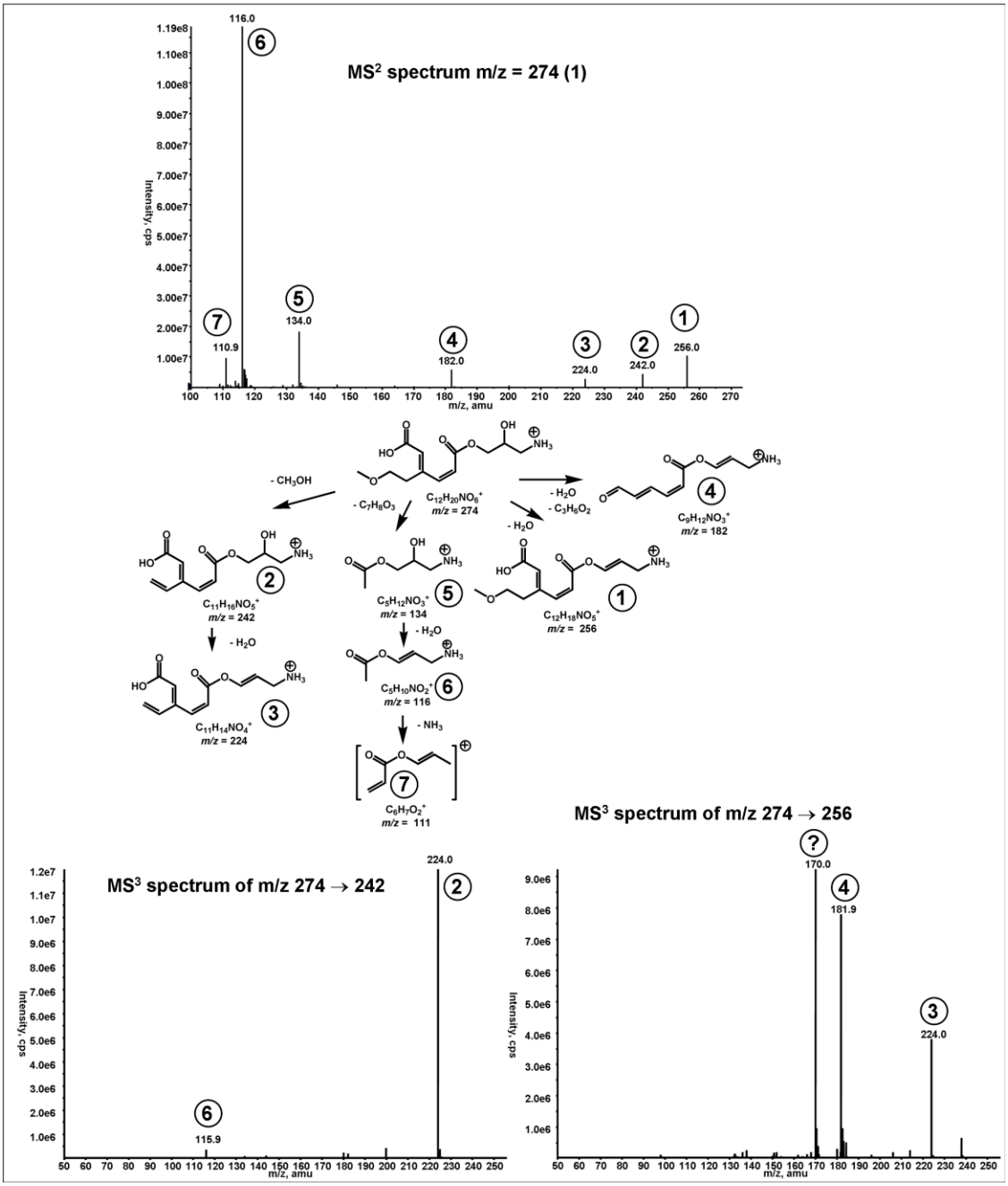
Scheme S 3-1: Proposed fragmentation pathway of a hydroxylamine as possible structure of M8/283/3 with MS^2 spectrum.



Scheme S 3-7: Proposed fragmentation pathway of one possible structure of M3/241 with MS^2 spectrum and MS^3 spectrum.

Text S 3-7: Proposed fragmentation pathway of M3/241.

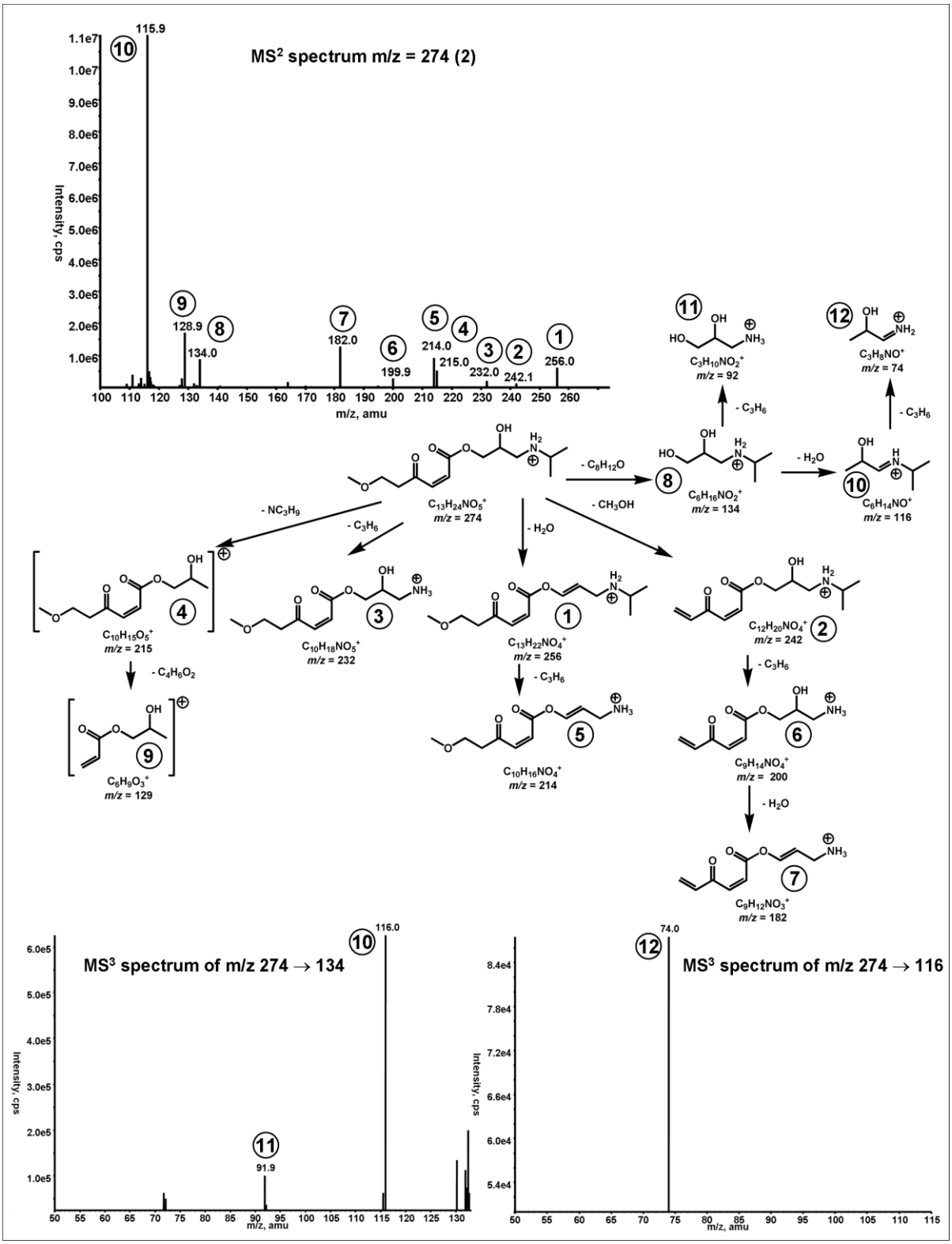
- Ethanolamine side chain intact due to :
  - Fragments 3 and 4
  - Loss of water (fragment 1)
  - Loss of 42 Da corresponding to isopropyl group (fragment 2).
- Proof for double hydroxylated ring: fragments 5 and 8.



Scheme S 3-8: Proposed fragmentation pathway of M3/273/1 with MS^2 spectrum and MS^3 spectra.

Text S 3-8: Proposed fragmentation pathway of M3/273/1.

- Ethanolamine side chain seems intact due to fragments 5 and 6 and the loss of water (fragments 1, 3, 6) but no loss of 42 Da corresponding to isopropyl group.
- Ether side chain intact as a loss of 32 occurs (fragment 2).
- Fragments 5 and 6 can only be explained if the proposed ester moiety is formed.
- Influence of pH change on chromatography:
  In reverse phase chromatography a change in eluent pH can lead to retention time shifts, which imply an existence of carboxyl groups (Schulz et al. 2008). Using non-buffered MilliQ water and acetonitrile as eluent instead of the acidified eluents, most of the OP peaks reacted the same way as metoprolol, they were not shifted but only broadened. One exception was M3/273/1 where a shift of 1.7 min was detected. M3/273/1 was also the only structures where we proposed a carboxyl moiety being formed, so that this experimental result is consistent with the proposed structure.



Scheme S 3-9: Proposed fragmentation pathway of M3/273/2 with MS^2 spectrum and MS^3 spectra.

Text S 3-9: Proposed fragmentation pathway of M3/273/2.

- Ethanolamine side chain intact due to:
  - Fragments 8, 10, 12
  - Loss of water (fragments 1 and 7)
  - Loss of 42 Da corresponding to isopropyl group (fragments 3, 5, 6, 11).
- Ether side chain intact as a loss of 32 occurs (fragment 2).

-

3.5.3 MS^2 and MS^3 spectra of unidentified OPs of metoprolol at pH 3 and pH 8

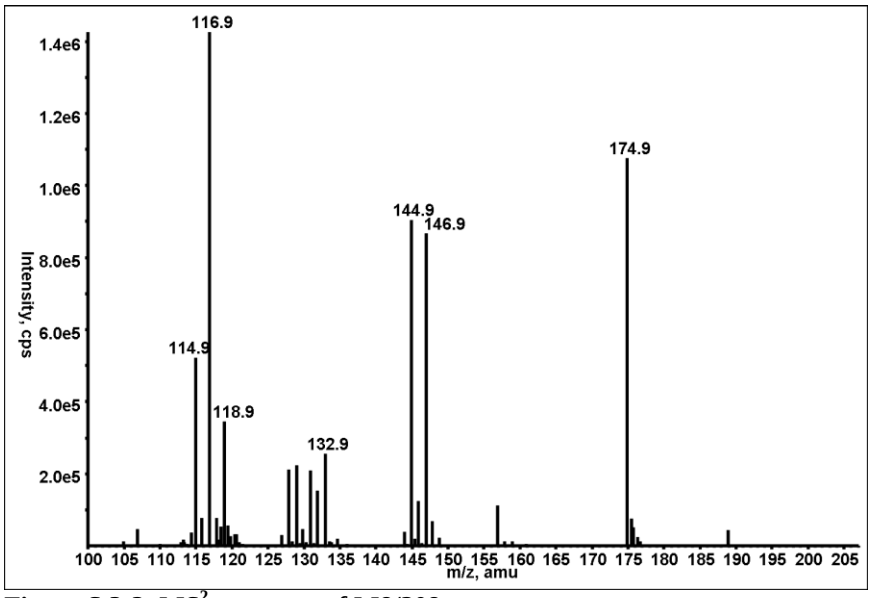


Figure S 3-2: MS^2 spectrum of M8/208.

