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Identification, Mechanisms and Kinetics of Macrolide Degradation Product Formation under Controlled Environmental Conditions

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Abstract

Erythromycin, azithromycin, clarithromycin and roxithromycin are antibiotics belonging to the widely used macrolide group. Their presence in the environment has been much investigated, despite the rapid degradation of Erythromycin to its spiroketal degradation product. In this study, the formation of macrolide degradation products was investigated in various aqueous solutions, each containing 100 µg/mL of the respective macrolide, under controlled artificial conditions: three phosphate buffer solutions (pH 5, pH 7 and pH 8.5), and a buffer solution at pH 7 with the addition of humic acids. Two solutions from natural sources were also examined: secondary effluent and tap water. The obtained degradation products were identified by their HRMS and NMR spectra (for Erythromycin-spiroketal, obtained from pure compounds isolated by preparative HPLC) as: N-oxide, N-desmethyl and N-didesmethyl forms of all examined macrolides. These degradation products were obtained only under irradiation by sunlight, while the Erythromycin-H_2O degradation products were also obtained in the shade. The secondary effluent was the most significant medium for achieving macrolide degradation products. According the degradation product's $t_{1/2}$ values obtained in the secondary effluent, the azithromycin was most rapidly degraded (23 hours). Furthermore, results suggested that the degradation process was activated by sunlight irradiation energy, and that the degradation mechanism started with the transfer of an electron from the amine group to O_2 to produce the radical ions $RMe_2N^{\cdot+}$ and $O_2^{\cdot-}$ as intermediates and production of the N-oxide and N-desmethyl macrolide degradation products. The kinetics of macrolide degradation was calculated as a first-order reaction.

Keywords: Macrolide; Degradation product; Secondary effluent; Humic acid; Photodegradation; LC-HRMS

Introduction

Degradation products (DPs) obtained from antibiotic residues are a recognized but mostly under-studied group of contaminants [1,2]. They may find their way into the aquatic environment where they are widely dispersed and persist for much longer than previously thought [3,4]. Moreover, drug DPs may be the result of natural biodegradation and/or chemical degradation (including advanced oxidation processes) during wastewater treatment. These DPs are suspected of being more resistant to degradation, and potentially more toxic, than their parent compounds [5-8]. For instance, the macrolide DPs N-desmethyl and N-didesmethyl macrolides have been reported to be biologically active [9,10].

The macrolides are an important group of antibacterial compounds that are commonly used for the treatment of upper and lower respiratory-tract infections. Erythromycin (ERY) is the first and most widely prescribed, orally administered member of this group. Due to its severe side effects, pharmaceutical manufacturers modified this compound to produce three other macrolide drugs: clarithromycin (CLA), roxithromycin (ROX) and azithromycin (AZI), which are widely used in livestock and human medicine. This group of molecules consists of a 14-membered (ERY, CLA and ROX) or 15-membered (AZI) lactone ring, with 10 asymmetric centers and 2 groups of sugar residues: L-cladinose and D-desosamine (Figure 1). ROX and AZI are distinguished from ERY by modifications in ERY's 14 lactone membered ring that prevent production of the undesirable DP "ERY-spiroketal", which is obtained after internal ketalization processes [11]. Additionally, CLA is distinguished from ERY by replacing the R^2 position from OH to O-CH_3 (Figure 1).

Macrolide DPs are frequently found in aquatic environments and their presence has been widely investigated, with a focus on their parent compounds ERY, CLA, ROX and AZI [12-18]. Moreover, several studies have reported detection of the ERY DP ERY-H_2O, which is probably the spiroketal product [14,19-22]. In fact, ERY-spiroketal is the only macrolide DP ever detected in the environment. In addition, a non-environmental study designed to obtain macrolide DPs recently revealed a potential effect of ERY on its tissue distribution and bioaccumulation in fish, and its metabolisis via demethylation to its N-desmethyl and N-didesmethyl DPs [23]. Studies analyzing related manufactured drug substances in macrolides found N-desmethylerythromycin E, erythromycin E N-oxide, anhydroerythromycin C, N-desmethylerythromycin B, anhydro-N-desmethylerythromycin A and pseudoerythromycin E enol ether [24]. Others, examining benzamycin which is a combination of benzoyl peroxide and ERY by liquid chromatography–mass spectrometry (LC-MS) showed the underlying oxidation process that produces DPs such as ERY-desmethyl and ERY-N-oxide [25].

In all of the studies in which macrolide DPs have been obtained, these compounds were synthesized. Freiberg [9] synthesized macrolides in which the two 3-dimethyl amino substituents of the desosamine and mycaminose moieties were N-demethylated and N-didemethylated by reaction with a halogen (preferably iodine) in the presence of a base to control pH. Napoletano [26] synthesized ERY, CLA and AZI DPs using UV irradiation, describing the synthesis of N-desmethylated macrolide DPs using methanol with sodium acetate and iodine. Similarly, Jakopović et al. [27] produced N-oxide, N-desmethylated, and N-didesmethylated CLA and AZI DPs.

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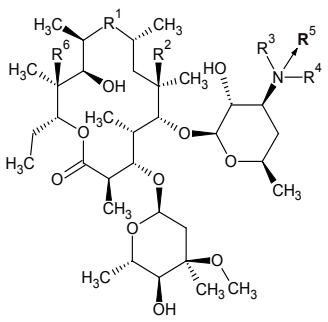


A = desosamine

B = cladinose (3-O- methylmycarose)

Macrolide name	R^1	R^2	R^3	R^4	R^5	R^6	pKa
ERY	C=O	OH	CH_3	CH_3		OH	8.8
ERY-N-desmethyl	C=O	OH	CH_3	H		OH	
ERY-N-didesmethyl	C=O	OH	H	H		OH	
ERY-N-oxide	C=O	OH	CH_3	CH_3	O	OH	
ERY-spiroketal	C	O	CH_3	CH_3		O	
ERY-spiroketal N-desmethyl	C	O	CH_3	H		O	
ERY-spiroketal N-oxide	C	O	H	H	O	O	
CLA	C=O	OCH_3	CH_3	CH_3		OH	8.9
CLA-N-desmethyl	C=O	OCH_3	CH_3	H		OH	
CLA-N-didesmethyl	C=O	OCH_3	H	H		OH	
CLA-N-oxide	C=O	OCH_3	CH_3	CH_3	O	OH	
AZI	CH3 N-CH_2	OH	CH_3	CH_3		OH	8.7/9.5
AZI-N-desmethyl	CH_3 N-CH_2	OH	CH_3	H		OH	
AZI-N-didesmethyl	CH_3 N-CH_2	OH	H	H		OH	
AZI-N-oxide	CH_3 N-CH_2	OH	CH_3	CH_3	O	OH	
ROX	C=N-OCH_2-CH_2-OCH_3	OH	CH_3	CH_3		OH	9.2
ROX-N-desmethyl	C=N-OCH_2-CH_2-OCH_3	OCH_3	CH_3	H		OH	
ROX-N-didesmethyl	C=N-OCH_2-CH_2-OCH_3	OCH_3	H	H		OH	
ROX-N-oxide	C=N-OCH_2-CH_2-OCH_3	OCH_3	CH_3	CH_3	O	OH	

Figure 1: Structures of macrolides and their degradation products.

