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Degradation profile of nepafenac in aqueous solution and structural characterization of a novel degradation product

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Abstract: The stability of the anti-inflammatory drug nepafenac was investigated in aqueous solutions containing hydroxypropyl-β-cyclodextrin at three different values of pH and degradation products were identified. (2-Amino-3-benzoyl)-oxoacetic acid, previously not reported as nepafenac-related impurity, was isolated and structurally characterized by NMR and ESI-MS analyses. It was also shown that the formation of this α-ketoacid from nepafenac in alkaline water/organic cosolvent solution occurs through an aerobic oxidation of the key intermediate 7-benzoyl-1,3-dihydro-indol-2-one, which in some extent is protected from oxidation in the presence of the cyclodextrin additive.

Keywords: Nepafenac; Drug stability; HPLC; α-Ketoacid; Benzylic oxidation

1. Introduction

Nepafenac [2-(2-amino-3-benzoylphenyl)acetamide], **1** has been recently introduced in the market as a non-steroidal anti-inflammatory prodrug for ophthalmic use [1], providing a novel drug delivery mechanism based on the release of the pharmacologically active metabolite amfenac, **2** in different eye segments, especially in the retina and choroid, where the activity of intraocular hydrolases is higher than in the cornea [2].

The rapid bioactivation of nepafenac to amfenac [3], which is a potent inhibitor of both COX-1 and COX-2 activities [4], and hence of prostaglandin synthesis, makes it indicated for the prevention and treatment of pain and inflammation associated with cataract surgery [5] and other ocular diseases [6-7]. For its uncharged structure at physiological pH nepafenac exhibits superior corneal permeability compared to amfenac and it is rapidly distributed to the anterior chamber and to posterior segment tissues, so minimizing surface accumulation and complications observed with other NSAIDs.

Since nepafenac is practically insoluble in water, it is formulated as a highly viscous 0.1% ophthalmic suspension (Nevanac®, Alcon) to be administered in multiple doses a day and recent studies have

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been focused on the development of novel nanocarriers in order to increase the bioavailability of the drug [8-11]. A 0.1% solution of nepafenac was obtained in the presence of hydroxypropyl-β-cyclodextrin as additive and a permeation rate 18 times higher than Nevanac® was reported for this type of solution formulation [12].

Nepafenac is stable in the solid state but different degradation compounds have been reported to be formed in water/organic solvent (CH_3CN or MeOH) mixtures under different stress conditions and the structural identification of the impurities has been carried out by LC-MS analyses aided by the comparison with reference samples [13-15]. However, some reported data appeared in contrast to each other; as an example, subjecting **1** to acidic stress conditions (4 mM HCl, 25°C, 1h) Runje *et al.* [15] recovered 95.2% of the starting concentration while Shrimali *et al.* [14] observed the complete degradation of the drug in 6 mM HCl (25°C, 0.5h), even if in both cases compound **3** was identified as the major impurity. In both these works hydroxy-nepafenac **4** was given as the main formed compound under alkaline conditions (4 mM NaOH, 25 °C, 1h or 0.6 M NaOH, 25 °C, 6h) while the sodium salt of amfenac was reported by Lipiec-Abramska *et al.* [13] in 1M NaOH (40 °C, 7h).

