Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 733–738

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Identification of a process impurity formed during synthesis of a nevirapine analogue HIV NNRT inhibitor using LC/MS and forced degradation studies

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ARTICLE INFO

Article history:
Received 17 October 2008
Received in revised form 8 January 2009
Accepted 9 January 2009
Available online 20 January 2009

Keywords:
Process impurity
Forced degradation
Oxidative degradation
LC/MS
Mechanism of formation

ABSTRACT

Impurities in pharmaceutical products do not enhance the desired therapeutic effect and may, of course, have adverse effects. Impurities must therefore be limited or controlled for quality and safety considerations. Structural identification of an impurity is the first step in understanding the chemistry of its formation and subsequently controlling the impurity. In this article, the chemical structure of an unknown by-product formed during the synthesis of a nevirapine analogue HIV NNRT inhibitor was identified using a combination of low resolution, high resolution and H/D exchange LC/MS and LC/MS/MS. The origin of the impurity was investigated through a series of photo- and oxidative stress studies. It was concluded that this impurity is formed *via* a side-reaction of the last intermediate with the oxidant used in the synthesis.

1. Introduction

Impurities in drug substances or drug products are chemical entities that are brought into the drug substances or drug products either from the manufacturing processes (process impurities and residual solvent, etc.) or due to degradation of the drug substances during manufacturing or storage (degradation products). Impurities likely have no therapeutic effect and may potentially have adverse effects; not surprisingly, the levels of impurities in pharmaceutical products (drug substances and drug products) are strictly regulated. Impurities present in pharmaceutical products must be reported, identified and qualified according to the respective reporting, identification and qualification thresholds [1–4]. According to ICH guideline Q3A (R2) and Q3B (R2) [1,3], process impurities can include starting materials, intermediates, chemical reagents/ligands/catalysts, by-products and degradation products formed during manufacturing process.

Identification of an impurity is the first step towards optimization of the chemical process, or understanding of degradation pathway to eventually reduce the level of the impurity for quality and safety considerations.

This article presents a case study for elucidation of the chemical structure and understanding of the origin of an unknown impurity observed during analytical release testing of certain batches of a nevirapine analogue HIV NNRT (non-nucleoside reverse transcription) inhibitor. Efforts for structural identification were triggered because the impurity was present at levels close to the ICH identification threshold (0.1%) for impurities in new drug substances [1] in various batches of drug substance at release. Preliminary study indicated that this impurity was only observed in the final drug substance and was not observed in any of the previous intermediates. Furthermore, this impurity did not grow on stability. Therefore, it can be reasonably concluded that the unknown was formed during the last step of the chemical synthesis of the nevirapine analogue as shown in Scheme 1. In the last step, intermediate **1** is oxidized to form the desired nevirapine analogue **2** [5]. During the manufacturing process, a photo-degradation product **3** can be formed from compound **2** through a photo-catalyzed rearrangement [6]. It was postulated that this impurity could be formed through either a side-reaction of **1** or further/over oxidation of **2** or **3** [7], but the actual origin of the unknown impurity was not clear.

2. Experimental

2.1. Materials

All solvents (methanol, water and acetonitrile) were Omnisolv® HPLC grade purchased from EDM (Gibbstown, NJ). Formic acid, 99%, hydrogen peroxide, 30 wt.% solution in water, 3-chloroperoxybenzoic acid, 77%, and rose bengal were purchased from Sigma–Aldrich (St. Louis, MO). Compounds **1**, **2** and **3** and the drug substance samples were obtained internally.

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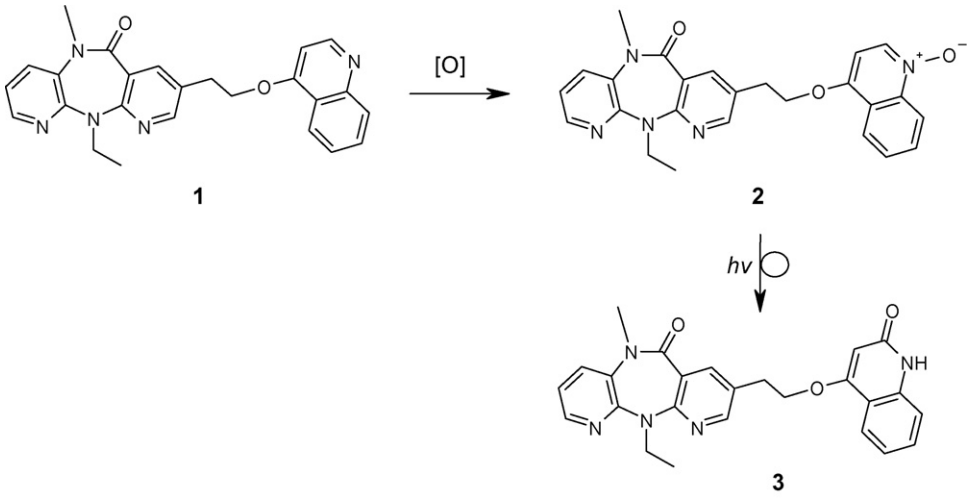
doi:10.1016/j.jpba.2009.01.010