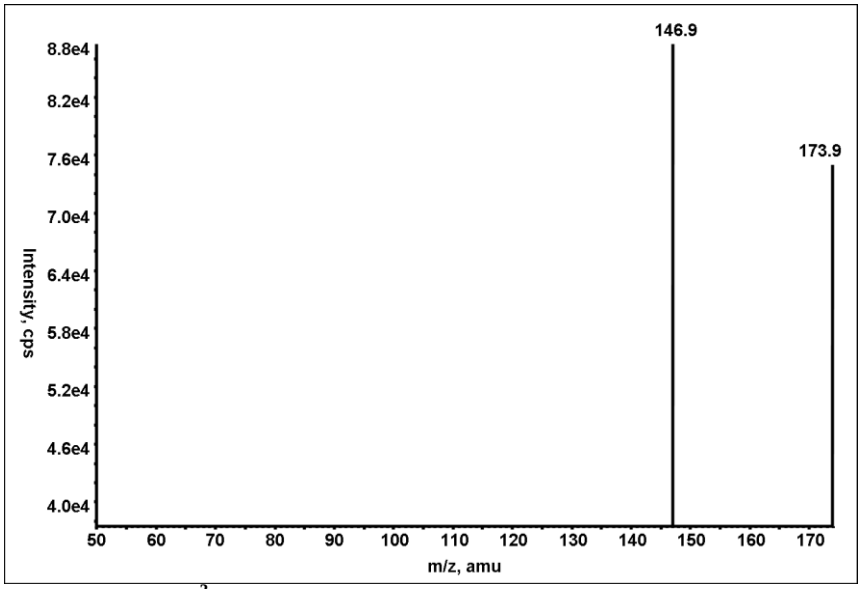


Figure S 3-3: MS^3 spectrum of production m/z = 175 of M8/208.

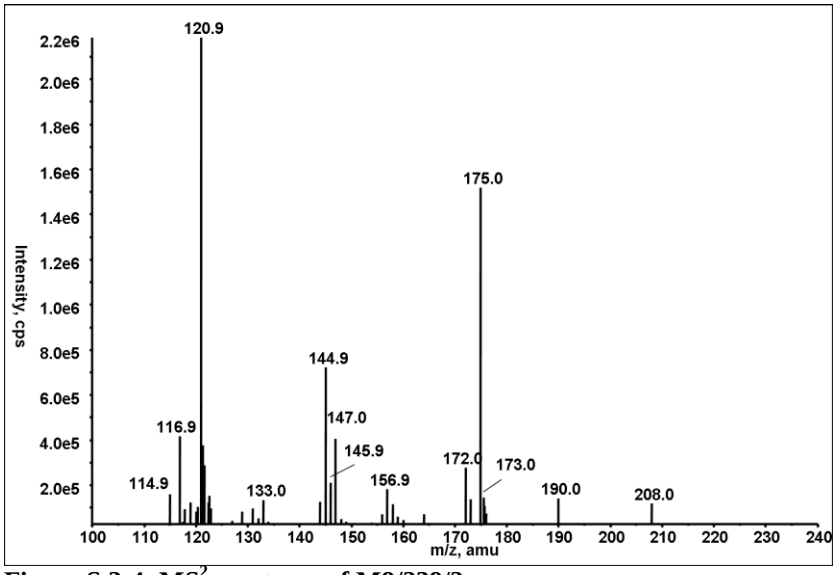


Figure S 3-4: MS^2 spectrum of M8/239/2.

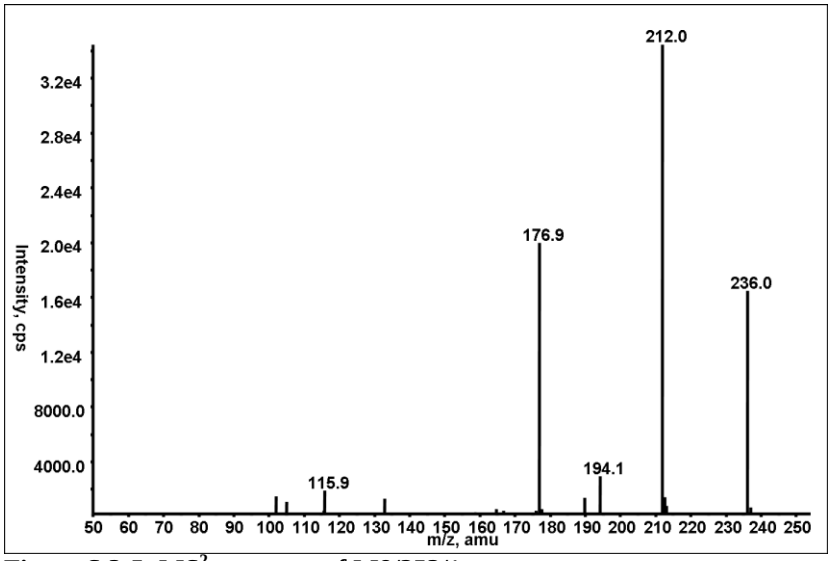


Figure S 3-5: MS^2 spectrum of M8/253/1.

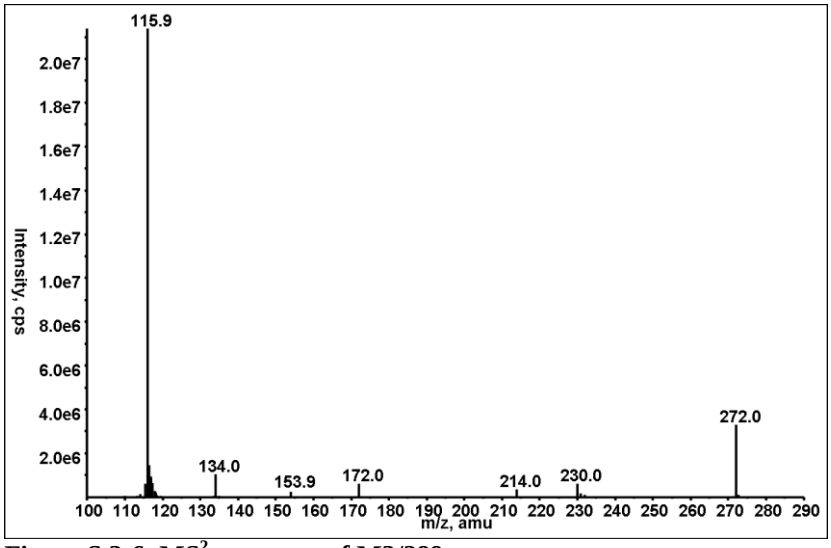


Figure S 3-6: MS^2 spectrum of M3/289.

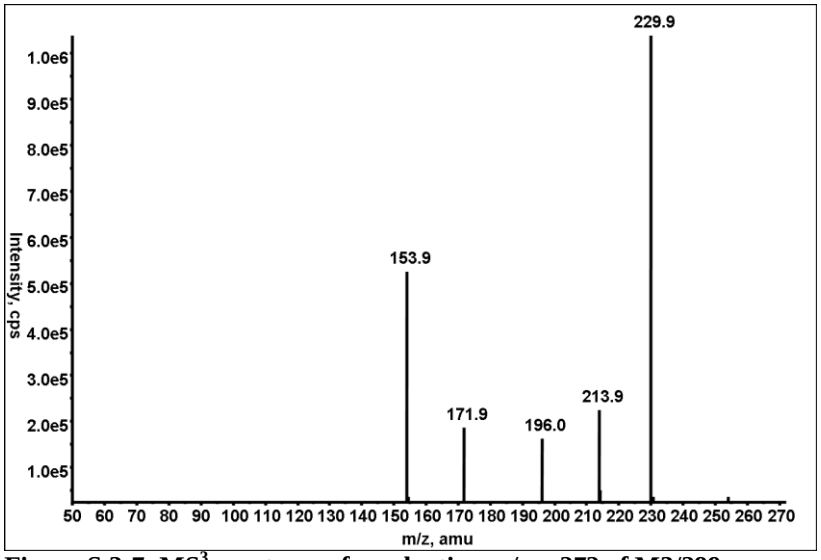


Figure S 3-7: MS^3 spectrum of production m/z = 272 of M3/289.

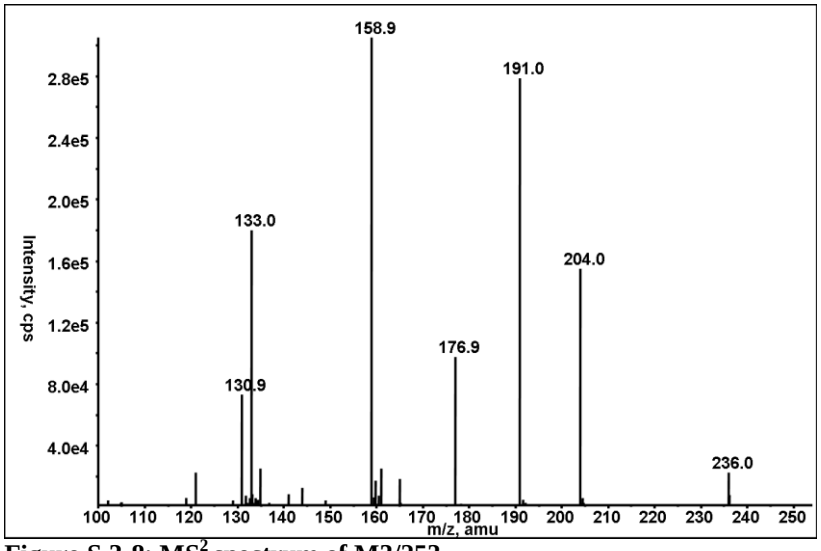


Figure S 3-8: MS^2 spectrum of M3/253.

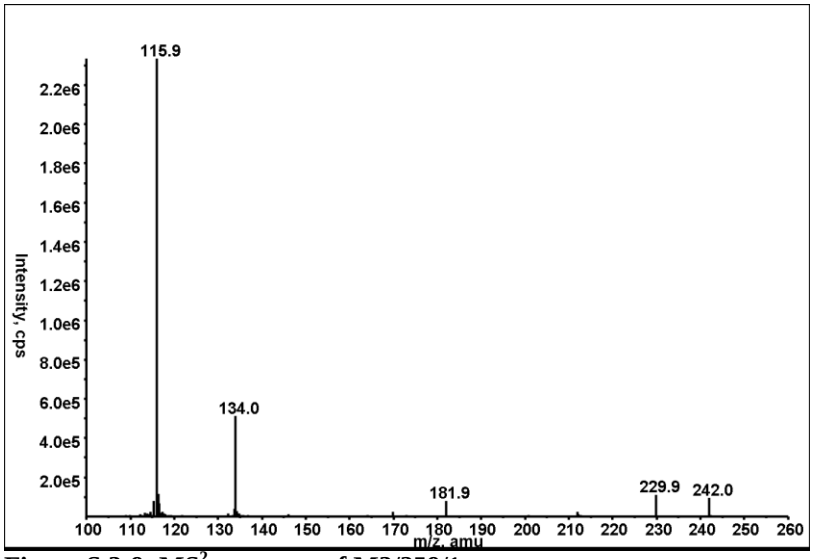


Figure S 3-9: MS^2 spectrum of M3/259/1.