To the best of our knowledge, there has been no report on the chemical behavior or identification of macrolides under controlled environmental conditions. Thus, the main objective of this study was to use controlled environmental conditions to obtain selected macrolide DPs, to verify their chemical structure, and to understand their degradation mechanisms.

Experimental

Standards and reagents

ERY (95.5%), AZI (96–102%), CLY (96-102%) and ROX (>90%, HPLC grade) analytical standards were purchased from Fluka (Israel). Acetonitrile, methanol, ethanol and water (all ULC/MS grade) were purchased from Bio-Lab (Israel). Humic acid was purchased from Fluka. Ammonium formate (>98%) was purchased from Fisher and ammonia (32%) from Merck. Phosphoric acid (H_3PO_4, 80-90%) was purchased from Fluka, sodium phosphate (98-100.5%) from Riedal-de Haen and hydrogen peroxide (H_2O_2, 30 wt. % in water) was purchased from Sigma. Tap water (TW) was collected from the Hydrochemistry Laboratory at Tel Aviv University. Field secondary effluent (SE) was taken from the Shafdan wastewater-treatment plant.

Obtaining DPs under sunlight irradiation and shade

Four solutions containing, respectively, ERY, CLA, AZI and ROX (each at a concentration of 100 µg/mL) were prepared. Three different phosphate buffer solutions were examined under controlled artificial conditions pH 5, pH 7 and pH 8.5 and a fourth phosphate buffer solution at pH 7 contained 5 mg/L humic acid. The three different pH's were selected to examine the behavior of macrolides under natural environmental conditions, which typically present a pH range of 5 to 8.5. In addition, two solutions from natural sources were examined SE and TW to simulate environmental conditions. The initial, non-environmental macrolide concentration of 100 µg/mL was chosen because it was high enough to enable monitoring DPs at the various obtained concentrations, but low enough to avoid intermolecular reactions. The sample solutions were prepared in sealed Pyrex glass bottles under natural sunlight (winter at 18°C, latitude: 32°, altitude: sea level), and in the shade (as a control). They were kept for 14 days (336 h), with sampling at 0, 2, 6, 32, 120 and 336 h. These times were chosen after preliminary tests to determine the optimal period for the degradation process. Each experiment was run in triplicate and relative standard deviation (RSTD) was calculated.

N-oxide, N-desmethyl and N-didesmethyl ERY, CLA, AZI and ROX DPs were obtained only under solar irradiation. These DPs were also obtained for ERY-H_2O (Table 1).

Analytical measurements

LC-MS analysis of macrolides and their DPs (after exposure to sunlight irradiation) was performed by high-performance liquid chromatography (HPLC, Agilent 1100) coupled to MS (Q-Tof, Waters, model Premier) via an ESI interface in positive mode, using a C18 ACE column (250 × 2.1 mm, 5 µm particle size). The column temperature was set to 28°C, the flow rate to 0.5 mL/min, and the injection volume was 10 µL. The HPLC mobile phase consisted of water with ammonium formate (0.05 M) adjusted to pH 8 with ammonia (A) and acetonitrile (B). The elution gradient was initiated with 20% B, increased to 80% over 14 min, and then held at 80% for 5 min. ^1H and ^{13}C nuclear magnetic resonance (NMR) analyses were carried out for ERY and ERY-spiroketal using a Bruker 500 MHz model ADVANCE II. The samples were dissolved in $CDCl_3$.

Sample preparation for N-oxide DPs

The four N-oxide DPs for ERY, CLA, AZI and ROX, respectively, were prepared and used as markers. In this procedure, 1 g of macrolide (ERY, CLA, AZI or ROX) was dissolved in 8 mL of methanol and 2 mL of H_2O_2 (30% in water), then heated to 60°C for 4 h. The four products were diluted to the appropriate concentration and then injected into the LC-MS. According to this analysis, the four N-oxide DPs were obtained at high conversion (more than 90% according to area percent).

Sample preparation of ERY-H_2O for NMR analysis

ERY-H_2O [peak at retention time (RT)=10.7 min] was isolated from the ERY working solution after its degradation (at pH 5), using semi-preparative HPLC (Agilent 1100) with a Vydac C18 column (250 mm length, 10 mm I.D. and 10 µm particle size). The mobile-phase composition and the elution-gradient program were the same as for the HPLC analytical method (section 2.3), except that the flow rate was set to 5 mL/min. The obtained fractions were lyophilized; they were chromatographically similar before and after the lyophilization procedure. The isolated ERY-H_2O was identified by MS and NMR spectra and used as a marker for the ERY-spiroketal compound (Tables 3-5).

Name	Azithromycin				Clarithromycin				Roxythromycin			
	DIDES	N-Oxide	DES	Parent	N-Oxide	DIDES	DES	Parent	DIDES	DES	N-Oxide	Parent
$[MH]^+$	721.49	765.51	735.50	749.52	764.48	720.45	734.47	748.48	809.5	823.52	853.53	837.53
RT (min)	8.93	9.23	9.26	9.95	10.50	10.65	11.02	11.83	11.02	11.33	12.07	12.31
Phosphate Buffer pH 5	n/d	n/d	n/d	100%	n/d	n/d	n/d	100%	n/d	0.4%	n/d	99%
Phosphate Buffer pH 7	n/d	n/d	n/d	100%	0.4%	n/d	0.8%	99%	n/d	0.5%	n/d	99%
Phosphate Buffer pH 8.5	0.8%	0.8%	8.4%	90%	0.4%	n/d	0.7%	98%	n/d	4.2%	0.6%	94%
Humic acids (5mg/L) pH 7	0.8%	1.1%	10.3%	88%	0.3%	n/d	1.7%	98%	n/d	3.7%	0.6%	96%
TW (measured pH 8.2)	2.3%	n/d	11.3%	86%	0.2	n/d	0.8%	99%	n/d	4.0%	n/d	96%
SE (measured pH 7.7)	23%	1.4%	76%	n/d	4.6%	0.5%	79%	16%	3.2%	86%	3.7%	8.5%

DES – N-desmethyl, DIDES – N-didsmethyl, n/d - not detected (<0.1%)

Table 1: Azithromycin, clarithromycin and roxithromycin degradation products (area%) under various conditions after 14 days under sunlight irradiation. TW, tap water; SE, secondary effluent.