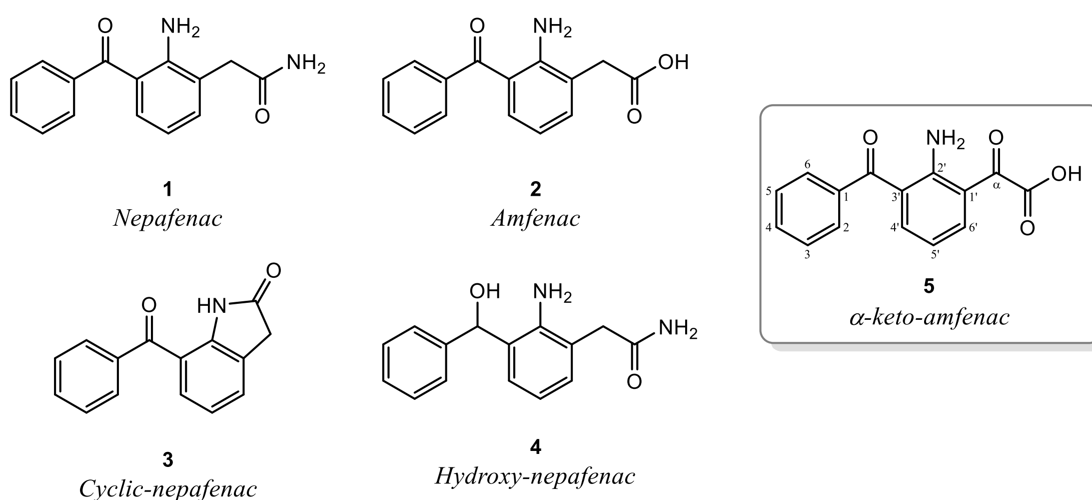


Figure 1. Chemical structures of nepafenac and some related degradation products

In the course of a study aimed to the development of an ophthalmic formulation of nepafenac in aqueous solution, we were interested in the degradation profile of **1** and a careful HPLC analysis of its related impurities was undertaken at different values of near physiological pH. Apart from the known degradation products, we isolated compound **5**, previously not reported as nepafenac-related impurity, and its structural characterization was carried out by NMR and HR-MS analysis. Further investigation on the formation of the ketoacid **5** was also carried out and here we report the obtained data.

2. Material and Methods

2.1. General

55 Nepafanec was kindly given by Old Pharma International and standard of compounds **2**-**4** were obtained from Toronto Research Chemicals. Hydroxypropyl-β-cyclodextrin was obtained from Roquette. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was from Sigma. ^{1}H- and ^{13}C-NMR spectra were recorded in d_6-DMSO (Eurisotop) on a Bruker Avance™ 400 instrument at 400.13 and 100.03 MHz respectively and chemical shifts (δ) are relative to the residual solvent 60 peak. 2D-experiments were carried out using an inverse multinuclear probe with pulse-field Z-gradient and standard Bruker pulse sequence programs. Column chromatography was performed on silica gel 60 (Merck, 40-63 μm) using the specified eluents.

High resolution mass spectra (HR ESI-MS) were acquired by a Thermo Scientific Exactive Plus Orbitra MS instrument (Thermo Fisher Scientific, Inc., San Jose, CA) using a heated electrospray 65 ionization (HESI II) interface. Mass spectra were recorded operating in negative ion mode in the *m*/*z* range 60-1000 at a resolving power of 25000 (full-width-at-half-maximum, at *m*/*z* 200, RFWHM), using automatic gain control target of 1.0 × 106 and a C-trap inject time of 100 ms under the following conditions: capillary temperature 325 °C, nitrogen as nebulizer gas, source voltage -4.5 kV; capillary voltage -82.5 V; tube lens voltage -100 V. The Orbitrap MS system was tuned and calibrated in 70 negative modes, by infusion of solutions of a standard mixture of sodium dodecyl sulfate (Mr 265.17 Da) sodium taurocholate (Mr 514.42 Da) and Ultramark (Mr 1621 Da). Data acquisition and analysis were performed using the Excalibur software.

Melting point are uncorrected. HPLC analyses were carried out on an Agilent 1260 Infinity instrument equipped with a refrigerated autosampler, thermostatted column compartment and UV-75 vis detector connected with OpenLAB software.

