Chlorination of 5-Fluorouracil: Reaction Mechanism and Ecotoxicity Assessment of Chlorinated Products

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Abstract

What happens to drugs in the chlorinating environment? Degradation products may vary in pharmacological profiles and in ecotoxicity potentials compared to the parent compound. This study combines synthesis, NMR spectroscopy, quantum chemical calculations, and toxicity experiments on *Daphnia magna* to investigate chemical fate of antineoplastic drug 5-fluorouracil (5-FU) in chlorinated environment, which is common in waste-water treatment procedures, but also endogenous in activated neutrophils. A reduction of toxicity (EC$_{50}$ after 48 hours is 50% higher than for the parent 5-FU) was observed after the first chlorination step, in which a chlorohydrin 5-chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil was formed. Further chlorination leads to N-chlorinated intermediate, that undergoes the pyrimidine ring opening reaction. The final product, 2-chloro-2-fluoro-3,3-dihydroxypropanoic acid was obtained after the loss of the chlorinated urea fragment. This is the most potent compound in the reaction sequence, with toxicity parameter EC$_{50}$, after 48 hours, more than twice lower compared to the parent 5-FU. Clearly, the contact time between chlorinating species and degradation products provide different ecotoxicological properties of reaction mixtures. Interplay between experimental and theoretical procedures, to properly describe reaction pathways and provide more information on toxicity profiles, is a way forward in environmental science research.

Keywords

5-fluorouracil, hypochlorous acid, ecotoxicity, reaction mechanism, DFT calculation
1. Introduction

5-Fluorouracil (5-FU) is a pyrimidine antimetabolite introduced in the clinic as an anticancer drug (Jordan, 2016). It is one of the most widely prescribed cytostatic pharmaceutical for the last 60 years. Up to 30% of its administered dose is excreted as the parent form that enters the environment (Kosjek and Heath, 2011). The measured environmental concentrations of 5-FU range from 5 – 100 ng/L in wastewater treatment plant influents (Kosjek et al., 2013), and may amount to more than 100 µg/L in hospital wastewaters (Mahnik et al., 2004). 5-FU is relatively stable in water, but undergoes different transformation reactions induced by chemical water treatments, such as ozonolysis or chlorination. The latter is the most widely used method for chemical treatment and disinfection of water (USEPA, 2004). Since 5-FU has been identified in wastewaters and surface waters (Mahnik et al., 2007) the potential exists for the formation of its disinfection byproducts during water chlorination.

The reaction between 5-FU and hypochlorous acid (HOCl) is the fundamental process which can occur in activated neutrophils in cancer patients (Winterbourn et al., 2016) or during chemical treatment of (hospital) wastewaters (Deborde and Gunten, 2008; Acero et al., 2010). Therefore, the chlorination of 5-FU is of utmost importance in medicinal and environmental chemistry. The elementary chemical reaction, the one with no enzyme assistance, is simple yet so intricate process. Mechanistic details underlying a HOCl-induced transformation of 5-FU have not been resolved. In addition, chlorinated products are unknown or only tentatively assigned, and their environmental effects have not been investigated. For this reason the chemical fate of 5-FU under chlorination conditions should be revisited.

We set to investigate the chlorination mechanism which gives rise to stable products. By using NMR spectroscopy coupled to high-level computational techniques, the relevant
reaction profiles were described in details. The chlorinated products were isolated and their
ecotoxicological effects were studied in acute immobilization assays with crustacean *Daphnia
magna*.

In this work the interplay between experimental and theoretical methods has been
shown as an efficient approach in solving some environmental problems. The results on the
chlorination of 5-FU are relevant for a series of pyrimidines, and for nucleobase derivatives in
particular.

2. Material and Methods

2.1. **General procedures and equipments**

The syntheses were carried out in distilled water, and kinetic experiments in the
phosphate buffer. Melting point was determined with a Büchi apparatus. For HPLC-MS
analysis, ultra high-speed single quadrupole mass spectrometer with ultra high-speed liquid
chromatography, Prominence UFLC+LCMS-2020 from Shimadzu Corp. (Kyoto, Japan) was
used. Separation was performed on a Zorbax SB C18 (150x2.1mm 3.5µm; Agilent
Technologies Deutschland GmbH, Waldbronn, Germany) narrow bore LC column with 0.1%
formic acid in water/ 0.1% formic acid in methanol as isocratic mobile phase in ratio (10/90)
with flow-rate of 0.2 mL min$^{-2}$. MS scanning in the range m/z 50-350 for negative and
positive electrospray ionisation was used (mass spectra recorded in positive ion mode in SI).
The interface parameters were: temperature of electrospray probe (ESI) = 350 °C, curved
desolvation line (CDL) temperature or temperature of the heated capillary = 250 °C, the
nebulizing and drying gas was N$_2$ with flow-rate 1.5 L min$^{-2}$, 15 L min$^{-2}$, respectively, and
the block temperature was set at 200 °C. The $^1$H, $^{13}$C, and $^{19}$F NMR spectra of DMSO-d$_6$,
D$_2$O, or CDCl$_3$ solutions were recorded on a Varian INOVA 400 spectrometer. The spectrometer operated at 399.6 MHz ($^1$H), 375.9 MHz ($^{19}$F), and 100.5 MHz ($^{13}$C). Chemical shifts in the $^1$H NMR and $^{13}$C NMR spectra were expressed in parts per million (ppm) vs. TMS as the external standard, and $^{19}$F chemical shifts were referenced to CFCl$_3$ as the external standard.