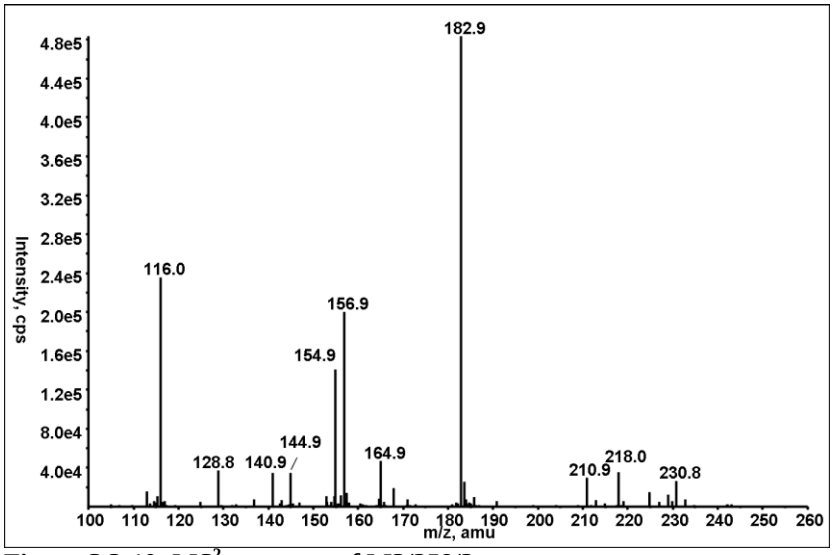


Figure S 3-10: MS^2 spectrum of M3/259/2.

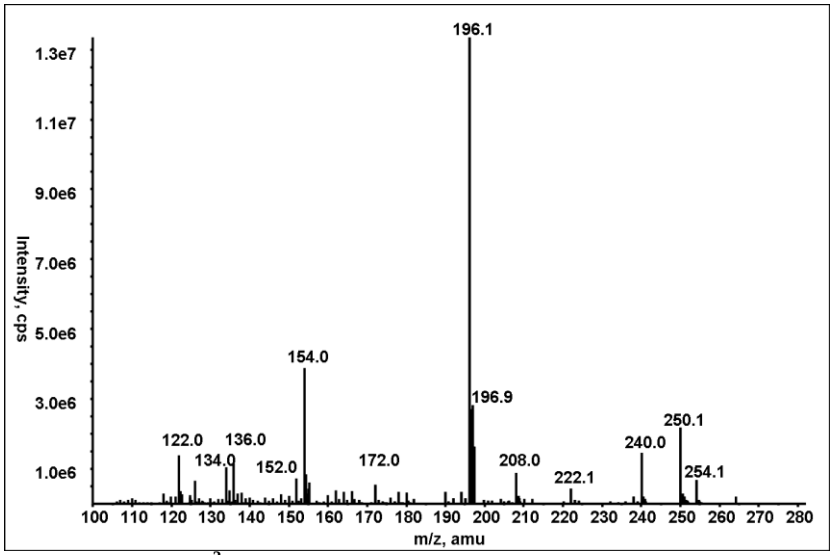


Figure S 3-11: MS^2 spectrum of M3/281.

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4 Ozonation of Propranolol: Formation of Oxidation Products

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Submitted to Environmental Science and Technology

Abstract

Major oxidation products (OPs) of the beta blocker propranolol, formed during ozonation in aqueous solution were identified and oxidation pathways were proposed. Ozonation led to a high number of OPs being formed. In total, chemical structures were elucidated for seven different nominal masses (including constitutional isomers, approximately 15 signals), while at least eight other OPs and their isomers (~ 30 signals) remained unidentified. The structural elucidation was performed via liquid chromatography coupled with hybide triple quadrupol with linear ion trap (LC-Qq LIT MS). The primary ozonation product, OP-291, was formed by ozone attacking the naphthalene ring, which resulted in the ring opening and two aldehyde moieties being formed. OP-291 was further oxidized to OP-307, which was then oxidized to OP-281. Experiments were performed at pH 3 and pH 8, as well as in the presence and absence of a radical scavenger. Ozonation of wastewater treatment plant (WWTP) effluents spiked with propranolol (10 µM) led to the same OPs being formed as observed in the experiments with deionized water. Therefore, ozonation of WWTP effluent is resulting in the formation of a high number of OPs with an elevated toxic potential (i.e. formation of aldehydes).

4.1 Introduction

Propranolol, a nonselective beta-adrenergic receptor blocker, is used in the treatment of cardiac malfunctions (e.g. hypertension or angina pectoris). Although only about 1 % of the

applied propranolol dose is excreted unchanged (Walle et al. 1985), it was found in WWTP effluents at concentrations up to 0.5 µg/L (Bendz et al. 2005, Gros et al. 2006, Ternes et al. 2003). Several studies focusing on the toxicological potential of propranolol signify an environmental relevance. Huggett et al. (2002) showed an effect on the reproduction and steroid levels in medaka (*Oryzias latipes*) at propranolol concentrations as low as 0.5 µg/L for a 4-week exposure experiment. For mixtures of beta blockers toxicological effects were also found at lower concentrations. Thus, even lower concentrations of propranolol might significantly contribute to the overall toxic potential of a mixture of beta blockers or other micropollutants present in the aquatic environment. This might be of even more concern if the concentrations of beta blockers increase in the aquatic environment, due to demographic reasons with an increasing percentage of older people, as expected for Germany, in the next few decades.

Ozonation was found to be an efficient technique for the attenuation of micropollutants such as pharmaceuticals (Huber et al. 2005, Suarez et al. 2007, von Gunten 2003). Nevertheless, with economically relevant ozone doses it is not possible to mineralize micropollutants. As a consequence, oxidation products (OPs) with unknown toxicological and ecotoxicological potentials are formed. Only for a few micropollutants have the main OPs during ozonation been identified (e.g. carbamazepine (McDowell et al. 2005), 17α-ethinylestradiol (Huber et al. 2004), diclofenac (Sein et al. 2008) or iomeprol (Seitz et al. 2008)). The techniques to identify the chemical structures of OPs used in these studies were LC-MS, LC-tandem MS, and NMR analysis. Especially for NMR analysis, isolation of high amounts of purified OPs (1-10 mg) is essential. In the current study an identification procedure was applied without NMR, using LC-UV, LC-tandem MS, and MS^3 with a linear ion trap. In cases where isolation was impossible, due to separation problems or to the formation of unstable OPs, this procedure is a possibility to at least attain possible chemical structures of the formed OPs.

Propranolol (refer to Table 4-2) has two functional moieties which are reactive towards ozone: i) the naphthalene ring activated by the electron donating oxygen and ii) the secondary amine-group. The protonated and positively charged amine moiety does not react with ozone. At pH 8 only about 3% of the propranolol amino group is deprotonated due to a pK_a (amino group) of 9.5. However, compared to other beta blockers, propranolol reacts very fast at an acidic pH in the protonated form with ozone (Benner et al. 2008). This indicates the main reactions are also occurring at the naphthalene moiety at high pH values. A study of naphthalene ozonation in a H_2O/MeOH mixture (50/50) indicated several oxidation pathways for the initial ozone attack (Legube et al. 1986). Possible reactions were either electrophilic substitution resulting in hydroxylation or a ring opening via a 1,3-dipolar cycloaddition as described in the Criegée mechanism (Dowideit and von Sonntag 1998).

It has been documented that changes in pH have an influence on the potential for OH radical formation. At an acidic pH, only a small proportion of OH radicals are formed, while at higher pH values OH radical formation is preferred (von Gunten 2003). To investigate the influence of increasing pH and OH radical reactions on OP formation, experiments were performed at pH 3 and pH 8, and with and without the addition of tertiary butanol (*t*-BuOH) as a radical scavenger. Although, ozonation of WWTP effluent is an efficient tool to attenuate the discharge of beta blockers in the environment (Benner et al. 2008), the formation of OPs needs to be elucidated to be able to predict the toxicological potential of this advanced treatment technology.

The objective of this study was to identify potential OPs of propranolol at two different pH values in deionized water as well as in WWTP effluent.

4.2 Materials and Methods

4.2.1 Sample preparation

The aqueous ozone stock solution (~ 0.7mM) was prepared by sparging ozone containing oxygen through deionized water cooled with an ice-bath (Bader and Hoigne 1981). The concentration of the ozone stock solution was measured directly by a UV spectrometer at 258 nm using $\varepsilon_{(O3)}$=3000 $M^{-1}cm^{-1}$.

Ozone stock solution was added to the reaction solution containing propranolol (100 µM) and phosphate buffer (50 mM) at pH 3 or 8, resulting in an ozone:propranolol ratio of 1:5, 1:3, 1:1, 2.5:1, 5:1, 10:1. To compare the influence of OH radicals to OPs formation, samples were prepared with and without the addition of *t*-BuOH (100 mM) as a radical scavenger.

In addition, the effluent from a local municipal WWTP (DOC = 8.5 mg/L, pH 8.4) was spiked with propranolol (10 µM) and an ozone stock solution was added to obtain ozone:propranolol ratios of 3:1, 6:1, 12:1.

4.2.2 Determination of molecular weights and fragmentation by mass spectrometry (MS)

An optimized gradient (Supporting Information (SI) Table S4-1) was used to determine the molecular weights of the OPs by Q1 scans using electrospray ionization (ESI) tandem MS. Because ionization of the OPs in negative mode was not successful, all measurements were carried out in the positive mode.

For structural elucidation, the MS fragmentation pathways of the OPs were studied by performing product ion scans using the linear ion trap of the LC tandem MS (EPI; enhanced product ion) and MS^3 scans. The obtained product ions were incorporated into a LC tandem MS method using multiple reaction mode (MRM) transitions. The detailed method is shown in SI (Table S4-1 to S4-3).

4.3 Results and Discussion

4.3.1 Identification of nominal masses of OPs

The formation of several OPs was detected by LC-UV in ozonated deionized water spiked with propranolol depending on the ozone:propranolol ratio applied. Figure 4-1A shows an example at pH 8 with the addition of *t*-BuOH. Using LC-Q1 scans (Figure 4-1B) with an advanced eluent gradient (refer to Table S4-1) an elevated number of OPs were observed. In total, more than 40 signals with 15 different nominal masses were detected. This is a surprising high number considering that ozonation reactions are usually known to be specific because only molecule sites with enhanced electron densities are attacked (von Gunten 2003).