Name	Erythromycin								
	DIDES	DES-H2O(1) (spiroketal)	DES	N-Oxide	Parent	DES-H_2O(2)	N-Oxide -H_2O	ERY-H_2O(1) (spiroketal)	ERY-H_2O(2)
$[MH]^+$	706.45	702.44	720.45	750.46	734.47	702.44	732.45	716.46	716.46
RT (min)	8.86	9.10	9.10	9.30	9.84	9.91	10.20	10.89	12.47
Phosphate Buffer pH 5	n/d	n/d	n/d	n/d	5.5%	0.5%	0.5%	90%	2.0%
Phosphate Buffer pH 7	n/d	n/d	n/d	3.8%	7.5%	0.5%	0.5%	83%	3.5%
Phosphate Buffer pH 8.5	n/d	n/d	0.3%	0.2%	48%	n/d	n/d	17%	26%
Humic acids (5mg/L) pH 7	n/d	n/d	n/d	3.0%	4.5%	0.5%	1%	83%	4.0%
TW (measured pH 8.2)	n/d	n/d	0.5%	n/d	95%	n/d	n/d	2.1%	n/d
SE (measured pH 7.7)	1.0%	2.0%	70%	5.5%	19.5%	n/d	n/d	n/d%	n/d

DES – N-desmethyl, DIDES – N-didsmethyl, n/d - not detected (<0.1%)

Table 2: Erythromycin degradation products (area %) obtained under various conditions after 14 days under sunlight irradiation.

Quality Control

All data were obtained in triplicate and the deviations (RSTD) were always less than 20%, and usually less than 10%. The correlations (R^2) were higher than 0.98 (except for experimental conditions with a very low degradation process) for the results obtained in the experiments run under sunlight.

Kinetics calculations

Kinetics calculations were based on the following equations:

k_1 calculation

$\ln [C] = -k_1 t + \ln[C_0]$, first order,

$[C_0]_{ERY, CLA, AZI, ROX} = 0.136, 0.133, 0.134, 0.120$ mM, respectively

where:

[C] (mM)=macrolide concentration at a given time

$[C_0]$ (mM)=macrolide concentration at starting time

k_1 (hr^{-1})=macrolide degradation-rate constant

t=time (hr)

Results and Discussion

The macrolide DPs were obtained in controlled laboratory experiments under sunlight irradiation.

Macrolide DPs laboratory experiments

A variety of macrolide DPs have been produced under controlled environmental conditions. The main DPs of ERY, CLA, AZI and ROX (obtained only under sunlight irradiation) were: N-oxide, N-desmethyl and N-didesmethyl (Figure 1). All results were obtained in triplicate and calculated as area percentage obtained from the MS detector. Tables 1 and 2 present the results after 336 h. In addition, DP's identity was confirmed by their MS spectra (Tables 4 and 5).

Three different phosphate buffer solutions (pH 5, 7 and 8.5), containing 100 µg/mL of each macrolide drug (prepared separately), were examined under controlled artificial conditions. In addition, the pH 7 macrolide-containing solution was also examined with the addition of humic acid. Solutions from natural sources SE and TW were also examined (Tables 1 and 2).

According to our observations, the photodegradation mechanism responsible for these DPs has never been presented. Chen et al. [28] investigated the autoxidation of tertiary amines using trimethylamine as a model, at a temperature of 100°C and pressure of 153 atm of O_2 to obtain the N-desmethyl and N-oxide products (Figure 2a). That study presented a mechanism that starts with electron transfer from the amine group to O_2 to obtain the radical ions Me_3N^+ and O_2^- (a second-order reaction). In the present study, sunlight irradiation at room temperature was used to obtain the N-oxide and N-desmethyl

	ERY		ERY-spiroketal		
Site	d_C (ppm)	d_H (ppm)	d_C (ppm)	d_H (ppm)	d_C (ppm) differences
1	175.91	-	181.31	-	5.4
2	44.96	2.88	47.54	3.42	2.6
3	80.02	3.94	77.63	4.38	-2.4
4	39.51	1.97	44.32	2.11	4.8
5	83.68	3.56	87.15	3.48	3.5
6	74.96	-	82.59	-	**7.6**
7	38.55	1.90, 1.70	42.91	2.42, 1.52	4.4
8	45.14	2.49	42.88	2.33	-2.3
9	**221.90**	**-**	**117.57**	**-**	**-104.3**
10	37.96	3.09	50.97	3.10	**13.0**
11	68.93	3.50	87.86	3.53	**18.9**
12	74.73	-	83.86	-	**9.1**
13	77.32	5.05	82.86	5.19	5.5
14	21.16	1.93, 1.50	25.4	2.02, 1.54	4.2
15	10.69	0.85	11.56	0.87	0.9
16	15.94	1.19	14.52	1.12	-1.4
17	9.18	1.12	18.45	1.23	**9.3**
18	26.90	1.47	28.56	1.45	1.7
19	18.32	1.17	12.68	1.09	-5.6
20	12.04	1.15	15.17	1.33	3.1
21	16.23	1.14	25.37	1.32	**9.1**
1'	103.23	4.39	103.64	4.31	0.4
2'	70.99	3.24	70.04	3.28	-0.9
3'	65.56	2.71	66.30	2.86	0.7
4'	28.90	1.73, 1.26	31.9	1.70, 1.25	3.0
5'	68.93	3.46	69.53	3.56	0.6
6'	21.40	1.23	21.35	1.28	0.0
3'-$N(CH_3)_2$	40.33	2.32	40.23	2.57	-0.1
1''	96.37	4.90	96.75	5.23	0.4
2''	35.01	2.31, 1.58	35.99	2.30,1.56	1.0
3''	72.68	-	74.57	-	1.9
4''	78.01	3.02	79.73	3.03	1.7
5''	65.68	4.01	66.96	4.01	1.3
6''	18.63	1.29	18.45	1.16	-0.2
7''	21.52	1.25	21.84	1.23	0.3
3''-OCH_3	49.52	3.32	50.01	3.29	0.5

ERY (^1H and ^{13}C in $CDCl_3$) (EL-Bondkly et al.)