2.2. Preparation of chlorinated products

All solutions were prepared using deionized, carbon filtered water with a chlorine demand of < 10 μmol/L. All reagents were of analytical grade. 5-FU (99%) was purchased from Alfa Aesar. For chlorination experiments, a 15% solution (>15% as Cl) of reagent grade sodium hypochlorite (NaOCl) was obtained from Alfa Aesar. The solution was standardized weekly using an iodometric titration, and the concentration was found to be stable over a period of months. pH was controlled by use of 0.1 M phosphate buffer system in which the pH was adjusted with NaOH.

5-Chloro-5-fluoro-6-hydroxy-5,6-dihydrouracil (3a): Cl$_2$ was bubbled into a suspension of 5-flourouracil (1; 1.0 g, 7.7 mmol) in 15 mL of water at 25 °C until a clear solution was obtained. The solvent was evaporated to dryness. The resulting white solid was recrystallized from acetone giving 1.2 g (85%) of 3a. White crystals; m.p. 144 °C. C$_4$H$_4$ClF$_2$N$_2$O$_3$ (182.54): calcd. C 26.32, H 2.21, Cl 19.42, F 10.41, N 15.35, O 26.29; found C 26.30, H 2.22, N 15.41. $^1$H NMR (400 MHz, DMSO-$d_6$, 25 °C): δ = 11.1 (s, 1H, N3-H), 8.9 (s, 1H, N1-H), 7.3 (br, 1H, C6-OH), 5.0 (dd, $^3$J$_{H,H}$ = 5.1 Hz, $^2$J$_{H,F}$ = 1.9 Hz, 1H, C6-H) ppm. $^{13}$C NMR (100 MHz, DMSO-$d_6$, 25 °C): δ = 163.2 (d, $^3$J$_{F,C}$ = 27.5 Hz, C4), 151.2 (C2), 97.4 (d, $^2$J$_{F,C}$ = 254.3 Hz, C5), 77.2 (d, $^3$J$_{F,C}$ = 26.3 Hz, C6) ppm. $^{19}$F NMR (376 MHz, DMSO-$d_6$, 25 °C): δ = -137.1 (d,
$^{3}J_{HF} = 1.9$ Hz, C5-F) ppm. MS (ESI-): m/z calcd. for C_4H_4ClFN_2O_3 [M - H]^- 181.53; found 181.52.

2-chloro-2-fluoro-3,3-dihydroxypropanoic acid (11): Cl_2 was bubbled into a solution of chlorohydrin (3a; 300 mg, 1.6 mmol) in 10 mL of water at room temperature, and the course of the reaction was followed by $^{19}$F NMR (reaction completed in ca. 45 min). The solvent was evaporated to dryness, and 240 mg (95%) of the clean oily product 11 was obtained.

C_3H_4ClFO_4 (158.51): calcd. C 22.73, H 2.54, Cl 22.37, F 11.99, O 40.37; found C 22.70, H 2.57. $^{1}$H NMR (400 MHz, DMSO-d$_6$, 25 °C): $\delta = 5.0$ (d, $^{3}J_{HF} = 14.1$ Hz 1H, C3-H) ppm. $^{13}$C NMR (100 MHz, DMSO-d$_6$, 25 °C): $\delta = 166.2$ (d, $^{3}J_{FC} = 27.9$ Hz, C1), 104.6 (d, $^{2}J_{FC} = 263.9$ Hz, C2), 90.4 (d, $^{3}J_{FC} = 20.9$ Hz, C3) ppm. $^{19}$F NMR (376 MHz, DMSO-d$_6$, 25 °C): $\delta = -138.9$ (d, $^{3}J_{HF} = 14.1$ Hz, C2-F) ppm. MS (ESI-): m/z calcd. for C_3H_4ClFO_4 [M - H]^- 157.50; found 157.51.

