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Newly Discovered Irbesartan Disinfection Byproducts via Chlorination: Investigating Potential Environmental Toxicity

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Abstract: Irbesartan belongs to the Sartan family, whose members are used in the treatment of arterial hypertension and kidney disease among patients with hypertension and type 2 diabetes mellitus as part of a treatment based on antihypertensive drugs. This drug has reached surface waters, accumulating to the extent of being considered an emerging pollutant, along with other substances from the same class. Wastewater treatment plants, which constitute the main environmental source of this compound, fail to completely reduce its presence in wastewater and generate additional toxic byproducts through the chlorine-based disinfection process. This study provides a comprehensive investigation into the chlorination mechanisms of irbesartan, revealing the identity of twelve new byproducts, which were characterized using NMR and mass spectrometry (MS-TOF). The other six byproducts were published in a previous study, allowing for the confirmation of some aspects of the supposed mechanisms of degradation, along with the identification of those that had only been hypothesized. An ecotoxicological assessment of a mixture and isolated byproducts was performed using *Raphidocelis subcapitata* for algal growth inhibition, *Daphnia magna* for immobility, and *Aliivibrio fischeri* for luminescence inhibition. The results revealed the variable toxicity of irbesartan and its byproducts. Different organisms exhibited varying sensitivities to the byproducts, with *Aliivibrio fischeri* being the most sensitive. The coexistence of multiple byproducts in the environment, their high toxicity, and their potential interactions highlight the significant environmental risks associated with chlorination and its derivates. Our study highlights the ongoing debate surrounding the generation of disinfection byproducts.

Keywords: irbesartan; chlorination; disinfection byproducts; artificial freshwater; acute toxicity test; chronic toxicity test

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1. Introduction

Medical advances have improved human lives, but, at the same time, high industrial production of medicines and the absence of specific methods for their elimination have led to a high level of pollution of both surface waters and, in some cases, soil, plants, and animals [1]. Most of the medicines sold every year worldwide end up in the environment. The causes of this phenomenon are manifold and range from the incorrect disposal of expired products to the industrial discharge of production processes; however, patients may be the biggest cause of this type of pollution. In fact, when a drug is taken, it is absorbed by the body and enters the body's circulation to be distributed and sent to the target site, where it performs its function. Many of the medicines taken, however, are

partially expelled without being metabolized [2]. A worrying fact is that the release of drugs into the environment has a higher frequency than the inactivation times of the compounds, which renders them pseudo-persistent. In most cases, the environmental concentrations of pharmaceuticals are too low to directly affect human lives; however, the presence of any drug in superficial waters can affect the local fauna continuously exposed to the drug through contact with the water, leading to the propagation of bioaccumulation problems along the food chain [3]. Pharmaceutical waste, together with sewage, reaches wastewater treatment plants, where the organic loads are degraded and the water is cleaned, but the fate of pharmaceuticals depends on their structural composition and reactivity towards the process used to treat the waste, which can lead to more toxic compounds. Downstream, the waters from wastewater treatment plants are still rich in active ingredients [4–7], which are poured into channels that carry the pollutants to superficial waters [8–13]. The fates of drugs in the environment can be very different; in fact, they can simply degrade or persist for a long time (up to 20 years), resulting in accumulation. Irbesartan is considered the progenitor of the Sartan family and is used alone or in combination with other drugs to treat high blood pressure. It can also be used in cases of kidney problems caused by type 2 diabetes mellitus and hypertension and in the treatment of congestive heart failure. Irbesartan, which has been on the market for about 19 years and for which there is an annual production of about 20,000 kilos, is an orally active substance that is absorbed quickly and effectively [14]. After it has exerted its biological effect and been metabolized in the liver, irbesartan is mainly eliminated through the feces, although around 20% is found in the urine.

In a recent study conducted on Italian wastewater, sartans were the emerging contaminants found most frequently and at the highest concentrations. In particular, irbesartan was one of the sartans detected in the highest number among the samples analyzed [12]. There have been several studies on the elimination, persistence, and toxicity of sartans [15,16], but there are no comprehensive data in the literature on irbesartan in relation to its persistence in the environment or its refractoriness to water or wastewater treatment. Irbesartan has been found in samples of surface waters in Africa and Europe at concentrations just below 1 μg/L [17] and, not infrequently, even in drinking water, with concentrations up to 3.0 ng/L [18]. Due to advanced oxidation processes (AOPs), 21% of irbesartan resists normal wastewater purification treatments and is thus released into the environment, while 79% is partially mineralized or transformed into degradation byproducts [19].

In a prior study, the disinfection pathway of irbesartan was examined based on the typical chlorination process employed in wastewater treatment plants (WWTPs) [16,20]. Six disinfection byproducts (DBPs) were isolated and then completely characterized via a combination of mass spectrometry (MS) and nuclear magnetic resonance (NMR) data. Just over 75% of the irbesartan underwent complete mineralization, 15% was recovered unchanged, and 6% had been transformed into DBPs. Similarly, toxicity caused by irbesartan's chlorinated derivatives was studied, with one report relating increased negative effects. Furthermore, a mechanism was proposed wherein the isolated products are obtained by passing through a series of possible intermediates.

In this paper, the isolation and structural determination of twelve new DBPs are reported (Figure 1). This study confirms the proposed mechanism of formation of the already known DBPs, wherein some of those compounds that had previously simply been hypothesized to be possible intermediates are identified as DBPs. Finally, to assess irbesartan's reactivity and biological impact during chlorination, a toxicity study of the DBPs was performed using *Daphnia magna*, *Aliivibrio fischeri*, and *Raphidocelis subcapitata*, i.e., three model organisms that cover different trophic levels (algae, bacteria, and crustaceans).