2.2. HPLC Chromatography

A validated HPLC method was developed for the separation of **1** and its impurities on a Waters XTerraMS C-18 column (250 mm × 4.6 mm i.d.), equipped with a XTerraMS guard column and thermostatted at 35 °C, setting the UV-detection at λ 245 nm. The column was eluted at flow 1.0 80 mL/min with mixtures of mobile phase A (20 mM ammonium formate buffer pH 4.0) and mobile phase B (CH_3CN). Solvents were HPLC gradient grade and mobile phases were degassed before use. The following gradient program was applied: time 0 min, 70% A, 30% B; time 20 min, 70% A, 30% B; time 30 min, 10% A, 90% B; time 35 min, 10% A, 90% B; time 38 min, 70% A, 30% B; post run 5 min. The nepafenac-related impurities were quantified by external calibration with analytical 85 standards in the concentration range 0.5–100 μg/mL and the method was validated

HPLC assay for the quantification of **1** was carried out on a EVO Kinetex (Phenomenex) column (150 mm × 4.6 mm i.d.) isocratically eluted at 25 °C, flow 0.7 mL/min, with a 35:65 (v/v) mixture of CH_3CN and 20 mM ammonium formate buffer at pH 6.0. The method was validated and the amount of **1** was quantified by external calibration with analytical standard in the concentration range 30-70
90 μg/mL. The correlation coefficients of the detector linearity for all the calibration curves were >0.9999. Mass balance of all the samples was in the range 95-99%. Statistical evaluation of the data was performed by using one-way analysis of variance (ANOVA).

2.3. Stress test at different pH values

Nepafenac (0.1% w/w) and hydroxypropyl-β-cyclodextrin (6% w/w) were dissolved in 12.6 mM
95 Na_2HPO_4 and H_3PO_4 was added to adjust the pH to the set values (pH 6.8, 7.2, 7.6). The solutions were prepared in sterile conditions and maintained at 60 °C in sterile polypropylene vials. Aliquots of solutions (1.0 mL) were withdrawn at suitable intervals and stored at –16 °C until they were analyzed. For nepafenac assay three independent samples were prepared diluting 0.25 mL of the withdrawn aliquots to 5.0 mL with distilled water and analyzed by HPLC. For the determination of
100 the impurities the withdrawn solution was used without dilution and HPLC analyses were carried out in triplicate.

Three data sets (pH 6.8, 7.2 and 7.6) for the concentration of nepafenac at different time were built and compared by Bonferroni multiple comparison test (OriginPro 8.5) to assess the significance of the differences between the three groups and a p-value ≤ 0.05 was accepted as significant.

105 *2.4. Hydrolytic degradation of **1** in acid/basic conditions*

Nepafenac **1** was dissolved in H_2O/CH_3CN 1:1 (v/v) at 1.0 mg/mL concentration and the solution was used for the tests of hydrolytic degradation. The reactions were carried out in dark to exclude possible photolytic degradation and samples were refrigerated at –16 °C until HPLC analysis.

Acid conditions: To a solution of **1** (5 mL) 0.5N HCl (60 μL) was added and the mixture was stirred
110 at room temperature for 3 h. The reaction was quenched by addition of 0.5N NaOH (60 μL); then, an aliquot (100 μL) of the neutral solution was diluted with 20 mM ammonium formate buffer (100 μL) at pH 4.0 before the HPLC analysis.

Alkaline conditions: To a solution of **1** (5 mL) 4N NaOH (750 μL) was added and the mixture was stirred at room temperature for 6 h. The reaction was quenched by addition of 8N HCl (375 μL); then,
115 an aliquot (100 μL) of the neutral solution was diluted with 20 mM ammonium formate buffer (100 μL) at pH 4.0 before the HPLC analysis.

