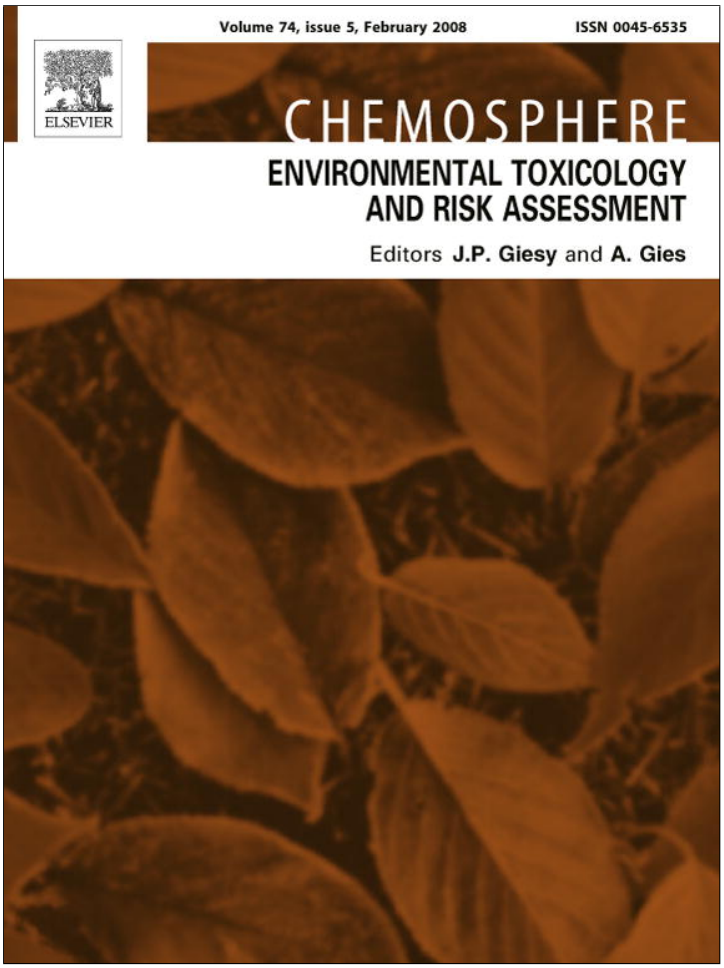

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Unusual products of the aqueous chlorination of atenolol

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ABSTRACT

The reaction of the drug atenolol with hypochlorite under conditions that simulate wastewater disinfection was investigated. The pharmaceutical reacted in 1 h yielding three products that were separated by chromatographic techniques and characterized by spectroscopic features. Two unusual products 2-(4-(3-(chloro(2-chloropropan-2-yl)amino)-2-hydroxypropoxy)phenyl) acetamide and 2-(4-(3-formamido-2-hydroxypropoxy)phenyl) acetamide were obtained along with 2-(4-hydroxyphenyl) acetamide. When the reaction was stopped at shorter times only 2-(4-(3-amino-2-hydroxypropoxy) phenyl) acetamide and the dichlorinated product were detected. Tests performed on the seeds of *Lactuca sativa* show that chlorinated products have phytotoxic activity.

1. Introduction

Reuse of wastewater from municipal STP for crop production is an interesting way to preserve the water supplies. Use of these effluents depends mainly on their quality: appropriate concentrations of components such as nutrients, salts, and metals; and absence of phytotoxic chemicals. Pharmaceuticals and personal care products (PPCPs) are considered emerging contaminants of the aquatic ecosystem (Kummerer, 2004), because they are continuously released into the aquatic environment and are not efficiently removed by conventional wastewater treatment processes (Heberer, 2002; Soulet et al., 2002; Andreozzi et al., 2003). Consequently, they have been detected in surface and in sewage treatment plants (STP) waters in many countries (Ternes, 1998; Kolpin et al., 2002; Calamari et al., 2003; Kummerer, 2004).

Recently, there is a lot of concern regarding the fate of these emerging pollutants during disinfection processes, required for water recycling or get drinking water (Pinkston and Sedlak, 2004; Bedner and MacCrehan, 2006a), in sewage treatment plants.