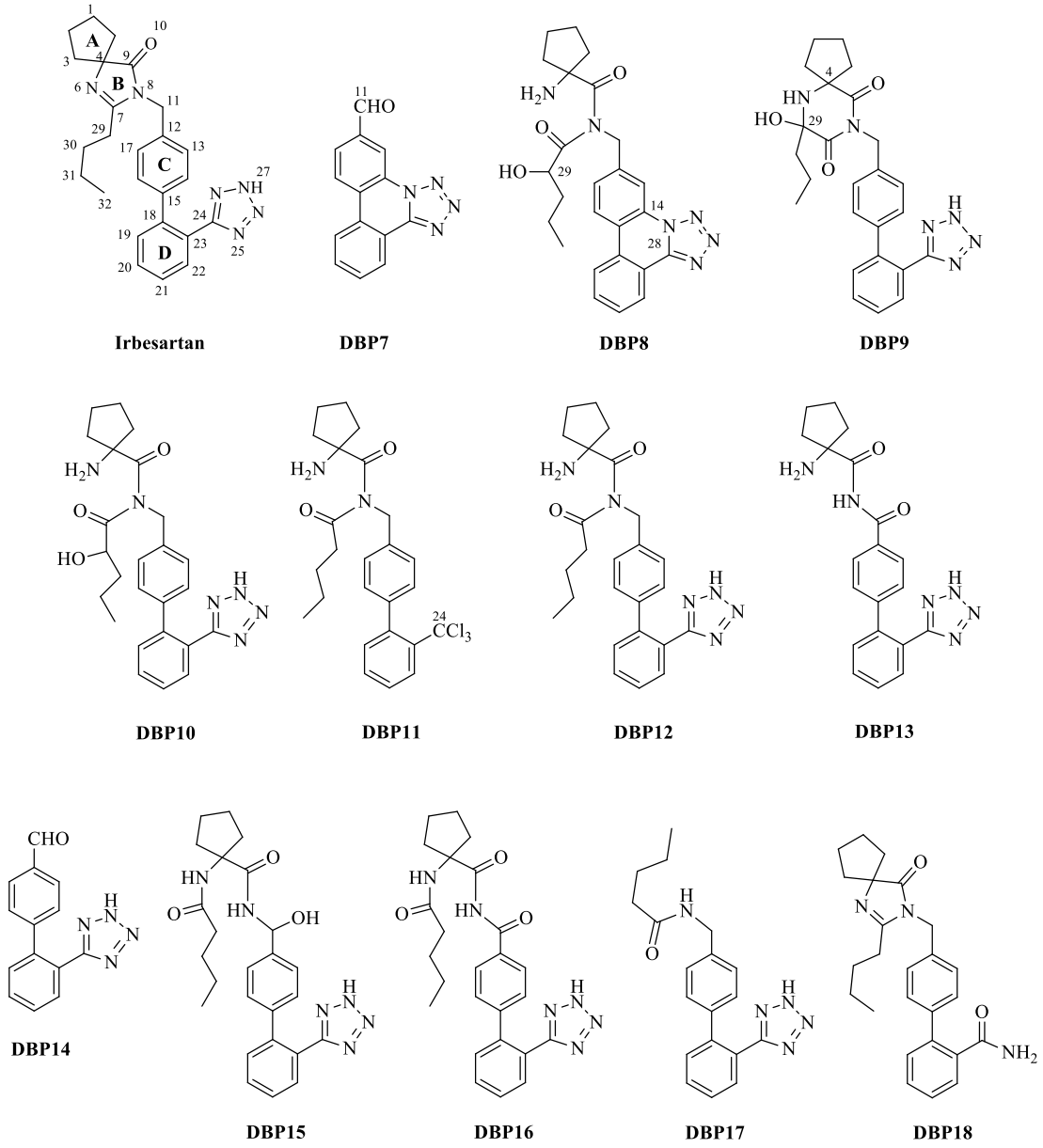


Figure 1. Chemical structures of irbesartan and its disinfection byproducts DBP7–DBP18, with the numbering of atoms and rings.

2. Materials and Methods

2.1. Drug and Reagents

Irbesartan (99.5%) was purchased from Sigma Aldrich (Milan, Italy). All the other chemicals and solvents were purchased from Fluka (Saint-Quentin Fallavier, France), were of HPLC grade, and were used as received. For the toxicity assessment, a reference toxicant (potassium dichromate) and salts used for the preparation of artificial freshwater ($CaCl_2 \times 2H_2O$, $MgSO_4 \times 7H_2O$, KCl, $NaHCO_3$) were employed. Double-distilled water (Microtech, Pozzuoli (NA), Italy) was used to prepare the dilution water and treatments.

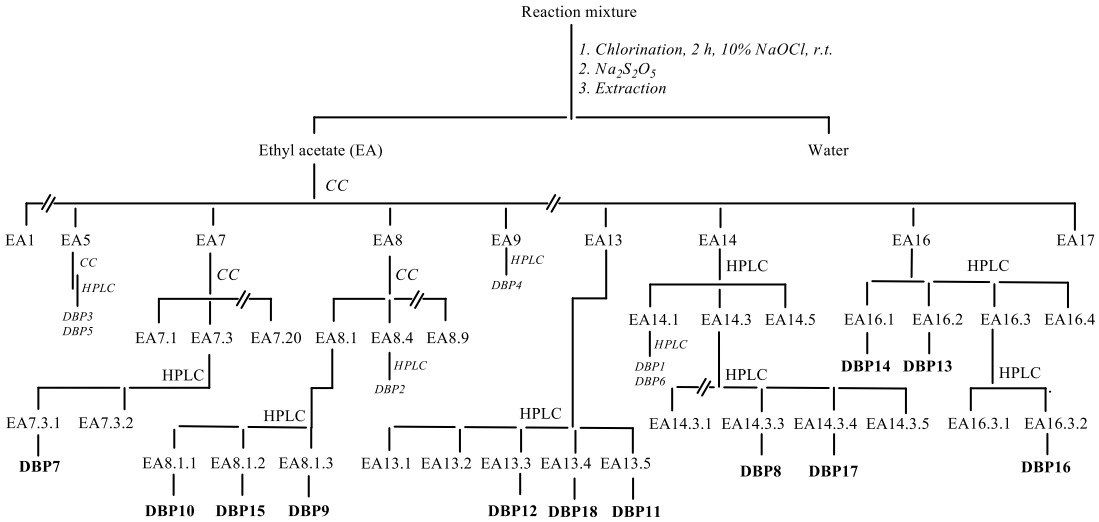
2.2. Apparatus and Equipment

Normal-phase chromatography (NPC) was performed using silica gel 60 (0.015–0.040 mm) (Merck KGaA, Darmstadt, Germany) for column chromatography. Reversed-phase chromatography was carried out using an HPLC Shimadzu LC-8A system with an RP Gemini C18-110A column (10 µm, 250 mm × 21.2 mm i.d. Phenomenex, Bologna, Italy). The NMR spectra were

recorded using a Varian Inova (operated at 500 MHz) and cross-referenced (in ppm) with the residual solvent signals. The mass-spectrometric analyses were performed using a Voyager De Pro MALDI. UV vis spectra were recorded on a JASCO V-750 UV–Visible Spectrophotometer.