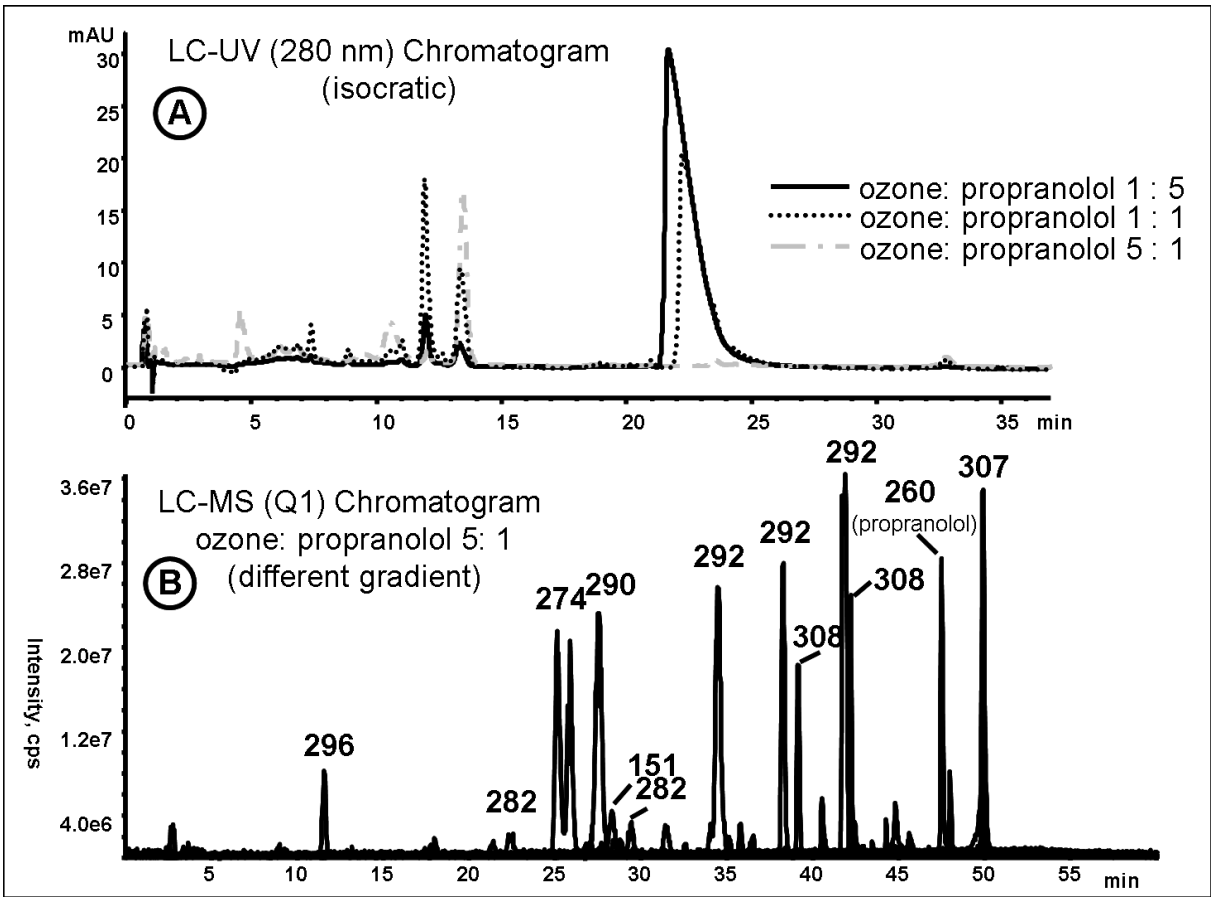


Figure 4-1Chromatograms of ozonated deionized water spiked with propranolol at pH 8 with the addition of *t*-BuOH, A: LC-UV chromatogram at three different ozone:propranolol ratios, B: LC-(+)ESI Q1 scan at an ozone:propranolol ratio (5:1).

To investigate the influence of increasing pH and OH radical formation on the OP formation, experiments were performed at pH 3 and pH 8, both with and without the addition of *t*-BuOH (100 mM) as radical scavenger. OH radical formation was tracked by addition of pCBA (2 µM). Table 1 provides the relative loss of pCBA in the different samples.

Surprisingly, even in the experiments with the addition of t-BuOH the losses of pCBA of up to 9 % indicated the presence of OH radicals. Due to the high consumption of OH radicals not only by pCBA but mainly by t-BuOH and propranolol (refer to rate constants in Table 1) even the very small attenuation of pCBA (e.g. 3 % at pH 8 in scavenged experiment) implies the formation of OH radicals. This assumption is confirmed by the high number of OPs formed.

Table 4-1: Propranolol (100 µM) and pCBA (2 µM) Attenuation in Samples at pH 3, pH8 with and without Addition of t-BuOH as well as in WWTP Effluent (10 µM propranolol).

experiment	ozone:propranolol ratio	decrease pCBA [%]	decrease propranolol [%]
pH 3 scavenged (100 mM t-BuOH)	1:5	1	0
	1:3	0	8
	1:1	0	55
	2.5:1	1	100
	5:1	4	100
	10:1	9	100
pH 3 non scavenged	1:5	1	8
	1:3	2	10
	1:1	0	71
	2.5:1	25	100
	5:1	36	100
	10:1	66	100
pH 8 scavenged (100 mM t-BuOH)	1:5	1	5
	1:3	0	18
	1:1	0	42
	2.5:1	0	100
	5:1	0	100
	10:1	3	100
pH 8 non scavenged	1:5	3	3
	1:3	4	9
	1:1	17	36
	2.5:1	82	100
	5:1	100	100
	10:1	100	100
WWTP effluent (1µM pCBA)	3:1	23	97
	6:1	43	98
	12:1	45	100

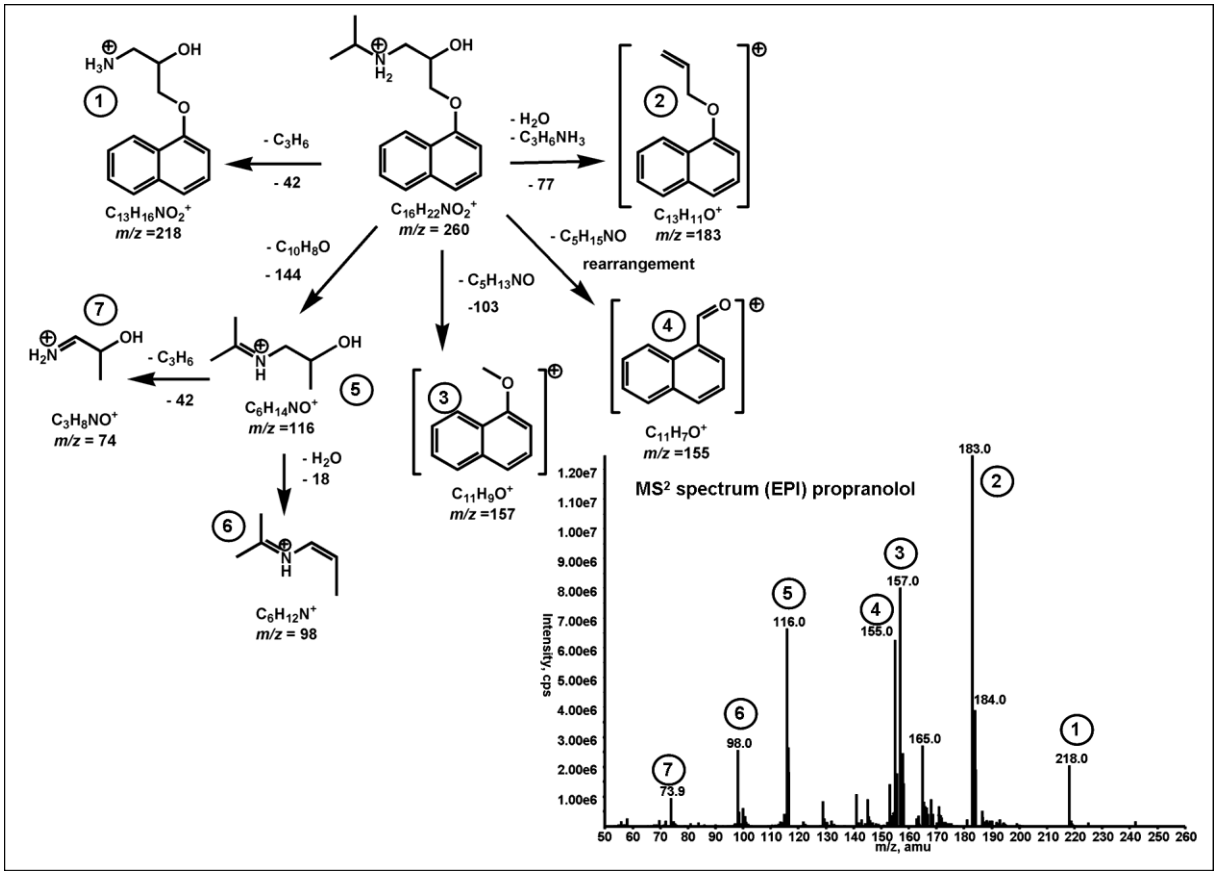
$k_{app,OH,\ pCBA} = 5 \cdot 10^{9}\ M^{-1}\ s^{-1}$; $k_{app,OH,\ t\text{-}BuOH} = 5.9 \cdot 10^{8}\ M^{-1}\ s^{-1}$; $k_{app,OH,\ propranolol} = 1 \cdot 10^{10}\ M^{-1}\ s^{-1}$

Although, literature always refer to excess of *t*-BuOH to be known to scavenge all OH radicals, these results show that in this experimental setup in addition to ozone, also a to a minor extent, OH radicals can be responsible for the formation of the propranolol OPs.

In addition to the peaks shown in Figure 1B, even more distinct OPs were found in the reaction systems without *t*-BuOH scavenge due to enhanced presence of OH radicals. Based on the relatively high intensities of OP signals observed in the Q1 scans as well as in the UV spectra, it can be assumed that the individual peaks account for the majoer oxidation products formed. An accurate quantitative mass balance of the OPs formed was impossible because pure compound standards were not available. The high number of OPs with low intensities overlapping the signals of the main OPs caused major challenges regarding isolation of the OPs.

4.3.2 Fragmentation pathways

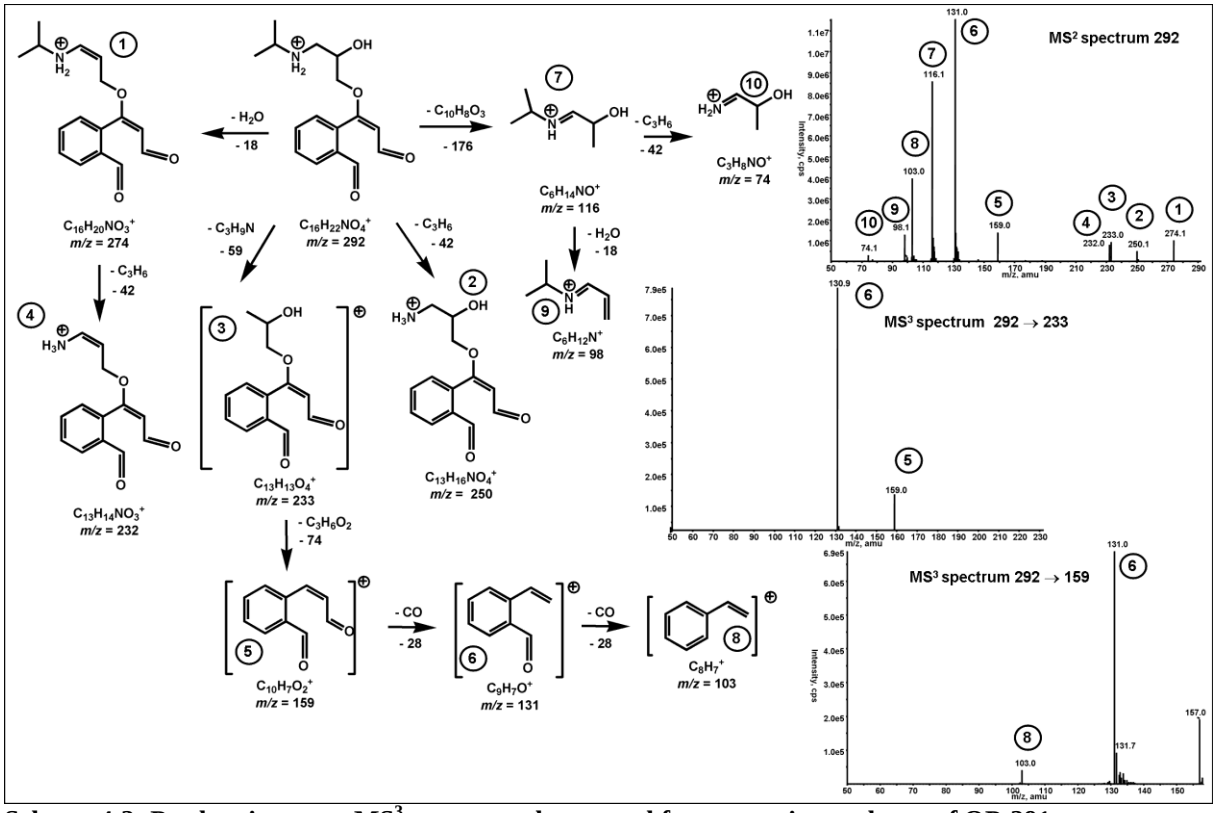
Propranolol. The product ion (MS^2) and MS^3 spectra of propranolol were completed and interpreted as a basis for elucidation of the chemical structures of the propranolol OPs.