ERY-spiroketal (^{13}C in MeOD, ^1H in $CDCl_3$) (Alam et al.)

Table 3: 1H and 13C NMR data of erythromycin (ERY) and ERY-spiroketal. In red: the most significant obtained shift between ERY and ERY-sprioketal.

products (Figure 2b) in different solutions (Tables 1 and 2), and the O_2 dissolved naturally in those solutions (first-order reaction, Figure 3 and Table 6). Production of the radical ions $RMe_2N^{+\cdot}$ and $O_2^{\cdot-}$ was activated by the sunlight irradiation energy (Figure 2b). The same mechanism can be suggested for the formation of the N-didesmethyl DP from the N-desmethyl DP (Figure 2c). Chen et al. [28] claimed that in the autoxidation process of N-oxide and N-desmethyl DP formation, one is not a side product of the other, meaning that N-desmethyl is not obtained from N-oxide. To examine this claim, an additional experiment was conducted. Artificially prepared CLA-N-oxide (section 2.3.1) was exposed to sunlight irradiation for 14 days. No N-desmethyl product was detected (Figure 2d). In support of this, Hill et al. [29] described CLA oxidation using meso-tetraarylmetalloporphyrins and NaOCl to obtain N-oxide and N-desmethyl products, and demonstrated that the latter was not obtained from CLA-N-oxide.

According to the above suggested mechanism, the macrolide DPs N-oxide, N-desmethyl and N-didesmethyl cannot, for the most part, be produced at low pH (5 and 7), due to unavailability of the amine's two non-bonding electrons as a result of its protonation according to its high pKa level (~9) (Table 1) [15]. In contrast, in a solution with high pH (8.5), the DPs were obtained, but at relatively low levels. The N-oxide DP was obtained at 0.2%, 0.8%, 0.4% and 0.6% for ERY, AZI, CLA and ROX respectively; the N-desmethyl DP was obtained at 0.3%, 8.4%, 0.7% and 4.2% for ERY, AZI, CLA and ROX respectively (Tables 1 and 2).

The addition of humic acid to the solution was expected to enhance production of the photodegradation products due to its ability to act as a sensitizer [30]. A comparison was made between solutions at pH 7 with and without humic acid to demonstrate the latter's effect. The humic acid certainly enhanced AZI degradation, based on the level of AZI N-desmethyl: 10.3% with humic acid and 0% without. As for the other macrolides, no significant effect of humic acid was observed (Table 1). The N-desmethyl DPs of all examined macrolides were obtained quite rapidly in SE: 70% for ERY, 76% for AZI, 79% for CLA and 86% for ROX after 2 weeks of sunlight irradiation. The N-didesmethyl DPs of all macrolides were also produced in SE, for at a level of 1.0% for ERY, 23% for AZI, 0.5% for CLA and 3.2% for ROX, after 2 weeks under sunlight irradiation. The relatively high AZI-N-didesmethyl production can be explained by the possible elimination of methyl from the N-methyl amine, which is part of the lactone ring (Figure 1). Nevertheless, no N-tridesmethyl DP was detected for AZI.

The N-oxide DP also appeared mainly in the SE, for ERY (5.5%), AZI (1.4%), CLA (4.6%) and ROX (3.7%). This can be explained by the fact that SE contains sensitizer molecules such as humic acids and others, which encourage the photodegradation process.

In the shade and in all experimental solutions, the macrolides AZI, CLA and ROX showed high stability with minor degradation, whereas ERY only showed high stability in the TW and SE solutions due to their relatively high pH (8.2 and 7.7 respectively). In contrast to the other solutions [phosphate buffer at pH 5 (90%), pH 7 (87%), pH 7 with humic acids (89%) and pH 8.5 (18%)], ERY degraded rapidly in the first 2 h, producing ERY-spiroketal. The second H_2O elimination product [ERY-H_2O(2)] degraded less to 2.0% (pH 5), 3.5% (pH 7), 4.0% (pH 7 with humic acids) and 26% (pH 8.5) (Table 2). According to these results, it can be concluded that production of the two ERY-H_2O elimination products is pH-dependent: the ERY-spiroketal is obtained mainly at low pH and ERY-H_2O (2) mainly at high pH.

Structural elucidation of the DPs

Structural elucidation of the macrolide DPs was carried out using the LC-HRMS and NMR techniques. The NMR analysis was carried out only for the ERY-H_2O (spiroketal product), following preparative separation and purification.

NMR analysis of ERY-H_2O

Definitive proof for the proposed ERY-H_2O structure was obtained by comparing the ^1H and ^{13}C NMR spectra of ERY and the isolated ERY-H_2O (RT=10.89 min; Table 3). Full assignment was performed for ^1H and ^{13}C spectra based on El-Bondkly et al. [31] for ERY and on Alam et al. [32] for ERY-spiroketal.

The most significant indication of the formation of ERY-spiroketal was disappearance of the ERY ketone carbon C(9) appearing at δ 221.90 ppm in the ^{13}C spectrum and the appearance of a new peak, related to C(9), at 117.57 ppm in the ERY-spiroketal spectrum a difference of 104.3 ppm upfield (Table 3).