2.3. Preparative Chlorination Procedure and Product Isolation

The preparative chlorination of irbesartan was achieved by reacting 1.0 g of the active ingredient (2.33×10^{-3} M) with a 10% hypochlorite solution that was determined via iodometric titration (molar ratio of irbesartan/hypochlorite at a 1:10 concentration) [21]. From the initial pH of 8.0, the solution reached pH 10.5 after 5 min and remained stationary. After 2 h (to facilitate maximum degradation of irbesartan and production of its byproducts), the free chlorine content was measured, which had decreased from its initial value of 10% to a value of 6%. The solution was then quenched with sodium sulfite and immediately extracted with ethyl acetate, yielding an EA fraction of 835 mg (Scheme 1), which was subjected to chromatography using silica gel column chromatography (CC) with a methylene chloride–methanol gradient (100:0 to 30:70, *v*/*v*). This process resulted in the isolation of 17 fractions. The fraction EA7 (138 mg), namely, eluted methylene chloride–methanol (95:5, *v*/*v*), was re-chromatographed through silica gel CC via elution with a gradient of petrol ether–acetone (90:10 to 70:30, *v*/*v*), yielding 20 subfractions. The subfraction EA7.3 (30 mg), eluted with petrol ether–acetone (90:10, *v*/*v*), was separated in two subtractions using preparative HPLC column elution with a gradient of CH_3COONH_4 (pH 3.8; 20 mM) and methanol (30:70 to 0:100, *v*/*v*). The subfractions EA7.3.1 contained DBP7 (1 mg). The fraction EA8 (101 mg), eluted with methylene chloride/methanol (90:10, *v*/*v*), was re-chromatographed with silica gel CC via elution with a gradient of petroleum ether–acetone (90:10 to 70:30, *v*/*v*), yielding 9 subfractions.



Scheme 1. Isolation of twelve identified disinfection byproducts.

The subfraction EA8.1 (30 mg), eluted with petrol ether–acetone (90:10, *v*/*v*), was purified via HPLC using a reversed phase column and elution with a gradient of CH_3COONH_4 (pH 4.0; 10 mM) and methanol (25:75 to 0:100, *v*/*v*), yielding 3 subfractions containing DBP10, DBP15, and DBP9 (10, 3, and 2 mg, respectively). The fraction EA13 (37 mg), eluted with methylene chloride–methanol (70:30, *v*/*v*), was separated into 5 subfractions under the same HPLC conditions as EA7.3, yielding DBP12, DBP18, and DBP11 (4, 3, and 16 mg, respectively). The fraction EA14 (205 mg), eluted with methylene chloride–methanol (60:40, *v*/*v*), was re-chromatographed with silica gel CC via elution with a gradient of methylene chloride–acetone (100:0 to 65:35, *v*/*v*), yielding 5 subfractions. The subfractions EA14.3 (23 mg) and EA14.4 (9 mg), eluted with methylene chloride–acetone (80:20, *v*/*v*), contained DBP8 and DBP17 (3 and 2 mg, respectively), which were purified via HPLC using a reversed phase column and elution with a gradient of CH_3COONH_4 (pH 4.0;

10 mM) and methanol (35:65 to 5:95, v/v). The fraction EA16 (37 mg), eluted with methylene chloride–methanol (50:50, v/v), was purified via preparative HPLC under the same conditions as EA7.3, yielding four subfractions, of which the first of the two contained DBP14 and the second contained DBP13 (5 and 8 mg, respectively). The fraction EA16.3 (15 mg), containing DBP16 (3 mg), was purified via preparative HPLC under the same conditions as EA7.3.

2.4. Ecotoxicity Assays

The toxicity of irbesartan and its byproducts was assessed using ecotoxicological tests, which were conducted in accordance with ISO guidelines. The experimental design, which followed an effect-driven approach [16], involved using a concentration of 5 mg/L of the drug, as well as a mixture of the various byproducts (DBPs) and their individual byproducts, to gain a better understanding of their fate and toxicity towards three organisms: *A. fischeri*, *R. subcapitata*, and *D. magna*.

The UNI EN ISO 6341:2013 [22] was followed to conduct an acute immobilization test using *D. magna*. The test involved exposing daphnids younger than 24 h to irbesartan and its DBPs and assessing immobilization after 24 h of exposure. The test was conducted in three parallel replicates on 6-well plates, and the number of immobilized or dead organisms was determined using stereomicroscopy. Toxicity was expressed as the percentage of immobilized or dead organisms relative to the total number of organisms exposed. The algal growth inhibition test was performed with *R. subcapitata*, for which ISO 8692:2012 was followed [23]. Algal cells were taken from an exponentially growing pre-culture, and toxicity tests were conducted in three replicates, maintained under continuous illumination, and manually stirred twice a day. After 72 h, the growth rate of algae was measured, and the inhibition rate of each solution was determined by comparing it to the growth rate of the control group. The level of algal growth inhibition (%) for each treatment replicate was calculated using the following equation: $I(\%) = ((\mu 0 - \mu T)/\mu 0) * 100$, where I (%) represents the percentage of the inhibition of the average specific growth rate, $\mu 0$ denotes the mean value for μ in the control (control growth rate), and μT represents the value for the growth rate in the treatment replicate. Negative values indicate stimulation of growth rather than inhibition. For the acute bioluminescence inhibition assay, the bacterium *A. fischeri* (NRRL-B-11177) was used, and the assay was carried out using a Microtox Model 500 analyzer, which was used in accordance with the international procedure (ISO 11348-3:2007) [24]. The bacteria were rehydrated with a reconstitution solution and exposed to solutions of irbesartan and its DBPs at 15 °C, and the bioluminescence intensity was measured after 30 min. The test was conducted in triplicate, for which a control was employed, and the pharmaceutical and toxic effects were expressed as the ratio of the decrease in bacterial light production to the remaining light. For every testing run, both negative and positive controls were incorporated. The negative tests were performed on chlorinated aqueous solutions comprising 0.01% of DMSO with L of sodium sulfite per liter of solution to eliminate residual chloride. Data were expressed as the effect (%) +/− standard deviation. The analysis was carried out using XLSTAT 2016.02.27444 Version (Addinsoft, Paris, France) and GraphPad Prism (GraphPad, San Diego, CA, USA).