Different processes are currently employed for the sterilisation of STP effluents before the reuse, i.e., UV irradiation, ozonation, chlorination, oxidant dosage as peracetic acid or ozone. The primary aim of these processes is reducing the presence of pathogens in treated waters below the limits imposed or recommended in the country (Lazarova et al., 1999; Liberti and Notarnicola, 1999).

Chlorination is the most widely used chemical process in different countries. Chlorine is added to water either as chlorine gas (Cl_2) or hypochlorite solution (NaOCl), and a mixture of HOCl and ClO^- is generally referred to as free available chlorine (FAC). Chlorine is a non-specific oxidant that has been shown to degrade various PPCPs to chlorinated, oxidized, and fragmented byproducts (Buth et al., 2007).

According to a recent research, acetaminophen, the most widely used painkiller in the world and the active ingredient in over-the-counter drugs, may be transformed into toxic compounds during chlorination in wastewater treatment plants (Bedner and MacCrehan, 2006a).

Treatment of fluoxetine and metoprolol with hypochlorite in conditions that simulate wastewater disinfection gives neutral N-chloramines (Bedner and MacCrehan, 2006b). While in recent literature most articles report the effects of chlorination on the persistence of pharmaceuticals in the environment, identification of potential transformation products has been limited to a few cases, for example cimetidine and sulfamethoxazole (Dodd and Huang, 2004; Buth et al., 2007).

Therefore, there is a need to assess the fate during the sterilisation treatments of the micropollutants present in STP effluents and to identify new or improved treatment processes that can

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guarantee the removal of the species, which are of some concern in view of a possible accumulation in crops or their presence in drinking waters.

In a recent investigation on the distribution and fate of residual pharmaceuticals in surface waters Castiglioni et al. (2006) reported that atenolol was one of the most abundant in the waters receiving the effluents of the STPs in Italy.

In this context we investigated the reaction of atenolol with hypochlorite under conditions that simulate wastewater disinfection. Our aim was to evaluate the phytotoxic effects of the drug and its chlorinated products on *Lactuca sativa*.

2. Experimental

2.1. Chemicals

Atenolol and 2-(4-hydroxyphenyl)acetamide (**2**) were purchased from Sigma. Sodium hypochlorite solution NaOCl (10% available chlorine) was purchased from Fluka. Water used in all experiments and in the preparation of aqueous buffers, was purified by a MilliQ filtration system (Millipore). Sodium thiosulfate, analytical reagent grade, was obtained from Carlo Erba. Methanol, acetonitrile, dichloromethane, and acetone were of HPLC grade.

2.2. Chlorination procedures

The sodium hypochlorite solutions were obtained for dilution of Fluka solution and the available chlorine in the solution was measured by iodometric titration method.

A solution of atenolol and hypochlorite in 1:1.5 molar ratio was prepared by dissolving 90 mg of drug in 1 L of a 452 μM sodium hypochlorite solution.

A solution of atenolol and hypochlorite in 1:8 molar ratio was prepared by dissolving 90 mg of drug in 1 L of a 2.75 mM sodium hypochlorite solution.

All chlorination experiments were performed at room temperature and the solution pHs were monitored during the reaction time. Final pH of reaction solutions did not vary by more than 0.3 units from starting values, which were 10.2 (for the experiment with 452 μM sodium hypochlorite solution) and 10.9 (for the experiment with 2.75 mM sodium hypochlorite solution).

Sodium thiosulfate solution (0.1 mM, $K_2Cr_2O_7$ iodometric titration) was used to quench residual free available chlorine in experiments conducted with excess of hypochlorite.

2.3. HPLC analysis

The chlorination reactions of atenolol was evaluated using an Agilent 1100 HPLC system equipped with a UV-absorbance detector. Chromatograms to monitor substrate degradation and products formation were obtained by injecting 20 μL samples on a Phenomenex Synergi Hydro RP 80A 250 × 4.6 mm, 4 μm particle size column with a mobile phase of water (A) and methanol/acetonitrile = 1/1 (B) whose composition was varied according the following gradient: t = 0, A = 90%, t = 15, A = 90%, t = 16, A = 80%, t = 26, A = 80%, t = 30, A = 50%, t = 43, A = 0%; the detection wavelength was set at 240 nm and the flow rate was 0.5 mL/min.