Name	Fragments	ERY	AZI	CLA	ROX
Parent	$[MH]^+$	**734.4713**	**749.5183**	**748.4838**	**837.5335**
	$[MH]^+$-H_2O	716.4688			
	$[MH]^+$-$2H_2O$	698.4579			
	$[MH]^+$-Clad	576.3818	591.4232	590.3889	679.4383
	$[MH]^+$-Clad-H_2O	558.3715			
	$[MH]^+$-Clad-$2H_2O$	540.3604			
	$[MH]^+$-Clad-$3H_2O$	522.3492			
	$[MH]^+$-Clad-$OCH_2O(CH_2)_2OCH_3$				573.3760
	Clad	158.1183		158.1221	
	$[(M/2)+H]^+$ z=2		375.2558		419.264
N-Oxide	$[MH]^+$	**750.4677**	**765.5155**	**764.4803**	**853.5284**
	$[MH]^+$-$OCH_2O(CH_2)_2OCH_3$				748.4897
	$[MH]^+$-H_2O	732.4518			
	$[MH]^+$-Clad	592.3673			
	$[MH]^+$-Clad-H_2O	574.3579			
	$[(M/2)+H]^+$ z=2		383.2537		
Desmethyl	$[MH]^+$	**720.4538**	**735.5018**	**734.4701**	**823.5194**
	$[MH]^+$-H_2O	702.442			
	$[MH]^+$-$2H_2O$	684.4321			
	$[MH]^+$-$OCH_2O(CH_2)_2OCH_3$				718.3818
			592.4066		
	718-CH_3				703.3809
	$[MH]^+$-Clad	562.3575	577.4067	576.3784	665.4236
	$[MH]^+$-Clad-H_2O	544.3472			
	$[MH]^+$-Clad-$2H_2O$	526.3369			
	$[MH]^+$-Clad-$3H_2O$	508.3257			
	$[MH]^+$-Clad-$OCH_2O(CH_2)_2OCH_3$				559.3606
			434.3111		
	Clad-CH_3	144.101	144.1016		
	$[(M/2)+H]^+$ z=2		368.2518		412.2583
Didesmethyl	$[MH]^+$	**706.4390**	**721.4846**	720.4559	**809.5029**
	$[MH]^+$-Clad	548.342	563.3903	562.3591	651.4084
	$[MH]^+$-Clad-$2H_2O$	512.3196			
	$[MH]^+$-Clad-$3H_2O$	494.3099			
M -H_2O (1) spiroketal	$[MH]^+$	**716.4612**			
	$[MH]^+$-H_2O	698.4497			
	$[MH]^+$-CH3	684.4348			
		658.4179			
	$[MH]^+$-Clad	558.3657			
	$[MH]^+$-Clad-H_2O	540.3535			
	$[MH]^+$-Clad-$2H_2O$	522.3433			
	$[MH]^+$-Clad-$3H_2O$	500.3229			
M -H_2O (2)	$[MH]^+$	**716.4612**		730.4780	
	$[MH]^+$-Clad	558.3666			
	Clad	158.1165			
N-Oxide -H_2O	$[MH]^+$	732.4569		746.4740	
Desmethyl -H_2O	$[MH]^+$	702.4420			

Table 4: Main MS fragments of macrolides erythromycin (ERY), azithromycin (AZI), clarithromycin (CLA) and roxithromycin (ROX) and their degradation products.

Further examination of the ^{13}C spectra indicated additional significant differences for the C(9)-adjacent carbon peaks C(10), C(11) and C(12), which were shifted downfield from δ 37.96, 68.93 and 74.73 ppm (for ERY) to 50.97, 87.86 and 83.86 ppm (for ERY-spiroketal), respectively. The two methyl carbon peaks, C (17) and C(21), also showed significant differences in chemical shifts: δ 9.18 and 16.23 ppm in ERY shifted downfield to δ 18.45 and 25.37 ppm in ERY-spiroketal, respectively (Table 3). The differences in chemical shifts of ^1H NMR spectral peaks of ERY and the spiroketal product were not as indicative as in the ^{13}C NMR spectra (Table 3).

LC-MS analysis

LC–MS analysis was carried out for macrolides and their DPs using HRMS to examine their structures. The main DPs of the four examined macrolides were identified according to their molecular masses, mass fragmentation, empirical formulas and relative retention times in the chromatographic column (Tables 4 and 5). The MS spectra of the detected parent macrolides consisted of their molecular masses $[MH]^+$, which were 734.4713 (ERY), 749.5183 (AZI), 748.4838 (CLA) and 837.5335 (ROX). Their spectra were also characterized by elimination of the cladinose residue to obtain the main fragment $[MH]^+$ without

Macrolide	Name	Measured m/z	Calculated m/z	Accuracy (mDa)	Accuracy (ppm)	DBE	$[MH]^+$ formula	RT (min)	
ERY	Parent	734.4713	734.4691	2.2	3.0	4.5	$C_{37}H_{68}NO_{13}$	9.84	
	N-Oxide	750.4677	750.4640	3.7	4.9	4.5	$C_{37}H_{68}NO_{14}$	9.30	
	Desmethyl	720.4538	720.4534	0.4	0.6	4.5	$C_{36}H_{66}NO_{13}$	9.10	11.26
	Didesmethyl	706.4390	706.4378	1.2	1.7	4.5	$C_{35}H_{64}NO_{13}$	8.86	
	M -H_2O	716.4612	716.4585	2.7	3.8	5.5	$C_{37}H_{66}NO_{12}$	10.89	12.47
	N-Oxide -H_2O	732.4569	732.4534	3.5	4.8	5.5	$C_{37}H_{66}NO_{13}$	10.20	
	Desmethyl -H_2O	702.4420	702.4429	-0.9	-1.3	5.5	$C_{36}H_{64}NO_{12}$	9.10	9.91
AZI	Parent	749.5183	749.5164	1.9	2.5	3.5	$C_{38}H_{73}N_2O_{12}$	9.95	
	N-Oxide	765.5155	765.5113	4.2	5.5	3.5	$C_{38}H_{73}N_2O_{13}$	9.23	
	Desmethyl	735.5018	735.5007	1.1	1.5	3.5	$C_{37}H_{71}N_2O_{12}$	9.26	
	Didesmethyl	721.4846	721.4851	-0.5	-0.7	3.5	$C_{36}H_{69}N_2O_{12}$	8.93	
	Parent	749.5183	749.5164	1.9	2.5	3.5	$C_{38}H_{73}N_2O_{12}$	9.95	
CLA	Parent	748.4838	748.4847	-0.9	-1.2	4.5	$C_{38}H_{70}NO_{13}$	11.83	
	N-Oxide	764.4803	764.4796	0.7	0.9	4.5	$C_{38}H_{70}NO_{14}$	11.50	
	Desmethyl	734.4701	734.4691	1.0	1.4	4.5	$C_{37}H_{68}NO_{13}$	11.02	
	Didesmethyl	720.4559	720.4534	2.5	3.5	4.5	$C_{36}H_{66}NO_{13}$	10.65	
ROX	Parent	837.5335	837.5324	1.1	1.3	4.5	$C_{41}H_{77}N_2O_{15}$	12.31	
	N-Oxide	853.5284	853.5273	1.1	1.3	4.5	$C_{41}H_{77}N_2O_{16}$	12.07	
	Desmethyl	823.5194	823.5167	2.7	3.3	4.5	$C_{40}H_{75}N_2O_{15}$	11.33	
	Didesmethyl	809.5029	809.5011	1.8	2.2	4.5	$C_{39}H_{73}N_2O_{15}$	11.02	

Table 5: Measured m/z, calculated m/z, accuracy (mDa and ppm), double-bond equivalent (DBE), formulae of protonated ion and retention time (RT) of macrolides erythromycin (ERY), azithromycin (AZI), clarithromycin (CLA) and roxithromycin (ROX) and their degradation products.