3. Results and Discussion

3.1. Chlorination Experiments

An approximately 10^{-5} M solution of irbesartan, a concentration comparable to that at which emerging micropollutants are detected in the environment, was used to monitor (via HPLC) the main disinfection byproducts formed during a 30 min reaction carried out at room temperature and at a concentration of approximately 10–20 mg/L of the active chlorine solution, constituting conditions close to those of a typical wastewater treatment plant [25,26]. Three chlorination tests, with irbesartan solutions always in the range of 10^{-5} M, qualitatively provided the same degradation pattern. The disinfection byproducts were identified by comparing their retention times with those obtained in

the preparative experiments (Figure 2). The concentrations of byproducts DBP7–DBP18 reached a maximum after 2 h, with percentages of 0.1, 0.3, 0.2, 1.0, 1.6, 0.4, 0.8, 0.5, 0.3, 0.3, 0.2, and 0.3% *w*/*w*, which could reflect the real percentages.

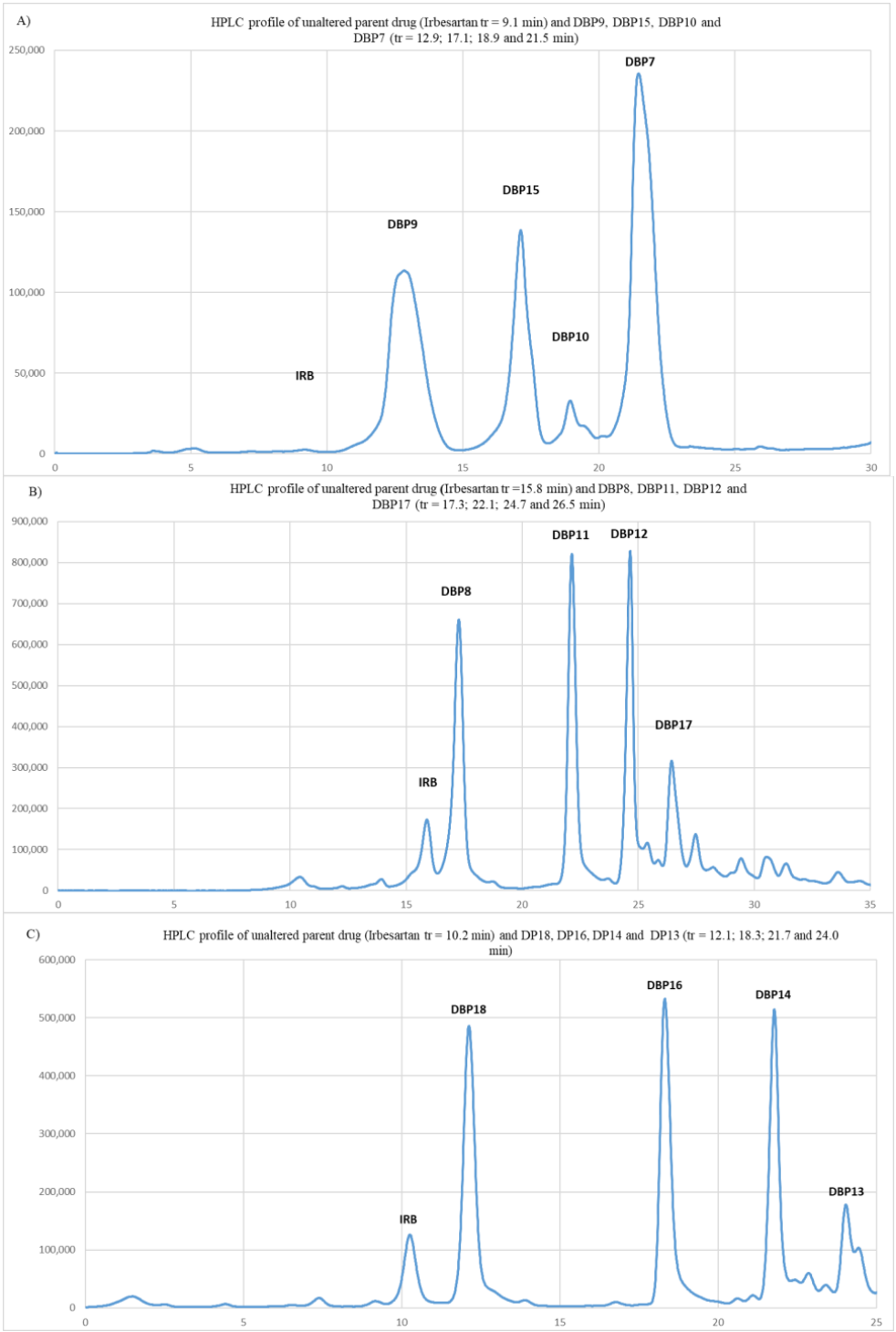


Figure 2. HPLC profiles of disinfection byproducts DBP7, DBP9, DBP10, and DBP15 (**A**); DBP8, DBP11, DBP12, and DBP17 (**B**); and DBP13, DBP14, DBP16, and DBP18 (**C**), along with the unaltered parent drug.

3.2. Structural Elucidation

All the byproducts described below have been obtained with a degree of purity greater than 95% in order to be able to describe them by means of accurate NMR and mass

spectrometry analyses. Compounds DBP7–DBP13 and DBP15–DBP16 are new compounds, which have been isolated for the first time.

3.2.1. Structural Elucidation of DBP7

The MS-TOF analysis showed a molecular ion peak at m/z 249.11 $[M + H]^+$ corresponding to the compound with the molecular formula $C_{14}H_8N_4O$. The ^1H NMR spectrum did not show the presence of an *n*-butyl side chain, an A ring, or the proton H-11, just as the ^{13}C NMR spectrum did not show the corresponding carbons nor the signals of carbons C-7 and C-9 (the B ring). In the ^1H NMR spectrum, only aromatic signals were present, specifically only seven protons at 9.19, 8.73, 8.28, 8.61, 7.98, 7.92, and 8.86 ppm, as well as the signal of an aldehyde proton at 10.30 ppm. The HSQC spectrum allowed for the correlation of the aforementioned signals with the carbons at 127.19, 126.57, 125.41 120.18, 125.09, 130.95, 124.03, and 190.01 ppm. Based on the 1H-1H COSY and the HMBC spectra, the first three signals were attributed to the protons H-13, H-16, and H-17 of 1,2,4-trisubstituted benzene (the C ring). The next four were attributed to the protons H-19, H-20, H-21, and H-22 of 1,2-disubstituted benzene (the D ring), while the last one was attributed to the aldehyde proton H-11. Evidently, the starting product has undergone hydrolysis of the C-7/N-8 and N-8/C-9 bonds [27,28], along with the partial oxidation of carbon C-11 and the concomitant closure of a new six-term ring. The latter could result from an aromatic nucleophilic substitution reaction on the C-14 carbon of the previously chlorinated aromatic B-ring via the adjacent N-28 nitrogen of the tetrazole ring [29]. The plausible mechanism of DBP7 formation from irbesartan is shown in Figure 3, and its NMR assignments are shown in Table S1.