*2.5. Isolation of (2-amino-3-benzoyl)-oxoacetic acid, **5***

A solution of **1** (150 mg, 0.59 mmol) in 50 mL of H_2O/CH_3CN 1:1 (v/v) was subjected to alkaline treatment as above and after neutralization the solvent was removed in vacuo. The residue was
120 purified by column chromatography on silica gel eluting firstly with EtOAc/*n*-hexane 3:7 (v/v) containing 0.02% AcOH in order to separate less polar compounds. Subsequent elution with EtOAc/MeOH 9:1 (v/v) containing 0.02% (v/v) AcOH afforded pure compound **5** (52 mg, 0.19 mmoli, 33% yield) as yellow powder, mp 204-205 °C (dec.); Rf 0.19 (EtOAc/MeOH 9/1 with 0.02% AcOH).
125 ^{1}H-NMR: δ 6.59 (t, J = 7.6 Hz, 1H, H-5'), 7.50-7.60 (m, 6H), 7.86 (dd, J = 7.6 and 1.2 Hz, 1H, H-6'), 8.66 (br s, 2H, $-NH_2$); ^{13}C-NMR: δ 112.8 (C-5'), 116.0 (C-3'), 118.5 (C-1'), 128.3 (C-3 and C-5), 128.7 (C-2 and C-6), 131.4 (C-4), 139.5 (C-4' and C-1), 140.5 (C-6'), 152.8 (C-2'), 168.8 (-COOH), 197.7 (CO), 200.1 (α-CO). ESI-MS: *m/z*: 268.0618 $(M - H)^-$, calcd. for $C_{15}H_{10}NO_4$: 268.06043.

130 *2.6. Reactions in alkaline aqueous solution (Table 1)*

The substrate of choice, **1**, **2** or **3** (10 mg), was dissolved in $H_2O:CH_3CN$ 1:1 (v/v) mixture (10 mL) and K_2CO_3 or $NaHCO_3$ (1:1.5 molar ratio substrate:base) was added. The solution was maintained at the set temperature (25 or 60 °C) for the specified time and quenched by addition of the suitable amount of 0.5N HCl. Then, an aliquot (100 µl) of the neutral solution was diluted with 20 mM
135 ammonium formate buffer (100 µl) at pH 4.0 and analyzed by HPLC using the method for the determination of the impurities.

3. Results and discussion

Since nepafenac at the pharmacologically active concentration (0.1% w/w) was not soluble in phosphate buffer, hydroxypropyl-β-cyclodextrin (6% w/w) [12] was added to obtain a clear aqueous
140 solution and pH was adjusted with H_3PO_4. Three different solutions at pH 6.8, 7.2 and 7.6 were prepared in sterile conditions and subjected to stress test (60°C, 240 h) in order to generate representative samples for checking drug stability [16-17].

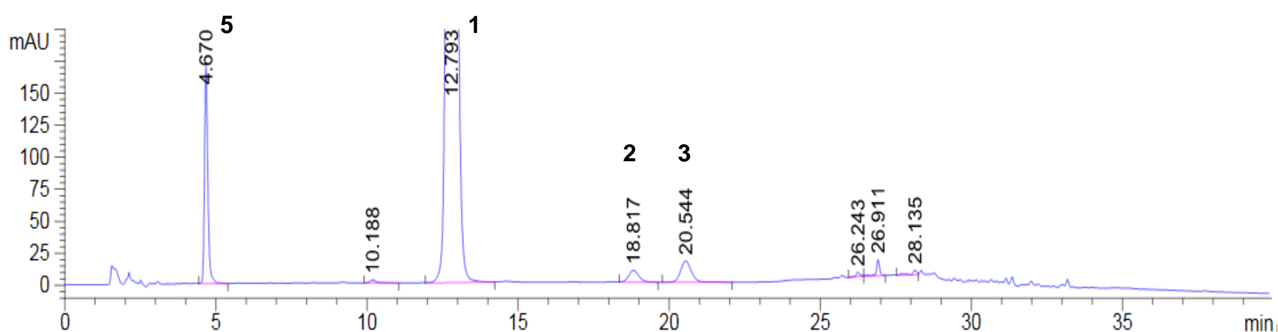


Figure 2. HPLC chromatogram of a sample of **1** subjected to stress test at pH 6.8 for 240 h

The time-course concentration of **1** was monitored at different intervals by RP-HPLC using a CH_3CN/formate buffer at pH 6.0 mixture as mobile phase, whereas optimal separation of the
145 degradation products was achieved at pH 4.0, since in these conditions acidic compounds were eluted as sharper peaks due to better suppression of their ionization. The HPLC chromatogram of a sample of **1** subjected to stress test at pH 6.8 for 240 h is shown in Figure 2 as a representative example of the separation between nepafenac and its degradation products.