Scheme 1. Last step of the chemical synthesis of compound **2** during which the unknown impurity was formed.

2.2. LC/MS and LC/MS/MS

Low resolution LC/MS(/MS) and H/D exchange LC/MS(/MS) experiments were performed on a Waters Quattro Ultima mass spectrometer coupled with an Agilent 1100 HPLC system operating under positive ion APCI (Atmosphere Pressure Chemical Ionization). High resolution and low resolution analyses of forced degradation samples were carried out on a Thermo Finnigan LTQ-FT-Ultra mass spectrometer interfaced with an Agilent 1100 HPLC system operating also under positive APCI mode. High resolution measurements were performed under 100,000 resolution power (FWHM). The HPLC parameters were as follows: column: Zorbax SB-CN, 5 μm, 4.6 mm × 150 mm; mobile phase (A): 0.1% formic acid, mobile phase (B): acetonitrile with 0.1% formic acid; mobile phase gradient: 90% A–90% B in 15 (for Ultima experiments) and 20 min (for LTQ-FT experiments); flow rate: 1 ml/min. H/D exchange LC/MS and LC/MS/MS experiments were carried out using D_2O for H_2O in the mobile phase described above.

2.3. Forced degradation studies

2.3.1. Photo-stress studies

Photo-stress studies were carried out using an Atlas XLS+ Suntest light chamber with a Xenon lamp (wavelength range 300–800 nm). In the first experiment, stock solutions of **1**, **2** or **3** were transferred into suitable transparent vials, and stressed for 21 h at 500 W/m^2 (about 2× the ICH condition [8]), respectively. In the second experiment, a rose bengal solution was added to the sample solutions at about equal molar ratio. The stressed samples were then diluted with a mixture of mobile phase A and B (50/50, v/v) for LC/MS analysis.

2.3.2. Oxidative stress studies

Stock solutions of compounds **1, 2** or **3** were prepared by dissolving known amounts of standard materials of **1**, **2** or **3** respectively in acetonitrile. The final concentrations of **1, 2** and **3** stock solutions were between 0.5 and 1 mg/ml. 0.5 ml of stock solution **1**, **2** or **3**