3.2.2. Structural Elucidation of DBP8

In the ^1H NMR spectrum, the compound DBP8, like DBP7, showed the presence of only seven aromatic protons, indicating the formation of a new six-term ring. The 1D and 2D NMR data also show the presence of an A ring and the proton H-11. In the HMBC spectrum, these features (at 5.16 and 4.94 ppm) were correlated with the two carbons at 186.43 and 159.43 ppm, which were identified as carbonyls C-9 and C-7, respectively. In fact, if C-9 was correlated with the protons H-3 and H-5 at 1.91 ppm, carbon C-7 was correlated with the protons at 4.34 and 1.27 ppm. These last protons were identified as H-29 and H-30, respectively, indicating the presence of the *n*-butyl side chain, which, however, was oxidized to carbon C-29 in alpha to carbonyl C-7.

3.2.3. Structural Elucidation of DBP9

The NMR data regarding the compound DBP9 show the presence of the A, C, and D rings; the protons H-11 (at 5.25 and 46.84 ppm); and carbons identified as C-9 and C-7 at 187.48 and 158.02 ppm, respectively. In the HMBC spectrum, both carbonyls were correlated with protons H-11, but only the first of which was correlated with protons H-3 and H-5, and only the second of which was correlated with the protons at 2.67 ppm, identified as proton H-30. The 1D and 2D NMR data show the *n*-butyl side chain but with a quaternary carbon at position C-29. In fact, the ^{13}C NMR spectrum showed the presence of a carbon at 83.09 ppm, which was identified as an oxygenated quaternary carbon engaged in the closure of a six-term cycle with the amino function at carbon C-4. The compound DBP9 was, therefore, very similar to the compound DBP2 reported in a previous article on the degradation products of irbesartan [16], for which the formation of a new bond between the positions N-28 and C-14 did not occur.

3.2.4. Structural Elucidation of DBP10

Compound DBP10 was very similar to compound DBP8, but it did not have the bond between the positions N-28 and C-14. Indeed, the ^1H NMR spectrum showed the presence of eight aromatic protons, attributed to two 1,4-disubstituted rings.

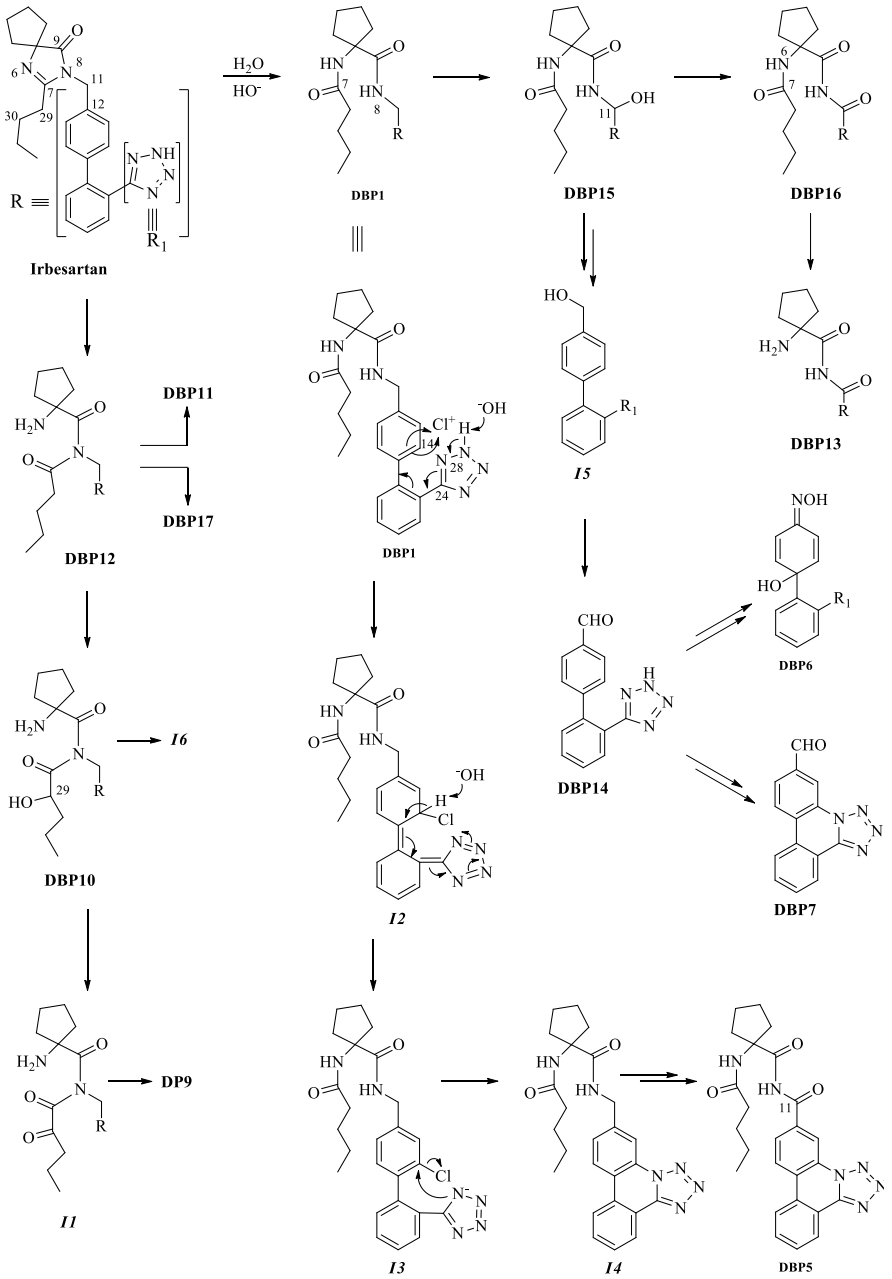


Figure 3. Plausible mechanism for the formation of DBP1, DBP5–DBP7, DBP10, and DBP12–DBP16.