Nepafenac **1** was found relatively stable at pH 7.2 with about 5% decrease of the initial concentration
150 over 10 days while slightly higher recovery (97%) was measured at pH 7.6 and statistically significant differences between these two sets of data were observed after 96 h under thermal stress. In the solution at pH 6.8, instead, the drug suffered more degradation resulting in 87% recovery of the starting **1** at the end of the experiment (Figure 3).

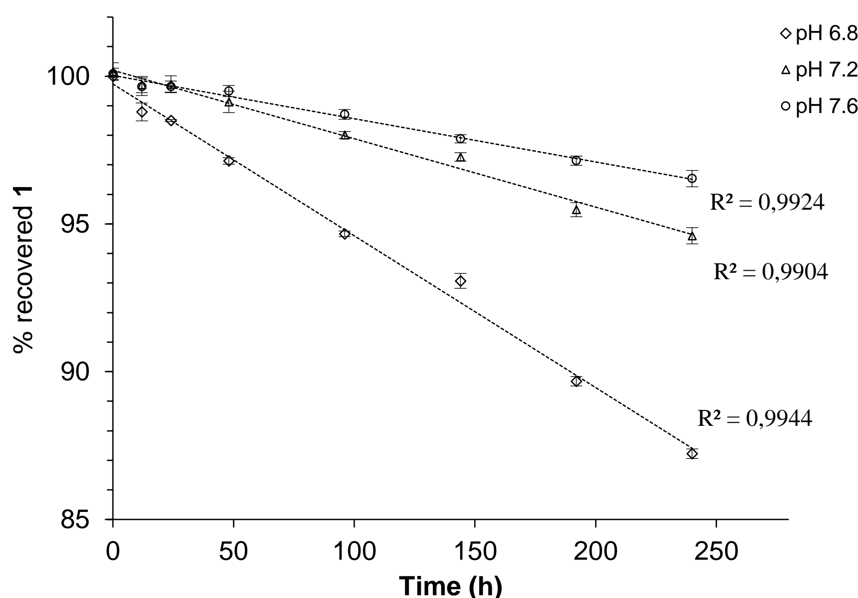


Figure 3. Time variation of % initial concentration of nepafenac in samples subjected to stress conditions (see Experimental section) at different values of pH

Degradation products detected at t_R around 19.0 and 20.5 min were identified as compounds **2** and **3**,
155 respectively, by co-injection of the samples with reference standards. The concentration of amfenac **2**, deriving from hydrolysis of the amide group of **1**, was quite similar and below 1% of the total composition in all the tested solutions. Compound **3** was mainly revealed at pH 6.8 reaching about 0.8% of the total impurities after 240 h and at pH > 7.0 its formation was almost suppressed.

Although alcohol **4** has been previously reported as degradation product of **1** in alkaline medium [15-
160 16] it was not detected in our samples and co-injection with a reference standard of **4** resulted in the presence of an additional distinct peak in the chromatogram at about t_R 5.5 min. Instead, a degradation product eluting at t_R 4.7 was observed in all the samples, with a maximum concentration of 5.8% in the solution at pH 6.8 after 240 h. The DAD-UV spectrum of this peak showed an intense band

centered at λ 405 nm, with a marked bathochromic shift compared to the carbonyl absorption of compounds **2-4** (Figure 1-SI).

In the attempt to obtain increased amount of this unknown degradation product **5** for the determination of its molecular structure, two samples of **1** were dissolved in $CH_3CN:H_2O$ 1:1 (v/v) mixture and subjected to forced degradation in acid and alkaline hydrolytic conditions, respectively, in the conditions reported by Shrimali *et al.* [14].

In the acidic solution (6 mM HCl, 3h, rt) nepafenac was exclusively converted into the cyclic derivative **3** (>95%) and compound **5** was detected at about 0.1% concentration. Instead, the chromatographic profile of the alkaline solution (0.52 N NaOH, 6 h, rt) showed the presence of **5** as main degradation product (36%) together with unchanged drug (51%) and low amounts of compounds **2** and **3** (about 9% and 3%, respectively).