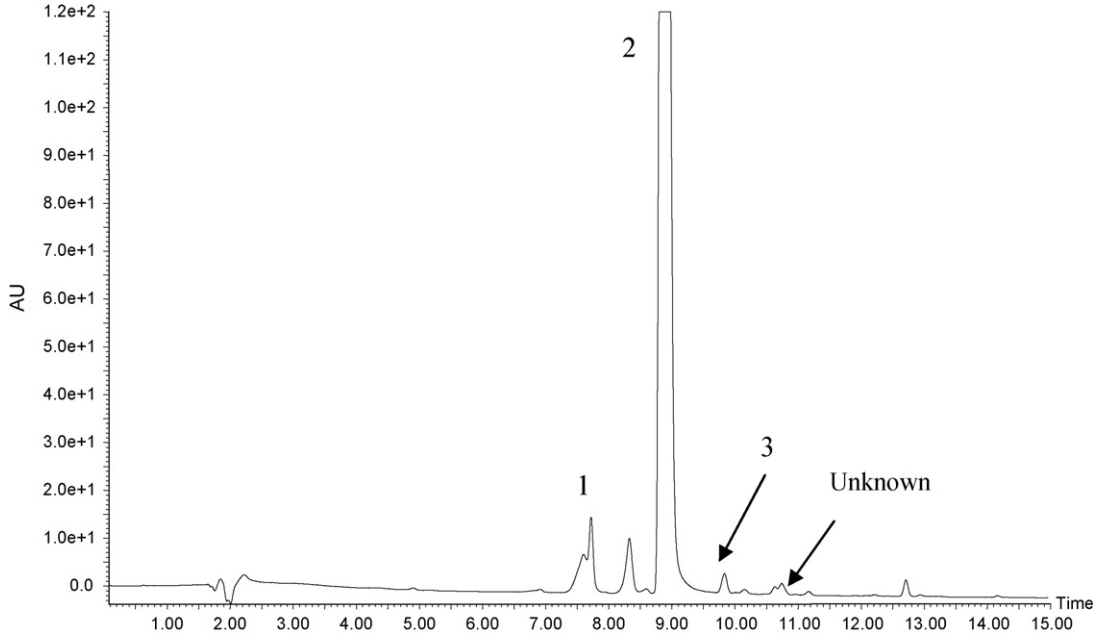


Fig. 1. A representative LC/UV chromatogram of the nevirapine analogue HIV NNRT inhibitor at release. Peaks are labeled with the number code given in Scheme 1. Note that other peaks were also identified and understood, however, these are out of the scope of this study.

was mixed with equal volume of a 30% H_2O_2 solution, respectively, in an amber HPLC vial (to prevent photo-degradation of compound **2**). The solutions were left on the HPLC autosampler at ambient temperature. The unstressed (control) and stressed solutions (with H_2O_2) were injected directly onto the mass spectrometer respectively for LC/MS analysis.

MCPBA (3-chloroperoxybenzoic acid) experiments were performed by mixing 50.0 ml of **1, 2** or **3** stock solutions with 25.0 ml of MCPBA stock solution, and 25.0 ml of water, 25.0 ml of 1 M NaOH or 25.0 ml of 1 M HCl, respectively. The solutions were incubated at 50 °C. The samples were injected at time 0 and 4 h time points.

3. Results and discussion

3.1. Structure of the unknown impurity

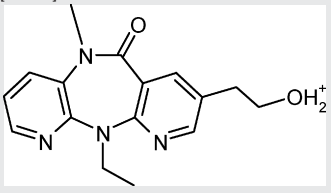
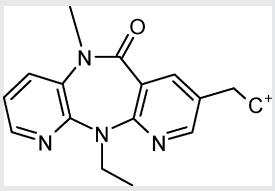
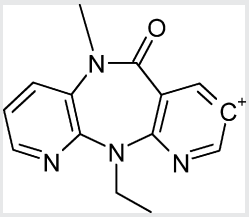
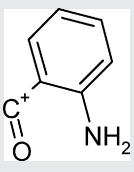
Fig. 1 shows a representative LC/UV chromatogram of a finished drug substance batch of compound **2**. The unknown impurity was present at about 0.1% (area) relative to compound **2**. The *m*/*z* value of the protonated molecule of the $[M+H]^+$ measured by APCI/MS is 418. Hence, the nominal molecular weight of this impurity is 417, which is 24 a.m.u. lower than that of compound **2** (441). The accurate mass of the protonated molecule of the unknown impurity (418.18729, see Table 1.) indicated that this impurity has a chemical formula of $C_{23}H_{23}N_5O_3$. Comparing to the chemical formula of compound **2** ($C_{25}H_{23}N_5O_3$), the impurity molecule contains two carbon atoms less than compound **2**.

The exchangeable protons are those that are attached to oxygen (e.g. -OH and -COOH), nitrogen (e.g. $–NH_2$ and –NHR), or sulfur (e.g. –SH) atoms. Those protons can be exchanged during the H/D exchange LC/MS experiment by deuteriums to form deuterated molecules in the mass spectrometer. As a result, the *m*/*z* values of the molecular ions and in some cases the fragment ions are shifted in the mass spectrum. The numbers of exchangeable protons can therefore be calculated from the difference between the *m*/*z* values of the deuterated and protonated molecules according to the following formula [9]: number of exchangeable protons = $\Delta m - 1$, where Δm is the difference between the deuterated and protonated molecules.

From the H/D exchange experiments, the deuterated molecule of the unknown has an *m*/*z* value of 421 and the deuterated molecule of compound **2** has an *m*/*z* value of 443. Thus, the number of exchangeable protons in the unknown and compound **2** can be calculated to be 2 and 0, respectively. These results indicate that OH or NH bonds are present in the unknown impurity, while those functional groups are not present in any of the compounds **1**, **2** and **3**.