Scheme 4-1: Proposed Fragmentation Pathway of Propranolol

The fragmentation pathway of propranolol (*m/z* = 260) is illustrated in Scheme 4-1. The loss of 42 Da indicates the cleavage of the isopropyl group and the neutral loss of 103 Da corresponds to a C-C bond cleavage on the side chain. A loss of 77 Da results in a *m/z* 183 fragment ion and corresponds to the cleavage of water, isopropene and ammonia (18 Da + 42 Da + 17 Da). The *m/z* 116, 98 and 74 fragment ions correspond to different moieties of the side chain being cleaved. These fragments are important for the comparison with the spectra of the OPs, as they provide information about the status of the side chain after oxidation. The methoxynaphthalene fragment ions *m/z* 157 and 155, resulting from a rearrangement of the C-O moiety, exhibited an unchanged aromatic ring system. A similar rearrangement was found for metoprolol as well as some of metoprolols OPs (Benner and Ternes 2009).

OP-291. Several peaks with a nominal mass of 291 were found. Their MS^2 and MS^3 spectra exhibited equal patterns. Scheme 4-2 illustrates one example for the possible isomers shown in Table 4-2. The OP-291 resulted from the addition of two oxygen atoms (+ 32 Da) to the propranolol molecule. In the MS^2 spectra the neutral loss of 42 Da, 59 Da (42 Da + 17 Da) and the occurrence of the *m/z* 116 fragment ion indicated that the isopropyl-amine moiety of the side chain remained unchanged (Scheme 4-2). The single loss of water (-18 Da) confirmed that only one hydroxyl group next to a removable proton is present.



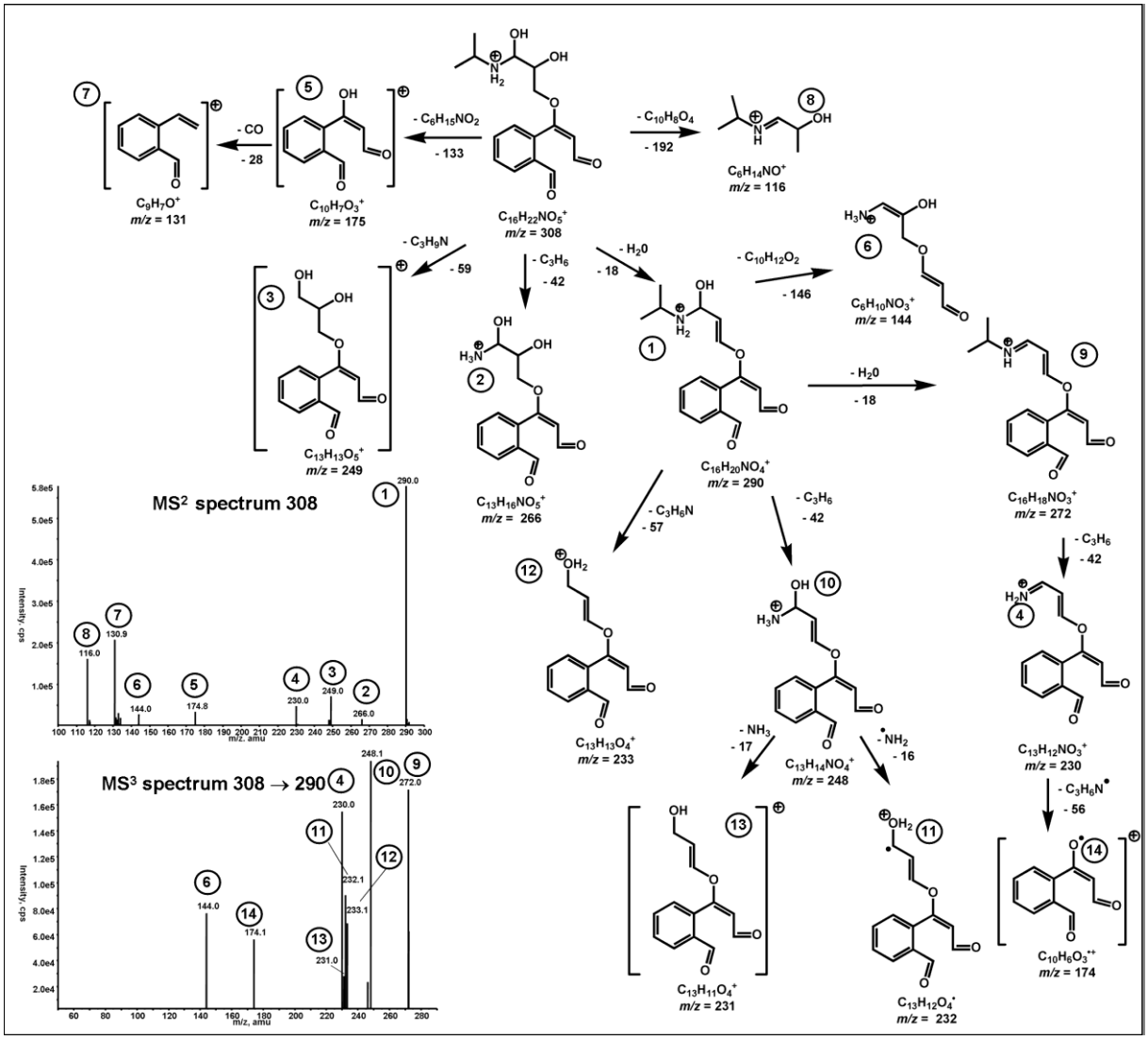
Scheme 4-2: Product ion scan, MS^3 spectra and proposed fragmentation pathway of OP-291.

The *m/z* 159 fragment ion implied that oxidation took place at the naphthalene moiety. The proposed formation of two aldehyde moieties instead of a double hydroxylation on the ring was supported by the fragment ion *m/z* 131 (159 Da -28 Da; loss of CO). The MS^3 spectrum of *m/z* 233 ion illustrated fragment ions *m/z* 159 and 131 were formed, and the MS^3 of the fragment ion *m/z* 159 led to the formation of *m/z* 131 and *m/z* 103 fragment ions. These fragments supported the proposed fragmentation pathway. The fragment ion *m/z* 103 resulted

from the loss of two carbon monoxide (CO) molecules, and confirmed the presence of two aldehyde moieties.

OP-307. The difference in molecular weight of propranolol and OP-307 is 48 Da corresponding to an addition of three oxygen atoms. Figure 4-1B illustrates that at least two signals were detected with a *m/z* of 308 and that the MS^2 and MS^3 spectra of these OPs were very similar, a formation of isomers can be assumed. The product ion scan (MS^2) showing the fragment ion *m/z* 116 and a single loss of water (fragment ion *m/z* 290) could signify an unmodified side chain (Scheme 4-3). However, the MS^3 spectra of the fragment ion *m/z* 290 showed an intense fragment at *m/z* 272, indicating a second loss of water. This can be caused if a second hydroxyl moiety is present next to a removable proton. The mass transition from fragment ion *m/z* of 308 to *m/z* of 266 represented the cleavage of an unchanged isopropyl group which implied that the hydroxylation did not take place at the isopropyl moiety.

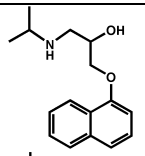
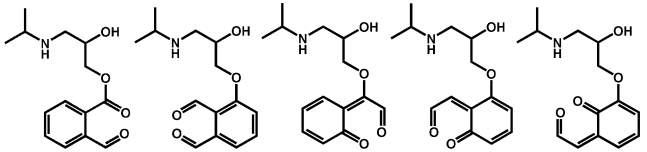
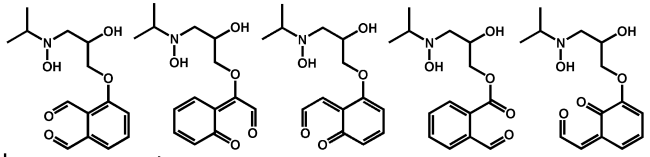
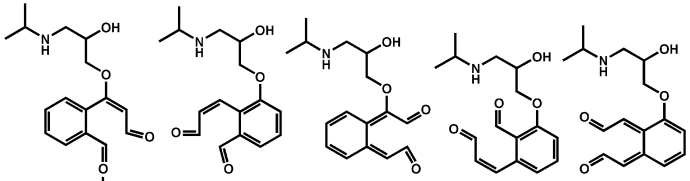
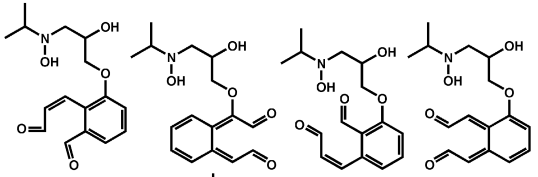
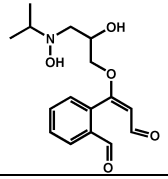
Hydroxylation can either occur by a hydroxylation at the methylen moieties next to the hydroxyl group already present in propranolol or at the amine moietiy, leading to a formation of a hydroxyl amine. A hydroxylation next to the ethoxy group would form a very unstable hemiacetal and a hydroxylation next to the amine moiety would cause an even more unstable hemiaminal. Hence, it is very likely that the amine moiety was directly hydroxylated. Scheme 3 presents the proposed fragmentation pathway of the hydroxylamine OP-307. Depending on the pH hydroxylamines are in an equilibrium with their deprotonated form, the *N*-oxides. As the eluent of the LC/MS system was always acidic (pH 2-3), the products detected were the hydroxylamines. The formation of the proposed fragment ion 3 (*m/z* = 249 Da) is only possible if a Meisenheimer rearrangement is considered, such as the Meisenheimer rearrangement reported for *N*-oxides at high temperatures (March 1985) .



Scheme 4-3: Product ion scan, proposed fragmentation pathways, and MS^2 and MS^3 of OP-307-RT43.

In case of the signal with a retention time (RT) of 42.8 min, it was possible to identify the exact position of the aldehyde moieties in the aromatic system. Fragment 6 (m/z = 144) appearing in the product ion scan as well as in the MS^3 spectra of the fragment ion *m/z* 290 suggested a ring opening of the naphthalene moiety at the 3,4-position (Scheme 4-3). Scheme 4-3 illustrates the general fragmentation pathway for all OP-307 isomers as described above.