A preparative reaction in alkaline conditions was then carried out to obtain compound **5**, which was isolated (33% yield) as a yellow solid sparingly soluble in most organic solvents and spectroscopically characterized. The ^{1}H-NMR spectrum (d_6-DMSO) of **5** showed resonances only in the 6.5-8.00 ppm aromatic region, accounting for eight protons, and a broad singlet at lower field (δ 8.7 ppm) related to two protons on nitrogen. The presence of all the expected aromatic protons and the lack of a methylene singlet at about 3.5 ppm, diagnostic for the acetamide chain in the spectrum of **1**, indicated a structural modification in this part of the molecule.

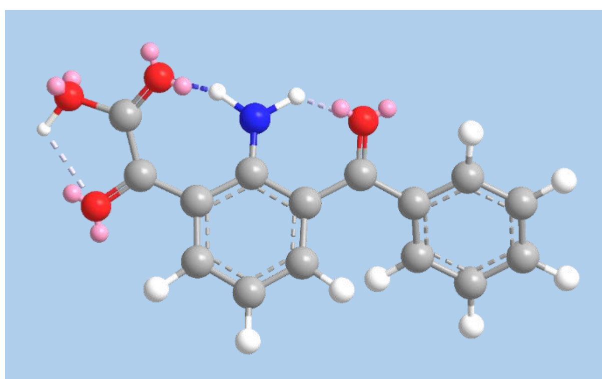


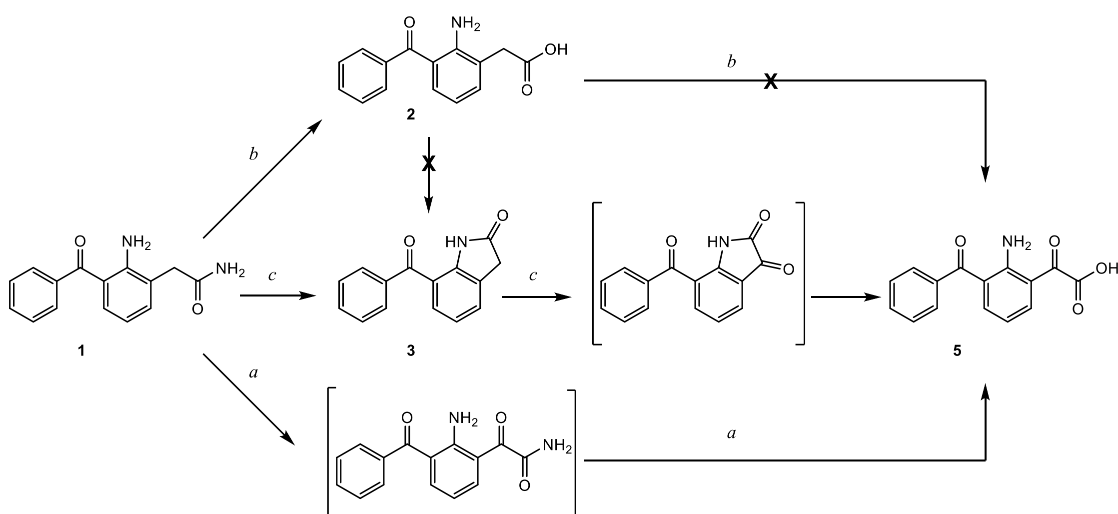
Figure 4. Conformational minimum (MM2 calculation) of compound **5**

The ^{13}C-NMR and DEPT-135 spectra of **5** confirmed this hypothesis and revealed the presence of three carbonyl groups, two of which associated to ketone functionalities on the basis of their high chemical shifts (197.7 and 200.1 ppm). Full assignment of the resonances was aided by 2D-NMR spectra (Figure 2-SI) and the long-range correlation of the broad singlet at 8.7 ppm with both carbons C-1’ and C-3’ was crucial for the attribution of these protons to the amino group. Compared to nepafenac, in compound **5** these protons experienced a sensible deshielding (Δδ = +1.6 ppm), probably due to anisothropy or hydrogen bonding effects related with the substituent in the side chain.