3.2.5. Structural Elucidation of DBP11

The 1D and 2D NMR data indicate the opening of the B ring via the hydrolysis of the bond C-7/N-6. Indeed, in the HMBC spectrum, carbonyl C-7 at 176.01 ppm was correlated with the signals at 2.49 and 1.54 ppm, which were identified as protons H-29 and H-30 of the *n*-butyl side chain, while carbonyl C-9 at 180.05 ppm was correlated with the signals at 2.12 and 1.96 ppm, which were identified as protons H-3 and H-5 of the cyclopentane group. MS-TOF analysis showed a molecular ion peak at m/z 495.88 $[M + H]^+$ in addition to the signals at m/z 494.13, 496.15, and 498.13, corresponding to the molecular formula $C_{25}H_{29}Cl_3N_2O_2$ and to the presence of three chlorine atoms. Indeed, in the HMBC spectrum, proton H-22 at 8.24 ppm was correlated with the carbons at 134.75 and 141.89 ppm, which were identified as carbons C-20 and C-18, respectively, and with carbon C-24 at 97.40 ppm, which, evidently, was obtained from the degradation of the tetrazole ring. The plausible mechanism behind the formation of DBP11 from irbesartan is shown in Figure 4, and the NMR assignments of DBP11 are shown in Table S5.

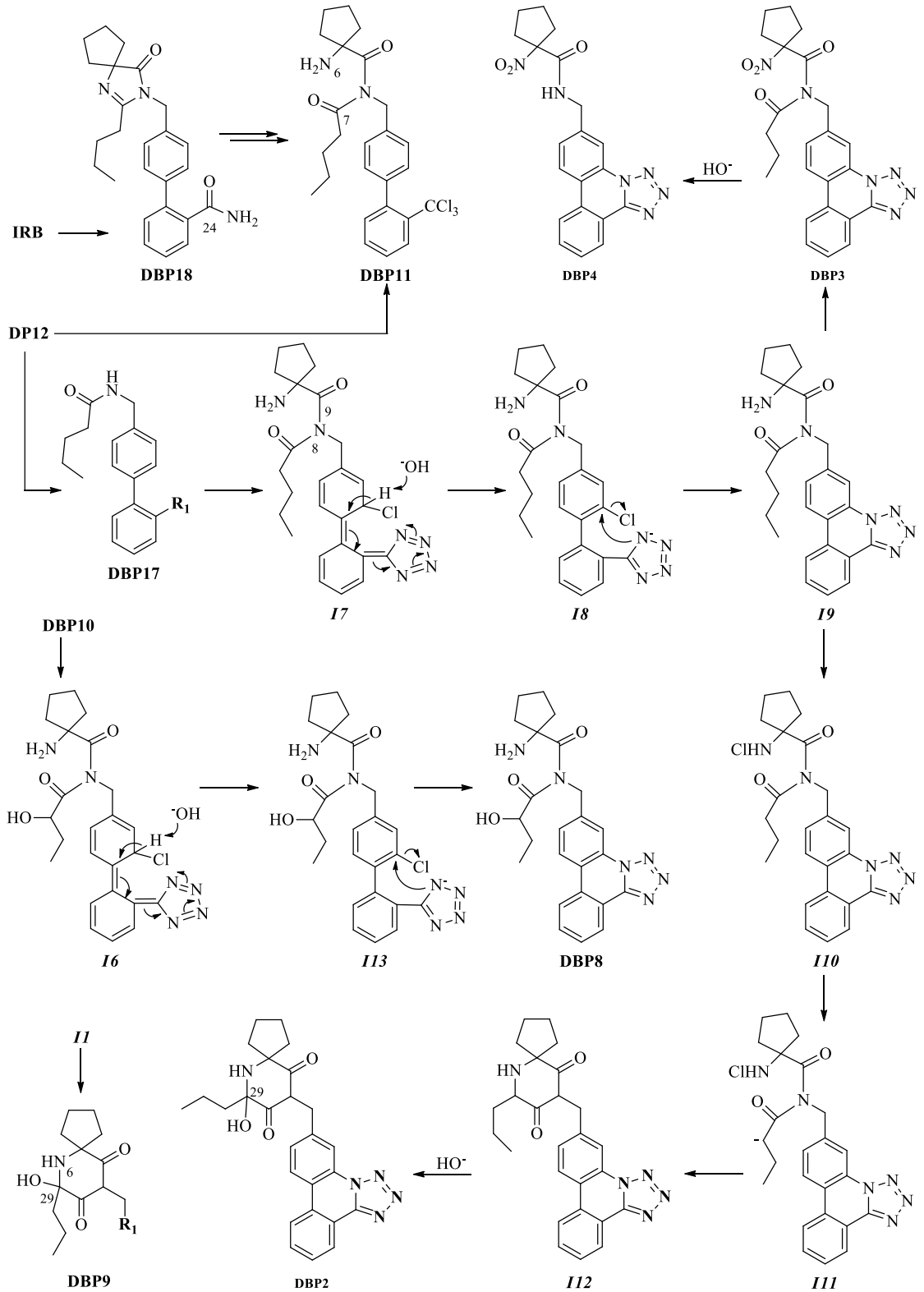


Figure 4. Plausible mechanism behind the formation of DBP2–DBP4, DBP8–DBP9, DBP11, and DBP17–DBP18.

3.2.6. Structural Elucidation of DBP12

The nuclear magnetic resonance (NMR) spectra obtained were consistent with those previously documented in the literature [30].

3.2.7. Structural Elucidation of DBP13

The MALDI-TOF analysis of DBP13 showed a molecular ion peak at m/z 377.18 $[M + H]^+$ corresponding to the compound with the molecular formula $C_{20}H_{20}N_6O_2$. The NMR and mass spectra data are like those of DBP12 but without the C_5 chain bound to the nitrogen N-8 and with carbon C-11 oxidized at the carbonyl group. This last one at 166.70 ppm was correlated in the HMBC spectrum with protons identified as H-13/H-17 at 7.84 ppm.

3.2.8. Structural Elucidation of DBP14

The NMR data regarding compound DBP14 are very similar to those of compound DBP7 but without the bond between the positions N-28 and C-14. Evidently, the starting product was only hydrolyzed to the bonds C-7/N-8 and N-8/C-9 and partially oxidized at carbon C-11 [31].

3.2.9. Structural Elucidation of DBP15

The MS-TOF analysis showed a molecular ion peak at m/z 463.26 $[M + H]^+$ corresponding to the compound with the molecular formula $C_{25}H_{30}N_6O_3$. The NMR data regarding DBP15 (Table S9) are very similar to those for DBP1 reported in the study by Romanucci et al. [16]. The difference is due to carbon C-11, present as methylene in compound DBP1 and as partially oxidized methine in DBP15. In fact, the ^{1}H NMR spectrum shows a singlet signal of a proton at 6.66 ppm, bound in the HSQC spectrum to the carbon at 83.04 ppm and correlated in the HMBC spectrum with carbonyl carbon C-9, as well as with carbons C-12 and C-13/C-17 at 169.00, 135.15, and 127.53 ppm, respectively.