Table 4-1: Retention Times in the Optimized Gradient and Proposed Structures of Propranolol and the Main OPs.

m/z ratio	RT [min]	Molecular formula	Abbreviation	Proposed structures (all possible constitutional isomers are shown)
260	48.5	$C_{16}H_{21}NO_2$ (Propranolol)		
266	32.5[b]	$C_{14}H_{19}NO_4$	OP-265	
282	19.0[a,c], 20.3[a,b,c] 22.0[a,b] 26.9[a,b,c]	$C_{14}H_{19}NO_5$	OP-281	
292	32.3[a,c] 40.3[a,b,c] 41.8[a,b,c]	$C_{16}H_{21}NO_4$	OP-291	
308	20.1[a] 38.8[a,b,c] 40.9[a]	$C_{16}H_{21}NO_5$	OP-307	
308	42.8[a,b,c]	2-{1-[2,3-dihydroxy-3-(propan-2-ylamino)propoxy]-3-oxoprop-1-en-1-yl}benzaldehyde	OP-307-RT43	

[a] found in experiment at pH 8 [b] found in experiment at pH 3 [c] found in experiment in the matrix sample (WWTP effluent)

4.3.3 Oxidation product formation pathways.

OP-291. The main ozonation product, OP-291, is formed by an ozone attack on each side of a double bond of the activated aromatic ring system. Following the well-known Criegee mechanism (Dowideit and von Sonntag 1998) the ring is opened via a 1,3-dipolar cycloaddition and two aldehyde groups are formed (Scheme 4-4). Several signals were found with *m/z* 292, but at distinct retention times, showing the same MS^2 and MS^3-fragmentation patterns. This implied the formation of constitutional isomers, presumably due to a 1,3-dipolar cycloaddition at one of the five possible sites of the activated naphthalene ring. However, due to the similar fragmentation patterns it was impossible to allocate the different isomers to the retention times. This might only be possible by isolation of every single OP in a quantity (approx. 1-10 mg) sufficient for NMR analysis, which was not possible in the current study.

OP-307. One possible reaction might be the oxidation of the aldehyde moiety to a carboxyl moiety (McDowell et al. 2005). However, the MS^2 product ion scans of OP-307 implied a different chemical structure, and the molecules with acidic moieties would have been detected in negative ionization mode. The OPs detected in the positive mode resulted most probably from a hydroxylation of OP-291 at the amine moiety. This reaction is known to occur mainly via an ozone oxidation mechanism.

A comparison of the peak areas of OP-291 (most intense signal at RT 41.8 min) and OP-307 (most intense signal at RT 38.8) in the four different reaction systems implies an influence of OH radical reactions to the formation of these OPs (Figure 4-2). Although they were found in all systems, the relative yield of the formed OPs differed significantly. At pH 3, OP-291 was formed to a much higher extent than OP-307, while at pH 8 the OP-307 formation was significantly increased. Comparing the formation of OP-291 in the scavenged and non scavenged reaction systems at the pH 8 confirmed a higher relative yield in the scavenged system. This confirmed that OP-291 is formed by an ozone attack of propranolol.

At lower pH, the stability of ozone is increased and the OH radical exposure is decreased (OH radicals promote ozone decay (von Gunten 2003)). In contrast, OP-307 is mainly found at pH 8, at a pH with a higher percentage of the deprotonated amine moiety, supporting the hydroxylamine formation. The higher yield without *t*-BuOH compared to the scavenged system indicates, that the presence of OH radicals increased the formation of OP-307, supporting an additional formation pathway via OH radicals attack.

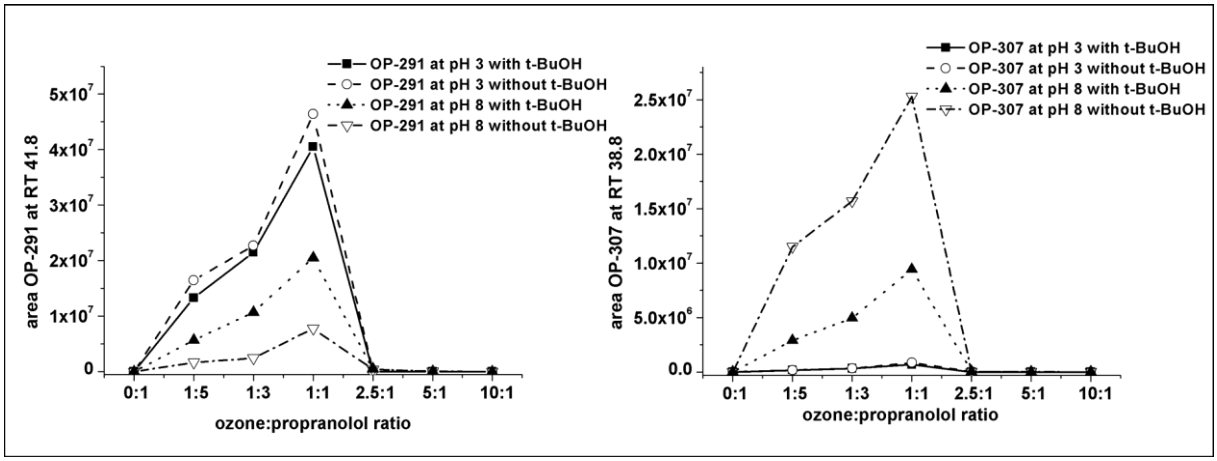
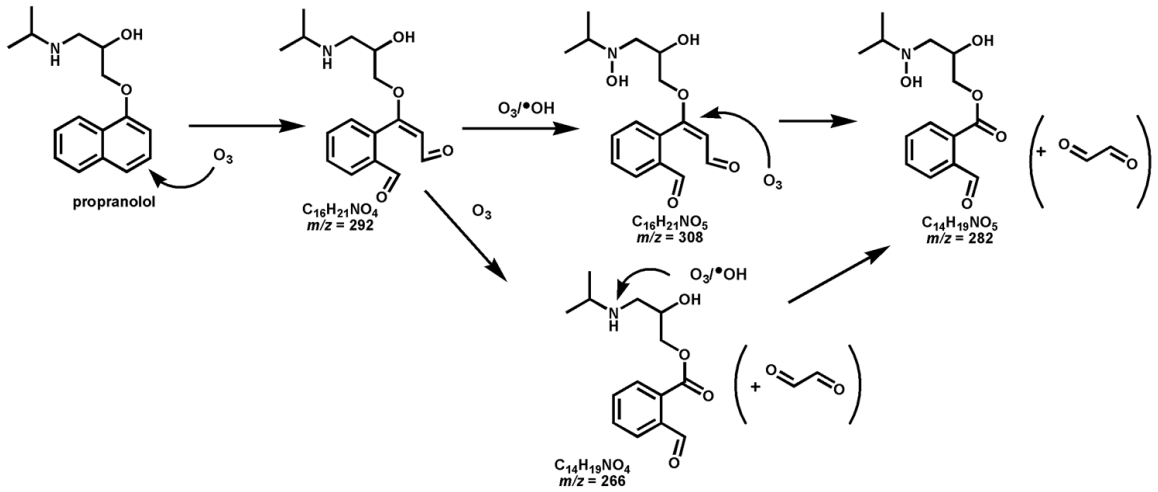


Figure 4-2: Peak areas of OP-291 and OP-307 in the batch experiments.

The OP formation of metoprolol by ozonation was different (Benner et al. 2008). OPs formed at pH 3 did not occur at pH 8 and *vice versa*, which can be explained by the slow ozone reaction rate and a relatively fast reaction rate with OH radicals at a higher pH. The naphthalene ring of propranolol appears to have a high reactivity, even at low pH values, and ozone and OH radical oxidation is likely to occur always, but to different extents (Benner et al. 2008).



Scheme 4-4: Reaction scheme for the oxidation of propranolol forming OP-291, OP-307, OP-281 and OP-265..

OP-265. OP-265 can be formed, if OP-291 was attacked by ozone at the double bond in α-position of the aldehyde groups, and thus a muconic aldehyde was cleaved according to the Criegée mechanism. OP-265 was detected only at pH 3, supporting the proposed reaction pathway. The chemical structures proposed (Table 4-2) were confirmed by the MS^2 spectra (refer to SI Scheme S4-2). Even though the formation of several isomers of OP-265 is possible, only one brought signal was detected. This could be explained by the insufficient separation of isomers during reverse phase chromatography or oxidation of only one certain isomer being dominant due to higher activation of the conjugated system in *ortho*-position of the ether bond.

OP-281. For the OP with *m/z* 282, there were two possible precursors: either OP-307, which is attacked by ozone and reacts the same way as described for the formation of OP-265 or OP-265 which oxidized in the same way as described for OP-307 (Scheme 4-4; MS data refer to SI, Scheme S 4-1). However, at pH 3 this oxidation of the amine is much less probable in favor of the other pathway via ozone reaction of OP-307.

4.3.4 Non-elucidated structures

The chromatogram in Figure 4-1 as well as Table S4 provides several OPs, for which the chemical structures could not be proposed, even though MS^2 and MS^3 spectra could be obtained for most of them. Further investigation with high resolution MS and NMR analysis is essential to elucidate these chemical structures.

4.3.5 Oxidation product formation in wastewater matrix

One possible application of ozone is the ozonation of WWTP effluents as post-treatment to reduce micropollutant discharges into surface waters. To investigate the matrix influence of treated wastewater on OP formation, a WWTP effluent (DOC = 8.5 mg/L, pH 8.4) was spiked with propranolol (10 µM; 2.6 mg/L) and was then ozonated with different ozone doses. A relative high concentration of propranolol was spiked because the samples had to be analyzed by direct injection, since enrichment by solid phase extraction (SPE) failed due to the high polarities of the OPs formed (description of extraction procedure is in SI Text S4-1). Ozone:propranolol ratios were selected to be 3:1 (1.35 mg O_3/L), 6:1 (2.6 mg O_3/L) and 12:1 (4.8 mg O_3/L).

To determine possible OH radical formation, *para*-chlorobenzoic acid (pCBA) (1µM) was added and analyzed by LC-UV. Using a MRM-method developed with the neat propranolol solutions, OP formation was measured. The pCBA analysis demonstrated significant OH radical formation (Table 4-1) during ozonation of the WWTP effluent.

Most of the OPs identified in the experiment with deionized water could also be found in the treated wastewater (Figure 4-3).

At the highest ozone dose of 4.8 mg O_3/L (~ 0.5 x DOC of the effluent) most of the OPs reached their maximum concentrations in the reaction systems. This indicates that most of them could be further oxidized with higher ozone doses. However, low ozone doses for

disinfection purposes (~ 2 mg O_3/L) might lead to an elevated formation of these OPs. This illustrates that the ozonation of a WWTP effluent containing compounds with activated aromatic moieties, especially naphthalene derivatives, result in the formation of a high number of products with unknown toxicological and ecotoxicological potential. The OPs of propranolol mainly contained aldehyde moieties, and several substances with aldehyde functions (e.g. the α,β-unsaturated carbonyl 4-hydroxynonenal) are known to interact with DNA and show genotoxic and carcinogenic properties (Richardson et al. 2007, Kuchenmeister et al. 1998, Roberts et al. 2003, Eckl et al. 1993). Bifunctional aldehydes such as glutaraldehyde even showed DNA-protein crosslinking, which could lead two false negative results in a commet assay, monitoring genotoxicity (Kuchenmeister et al. 1998). Therefore, the results from this study clearly illustrate the importance of investigating the formation of transformation products during treatment to better assess the impact these treatment technologies have on removing micropollutants before entering the environment.