Isolation of the atenolol chlorination products was accomplished by semi-preparative HPLC using a Phenomenex Luna C18 250 × 10.0 mm, 10 μm particle size column with the same mobile phase used for analytical HPLC, a flow rate of 1.4 mL/min and detection at 240 nm.

HPLC/MS analyses were performed using an Agilent 1100 chromatographic system, coupled to electrospray ionisation mass spectrometry (ESI). The LC system was equipped with a Phenomenex Synergi Hydro RP 80A (250 × 4.6 mm, particle size 4 μm). The mobile phase was made of eluent A (0.1% formic acid in ultrapure water) and B (methanol/acetonitrile = 1/1) under the same gradient conditions as above. The flow rate of mobile phase was 0.5 mL/min. The analyses were done in the positive mode (API-ES). The capillary voltage was 3.0 kV. Nitrogen as drying gas was set at 12.0 L/min with a temperature of 350 °C.

All ions in ESI positive were the result of protonation of the intact molecules $[M+H]^+$. Chromatogram at 10 min: retention time = 8.35 min (m/z 225, compound **4**), retention time = 14.22 min (m/z 267, atenolol), retention time = 33.96 min (m/z 259, compound **6**), retention time = 39.96 min (m/z 293, compound **7**), retention time = 44.64 min (m/z 301, compound **5**).

2.4. Isolation of chlorination products

After 1 h, the chlorination solution of atenolol/hypochlorite 1:1.5 was evaporated and analyzed by ^{1}H NMR and TLC (Kieselgel 60 F_{254} plates with 1 mm layer thickness, Merck).

The residue was chromatographed on preparative TLC [eluent:dichloromethane/acetone (8/2), two runs] to give two products **1** (16%) and **2** (9%), and a mixture. This mixture contained mainly product **3**, which was purified by semi-preparative HPLC. The isolated products were subjected to spectroscopic studies.

A similar experiment was stopped after 30 min and the chlorination solution was evaporated and analyzed by ^{1}H NMR and TLC. TLC of the residue, treated as above, gave only two products **1** and **4**.

2.5. Spectroscopic analyses

Electrospray ionization (ESI) mass spectra of products isolated were acquired using a LC/MS Agilent 1100 LC equipped with ESI detector in positive mode (ESI^+). NMR spectra in CD_3OD were recorded on a Varian Inova-500 instrument operating at 499.6 and 125.6 MHz for ^{1}H and ^{13}C, respectively. UV/Vis spectra were recorded in methanol on a Perkin–Elmer Lambda 7 spectrophotometer. IR spectra were recorded on a Jasco FT/IR-430 instrument.

2.6. Toxicity testing

Bioassays used Petri dishes (50 mm diameter) with one sheet of Whatman No. 1 filter paper as support. Germination and growth experiments were conducted in aqueous solutions at controlled pH, in four replicate experiments, repeated three times for three different periods. Seeds of *Lactuca sativa* L. (cv Napoli V. F.) collected during 2007, were obtained from Ingegnoli S.p.a. All undersized or damaged seeds were discarded, and the seeds to be assayed were selected for uniformity.

Toxicity tests were performed using the mixture obtained by chlorination of atenolol/hypochlorite 1:1.5 after 1 h. It was evaporated in vacuum and the residue was dissolved in methanol and filtrated on Millex (0.22 μm), and evaporated. Test solution of 3.0 ppm was prepared using 10 mM MES buffer (2-[*N*-morpholino] ethanesulfonic acid; pH 6.0), and subsequent dilutions gave the concentration of 0.3–0.003 ppm.

Pure compounds **1**–**3** were tested. Test solutions of compounds **1**–**3** (10^{-4} M) were prepared using 10 mM MES buffer. Concentrations of 10^{-5} to 10^{-7} M were obtained by dilution.

Parallel controls were performed. After the addition of 25 seeds and 2.5 mL of test solutions, the Petri dishes were sealed with Parafilm to ensure closed-system models. The seeds were placed in a growth chamber (KBW Binder 240) at 25 °C. Bioassays took 5 d and after growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process. This helped in the handling of plants, especially in accurately measuring root

and shoot lengths. Data are reported as differences (in %) from the controls (0%). Positive values represent stimulation of the parameter studied, and negative values represent inhibition.