	ERY		AZI		CLA		ROX	
Solution	k_1 (hr^{-1})	$t_{1/2}$ (hours)	k_1 (hr^{-1})	$t_{1/2}$ (hours)	k_1 (hr^{-1})	$t_{1/2}$ (hours)	k_1 (hr^{-1})	$t_{1/2}$ (hours)
SE	4.88E-03	142	3.04E-02	23	5.56E-03	125	7.60E-03	91
TW	6.60E-05	10500	3.20E-04	2310	2.76E-05	25109	9.00E-05	7700
pH 7 (HA)	*	*	3.90E-04	1733	6.01E-05	11531	5.30E-05	13076
pH 5	*	*	0	-	3.62E-06	191436	8.80E-05	7875
pH 7	*	*	0	-	2.74E-05	25292	2.00E-05	34650
pH 8.5	*	*	3.20E-04	2310	5.02E-05	13805	1.58E-04	4386

* Kinetic data not available

HA – humic acids

Table 6: Kinetics rate parameters of macrolides erythromycin (ERY), azithromycin (AZI), clarithromycin (CLA) and roxithromycin (ROX) in various solutions under sunlight irradiation. SE, secondary effluent; TW, tap water; HA, humic acid.

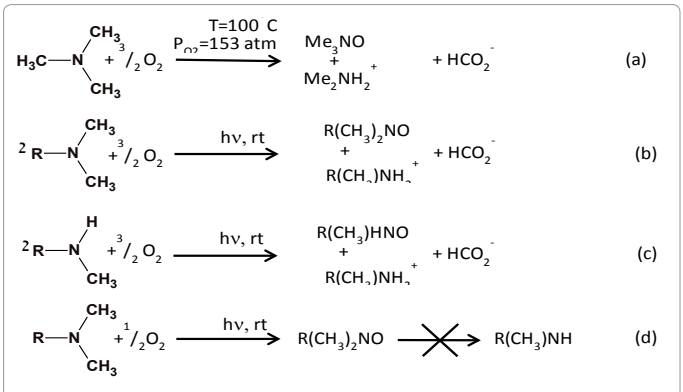


Figure 2: Mechanism underlying autoxidation of tertiary amines, demonstrated by trimethylamine (a), and by exposing tertiary amines at room temperature to sunlight irradiation (b, c and d).

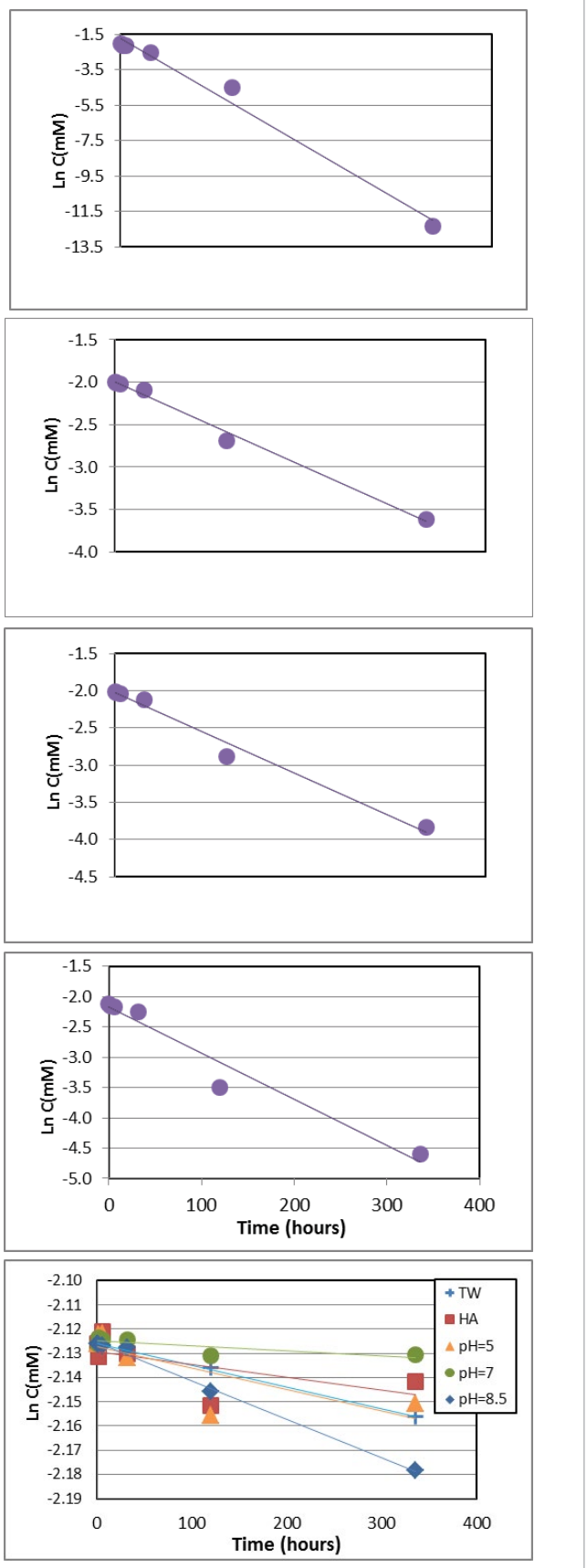
cladinose as presented by Chitneni et al. [24], Haghedooren et al. [25], Leonard et al. [33] and Barrett et al. [34].

Elimination of H_2O from the molecular mass was obtained mainly for ERY (as a significant fragment), due to its sTable product ERY-H_2O, compared to other macrolides. AZI has two amine groups (Figure 1), and it was therefore also characterized by the mass m/z 375.2558, which is a result of z=2 (two protonated amines).

The three main DPs, which were obtained only under sunlight irradiation, were N-oxide, N-desmethyl and N-didesmethyl. The spectra of the macrolide N-oxide contained mainly the molecular masses $[MH]^+$, which were 750.4634 (ERY), 765.5107 (AZI), 764.4791(CLA) and 853.5268 (ROX). These DPs' empirical formulas were confirmed by their HRMS spectra (Table 5). The N-desmethyl and N-didesmethyl DP spectra consisted of their molecular mass as well as their main fragment $[MH]^+$ without cladinose related to the elimination of cladinose (Tables 4 and 5). For ERY, due to its rapid formation to a spiroketal product, two additional photodegradation products were also obtained: ERY-N-oxide-H_2O ($[MH]^+$ 732.4529) and ERY-N-desmethyl-H_2O ($[MH]^+$ 702.4423) [35].