2.3. Daphnia magna Immobilisation Assay

The D. magna Straus clone MBP996 was purchased as Daphtoxkit F from the MicroBioTests Inc. (Mariakerke, Belgium). D. magna neonates were hatched from dormant eggs (ephippia) in Petri dishes containing standard culture media (SCM) and handled according to the supplier instructions. The SCM for D. magna was reconstituted in hard water containing CaCl_2×2H_2O (294 mg/L), MgSO_4×7H_2O (123.25 mg/L), NaHCO_3 (64.75 mg/L) and KCl (5.75 mg/L) at pH 7.8 ± 0.5. The SCM did not contain any organic compounds. Temperature was maintained at 20 ± 1 °C.

The immobilization assay was performed according to the HRN EN ISO 6341:2013 protocol. Neonates were not older than 24 h at the beginning of the test and were transferred in the
glass test vessels containing SCM (control daphnids) or substance 5-FU (1), chlorohydrin 3a, hydrate 11, or chloral hydrate diluted in SCM at predetermined concentrations (substance exposed daphnids). Transferring procedure was done in order to avoid any hurting of daphnids and causing false positive results. The substance exposure concentrations were prepared in the range 10-1000 mg/L (1), 100-1000 mg/L (3a), 85-150 mg/L (11) and 350-750 mg/L (chloral hydrate) by diluting the concentrated stock solution in SCM immediately prior to use. The physicochemical properties of the SCM were evaluated to obtain additional information regarding the toxicity test. The temperature, pH, conductivity and oxygen content of the test solutions were assessed at time 0 h and at the end of the exposure time (48 h).

Five *D. magna* neonates (24 h old) were added from Petri dish to each exposure vessels containing 10 mL of SCM (control) or 10 mL solution desired concentration. Daphnids were exposed for 24 and 48 h to the concentrations listed above in four replicates. No food or supplements were added during the exposure period. Neonate immobilization and/or behavioral abnormalities were assessed visually after 24 and 48 h of incubation. The immobilised daphnids were considered those lying on the bottom of the vessels and did not resume swimming within 10 sec after gentle agitation, while swimming in circles or trapping at surface of media were considered as abnormal behavior.

The EC$_{50}$ (i.e., effective concentrations that causes 50% immobilization of the test population) values with the 95% confidence intervals, NOAEC (no observed adverse effect concentration) and LOAEC (lowest observed adverse effect concentration) values were calculated based upon substance concentrations in SCM (Table 1).

2.4. Computational Methods
The quantum chemical calculations were performed using the Gaussian09 suite of programs (Frisch et al., 2009). Geometry optimization and frequency calculation were performed at the B3LYP/6-31+G(d) level (Lee et al., 1988; Becke, 1993). Improved energetics have been calculated using B2K-PLYP functional (Tarnopolsky et al., 2008). This double-hybrid DFT procedure in combination with 6-311+G(3df,2p) basis set shows the best overall performance for calculating barrier heights for water-catalyzed proton-transfer reactions (Karton et al., 2012). In case of chlorination of amides, the B2K-PLYP model accurately reproduces a high-level composite G3B3 results (Sakic et al., 2014) and has therefore been used throughout the text.

Gibbs energies of solvation were determined using the SMD continuum solvation model at the B3LYP/6-31+G(d) level (Marenich et al., 2009). The solvent relative permittivity of $\varepsilon = 78.4$ (water) was used. All other structures, reported throughout the text, include extra water molecules. We found that two explicit water molecules corresponded to „the ideal number of solvent molecules” (Pliego, 2004) for a reliable description of the corresponding potential energy surfaces. The relative energy of reactants complexed to an optimal number of water is set to zero.

In case of all transition state structures the two water molecules were found as an adequate number. Several configurations of explicit water molecules were located in each case, but only the lowest energy transition structures were used for the calculation of the barrier for the corresponding reaction (Sakic et al., 2014).

Calculations of NMR chemical shifts and spin couplings were performed at the GIAO-B3LYP/6-31+G(2d,p)//B3LYP/6-31+G(d) and BHandH/6-311++G(2d,p)// B3LYP/6-31+G(d) level, respectively (Gryff-Keller and Szczecinski, 2014).

3. Results and Discussion
3.1. The chlorination of 5-fluorouracil

The initial site of chlorination of 5-FU is a conflicting issue. In the early report by Miyashita et al. (1982) the chlorination of 5-FU by molecular chlorine (Cl₂) results in the formation of the C5-chlorinated product. Whereas the recent study by Li and coworkers (Li et al., 2015) suggests that 5-FU is chlorinated at the C6-position. To resolve the controversy over the reaction regioselectivity, an experimental procedure for chlorination of 5-FU was repeated and product structures were determined by the NMR spectroscopy and mass spectrometry (Table S5).