A similar but minor effect was also observed for H-6’ while resonances for amide protons, visible in
190 the spectrum of **1** as two separate signals, were not present in the spectrum of **5**. These NMR data are in agreement with a conformational minimum (MM2 calculation, figure 4) for (2-amino-3-benzoyl)-oxoacetic acid in which the amino group is engaged in intramolecular hydrogen bonds with the two flanking carbonyls and the H-6’ is deshielded by the third carbonyl group (Figure 4). The assigned structure was further confirmed by the presence of a molecular peak for the carboxylate anion in the
195 ESI-MS spectrum (negative mode).

Compound **5**, which has been previously reported in a study on the biological evaluation of potential prodrugs of amfenac [18] and characterized only by elemental analysis, up to date has not been identified as a degradation product of **1** or **2**.

The formation of **5** could result from a benzylic aerobic oxidation of **1** followed by the hydrolysis of
200 the amide group (Scheme 1, route *a*) or from the same steps in reverse order (Scheme 1, route *b*), but also the conversion of **3** into **5** via an isatin-type intermediate could not be excluded (Scheme 1, route *c*). Effective procedures for the preparation of α-ketoamides in the presence of base (and *n*-tetrabutylammonium salts) are known for *N*-mono or *N*-disubstituted arylacetamides as substrates using molecular oxygen [19-20] or other oxidants [21] whereas the synthesis of α-ketoacids by direct
205 conversion of the corresponding acids has not been reported up to date. Oxidation of 2-oxindoles to isatins with molecular oxygen has been recently reported [22] and it is well known that above pH 5 isatins suffer irreversible hydrolysis of the lactam bond [18, 23-24].



Scheme 1. Possible routes for the formation of α-ketoacid **5** from **1** in alkaline medium (compounds in square brackets are putative and were not identified in the solutions)

In order to get some insight into the mechanism of formation of **5**, parallel reactions were carried out using **1**, **2** or **3** as substrates in aqueous solutions (CH_3CN:H_2O 1:1 v/v) made alkaline with different
210 bases and the resultant mixtures were analyzed by HPLC. In the presence of NaOH amfenac **2** was

recovered unchanged after 6 h, but **3** quickly reacted to give **5** as almost exclusive product (94%) (Table 1, entries 2 and 3). The fast conversion of **3** into **5** was also observed using K_2CO_3 as base, either at 60 °C or room temperature (entries 7 and 8), while in the presence of $NaHCO_3$ the reaction occurred at lower rate and it was not significant at room temperature (entries 12 and 13).

215 Changing NaOH with K_2CO_3 or $NaHCO_3$ in the solution of **1** resulted in markedly decreased formation of **5** (compare entry 1 with entries 4 and 6).

Reactions of **3** in absence of oxygen or in the presence of Trolox as antioxidant [25] resulted in marked decrease of compound **5** (compare entry 7 with entries 9 and 10) indicating some involvement of intermediate free radicals.

Table 1. Influence of the reaction parameters on the formation of compound **5**[a]

Entry	Substrate	Base	Temp. (°C)	Time (h)	1 (%)[b]	2 (%)[b]	3 (%)[b]	5 (%)[b]
1	**1**	NaOH	25	6	51.4	9.3	2.9	36.3
2	**2**	NaOH	25	6	-	98.0	1.5	0.5
3	**3**	NaOH	25	6	-	2.8	0.3	94.0
4	**1**	K_2CO_3	60	6	89.9	0.8	6.8	2.5
5	**1**	K_2CO_3[e]	60	6	97.5	1.0	0.1	0.9
6	**1**	$NaHCO_3$	60	6	98.9	-	1.1	-
7	**3**	K_2CO_3	60	6	-	<0.1	6.6	92.4
8	**3**	K_2CO_3	25	72	-	<0.1	7.0	91.8
9	**3**	K_2CO_3[c]	60	6	-	<0.1	71.0	24.5
10	**3**	K_2CO_3[d]	60	6	-	<0.1	92.5	0.3
11	**3**	K_2CO_3[e]	60	6	-	0.4	57.8	24.3
12	**3**	$NaHCO_3$	60	6	-	<0.1	80.1	16.2
13	**3**	$NaHCO_3$	25	72	-	<0.1	97.5	0.3
14	**3**	$NaHCO_3$[e]	60	6	-	<0.1	91.4	3.0