Kinetics

During the experiments, the macrolides degraded with time mainly under sunlight irradiation, following first-order kinetics (Table 6, Figure 3). No kinetic data could be obtained for the experiments in the shade, due to the relatively high stability of the macrolides under these experimental conditions, except for ERY, which was rapidly degraded in the artificial solutions (different pHs) (section 3.1). Regarding the



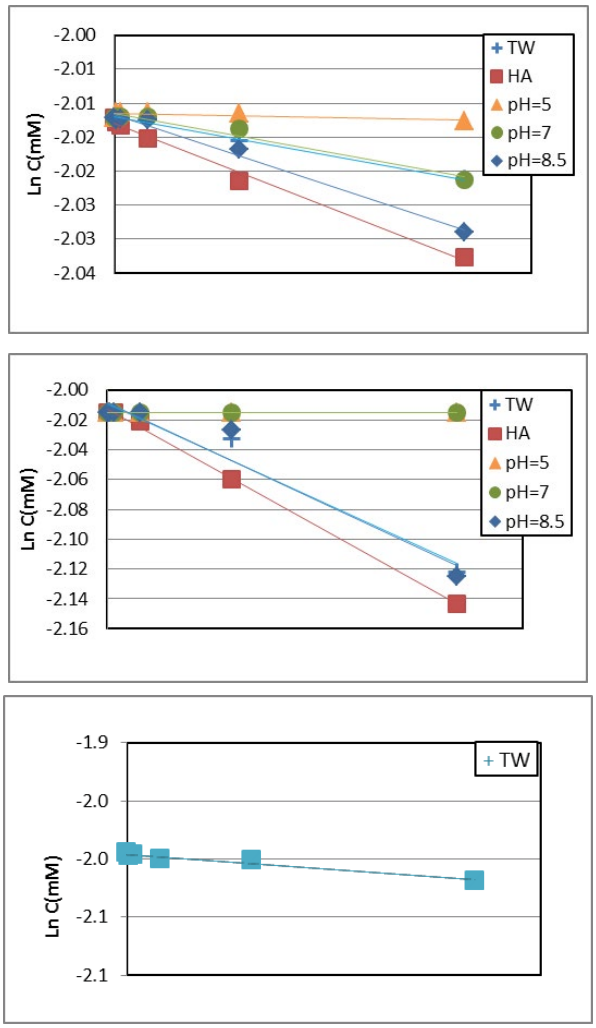


Figure 3: Degradation rates of macrolides spiked in various solutions and exposed to solar irradiation. (a, b, c and d) Erythromycin (ERY), azithromycin (AZI), clarithromycin (CLA) and roxithromycin (ROX) respectively, in secondary effluent. (a1, b1, c1 and d1) ERY, AZI, CLA and ROX, respectively, in phosphate buffer solutions at pH 5, 7 and 8.5 in the pH 7 solution with added humic acid (HA) and in tap water (TW).

kinetics data presented in Table 5 and Figure 3, the kinetics values for ERY could be obtained only in natural solutions (SE and TW). Production of the main macrolide DPs only under sunlight irradiation can be explained by the decomposition of the parent molecules due to photoactivation, with degradation that is much faster than in the shade; this is in contrast to ERY in artificial solutions, which rapidly decomposes to its main product ERY-spiroketal [11] (Table 2). In the present study, the kinetics results are demonstrated in various solutions under solar irradiation, producing k1 values ranging from 0.0304 hr^{-1} for AZI in SE (Figure 3b) to 0 for AZI at pH 5 and 7 (Figure 3b1; Table 6). It was anticipated that humic acids would act as a sensitizer [30] for the induction of photodegradation products (N-oxide, desmethyl and didesmethyl) in comparison to the other solutions. However, no additional effect was observed in CLA or ROX in the presence of humic acids (Figure 3c1 and d1, Table 6), while the k1 of AZI at pH 7 with the addition of humic acids was 0.000390 hr^{-1}, and without humic acids it was 0 (Figure 3b1, Table 6). As discussed in section 3.1, the degradation process is not favored at low pH due to the unavailability of the non-

bonding electrons of the amine group. As expected, the degradation rate in SE was relatively high, with k1 values of 0.00488 hr^{-1} (ERY), 0.0304 hr^{-1} (AZI), 0.00556 hr^{-1} (CLA) and 0.00760 hr^{-1} (ROX) (Figure 3a-d; Table 6).

Summary and conclusions

To the best of our knowledge, this is the first study to examine the formation of a variety of macrolide DPs under controlled environmental conditions, followed by a characterization and kinetics behavior analysis of macrolide degradation under solar irradiation. Three macrolide DPs (N-oxide, N-desmethyl and N-didesmethyl) were produced following exposure of macrolides to solar irradiation. Investigating the N-oxide and N-desmethyl DPs enabled us to understand the reaction mechanism governing DP formation through the intermediate radical ions RMe_2N^+ and O_2^-. The macrolides degraded rapidly under solar irradiation in the investigated aqueous solutions, mainly SE, whereas in the same solutions in the shade, macrolide degradation was negligible, except for ERY in the artificial solutions. The results of this study should direct further research into identified and suggested DPs, not only from macrolides but also from other drugs, which could potentially be found in aquatic environments. The characterization, presented herein, is expected to enable the detection of such DPs in various aquatic environments. Moreover, N-desmethyl and N-didesmethyl DPs are still biologically active, potentially increasing their toxicity to humans. Thus, further research is warranted to examine the environmental toxicity and stability of these compounds, which might, through exchange, form other DPs.