3.1.1. NMR analysis of the reaction mixture

The reaction between HOCl and 5-FU (1) was followed by ¹H and ¹⁹F NMR and the obtained spectra confirmed the formation of one product only (Fig 1). In the proton spectrum the resonance signal of C6-H at 7.50 ppm (d, ³J_{F,H} = 5.5 Hz) disappeared from the aromatic region, and the new doublet showed up at 5.22 ppm (d, ³J_{F,H} = 2.0 Hz). This upfield shift is consistent with the rehybridization of the C6-carbon atom from sp² to sp³. The multiplicity (doublet) of the signal in ¹H NMR spectrum confirms that the C6-hydrogen is coupled to the C5-fluorine in the chlorinated product.
Fig. 1. $^{19}$F NMR (376 MHz) spectra of the reaction mixture aliquots (5-FU + HOCl in water) taken at several time points. 5-FU concentration 0.05 mol/L, HOCl concentration 0.05 mol/L, pH = 6.7 (phosphate buffer, 0.1 mol/L). The stack-plot of spectra (with an offset included) were recorded over 30 min. The small inset shows $^1$H NMR (400 MHz) spectrum of the same reaction mixture; arrows point how signals change with time. All spectra were measured at 25 °C (a D$_2$O filled capillary used).

In the fluorine spectrum (Fig 1), during the course of the reaction, the corresponding doublet of 5-FU ($^3J_{F,H}$ = 5.4 Hz, at -169 ppm) was converted to a low-field doublet ($^3J_{F,H}$ = 2.0 Hz, at -139 ppm) of the chlorinated product. The small coupling constant observed in $^1$H and $^{19}$F NMR spectra of the product is indicative of a trans relation between C6-H and C5-F atoms. In the cis isomer the fluorine-hydrogen coupling constant should be much larger ($^3J_{F,H}$ > 10 Hz) (Legay, 2014; Robins et al., 1976).

To correctly assign the structure of the chlorinated product, we performed additional NMR analysis. If aqueous solution (or reaction mixture) of the chlorinated product is acidified, a new coupling pattern (dd, $^3J_{F,H}$ = 2.0 Hz, $^3J_{H,H}$ = 5.2 Hz) of a signal at 5.19 ppm appears in the spectrum (Fig S1). The doublet of doublets results from coupling of C6-H to both C5-F and N1-H. It was shown earlier that splitting of C6-H with amide proton in uracil
could be observed when exchange of N1-H was sufficiently slow (e.g. inhibited by the addition of acid) (Hurd and Reid, 1977; Kokko and Mandell, 1962). It is also possible to detect a coupling of C6-H with C6-OH if the chlorinated product is dissolved in d6-DMSO, and small amount of HCl is added. The corresponding coupling constant (3J_HH) of 4.9 Hz is very similar to that observed for interaction between C6-H and N1-H (3J_HH = 5.1 Hz), which results in the superposition of two doublets. The appearance of triplet of doublets at 4.97 ppm (Fig S2) is due to the additional coupling constant of 1.9 Hz for splitting between C6-H and C5-F.

![Chart 1](image)

**Chart 1.** Proposed structures of the initial product in the reaction between HOCl and 5-FU, which are considered in recently reported studies or in this work.

All this points to the chlorohydrin structure 3a in which the C5-position is chlorinated and the C6-position is hydroxylated (Chart 1). The cis isomer 3b may be rule out as a product on the basis of 19F-1H coupling constants (see above), and because of the unfavorable reaction pathway calculated for its formation (see calculations below). Structural properties of isomers 4a and 4b are also not in accordance with the measured NMR data. Finally, in case of C6-chlorinated fluorouracil (5), *i.e.* the product which was suggested by Li et al. (2015), the fluorine signal should be observed as a singlet in the coupled 19F NMR spectrum.

Key data from the experimental NMR study have been reproduced by DFT GIAO-NMR calculations (Gryff-Keller and Szczecinski, 2014). The 13C and 19F NMR chemical shifts and corresponding coupling constants were calculated for the parent 5-FU, for
chlorohydrins 3a – 4b as plausible product structures (Chart 1), and for the C6-chloro-5-fluorouracil (5) proposed earlier (see Table S1) (Li et al., 2015). $^{19}$F NMR chemical shifts were found as sensitive tool to assign the correct structure of the chlorohydrin product. By comparing experimental and calculated chemical shifts it is possible to discriminate between C5- and C6-chlorinated chlorohydrin structures 3 and 4, respectively. In chlorohydrins 3a and 3b, the calculated chemical shift for C5-F is -135.3 and -134.7 ppm, which is very close to experimental value of -137 ppm. In chlorohydrins 4a and 4b, however, the corresponding peaks are shifted by ~20 ppm downfield. In addition, the experimental value for one-bond fluorine-carbon coupling is 255.9 Hz, and this value can be reproduced only for C5-chlorinated structures 3a ($^{1}J_{F,C} = 256.3$ Hz) and 3b ($^{1}J_{F,C} = 249.2$ Hz). Therefore, the C6-carbon atom can be rejected as a possible site of chlorination of 5-fluorouracil.