2.7. Physical data for compounds 1, 3 and 4

Compound **1** [2-(4-(3-(chloro(2-chloropropan-2-yl)amino)-2-hydroxypropoxy)phenyl) acetamide]: ^{1}H NMR: δ (CD_3OD) 7.21 (d, J=9.0, 2H, H-2′ and H-6′), 6.90 (d, J=9.0, 2H, H-3′ and H-5′), 4.58 (m, 1H, H-2″), 4.09 (d, J=4.9, 2H, H-1″), 3.76 (dd, J=12.7, 6.8, 1H, H_a-3″), 3.64 (dd, J=12.7, 7.8, 1H, H_b-3″), 3.44 (s, 2H, H-2), 1.48 (s, 6H, H-6″ and H-7″). ^{13}C NMR: δ (CD_3OD) 177.9 (C-1), 159.7 (C-4′), 131.8 (C-2′ and C-6′), 129.9 (C-1′), 116.2 (C-3′ and C-5′), 104.0 (C-5″), 76.0 (C-2″), 71.1 (C-1″), 63.2 (C-3″), 43.0 (C-2), 27.4 and 25.8 (C-6″ and C-7″). ESI-MS: 299.0 $[M-HCl+H]^+$, 321.0 $[M-HCl+Na]^+$; IR (CH_3OH): ν_{max} (ZnSe): 3339, 3166, 2915, 1632, 1404, 1244, 1032, 828; UV λ_{max} (CH_3OH) nm (log ε): 222 (3.23), 276 (2.63), 283 (2.57).

Compound **3** [2-(4-(3-formamido-2-hydroxypropoxy)phenyl) acetamide]: ^{1}H NMR: δ (CD_3OD) ^{13}C NMR: δ (CD_3OD) 8.10 (s, 1H, H-5″), 7.21 (d, J=8.5, 2H, H-2′ and H-6′), 6.91 (d, J=8.5, 2H, H-3′ and H-5′), 4.02 (m, 1H, H-2″), 3.94 (m, 2H, H-1″), 3.53 (dd, J=13.7, 4.9, 1H, H_a-3″), 3.44 (s, 2H, H-2), 3.35 (dd, J=13.7, 6.8, 1H, H_b-3″). ^{13}C NMR: δ (CD_3OD) 177.8 (C-1), 164.7 (C-5″), 159.8 (C-4′), 131.7 (C-2′ and C-6′), 129.7 (C-1′), 116.2 (C-3′ and C-5′), 71.6 (C-2″), 70.2 (C-1″), 43.0 (C-2), 42.5 (C-3″). ESI-MS: 253.1 $[M+H]^+$, 275.1 $[M+Na]^+$, 291.0 $[M+K]^+$; IR ($CHCl_3$): ν_{max} (NaCl) 3002.4, 2941.6, 2827.1, 1737.0, 1607.3, 1503.5, 1342.6, 1095.1, 1021.8; UV λ_{max} (CH_3OH) nm (log ε): 218 (3.17), 230 (3.12), 276 (2.57), 283 (2.51).

Compound **4** [2-(4-(3-amino-2-hydroxypropoxy)phenyl)acetamide]: ^{1}H NMR: δ (CD_3OD) 7.22 (d, J=8.0, 2H, H-2′ and H-6′), 6.90 (d, J=8.0, 2H, H-3′ and H-5′), 3.95 (m, 2H, H-1″ and H-2″), 3.44 (s, 2H, H-2), 2.89 (dd, J=13.0, 2.9, 1H, H_a-3″), 2.76 (dd, J=13.0, 6.8, 1H, H_b-3″). ESI-MS: 225.0 $[M+H]^+$, 247.1 $[M+Na]^+$, 263.1 $[M+K]^+$; IR (CH_3CN): ν_{max} (ZnSe) 3490, 1635, 1242, 964; UV λ_{max} (CH_3OH) nm (log ε): 269 (4.74).

3. Results and discussion

In the chlorination experiments, using atenolol/hypochlorite 1:1.5 ratio, the drug was completely transformed after 1 h as proved by TLC and ^{1}H NMR analyses.