MS/MS results of the protonated molecule of the unknown (Fig. 2A) revealed that the unknown shares several common fragment ions *m*/*z* 299, *m*/*z* 281, and *m*/*z* 253, with compound **2** (spectrum not shown). These are the characteristic fragments of all compounds **1**, **2** and **3**, as shown in Scheme 2. MS/MS results of the deuterated molecule of the unknown *m*/*z* 421 (Fig. 2B) further confirmed that common fragment ions *m*/*z* 253 and *m*/*z* 281 do not contain exchangeable protons. The *m*/*z* 299 fragment contains one exchangeable proton because of the formation of an alcohol (–OH) after cleavage, which is changed to *m*/*z* 301 after H/D exchange (this portion of the molecule can only have an exchangeable proton as a fragment from compounds **1**, **2** or **3**). The fragment ion *m*/*z* 120 became *m*/*z* 122 after H/D exchange, indicating that this fragment may contain both the exchangeable protons in the unknown molecule (the calculation of exchangeable protons in a fragment ion

Table 1
Exact masses of the protonated molecule and major fragments of the unknown impurity.

Ions	Measured mass	Calculated mass	Proposed structure
m/*z* 418	418.18729	418.18737	$[M+H]^+$ of **4**
m/*z* 299	299.15039	299.15025	
m/*z* 281	281.13980	281.13969	
m/*z* 253	253.10843	253.10830	
m/*z* 120	120.04438	120.04439	

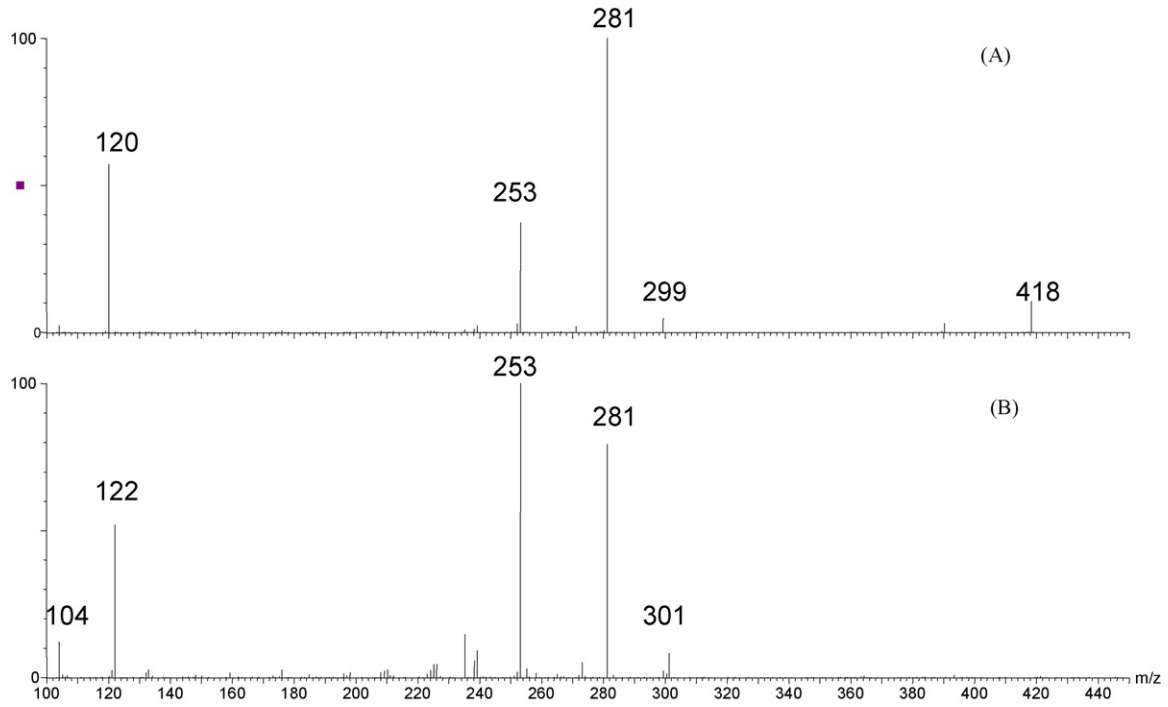
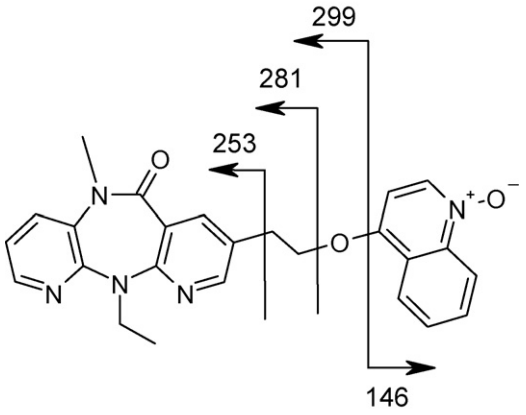