As well, it is straightforward to compare experimental and calculated $^{19}$F-$^{1}$H couplings to interpret the stereochemistry in C5-chlorinated chlorohydrins. The experimental value of 2.0 Hz for $^{19}$F-$^{1}$H splitting can be correctly reproduced only if trans-chlorohydrin 3a is taken into account (calculated $^{3}J_{F,H} = 2.9$ Hz). In the presumed cis-isomer 3b this value amounts to 7.8 Hz.

$^{13}$C NMR calculated chemical shifts are not informative (Table S1), as values for all chlorohydrins are scattered around experimental data, while calculated two-bond fluorine-carbon couplings do not offer reliable tool to discriminate between the proposed structures in Chart 1. In conclusion, only chlorohydrin structure 3a fits well to calculated and experimental NMR data.
Scheme 1. The reaction mechanism for chlorination of 5-fluorouracil anions N1 and N3.

Relative Gibbs free energies (ΔG$_{298}$ in kJ/mol, in parentheses) calculated at the B2K-PLYP/6-311+G(3df,2p)//B3LYP/6-31+G(d) level are given in italics (N1 is set to zero for the anionic pathway) and in underlined format (2 is set to zero for the neutral pathway). For clarity, the two explicit water molecules included in calculations are not presented.

3.1.2. Quantum chemical study of the chlorination of 5-FU

Different mechanisms, underlying the reaction between 5-FU and HOCl, were probed by the use of computational models. The epoxidation of the C5-C6 double bond in 5-FU, the one-step addition of HOCl on C5-C6 double bond, and C5- and C6-hydroxylation processes were considered, but calculated barriers for these alternative processes were prohibitively high, and therefore all are deposited in Supporting Information (Scheme S1).

According to quantum-chemical calculations the most favorable process involves HOCl and 5-FU anion as reactants (Scheme 1). The molecular form of 5-FU is a predominant species in a neutral aqueous solution (pK$_a$ of 5-FU is 7.93) (Diaz-Gavilan et al., 2006), but the anion form is expected to be much more reactive. It was shown that bromination of uracil
anion is several order of magnitude faster than the corresponding reaction with the molecular form of uracil (Tee and Berks, 1980).

The 5-FU anion exists as an equilibrium mixture of two monoanions, i.e. N3- and N1-species (Scheme 1) (Wierzchowski et al., 1965). They are present in aqueous solution in the ratio of 2 : 3, respectively (Abdrakhimova et al., 2014), and the observed pKₐ for 5-fluorouracil is a composite of the overlapping ionizations of the N1 and N3 positions.

Nucleophilic reactivities of these two anions, however, could be quite different. It has been suggested that the imide anion is much better solvated in water and is, therefore, significantly less reactive than the amide anion (Breugst et al., 2012).

Our computational results support these experimental findings. All calculations were performed at the B2K-PLYP/6-311+G(3df,2p)//B3LYP/6-31+G(d) level of theory, and all stationary points located at the potential energy surface include two explicit water molecules (not presented in Scheme 1). We located two transition state structures TSN₁ and TSN₃ (Scheme 1 and Fig S3) for the C5-chlorination of anions N₁ and N₃, respectively. The corresponding barriers are 27.1 and 88.4 kJ/mol, respectively, which supports a claim that amide anion N₁ is more nucleophilic towards Cl⁺ cation (values are given in Scheme 1). In both reactions the imine intermediate 2' is formed, which is 107.0 kJ/mol more stable than the starting reactants, i.e. the separated reactants N₁ and HOCl. The results from our calculations performed at different level of theory are presented in Table S2.

The involvement of the imine was confirmed earlier in the course of the bromination of uracil (Tee and Berks, 1980; Henderson et al., 2001), and here we suggest it as an intermediate in the course of chlorination of 5-fluorouracil. In water the imine 2 undergoes the addition of water which can result in either trans- or cis-chlorohydrin products (3a or 3b). Our computational results suggest that the anti-addition (i.e. water approaches the N1-C6 double bond at the side opposite to the C5-Cl bond) is a faster process ($\Delta G^\ddagger_{\text{anti}} = 52.6$ kJ/mol)
than the syn-attack of water ($\Delta G_{\text{syn}}^{\ddagger} = 61.0 \text{ kJ/mol}$). The respective transition state structures $\text{TS}_{3a}$ (for reaction 2 $\rightarrow$ 3a) and $\text{TS}_{3b}$ (for reaction 2 $\rightarrow$ 3b) are presented in Fig S4. The anti-addition of water to imine 2 results in trans-chlorohydrin 3a which is 12.1 kJ/mol more stable than the cis-isomer 3b (Scheme 1). Therefore, the formation of 3a is both thermodynamically and kinetically favored. This is in agreement with experimental findings that chlorination of 5-FU results in only one product, and fits to the measured NMR $^{19}\text{F}-^1\text{H}$ coupling data (see above) which indicate trans-stereochemistry of the chlorohydrin formed.