Chromatographic purification of reaction mixtures gave three main products (**1**–**3**, Fig. 1). Similar results were obtained using an excess of hypochlorite and after 1 h compounds **1**–**3** were detected. In an experiment the excess of hypochlorite was quenched by $Na_2S_2O_3$, and the HPLC analysis evidenced the absence of dichlorinated **1** and the presence of atenolol and products **2**–**4**. A reasonable hypothesis is that, in this case, dichlorinated **1** could be converted to atenolol and/or compound **4**. At shorter chlorination time (30 min) only dichlorinated **1** and compound **4** were detected.

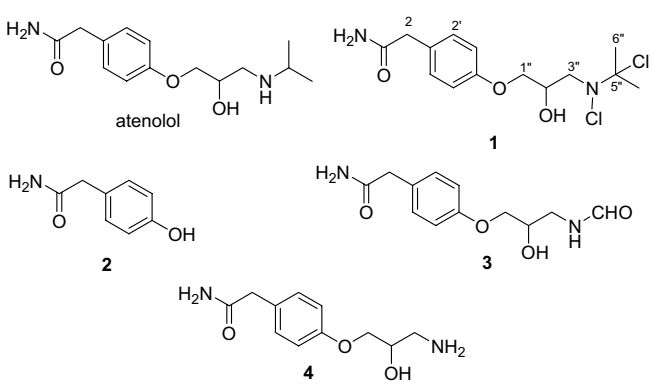


Fig. 1. Structures of atenolol and compounds **1**–**4**.

All compounds were identified by spectroscopic means.

The ESI mass spectrum, of product **1**, revealed peaks at m/z 321.0 and 299.0. These ions likely are $[M-HCl+Na]^+$ and $[M-HCl+H]^+$. The ^{13}C NMR spectrum showed twelve carbon signals that were assigned by DEPT experiment to two methyls, three methylenes and three methines. The ^{1}H NMR spectrum showed two doublets at δ 7.21 (2H, d, J=9.0 Hz, H-2′ and H-6′), 6.90 (2H, d, J=9.0 Hz, H-3′ and H-5′), of a 1,4-disubstituted aromatic ring. These protons were correlated in the HSQC experiment at δ 131.8 and 116.2 carbon signals, respectively. The COSY experiment showed homocorrelations between the proton signals at δ 4.09 (2H, d, J=4.9 Hz, H-1″), 4.58 (1H, m, H-2″), 3.76 and 3.64 (2H, dd, J=12.7, 6.8, 7.8 Hz, H-3″), correlated in the HSQC experiment to the carbons at δ 71.1, 76.0, and 63.2, indicated the presence of 3-aminopropyl-1,2-diol chains group. Furthermore, the ^{1}H NMR spectrum showed the H-2 methylene protons at δ 3.44, and the H-6″ and H-7″ at δ 1.48 as singlets. Long-range correlations between the H-1″ protons and the C-4′, C-2″, and C-3″ carbons, the H-3″ and H-6″/H-7″ and C-5″ quaternary carbon were present in the HMBC spectrum. All these spectral data were consistent with structure of 2-(4-(3-(chloro(2-chloropropan-2-yl)amino)-2-hydroxypropoxy)phenyl) acetamide assigned to product **1**.