3.2.10. Structural Elucidation of DBP16

Compound DBP16 was the corresponding oxidized product of DBP15. In fact, in the ^{13}C NMR spectrum, the signals of three carbonyl carbons appeared at 176.73, 168.48, and 168.42 ppm. In particular, in the HMBC spectrum, the third carbonyl carbon, identified as carbon C-11, was correlated with protons H-13 and H-17 of the 1,4-disubstituted aromatic C ring at 7.60 ppm. Compound DBP16 also corresponded to DBP5 reported by Romanucci et al. [16] but without presenting the bond between the positions N-28 and C-14.

3.2.11. Structural Elucidation of DBP17

The nuclear magnetic resonance (NMR) spectra obtained were consistent with those previously documented in the literature [30].

3.2.12. Structural Elucidation of DBP18

Compound DBP18 was very similar to irbesartan but with the tetrazole ring partially degraded to the point of performing an amide function. In fact, proton H-22 at 7.78 ppm, which, according to the HSQC spectrum, was bonded to the carbon at 130.73 ppm, was correlated in the HMBC spectrum with a carbonyl at 170.51 ppm, which was identified as carbon C-24. The MS-TOF analysis showed a molecular ion peak at m/z 404.54 $[M + H]^+$ corresponding to the molecular formula $C_{25}H_{29}N_3O_2$ [32].

3.3. Proposed Mechanism for the Formation of Disinfection Byproducts

It is possible to propose a mechanism that explains the acquisition of all 18 compounds isolated to date from the chlorination of irbesartan, including compounds DBP2–DBP5 and DBP7–DBP8 with a tetracyclic aromatic nucleus (Figures 3 and 4).

With the hydrolysis of the C-7/N-8 bond of irbesartan, compound DBP1 was obtained, which, via oxidation of the C-11 carbon, led first to the formation of the corresponding carbinol DBP15 and then to the corresponding oxidized compound DBP16. The hydrolysis of the N-6/C-7 bond of DBP16 led to the production of compound DBP13. From compound DBP15, through intermediate I5, product DBP14 was obtained, and from this, the DBP6 oxime and compound DBP7 were obtained.

In the authors' opinion, it can be hypothesized that under the strongly oxidizing reaction conditions employed, a neutral intermediate, I2, can be formed via the addition of chlorine to the aromatic C ring of compound DBP1 and the consequential loss of a proton from the tetrazole ring. The loss of the proton geminal to the chlorine atom would allow for the aromaticity of the ring to be restored and would yield intermediate I3 with a negative charge on the nitrogen of the tetrazole ring. This intermediate would evolve into intermediate I4 via an intramolecular nucleophilic attack, and from the latter, DBP5 would then be obtained by the oxidation of the benzyl carbon C-11.

Conversely, the hydrolysis of the C-7/N-6 bond of irbesartan leads to the formation of the byproduct DBP12, and from this, it is possible to obtain byproduct DBP10 via the oxidation of the *n*-butyl side chain at carbon C-29. The subsequent oxidation of carbon C-29 of the byproduct DBP10 would lead to intermediate I1, and from this, with an intramolecular nucleophilic attack, byproduct DBP9 would be obtained.

Byproduct DBP12 could yield DBP17 via the hydrolysis of the N-8/C-9 bond. In turn, byproduct DBP17 would lead to byproduct DBP3 through intermediates I7-I9 and to byproduct DBP2 through intermediates I7-I12. Finally, it can be hypothesized that byproduct DBP18 could be obtained via the oxidation of the tetrazole ring of the starting irbesartan. Byproduct DBP4 could be obtained from the corresponding DBP3 for the hydrolysis of the C-7/N-8 bond, and byproduct DBP8 could be obtained from DBP10 by incorporating the I6 and I13 intermediates.

3.4. Ecotoxicity Data

The original irbesartan compound was proven to be relatively non-toxic through the *D. magna*, *A. fischeri*, and *R. subcapitata* screening tests, with $EC_{50} > 100$ mg/L. The current research shows that the chlorination of irbesartan involves more complex DBPs. Our previous work found different DBPs derived from irbesartan chlorination, inducing significantly elevated toxicity in specific model organisms [25]. The ecotoxicological tests presented dynamic and variable toxicity of irbesartan and its byproducts (Figure 5). The lowest toxicity level occurred at an around −32% (indicating stimulation) concentration of DBP7 in the algal test, while the highest toxicity was at 84% of the mixture in the bacteria test.

Among the tested organisms, *A. fischeri* was the most sensitive to the DBPs, followed by *R. subcapitata* and, finally, *D. magna*, showing significant species-specific toxicity. According to a previous study, the sensitivity to DBP effects increases with decreasing trophic levels [33]. In contrast to species-specific toxicity differences, only DBP10 showed the same range of toxicity in all the tested organisms, but no unique physicochemical characteristics have been associated with contributing to the observed effects.

The observed sensitivity and, consequently, toxicity changes can be attributed to the new smaller molecules present following the cleavage of functional groups that renders them more or less reactive against non-target organisms [33,34]. This also aligns with previous studies that stated the introduction of a chlorine atom into a molecule or the formation of a chlorinated compound led to an increase in acute toxicity [35].

To further evaluate and interpret the toxicological relationships among the tested DBPs, the obtained results were compared to those reported in the study by Romanucci et al. [16] on *D. magna*. As mentioned previously, compounds DBP9, DBP15, and DBP16 were found to be very similar to DBP2, DBP1, and DBP5 [16], respectively. Although the toxicity of the new DBPs was lower than that of the DBPs previously reported [16], differences in the structures of the newly identified compounds compared to those previously reported could have led to different toxic effects. Even slight structural differences can significantly alter the physicochemical and biological properties of a compound, including its toxicity. Therefore, a comprehensive analysis of the ecotoxicity of all the identified DBPs, including the newly identified ones, is necessary to fully understand the potential health risks of irbesartan and its byproducts in the aquatic environment.