3.2. The chlorination of trans-chlorohydrin 3a

![Fig 2. $^{19}\text{F}$ NMR (376 MHz) spectra of the reaction mixture aliquots (3a + HOCl in water) taken at several time points. Chlorohydrin 3a concentration 0.05 mol/L, HOCl concentration 0.1 mol/L, pH = 6.7 (phosphate buffer, 0.1 mol/L). The stack-plot of spectra (with an offset included) were recorded over 40 min; arrows point how signals change with time. All spectra were measured at 25 °C (a D$_2$O filled capillary used).]
Scheme 2. The reaction mechanism for chlorination of the chlorohydrin 3a. Relative Gibbs free energies ($\Delta G_{298}$ in kJ/mol, in parentheses) calculated at the B2K-PLYP/6-311+G(3df,2p)/B3LYP/6-31+G(d) level are given in italics ($4N3$ is set to zero for anionic pathway only). For clarity, the two explicit water molecules included in calculations are not presented.

In basic aqueous medium (pH = 10-11) the chlorohydrin 3a is easily converted to glycol 8 (Scheme 2). Upon addition of NaOH to aqueous solution of 3a, only one signal (at -122.03 ppm) was observed in the $^{19}$F NMR spectra, while the corresponding $^1$H and $^{13}$C NMR data (Fig S8) were consistent with the structure of the glycol 8 (Scheme 2). The glycol 8 is, however, out of the scope of the present study and has not been tested for its ecotoxicity.

In neutral or slightly acidic medium, the chlorohydrin 3a undergoes N-chlorination induced by HOCl, which may result in either N1- or N3-chlorinated product (6 and 7 in Scheme 2). In the $^{19}$F NMR spectrum of the reaction mixture (Fig 2) a new signal appeared at -135.25 ppm, which suggested the formation of one product only (the same reaction occurs and the same product was detected when the parent 5-FU is subjected to a prolonged chlorination reaction time). In order to isolate and identify the unstable N-chlorinated intermediate, the reaction mixture was sampled and treated by chloroform. In this solvent the
chlorohydrin 3a is insoluble, but the N-chlorinated product is slightly soluble. In the $^1$H NMR of the extracted N-chlorinated product only one NH signal is observed at 8.10 ppm which corresponds to imide N3-proton (Fig S9). In addition, a new doublet ($^3J_{F,H} = 2.0$ Hz) appears in the spectrum at 5.36 ppm. Contrary to the chlorohydrin 3a, no coupling between C6-H and N1-H protons was observed when HCl is added to aqueous solution (see above). All this indicates that N1-position is chlorinated, i.e. that the reaction between HOCl and chlorohydrin 3a results in chloroamide 6 (Scheme 2).

The regioselective chlorination of amide N1-position (vs. imide N3-position) can be rationalized by computational results. Both N1- and N3-anionic forms of chlorohydrin 3a have been considered as a reactive species in the reaction with HOCl. The corresponding energy barriers for the addition of $Cl^-$ ion to N1- and N3-position are very similar (i.e. the corresponding transition states $2TS_{N1}$ and $2TS_{N3}$ are close in energy, Scheme 2 and Fig S5), and therefore the kinetic control cannot be accounted for the observed regioselectivity. However, the product of N1-chlorination ($5_{N1}$) was calculated more stable ($\Delta G_{298} = 29.3$ kJ/mol) than the corresponding product of N3-chlorination ($5_{N3}$), which strongly suggested that regioselectivity of N-chlorination in chlorohydrin 3a was thermodynamically driven.

![Scheme 3](image_url)

Scheme 3. The reaction mechanism for the intramolecular proton transfer and ring opening in 6 (set to zero), and subsequent hydrolysis of 9 resulting in the final product 11 (exists in equilibrium with the aldehyde form 10).
In aqueous medium the chloroamide intermediate 6 may be converted to the final product 11 (Scheme 3). It was demonstrated earlier that N-chlorination of the chlorohydrin intermediate promoted the pyrimidine ring opening in the uracil system (Young and Uden, 1994). It was also shown that water was essential for that reaction to occur.