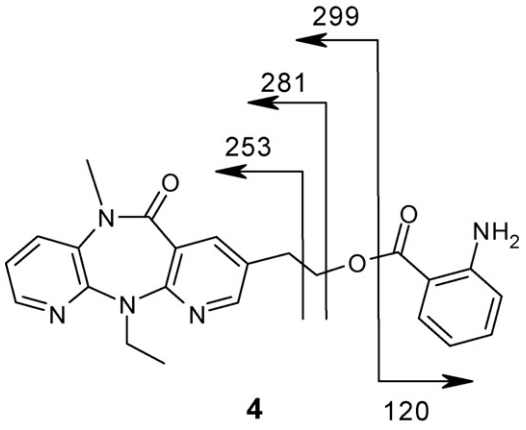
Fig. 2. (A) Product ion spectrum of *m*/*z* 418, protonated molecule of the unknown; (B) product ion spectrum of *m*/*z* 421, deuterated molecule of the unknown.



Scheme 2. Fragmentation pattern of compound **2**.

may be different from that in the protonated molecule because a fragment ion can carry a charge without protonation). Based on the fragmentation pattern of this type of molecule shown in Scheme 2, this fragment in the unknown molecule must correspond to the quinoline portion in compounds **1**, **2** and **3**. From the discussions above, the left side of the molecule of the unknown is unchanged from compounds **1**, **2** and **3** and does not contain any exchangeable protons. The quinoline portion must be modified during the oxidation step in the synthesis to form the unknown impurity. This modification must result in the loss of two carbon atoms and formation of two exchangeable protons.

Based on the structural information discussed above, structure **4** was proposed for the unknown as shown in Scheme 3. In addition to the structural evidences discussed above, this structure is also consistent with exact masses of the protonated molecule and all major fragment ions, as shown in Table 1. To further confirm the structure, an authentic sample corresponding to the proposed structure was independently synthesized. NMR analysis of the synthetic material was consistent with structure **4** [5].



Scheme 3. Proposed structure of the unknown and its fragmentation pattern.

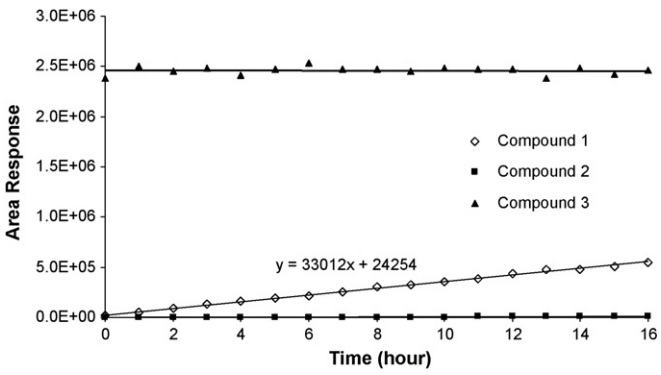
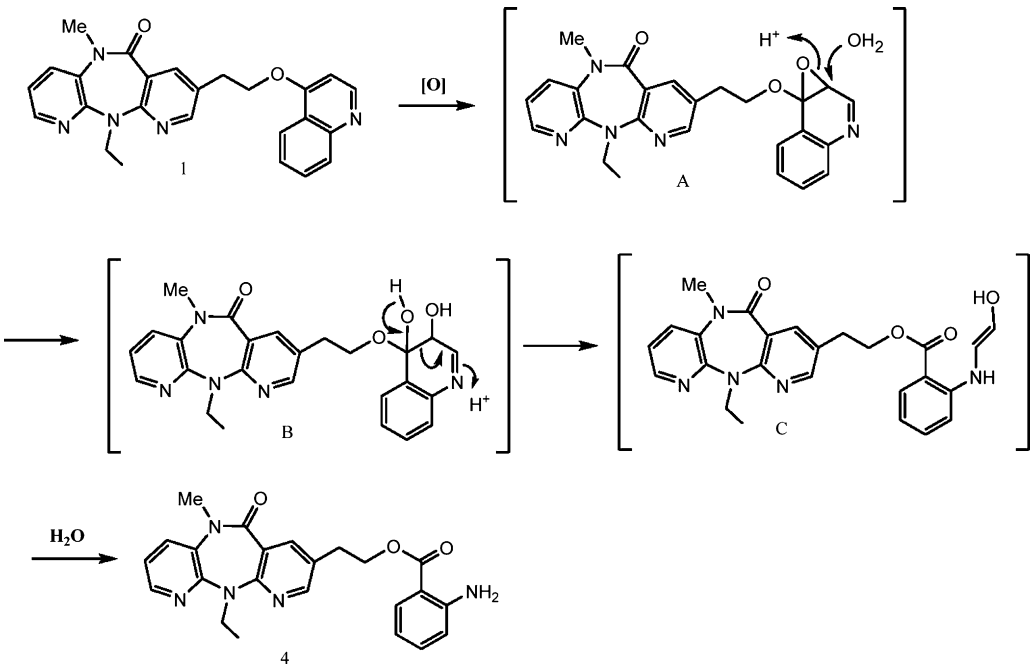