4.4 Acknowledgment

The authors thank Manoj Schulz for the support in MS data elucidation and Jennifer Lynne Kormos and Carsten Prasse for reviewing the manuscript. We thank Prof. Dr. Clemens von Sonnatg for the fruitful discussion about the hydroxyl amine formation. Financial support by EU Commission for the EU-project RECLAIM WATER (Project No. 018309) is greatly acknowledged.

4.5 Supporting information

Text S 4-10 :HPLC/UV separation.

Prior to MS detection an optimization of OP peak separation was performed with an Agilent 1100 HPLC system (Agilent Technologies, USA-Santa Clara) using a Synergi 4u Hydro-RP column, 3 mm i.d., 250 mm, 4 µm (Phenomenex®, Aschaffenburg, Germany) at room temperature. Milli-Q water and acetonitrile both consisting of 0.5 % aqueous formic acid were used as eluents. The UV system was operated at 254 and 280 nm.

Table S 4-2: Optimized liquid chromatography gradient used for LC-MS and LC/UV experiments

Step	time [min]	flow rate [µl/min]	A [%]	B [%]
0	0.0	400	95.0	5.0
1	30.0	650	90.0	10.0
2	40.0	750	80.0	20.0
3	50.0	800	60.0	40.0
4	55.0	400	95.0	5.0

A: Milli-Q water with 0.5 % formic acid; B: acetonitrile with 0.5 % formic acid

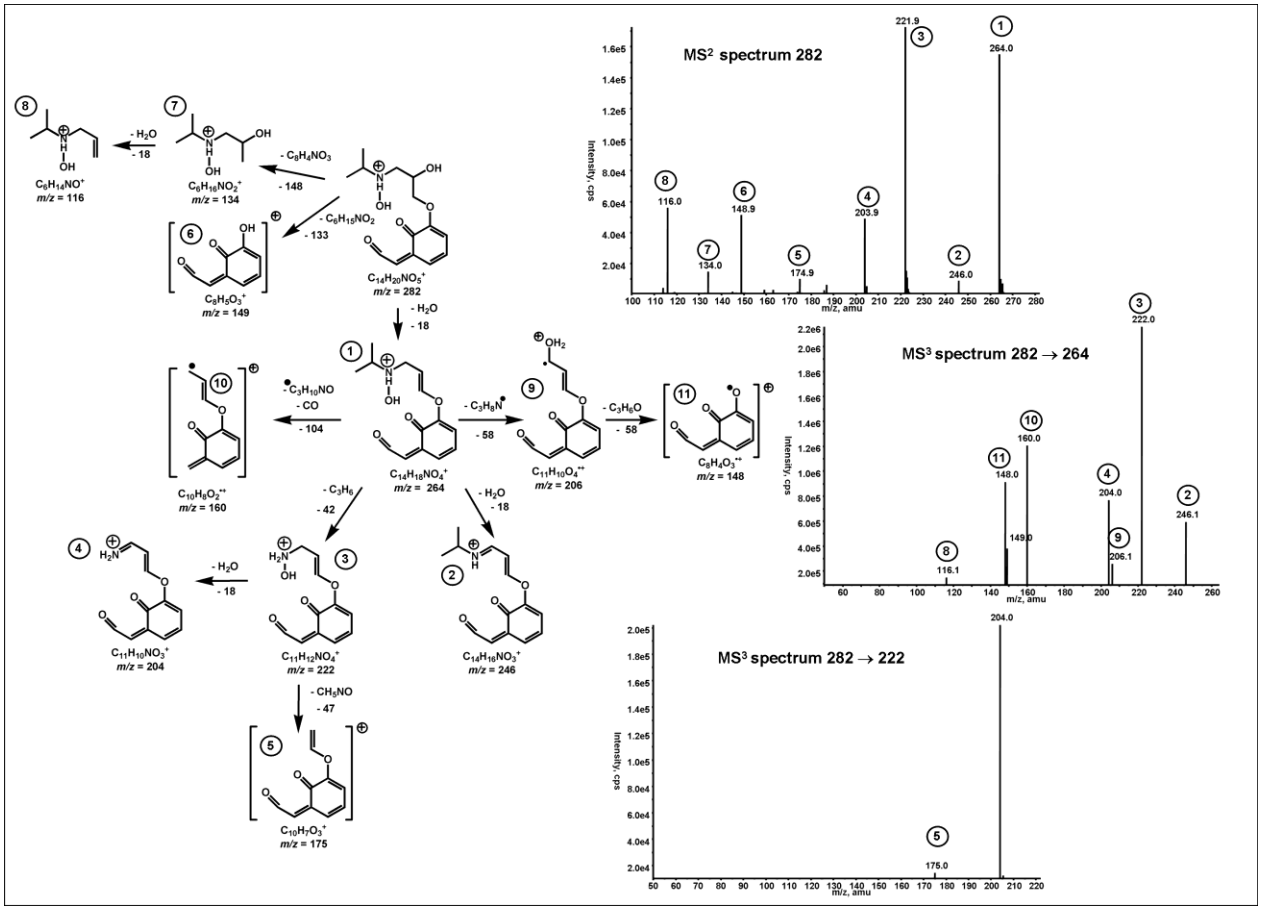
Table S 4-3: LCMS setup properties

LC Pump Properties	
Pump Model:	Agilent 1200 Binary Pump SL
Dead Volume (µl)	40.0
Columnoven Temperature	28 °C
Autosampler Properties	
Autosampler Model	Agilent 1200 High Performance Autosampler SL
Syringe Size (µl)	100
Injection Volume (µl)	50.0
Source Parameter	
Source and desolvation temperature	450.0 °C
collision activated dissociation gas	High
capillary voltage positive mode	5500.0
Scan Type	MRM (multiple reaction mode)
Polarity	Positive
Ion Source	Turbo Spray

Text S 4-11: Fragmentation pathway of OP-281.

Chemical structure confirmation of OP-281 was performed in a similar way as for OP-307. The elimination of two water molecules (fragment ion *m/z* 246), the loss of 60 Da (42 Da + 18 Da) corresponding to cleavage of a water molecule and the isopropyl moiety, as well as the fragment ion *m/z* 134 implied the same chemical structure for the side chain of OP-307.

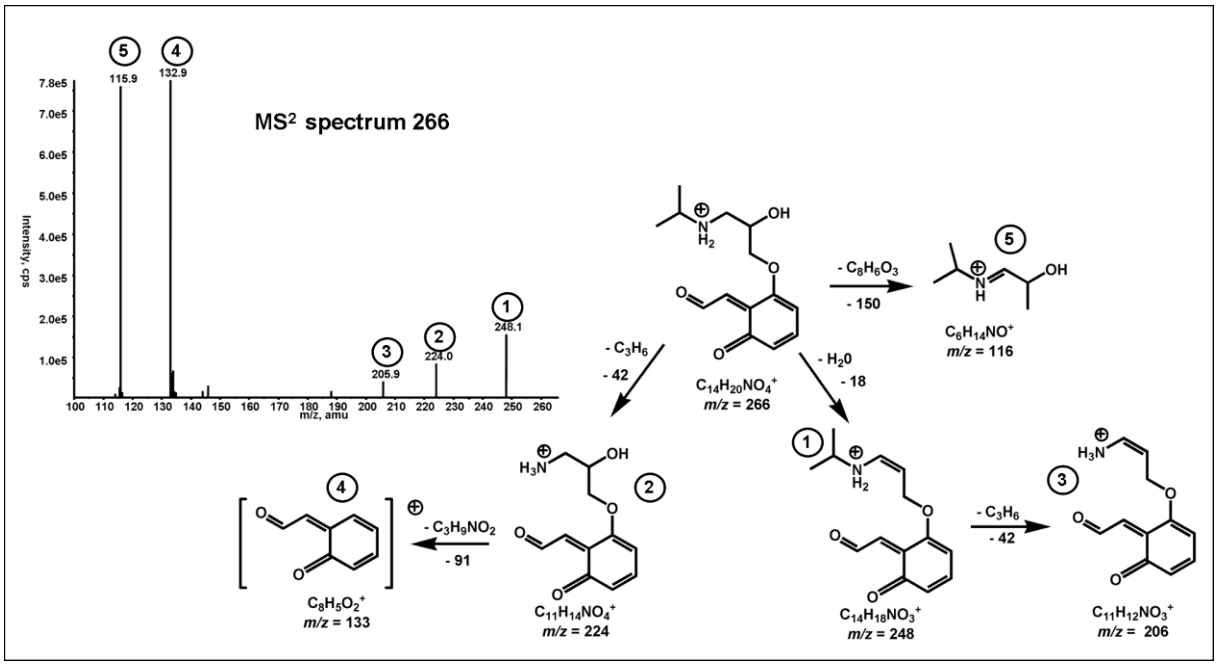
The *m/z* 149 and *m/z* 148 fragment ions indicated that the remaining molecule was smaller than for OP-307. The proposed structure shown in Scheme 4 could be confirmed by the MS^2 and MS^3 spectra. The MS^3 spectra of *m/z* 264 and *m/z* 222 fragment ions corroborated the proposed fragmentation pathway of *m/z* 264 → 222 → 204 → 175 (Scheme S 1).



Scheme S 4-6: Product ion scan and proposed fragmentation pathway for OP-281.

Text S 4-12: Fragmentation pathway of OP-265.

For OP-265 only a product ion scan (MS^2) could be recorded. The intensity of the higher mass fragments were too small for an accurate MS^3 spectrum. However, an interpretation of the MS^2 spectrum resulted in a proposed structure. The fragment ion *m/z* 116, the loss of an isopropyl group (loss of 42 Da) and a single elimination of water signified once more an unchanged side chain. The very intense and stable fragment ion at *m/z* 133 confirmed the proposed structure shown in Scheme S 2.



Scheme S 4-7: Product ion scan and proposed fragmentation pathway of OP-265.

Table S 4-4: Multiple reaction mode (MRM) transitions for propranolol OPs.