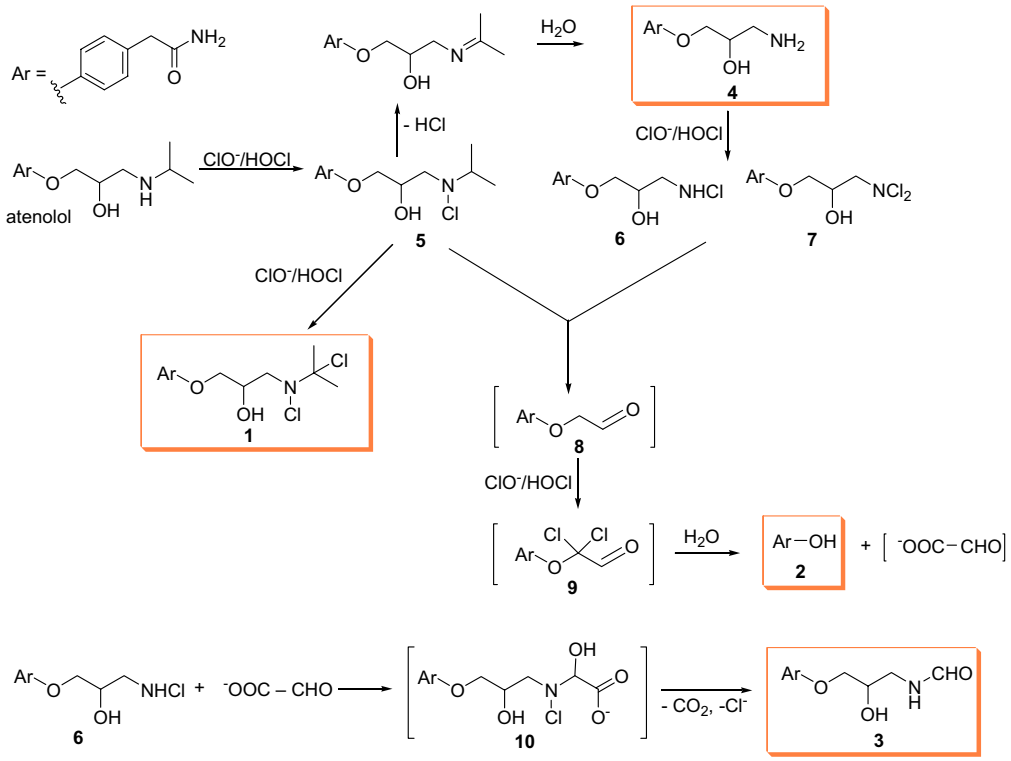
Product **2** was identified as 2-(4-hydroxyphenyl) acetamide by comparison of its spectral data with those of commercial sample.

The structure of 2-(4-(3-formamido-2-hydroxypropoxy)phenyl) acetamide was attributed to compound **3**. It had the molecular formula $C_{12}H_{16}N_2O_4$ according to the pseudo molecular ion at m/z 253.1 $[M+H]^+$ in its ESI-MS spectrum and the presence of 10 carbon signals in the ^{13}C NMR spectrum. A DEPT experiment defined the carbons as three methylenes, four methines, and three quaternary carbons. The ^{1}H NMR spectrum showed two doublets at δ 7.21 (2H, d, J=8.5 Hz, H-2′ and H-6′), 6.90 (2H, d, J=8.5 Hz, H-3′ and H-5′), of a 1,4-disubstituted aromatic ring. These protons were correlated in the HSQC experiment at δ 131.7 and 116.2 carbon signals, respectively. COSY experiment showed homocorrelations between the proton signals at δ 3.94 (2H, m, H-1″), 4.02 (1H, m, H-2″), 3.53 and 3.44 (2H, dd, J=13.7, 6.8, 4.9 Hz, H-3″), correlated in the HSQC experiment to the carbons at δ 70.2, 71.6, and 42.5, indicated the presence of 3-aminopropyl-1,2-diol chains group. Furthermore, the ^{1}H NMR spectrum showed the H-2 methylene protons at δ 3.44 and H-5″ proton at δ 8.10 (1H, s, H-5″). Long-range correlations between the H-1″ protons and the C-4′, C-2″, and C-3″ carbons, the H-3″ and C-5″ methine carbon, the H-5″ and C-3″ were present in the HMBC spectrum.

The structure of 2-(4-(3-amino-2-hydroxypropoxy)phenyl) acetamide was attributed to compound **4**. It had the molecular formula $C_{12}H_{16}N_2O_4$ according to the pseudo molecular ion at m/z 225.0 $[M+H]^+$ in its ESI-MS spectrum. The ^{1}H NMR spectrum showed two doublets at δ 7.22 and 6.90 of a 1,4-disubstituted aromatic ring; a multiplet at δ 3.95, a singlet at 3.44, two double doublets at 2.89 and 2.76 indicated the presence of 3-aminopropyl-1,2-diol chains group.

To get information on the possible mechanism involved in the production of compounds **1**–**3** HPLC/MS analyses were performed. Injection at 10 min of the chlorination reaction mixture evidenced the presence of amine **4**, *N*-chloroatenolol (**5**), *N*-chloroamine of **4**(**6**), and *N*,*N*-dichloroamine of **4**(**7**). All these compounds disappeared after 1 h of chlorination as proved by HPLC/MS analyses.