The chlorination of N1-position induced an intramolecular proton transfer in 6. The C6-OH proton is shifted to the N1-Cl group, which makes N1-C6 bond very labile and prone to the cleavage process $6 \rightarrow 9$. The amide bond in the short-lived intermediate 9 is hydrolyzed resulting in the elimination of the chlorinated urea. In this process the aldehyde 10 is produced, which is easily converted to the final product 11. None of the two intermediates, 9 and 10, were detected by $^1$H or $^{19}$F NMR during the course of the reaction.

These two reaction steps, $6 \rightarrow 9$ and $9 \rightarrow 10$, were investigated by means of DFT calculations which support the mechanism proposed in Scheme 3. The first transition state $T_{S9}$ corresponds to the structure in which the intramolecular proton transfer occurs concurrently with the pyrimidine ring opening (Fig S6). The calculated barrier for this process amounts to 126.3 kJ/mol. It is interesting to note that the calculated energy barrier for the analogous proton transfer (C6-O to N1) in the chlorohydrin 3a is much higher ($\Delta G^\ddagger = 149.1$ kJ/mol). Therefore, the chlorohydrin intermediate 3a is resistant to the ring opening process, unless the N1-position is chlorinated. The second transition state $T_{S10}$, which represents the rate determining step, corresponds to the hydrolysis of the amide (see Fig S7 for details). This is a two-step consecutive process which follows the general mechanism for amide hydrolysis reported earlier (Pliego, 2004; Bachrach and Dzierlenga, 2011). The calculated energy barrier for the hydrolysis step is somewhat high, but is lowered significantly when acid or base catalysis is included. Due to simplicity, only the neutral pathway was considered in the calculations (Scheme 3).
In short, the chlorohydrin product \(3a\) can be converted to hydrate \(11\), only after the chlorination at the N1-position. This is exactly what was recorded by our NMR experiment, in which all three species \(3a, 6,\) and \(11\) were observed simultaneously (Fig 2). This reveals that the reaction channel \(3a \rightarrow 6 \rightarrow 11\) is indeed operative.

The similar reaction sequence was established in case of uracil chlorination, in which the trichloroacetaldehyde (\(\text{Cl}_3\text{CCHO}\)), or chloral hydrate (\(\text{Cl}_3\text{CC(OH)}_2\)), was detected as the final product (Young and Uden, 1994). Chloral hydrate is structurally and electronically related to hydrate \(11\), which exists in equilibrium with its aldehyde form \(10\) (Scheme 3). In general, aldehydes are more stable than the hydrate counterparts, but electron-withdrawing groups may shift the equilibrium toward the latters (Smith, 2013). In case of the trichloroacetaldehyde, the three chlorine atoms withdraw electron density from the partially positive carbon atom, which destabilizes the carbonyl bond, whereas the aldehyde \(10\) is destabilized by the similar electron-withdrawing effect of carboxyl group, fluorine and chlorine atoms. Therefore, in both cases hydrates are predominate forms in the corresponding equilibria. For that reason the ecotoxicity properties of the two hydrates were compared in \textit{Daphnia magna} immobilization assays (see below).

### 3.3. Ecotoxicity results for \(1, 3a, 11\), and chloral hydrate

A number of data exist on the toxic effects of the parent 5-FU (\(1\)) on \textit{D. magna} immobilisation, with \(\text{EC}_{50}\) values ranging from 15 mg/L to 319 mg/L. Higher toxicity of 5-FU (15 – 36 mg/L) was reported in earlier studies (Zounkova et al., 2010; Cleuvers, 2002; Straub, 2010; Parrella et al., 2014; Zounkova et al., 2007), while the most recent result of \(\text{EC}_{50}\) (319 mg/L) was an order of magnitude higher, suggesting that 5-FU is only slightly toxic to \textit{D. magna} (Harris, 2015). In the latter study the large variation of \(\text{EC}_{50}\) values was discussed.
thoroughly, including details on protocols, growth media, solvents, etc. In our study we followed the protocol according to the HRN EN ISO 6341:2013, which is comparable to the procedure reported by Harris (Harris, 2015). Our result \( (EC_{50} = 285.8 \text{ mg/L}) \) approaches the most recent result \( (319 \text{ mg/L}) \), and shows that 5-FU induces only negligible toxicity to \( D. \ magn\a \) (Table 1). In addition, this is supported by an environmental risk assessment in which authors claimed that \( EC_{50} \) for 5-FU must be over 100 mg/L (Załęska-Radziwiłł et al., 2011).

### Table 1
Ecotoxicity of 5-FU (1), chlorohydrin 3a, the mixture of 1 and 3a, hydrate 11, and chloral hydrate (all values in mg/L).