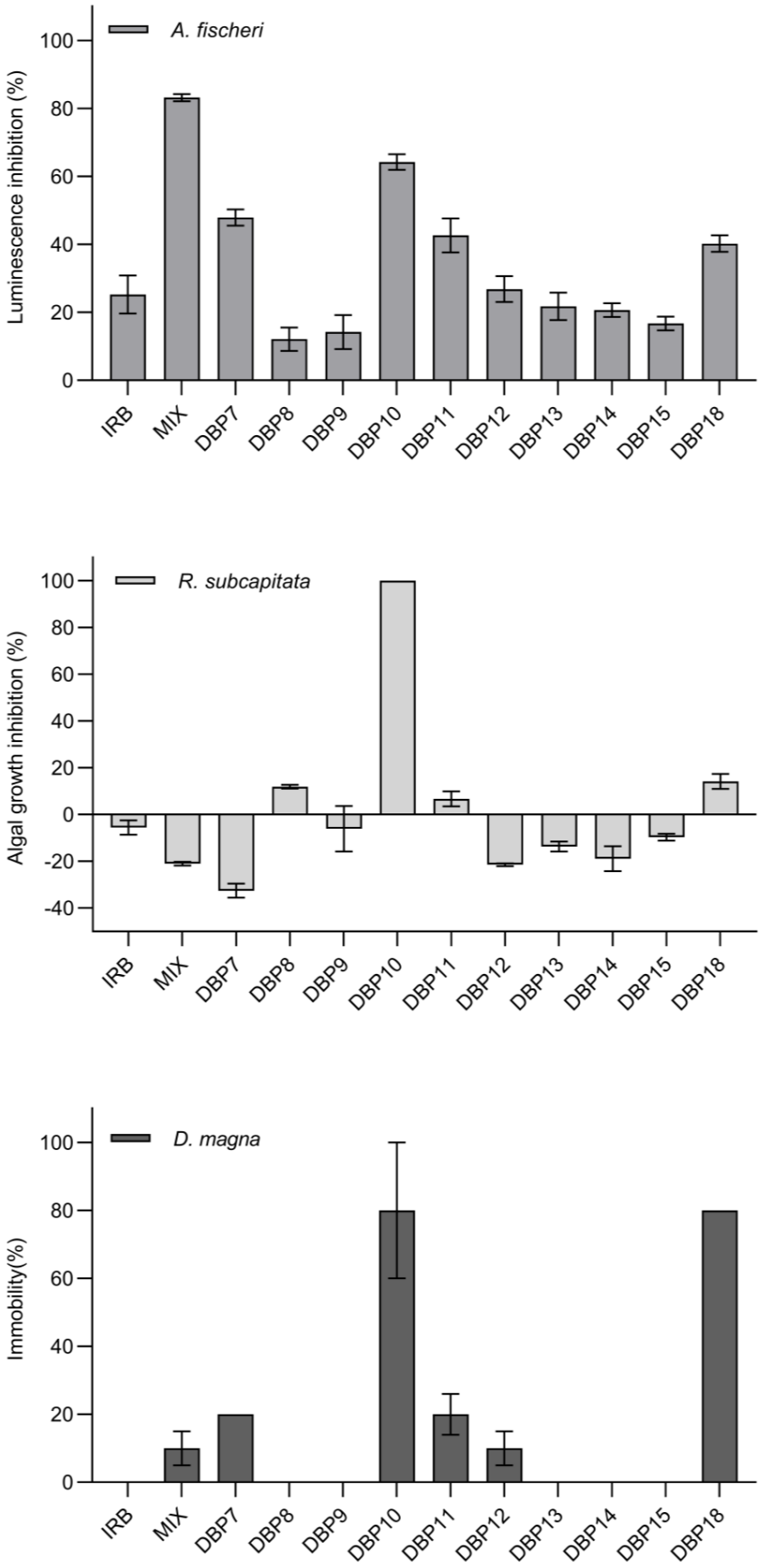


Figure 5. Toxicity results after exposure to irbesartan and its oxidation byproducts.

In addition, this study found that 64% of DBPs exhibited the stimulation (hormesis) of *R. subcapitata*. From an environmental perspective, it is crucial to evaluate the observed hormetic effect of disinfection byproducts (DBPs) on algal populations in water bodies that receive sewage. This evaluation should consider the occurrence of DBPs and algal blooms and assess the true nature of the contribution of DBPs to phytoplankton blooms [36]. It should be noted that the co-existence of many other DBPs in the environment, their high toxicity, and the potential interactions between them [37] suggest that chlorine products could pose high environmental risks.

4. Conclusions

This work provides a complete picture of the transformation of irbesartan, a known pollutant of surface and wastewater, when subjected to chlorination processes, such as those carried out in wastewater treatment plants. Twelve new disinfection byproducts were isolated and identified using mono- and bidimensional NMR spectra and MALDI TOF experiments. In particular, nine of these were isolated for the first time. Furthermore, by adding them to the library of compounds isolated in a previous work, it was possible to provide a complete overview of the byproducts and hypothesize a formational mechanism for each of them; this work is useful for predicting the behavior of other sartans under the same conditions, which, like irbesartan, are considered emerging pollutants. The products reported were derived from the hydrolysis of two different bonds of the imidazole-like ring or from the partial or complete oxidation of the C-11 carbon and/or the *n*-butyl side chain. Notably, the biphenyl nucleus did not undergo transformation apart from the formation of a new ring with the adjacent tetrazole, and, apart from one, no halogenated disinfection byproducts were isolated.

Regarding the detected toxicity via the biological assays, the DBPs showed a dynamic trend, resulting in further transformation. The transformation pathway provided an overview of the fate of irbesartan and its DBPs in the aquatic environment. Notably, a batch of DBPs was detected, which showed higher toxicity than the parent compound. The DBPs exhibited dynamic and variable toxicity, with different levels of toxicity observed in different organisms. *A. fischeri* was the most sensitive to the DBPs, followed by *R. subcapitata* and *D. magna*, indicating species-specific toxicity. About two-thirds (64%) of the DBPs exhibited a stimulating effect (hormesis) on the algal species R. subcapi-tata. This outcome raises concerns regarding the potential consequences of these DBPs on algal populations in water bodies that receive sewage, emphasizing the importance of further research and appropriate actions to safeguard water quality and the ecological equilibrium. Chlorine disinfection is not an optimal process for irbesartan removal because of its production of DBPs, which possess greater toxicity compared to the original chemical. It is important to note that the primary goal of water chlorination is to ensure the safety of drinking water by disinfecting it and preventing the spread of waterborne diseases. The presence of DBPs, including those derived from pharmaceutical compounds like irbesartan, highlights the need for comprehensive water treatment strategies that can address the removal of or reduction in both microbial contaminants and potentially harmful byproducts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13148170/s1, The 1D and 2D NMR characterization (Tables S1–S11) of the disinfection byproducts are available online.

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