Q1 →Q3 *m/z* (amu)	dwell time [ms]	Collision energy [V]	collision cell exit potential [V]	Entrance potential [V]	Declustering potential [V]
260 → 116 (propranolol)	25	27	10	10	50
260 →183 (propranolol)	25	25	10	10	50
266 → 174	100	35	10	10	50
266 → 248	100	35	10	10	50
266 → 146	100	35	10	10	50
282.3 → 148.9	100	30	10	10	50
282 → 120.9	100	50	10	10	50
282.3 → 115.9	100	30	10	10	50
292 → 130.9	100	50	10	10	50
292 → 158.9	100	30	10	10	50
292 → 116	100	35	10	10	50
308 → 130.9	100	50	10	10	50
308 → 115.9	100	30	10	10	50
308 → 219.9	100	35	10	10	50
transitions of unidentified OPs:					
151 → 132.8	100	30	10	10	50
151 → 104.9	100	30	10	10	50
151 → 77	100	35	10	10	50
167 → 148.8	100	30	10	10	50
167 → 85	100	35	10	10	50
175 → 128	100	35	10	10	50
175 → 144	100	35	10	10	50
175 → 155.9	100	30	10	10	50
218.3 → 157.8	100	35	10	10	50
246.3 → 157	100	35	10	10	50
274 → 256	100	35	10	10	50
274 → 214	100	35	10	10	50
274.4 → 143	100	50	10	10	50
274.4 → 132.9	100	50	10	10	50
274.4 → 116	100	30	10	10	50
290 → 148.9	100	30	10	10	50
290 → 146.9	100	50	10	10	50
296 → 278	100	35	10	10	50
296 → 133.9	100	30	10	10	50
296 → 130.9	100	30	10	10	50
296 → 116	100	35	10	10	50
307.3 → 219	100	30	10	10	50
314 → 286	100	35	10	10	50
314 → 198.9	100	30	10	10	50
314 → 115.9	100	30	10	10	50
324 → 149	100	35	10	10	50
324 → 282	100	35	10	10	50
324 → 116	100	35	10	10	50
324 → 147	100	35	10	10	50
338 → 115.9	100	30	10	10	50
349 → 116	100	30	10	10	50
379 → 133	100	30	10	10	50
379 → 177	100	30	10	10	50

Table S 4-5: List of unidentified OPs and the MS^2 and MS^3 fragments.

m/z ratio	RT [min]	MS^2 fragments (*m/z* ratio, in order of intensity)	MS^3 fragments (*m/z* ratio, in order of intensity)
152	24.8 [a,b]	133; 77; 105; 95	**133:** 105
168	26.3 [a,b]	121; 111; 149	
175	41.7[a]	128; 156; 174; 144; 133; 146; 116; 91;	**156:** 128
	43.7[a]	103; 129; 145; 157; 147; 101; 77	
274	23.9[a,b,c]	214; 196; 158; 232; 197; 200;184; 170; 168; 133; 256	**214:** 196 **256:** 214
	24.6[a,b,c]	214; 256; 196; 158; 184; 200; 168; 143; 133; 232	**256:** 143; 214; 170
	41.2[a,c]	116	
	45.8[a]	232	
280	37.6[b] (only low ozone dose)	147; 116; 134; 262; 98; 74	
290	25.7[a]	149; 212; 174; 230; 216; 248; 200; 182; 172; 186; 159; 202; 184; 242; 272; 82; 254	**212:** 184; 194 **248:** 246; 230; 159; 176; 212
	33.2[a]	248; 174; 220	
	34.2[a]	248;174; 220; 230; 262; 146; 272	
	34.7[b]	116;	
	36.6[a]	116; 187; 215; 213; 175; 159; 272; 201; 189; 98; 248; 262; 231; 157	
	40.7[b]	116; 213; 272; 185; 98; 249; 157; 197	
	48.9[b]	220; 219; 262; 244; 160; 272;	
	51.3[a]	220; 219; 262; 160; 244; 192; 272	
296	13.0[a]	134; 116; 135; 278; 144; 218; 260; 136; 250;	**278:** 144; 260; 218
	20.6[a]	134; 116; 135; 278; 146; 250; 144; 190; 218; 260; 174	
	34.1[a]	278; 116; 236; 98; 161; 145; 134; 114; 203; 219; 234; 260	**278:** 220
307	25.8[b]	173; 135; 147; 191; 163; 145; 136; 174; 119; 209; 91;	
	40.9[a]	156; 174; 128; 116; 133; 134; 144; 289; 157; 175; 271; 247; 98	
	47.5[b]	261; 233; 205;289; 133	
	50.0[a,b]	219; 261; 159; 131; 271; 289; 243; 191; 105	
	51.2[a,b,c]	219; 159; 261; 157; 243; 271; 289; 233; 247; 105; 131	
314	19.5[a,b]	134; 116; 181; 135; 296; 92	
	29.1[b]	181; 116; 134; 296; 98	
	42.5[b]	181; 272; 237; 199; 116; 98; 296; 255; 193; 145	**237:** 236; 181
324	12.9[b]	163; 134; 145; 116; 135; 89; 117; 279; 296; 264; 191; 306; 237	
	13.5[b]	163; 134; 145; 116; 89; 164; 279; 296; 264; 191; 306	
	23.0[b]	306; 116; 246; 288; 218; 264; 278; 279; 236; 174; 149;	**264:** 246; 218 **306:** 288; 218; 246 **218:** 174; 200; 188; 162; 189; 146; 191

m/z ratio	RT [min]	MS^2 fragments (*m/z* ratio, in order of intensity)	MS^3 fragments (*m/z* ratio, in order of intensity)
	26.0[a,b]	149; 134; 147; 116; 280; 279;: 173; 191; 306; 151; 262; 288	**306:** 288
	32.6[b]	162; 144; 163; 134; 174; 306; 116; 98; 147; 133; 102; 279; 220; 246; 288	
	32.2[b]	282; 147; 229; 306; 149; 98; 279; 185; 173; 217; 247; 116; 205; 264; 199; 157	**247:** 229 **282:** 264; 229; 247; 265; 217; 209; 185
	33.5[b]	282; 147; 229; 98; 306; 149; 185; 173; 279; 116; 187; 217;205; 264; 157	**282:** 264; 229; 247; 265; 217; 209; 149;
	35.0[b]	306; 247; 280; 222; 279; 264; 175; 191; 282; 149; 246; 204; 187; 185; 147; 205; 116; 238; 203	**306:** 247 **282:**280: 264; 247
	37.6[a]	147; 134; 116; 306; 279; 149; 174; 156; 216; 98; 265; 173; 237; 288	
	40.9[a]	116; 147; 173; 280; 134; 279; 135; 119	
	41.4[a]	174; 306; 156; 234; 216; 133; 147; 144; 146; 143; 115; 128; 246; 288; 279; 264	**306:** 234; 174; 216; 288; 143; 156; 246; 133; 235; 159;
338	14.5[a]	116; 292; 310; 276; 149; 74	
	24.9[a]	116; 145; 149; 310; 180; 74	
349	41.3[a]	174; 156; 331; 116; 216; 289; 266; 307; 234; 133; 248	**331:** 289; 174; 244; 256; 197 **289:** 247; 156; 230; 229; 174; 146; 158; 248; 214 **216:** 174; 156

[a] found in experiment at pH 8 [b] found in experiment at pH 3 [c] found in experiment in the matrix sample (WWTP effluent and influent)

Text S 4-13: OP formation in matrix samples.

WWTP effluent (DOC = 8.5 mg/L, pH 8.4) were spiked with propranolol (10 µM) and ozone stock solution was added to obtain propranolol:ozone ratios of 1:3, 1:6, 1:12. OH radical formation was monitored in the matrix, by addition of 1µM para-chlorobenzoic acid (pCBA) to the samples. Attenuation of pCBA was detected by HPLC-UV at a wavelength of 234 nm (28).

Text S 4-14: Solid phase extraction (SPE) of propranolol OP

Ozone stock solution (210 mL) was added to 0.5 L of a stirred metoprolol solution (100 µM; with 100 mM t-BuOH) (metoprolol:ozone ratio 1:5). After the ozone was consumed completely, the solution was adjusted to pH 7 with hydrochloric acid (25 %). ENV+ (200 mg, Isolute, Biotage), MCX (60 mg, Water Oasis, Waters, Massachusetts, USA), HLB (200 mg, Water Oasis, Waters, Massachusetts; USA) and C_{18}- cartridges (500 mg, Bakerbond SPE, JT Baker, Deventer, Holland) were used to extract 200 ml each. The filtrates of the different

extractions were collected and the cartridges were eluted with 2x2mL methanol. The eluate, the filtrate and the original sample were analysed with the above mentioned LC-MRM method.

A comparison of the peak areas in the original sample and the filtrate showed, that 0 % of the OPs was retained on any of the solid phase materials. 0 % OPs could be detected in the eluates.

4.6 References

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5 General Conclusions

In Chapter 2 the second order rate constants for the reaction of four beta blockers (acebutolol, atenolol, metoprolol and propranolol) with ozone and OH radicals were determined. The rate constants of acebutolol, atenolol and metoprolol showed strong pH dependence due to the amine moiety. A comparison with propranolol signified about two orders of magnitude higher reactivity of the naphthalene ring than determined for the beta blockers with phenolic moieties.

A very specific characteristic of the study described in Chapter 2 was ozonation of reverse osmosis (RO brine) concentrate. For the presence of high concentrations of DOC and micropollutants, the concentrate was seen as a worst case scenario. Although ozone has a lower stability in samples with elevated DOC concentration (46 mg/L) of the RO brine, the moderate ozone doses applied (5-10 mg/L) in our experiments were sufficient to remove beta blockers efficiently. Therefore, ozonation of WWTP effluents and brines is a very efficient tool to decrease the discharge of beta blockers into the aquatic environment.

Additionally, the ozone exposure measurements of samples from the specific treatment train of the investigated drinking water production plant in Wulpen demonstrated increased ozone stability and extended life time of the ozone due to prechlorination. This might have led to a more efficient oxidation of micropollutants. However, OH radical exposure for a given ozone exposure was decreased, which could have led to a decrease in the oxidation of compounds with low rate constants for the direct reaction with ozone.

With the determined rate constants an the ozone as well as OH radical exposures measured in these samples the extent of how much propranolol was oxidized could be predicted.

Chapters 3 and 4 mainly focused on the formation of oxidation products (OPs) during the ozonation of metoprolol and propranolol. In both cases a high number of OPs were formed.

The pH dependence of the metoprolol rate constant enabled us to compare the influence of protonated and non protonated species on the formation of OH radical precursors ($^{\bullet}O_2^-$). This confirmed that the direct ozone reaction with non protonated secondary amines in a pure reaction mixture led to a stoichiometric $^{\bullet}O_2^-$ formation. In addition, the reaction of the aromatic moiety (protonated metoprolol species) contributed to the $^{\bullet}O_2^-$ formation, but to a significantly lower extent.

The structural identification of the OPs of metoprolol and propranolol signified that not only direct ozone reactions, but also OH radical oxidation influence OP formation. This made it very difficult to predict OP formation for structurally similar compounds, as OH radical reactions are rather unspecific. Nevertheless, the awareness of this result might help to elucidate the chemical structures of new OPs and their formation pathways.

The ozonation of metoprolol and propranolol led to the formation of aldehydes, which are known to posses genotoxic potential. During an experiment, which simulated wastewater conditions, metoprolol formed hydroxylated aromatic moieties which are known to show estrogenic activity. Another compound with a potential for high reactivity is the free amine, formed by the cleavage of the isopropyl group of metoprolol.

In general, the OP formation studies showed that ozonation of wastewater leads to the formation of a high number of OPs with unknown toxicological and ecotoxicological properties. For instance, 99% of the applied dose of propranolol is excreted as metabolites containing the naphthalene ring (Walle et al. 1985), and therefore they might be present in several aquatic compartments. Unfortunately, an investigation of these compounds is not possible, as most metabolites are not currently available as analytical standards. However, the reactivity of propranolol could be an indication that a large number of potentially toxic OPs are formed only from these metabolites.

Ozonation is already widely used for drinking water disinfection, with the potential of these relatively low ozone doses to result in the formation of various OPs.

For elucidation of the toxicological potential of each OP with common toxicity tests, high amounts of the pure compound would be needed. However, a relatively novel approach, not requiring high amounts of the compound, is the combination of thin layer chromatography (TLC) and a *Vibrio fischeri* suspension (Weins and Jork 1996). These luminous bacteria indicate a toxicological potential if their luminescence is inhibited. An ongoing study is focusing on the identification of single compounds showing inhibition on the TLC plate.

5.1 References

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