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References

1. Lamm A, Gozlan I, Rotstein A, Avisar D (2009) Detection of amoxicillin-diketopiperazine-2',5' in wastewater samples. J Environ Sci Health A Tox Hazard Subst Environ Eng 44: 1512-1517.
2. Gozlan I, Rotstein A, Avisar D (2010) Investigation of an amoxicillin oxidative degradation product formed under controlled environmental conditions. Environ Chem 7: 435-442.
3. Perez-Parada A, Aguera A, Del Mar Gomez-Ramos M, Garcia-Reyes JF, Heinzen H, et al. (2011) Behavior of amoxicillin in wastewater and river water: identification of its main transformation products by liquid chromatography/electrospray quadrupole time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 25: 731-742.
4. Gozlan I, Rotstein A, Avisar D (2013) Amoxicillin-degradation products formed under controlled environmental conditions: Identification and determination in the aquatic environment. Chemosphere 91: 985-992.
5. Andreozzi R, Campanella L, Fraysse B, Garric J, Gonnella A, et al. (2004) Effects of advanced oxidation processes (AOPs) on the toxicity of a mixture of pharmaceuticals. Water Sci Technol 50: 23-28.
6. Arslan-Alaton I, Caglayan AE (2006) Toxicity and biodegradability assessment of raw and ozonated procaine penicillin G formulation effluent. Ecotoxicol Environ Saf 63: 131-140.
7. Fatta-Kassinos D, Kalavrouziotis IK, Koukoulakis PH, Vasquez MI (2011) The risks associated with wastewater reuse and xenobiotics in the agroecological environment. Sci Total Environ 409: 3555-3563.
8. Rizzo L, Meric S, Guida M, Kassinos D, Belgiorno V (2009) Heterogenous photocatalytic degradation kinetics and detoxification of an urban wastewater treatment plant effluent contaminated with pharmaceuticals. Water Res 43: 4070-4078.
9. Freiberg L (1973) Process for the demethylation of 3-amino macrolides. US Patent 3,725,385.
10. LeTourneau N, Vimal P, Klepacki D, Mankin A, Melman A (2012) Synthesis and antibacterial activity of desosamine-modified macrolide derivatives. Bioorg Med Chem Lett 22: 4575-4578.
11. Roth HJ, Fenner H (1994) Stuttgart: Struktur-Bioreaktivit at-Wirkungsbezogene Eigenschaften. 2nd edn. Georg Thieme Verlag. pp: 65-68.
12. Hilton MJ, Thomas KV (2003) Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr 1015: 129-141.
13. Wiegel S, Aulinger A, Brockmeyer R, Harms H, Löffler J, et al. (2004) Pharmaceuticals in the river Elbe and its tributaries. Chemosphere 57: 107-126.
14. Yang S, Carlson KH (2004) Solid-phase extraction-high-performance liquid chromatography-ion trap mass spectrometry for analysis of trace concentrations of macrolide antibiotics in natural and waste water matrices. J Chromatogr A 1038: 141-155.
15. Gobel A, McArdell CS, Suter MJ, Suter MJF, Giger W (2004) Trace Determination of Macrolide and Sulfonamide Antimicrobials, a Human Sulfonamide Metabolite, and Trimethoprim in Wastewater Using Liquid Chromatography Coupled to Electrospray Tandem Mass Spectrometry. Anal Chem 76: 4756-4764.
16. Schlusener MP, Bester K (2005) Determination of steroid hormones, hormone conjugates and macrolide antibiotics in influents and effluents of sewage treatment plants utilising high-performance liquid chromatography/tandem mass spectrometry with electrospray and atmospheric pressure chemical ionisation. Rapid Commun Mass Spectrom 19: 3269-3278.
17. Nebot C, Gibb SW, Boyd KG (2007) Quantification of human pharmaceuticals in water samples by high performance liquid chromatography-tandem mass spectrometry. Anal Chim Acta 598: 87-94.
18. Uslu MO, Jasim S, Arvai A, Bewtra J, Biswas N (2013) A Survey of Occurrence and Risk Assessment of Pharmaceutical Substances in the Great Lakes Basin. Ozone Sci Eng 35: 249-262.
19. Sacher F, Lange FT, Brauch HJ, Blankenhorn I (2001) Pharmaceuticals in groundwaters analytical methods and results of a monitoring program in Baden-Württemberg, Germany. J Chromatogr A 938: 199-210.
20. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, et al. (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: a national reconnaissance. Environ Sci Technol 36: 1202-1211.
21. Lee KE, Barber LB, Furlong ET, Cahill JD, Kolpin DW, et al. (2004) Presence and distribution of organic wastewater compounds in wastewater, surface, ground, and drinking waters, Minnesota, 2000-02. US Geol Surv Sci Investig Rep Num 47: 2004-5138.
22. Lindberg R (2006) Determination of Antibiotics in the Swedish Environment with Emphasis on Sewage Treatment Plants. p: 31.
23. Liu J, Lu G, Ding J, Zhang Z, Wang Y (2014) Tissue distribution, bioconcentration, metabolism, and effects of erythromycin in crucian carp (Carassius auratus). Sci Total Environ 490: 914-920.
24. Kumar Chitneni S, Govaerts C, Adams E, Van Schepdael A, Hoogmartens J (2004) Identification of impurities in erythromycin by liquid chromatography-mass spectrometric detection. J Chromatogr A 1056: 111-120.
25. Haghedooren E, Raju VSKK, Dehouck P, Govaerts C, Schepdael AV, et al. (2006) Investigation of degradation products in a topical gel containing erythromycin and benzoyl peroxide by liquid chromatography-mass spectrometry. J Pharm Biomed Anal 41: 165-175.
26. Napoletano M (2004) Macrolide compounds endowed with anti-inflammatory activity. World Intellectual Property Organization. PCT WO 2004/013153 A2.
27. Jakopović IP, Krajačić MB, Skugor MM, Stimac V, Pešić D, et al. (2012) Novel desosamine-modified 14- and 15-membered macrolides without antibacterial activity. Bioorg Med Chem Lett 22: 3527-3530.
28. Chen MJ, Linehan JC, Rathke JW (1990) Autoxidation of Trimethylamine in Aqueous Solutions. J Org Chem 55: 3233-3236.
29. Hill DR, Celebuski JE, Pariza RJ, Chorghade MS, Levenberg M, et al. (1996) Novel Macrolides via meso-Tetraarylmetalloporphyrin Assisted Oxidations. Tetrahedron Lett 37: 787-790.
30. Lee E, Glover CM, Rosario-Ortiz FL (2013) Photochemical formation of hydroxyl radical from effluent organic matter: role of composition. Environ Sci Technol 47: 12073-12080.

31. El-Bondkly AM, Abd-Alla HI, Shaaban M, Khaled A (2009) Genetic improvement and electrospray ionization-mass spectra of erythromycin A obtained from marine Streptomyces spp. Artab J Biotech 12: 175-186.

32. Alam P, Buxton PC, Embrey KJ, Parkinson JA, Barber J (1996) Assignments of the 1H and 13C NMR Spectra of Anhydroerythromycin A in Organic and Aqueous Solutions. Magn Reson Chem 34: 559-572.

33. Leonard S, Ferraro M, Adams E, Hoogmartens J, Van Schepdael A (2006) Application of liquid chromatography/ion trap mass spectrometry to the characterization of the related substances of clarithromycin. Rapid Commun Mass Spectrom 20: 3101-3110.

34. Barrett B, Borek-Dohalský V, Fejt P, Vaingátová S, Huclová J, et al. (2005) Validated HPLC-MS-MS method for determination of azithromycin in human plasma. Anal Bioanal Chem 383: 210-217.

35. Hendrickson WA, Ward KB (1975) Atomic models for the polypeptide backbones of myohemerythrin and hemerythrin. Biochem Biophys Res Commun 66: 1349-1356.