On the basis of the literature data (Armesto et al. 1998; Pinkston and Sedlak, 2004), the chlorination of atenolol should give N-chlorination of its secondary amino group and subsequent formation of primary amino derivative (**4**), by loss of HCl and hydrolysis of imine intermediate (Scheme 1). (*N*-Halo)-alcoholamines (**5**–**7**)



Scheme 1. Proposed reaction pathways for the reaction of atenolol with hypochlorite. Boxed structures represent isolated products. Bracketed structures represent proposed reaction intermediates.

also undergo fragmentation (Armesto et al., 1997) giving aldehyde (**8**) (Scheme 1). This product could give an haloform reaction with hypochlorite, as also observed for non-methyl ketones (Rothenberg and Sasson, 1996), to obtain the α,α-dichloroaldeyde (**9**). This latter could be hydrolyzed to product **2** and glyoxylate. Glyoxylate could further react with N–Cl amine **4** to give an intermediate (N–Cl)-hemiaminal (**10**) (Böhme and Sadanandam, 1973; Joo and Mitch, 2007), which decomposes to give product **3**. Formamide derivatives have been reported previously during chlorination of secondary alkyl amines but formation mechanisms remain unclear (Mitch and Sedlak, 2002). Formation of dichlorinated compound **1** could be due to the further chlorination of the *N*-chloroatenolol (**5**, Scheme 1). This substitution reaction of the hydrogen (H-5″) with Cl could compete with the elimination of HCl that gives the imine. As far as we know dichlorinated products weren't identified in the chlorination of secondary amines.

Atenolol, chlorination products (**1**–**3**), and chlorination mixture (1 h, after evaporation and desalification) were tested for their phytotoxicity on the seeds of *Lactuca sativa*. This species was selected as representative of main dicotyledon commercial crops (Macias et al., 2000). It has been used extensively as a test organism because of its fast germination and high sensitivity, and allows comparison of bioassay results for many different compounds (Leather, 1983; Yu and Matsui, 1994). The chlorination reaction mixture was tested on *L. sativa* at 3.0, 0.30, 0.030, and 0.003 ppm in order to evaluate the inhibitory or stimulatory effects on germination and seedling growth. The results expressed as % inhibition or stimulation respect to the control, showed that the mixture inhibited germination of ~35% at all concentrations tested, while slight effects were observed on root length (~9%). The shoot elongation was significantly reduced and 87% inhibition was observed at all tested concentrations. Atenolol and the chlorination products were also tested against *L. sativa* and the activities were compared with that of pendimethalin (P), a commercial pre-emergence herbicide widely used in agriculture. Aqueous solutions of atenolol and compounds **1**–**3**, ranging between 10^{-4} and 10^{-7} M, were tested on germination, root length and shoot length of treated lettuce seeds (Table 1). The effect observed was an inhibition of germination of *L. sativa* produced by compounds **1**–**3** higher than that observed for atenolol and comparable to that of pendimethalin. The root and

Table 1
Activity of atenolol and compounds **1**–**3**, and pendimethalin (**P**) on the germination, root elongation, and shoot elongation of *Lactuca sativa*

	Germination				Root elongation				Shoot elongation			
M	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Atenolol	−3	−11[a]	−18[a]	−22[a]	1	−8	1	8	8	−5	8	7
1	−33[a]	−32[a]	−33[a]	−30[a]	19[b]	32[b]	2	−10[b]	−10[b]	10	−6[b]	4
2	−19[a]	−28[b]	−29[a]	−27[a]	−7	−21	7	5	5	−7	−7	5
3	−29[a]	−32[a]	−30[a]	−36[b]	−14	−15	−6	1	1	4	8	−19
P	−36[b]	−38[a]	−40[a]	−41[a]	−78[a]	−78[a]	−78[a]	−78[a]	−78[a]	−87[a]	−87[a]	−87[a]

Values are presented as percentage differences from control and are significantly different with $P > 0.005$ for Student's t-test.
A positive percentage represents stimulation while negative values represent inhibitions.
[a] $P < 0.01$.
[b] $0.01 < P < 0.05$.

shoot lengths were slightly affected by the compounds in respect to the pesticide.

The results of the phytotoxic experiments indicate that atenolol and particularly its chlorinated reaction products may have negative effects on the plant growth. Tested concentrations are much higher than those eventually present in effluents of STP, but bioaccumulation, caused by continual irrigations, could make dangerous these products.

Atenolol could represent a model of basic drugs that contain secondary amines.

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