<table>
<thead>
<tr>
<th>Substance</th>
<th>NOAEC</th>
<th>LOAEC</th>
<th>( EC_{50}(24h) )</th>
<th>( EC_{50}(48h) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU (1)</td>
<td>5</td>
<td>10</td>
<td>-</td>
<td>285.8</td>
</tr>
<tr>
<td>Chlorohydrin 3a</td>
<td>50</td>
<td>100</td>
<td>-</td>
<td>425.5</td>
</tr>
<tr>
<td>1 + 3a (1 : 1)</td>
<td>65 (48 h)</td>
<td>85 (48 h)</td>
<td>-</td>
<td>116.7</td>
</tr>
<tr>
<td>Hydrate 11</td>
<td>100 (24h)</td>
<td>125 (24h)</td>
<td>138</td>
<td>122.0</td>
</tr>
<tr>
<td></td>
<td>50 (48 h)</td>
<td>85 (48 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloral hydrate</td>
<td>350</td>
<td>450</td>
<td>586</td>
<td>537.0</td>
</tr>
</tbody>
</table>

The same test with \( D. \ magna \) immobilization was carried out to assess the ecotoxicological effects of the chlorohydrin product 3a, which is the first chlorinated derivative of the 5-FU. Obtained data demonstrated that microcrustacean were more sensitive to the parent 5-FU than to its chlorinated product 3a with an \( EC_{50} \) (48 h) of 425.5 mg/L. It appears that chlorination of 5-FU results in the product which has much lower (eco)toxicity. According to the EU-Directive 93/67/ EEC (EC, 1996), the chlorohydrin product 3a may be classified as nontoxic.

In addition, we performed ecotoxicity test for the mixture of 5-FU and chlorohydrin 3a (1 : 1) and obtained an \( EC_{50} \) (48 h) of 116.7 mg/L. Therefore, the mixture of 5-FU and its chlorinated product is more toxic to \( D. \ magna \) than any individual component, which suggests a possible interaction between the two substances.
The prolonged chlorination of 5-FU gives rise to the hydrate 11 in which the pyrimidine ring opening occurred. This chlorinated byproduct was found more toxic to *D. magna* than either of the two precursors (5-FU and chlorohydrin 3a). The EC$_{50}$ data at 24 h and 48 h were 138 mg/l and 122 mg/l, respectively. We compared these results to the EC$_{50}$ value obtained for chloral hydrate, a well known sedative and hypnotic drug, which is structurally related to hydrate 11. The measured EC$_{50}$ values for 24 h and 48 h were 586 mg/L and 537 mg/l, respectively, which is in a good agreement with reported data for chloral hydrate ranging between 500 – 630 mg/L (Bringmann and Kuhn, 1982). Therefore, among the four tested single substances, the hydrate 11 is evidently the most harmful compound in terms of *D. magna* immobilization, which is used hereby as a toxicity assessment criterion.

Our results reveal a composite effect of chlorination on the ecotoxicity profile of 5-FU (1) and its chlorinated metabolites. The adverse effect of chlorinated species depends on the extent of chlorination, with the first chlorination product (chlorohydrin 3a) being less toxic than the parent, while the toxicity enhances by a prolonged chlorination (hydrate 11). The final chlorination product exists in a fast equilibrium between its aldehyde (10) and hydrate (11) form (see Scheme 3), and therefore is not easy to resolve which species is more relevant for the observed toxicity.

### 4. Conclusions

A detailed mechanism underlying the chlorination of 5-fluorouracil was explored by use of NMR analysis and quantum chemical calculations. In neutral aqueous solution the reactive species are N-anionic form of 5-FU and HOCl as a chlorinating agent. Both $^1$H and $^{19}$F NMR spectra of the reaction mixture confirm the formation of only one product, which
corresponds to a chlorohydrin structure 3a. With the finding that the chlorination occurs at the C5-position, the old controversy on the site of the 5-FU chlorination has come to a closure.

The chlorohydrin itself may undergo chlorination at the N1-position, which resulted in the formation of the hydrate 11. Therefore, the chlorination of the parent 5-FU produces at least two stable transformation species – chlorohydrin 3a as the primary product, and the hydrate 11, which emerged if the chlorination procedure with HOCl was prolonged.

To complete the whole reaction profile quantum chemical calculations, at the B2K-PLYP//B3LYP level were employed to describe the corresponding energy surface. We found this DFT model sufficient to support and interpret experimental data.

The two isolated chlorinated product are of relevance for the chemical fate of 5-FU under chlorinating conditions. For that reason, their ecotoxicological profile was assessed by *Daphnia magna* immobilization test. The first chlorinated product chlorohydrin 3a was less toxic than the parent 5-FU, suggesting the beneficial effect of chlorination. The second chlorinated product hydrate 11, however, exhibited increased ecotoxicity. Therefore, the existence (and related toxicity) of chlorinated products of 5-FU in treated wastewaters may be a function of contact time with a chlorinating agent, which is an interesting feature from the environmental point of view.

**Conflicts of interest**

The authors declare no conflict of interest.

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