Fig. 3. Area response of impurity **4** from the Extracted Ion Chromatograms (*m*/*z* 418) of compound **1**, **2** or **3** under H_2O_2 oxidative stress vs. stress time.



Scheme 4. Proposed mechanism of formation of impurity 4.

3.2. Origin of the unknown impurity

It had been previously established that the unknown impurity was formed during the oxidation step of the chemical synthesis of compound **2**. It was postulated that **4** might be a further degradation product of **2** or **3**. In order to investigate the origin and to understand the chemistry of formation of impurity **4,** a series of forced degradation studies were carried out.

3.2.1. Results of photo-degradation studies

In solution, compound **2** is extremely sensitive to light, rearranging to form lactam **3**. Thus, it is reasonable to assume that compound **4** is also a photo-degradation product. However, after 2× ICH exposure [8] of light, none of compounds **1**, **2** or **3** produced compound **4**. Under photo-irradiation, singlet oxygen could react with compounds possessing double bonds to form carbonyl degradation products [9]. This possibility was also ruled out by photo-stress of compounds **1**, **2** or **3** solutions, respectively, with rose bengal, a known singlet oxygen sensitizer [10]. In summary, compound **4** is not a photo-degradation product of compound **1**, **2** or **3**.

3.2.2. Results of oxidative degradation studies

Fig. 3 shows the kinetic results of oxidative degradation of compounds **1**, **2** and **3** with H_2O_2 under ambient conditions. It is clear that impurity **4** was not formed from either compound **2** or **3** under the H_2O_2 oxidative conditions (note that compound **2** used in this study is a purified reference standard and does not contain impurity **4** initially; compound **3** was an isolated material and contains 0.3 % of impurity **4** initially). Impurity **4** was only formed from compound **1.** The rate of formation of impurity **4** from compound **1** is linear with the stress time, indicating zero order kinetics under the given conditions.

In the chemical synthesis of compound **2** (Scheme 1), MCPBA was used as the oxidant at basic pH. In order to exclude any bias due to the use of a different oxidant or pH condition, stress studies with MCPBA as the oxidant at basic, neutral and acidic pH were also conducted. The results demonstrated that qualitatively both MCPBA and H_2O_2 generated the same reaction products at neutral pH. At basic pH, MCPBA still generates the same oxidation products but at a higher rate. At acidic pH, the oxidation does not occur, presumably due to the protonation of the nitrogen atom in the quinoline ring, which reduces the electron density of the double bonds. H_2O_2 was used for most of the studies at neutral conditions because MCPBA complicated the chromatography.

It can be concluded that impurity **4** was formed through an oxidative side-reaction of intermediate **1** during the oxidation step of the chemical synthesis of compound **2**. A proposed mechanism of formation is shown in Scheme 4. The first step of the reaction is likely the oxidation of the more electron-enriched double bond in the pyridine side of the quinoline ring in compound **1** to form an epoxide, which is then followed by hydrolysis of the epoxide to form a diol, which is further decomposed to form **4**. The same reaction does not occur for compounds **2** and **3** under the given conditions because in those compounds the addition of the oxygen reduced the electron density of the double bonds and therefore reduced their reactivity towards oxidation.

4. Conclusions

This paper described the identification of an unknown impurity **4** using a combination of low resolution, high resolution and H/D exchange LC/MS and LC/MS/MS. The origin of the impurity was investigated through a series of photo- and oxidative stress studies. It was concluded that this impurity is formed from a side-reaction of the last intermediate **1** with the oxidant used in the synthesis, not as postulated originally from the further oxidation/degradation of the final product **2** or its photo-degradant **3**.

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