[a]Experimental conditions: Substrate (10 mg), CH_3CN/H_2O (1:1 v/v, 10 mL), Base:1.5 molar excess, 25 or 60 °C
[b]Determined by HPLC using the method for the detection of the impurities
[c]The reaction was carried out under argon atmosphere
[d]Trolox (3 eqv.) was added to the solution and the molar excess of K_2CO_3 was increased to 5.8
[e]The substrate was dissolved in water in the presence of 6% hydroxypropyl-β-cyclodextrin instead of $CH_3CN:H_2O$ 1:1 v/v

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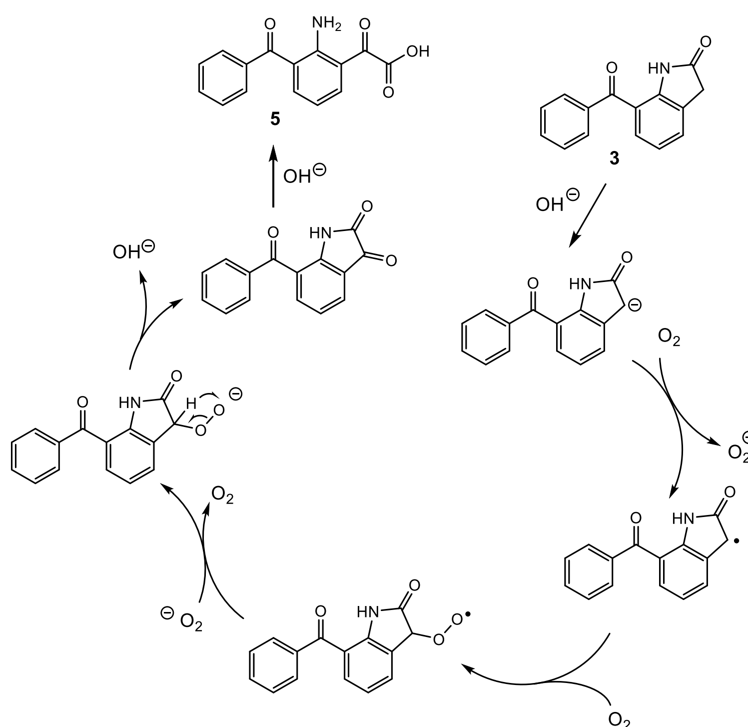
These data are in agreement with the hypothesis that compound **5** originates from a benzylic oxidation, affected by both temperature and strength of the employed base, of the key intermediate **3**, whose formation could be the limiting step in the conversion of **1** into **5** and is strictly related to the pH of solution. Indeed, lowering the pH promotes the cyclization of **1** or **2** to give **3**, but at the same

225 time further reactions are inhibited and neither **5** or its parent isatin form were detected in solutions

at pH < 4.5. Increasing the pH, on the other hand, should decrease the amount of **3** facilitating at the same time the formation of **5** and also the contribution of direct hydrolysis of **1** to **2** becomes not negligible so that the final composition of solution depends from the balance of the different reaction rates for the formation of **2**, **3** and **5**.

230 Concerning the mechanism of formation of **5**, it could be reasonably viewed as a base-catalyzed air oxidation, which is known for many compounds yielding stabilized carbanions upon dissociation and has been interpreted in terms of reaction sequences in which a one-electron transfer from a carbanion to an oxygen molecule to give the carbon radical and superoxide ion is a key step [26]. According this pathway, the hydroperoxides formed as primary products can then decompose or rearrange to the

235 final oxidized compounds [19, 27]. A proposed mechanism for the formation of **5** from **3** in alkaline aqueous solution is shown in Scheme 2.



Scheme 2. Proposed mechanism for the formation of **5** in alkaline aqueous solution

Interestingly, when **3** was dissolved in water/hydroxylpropyl-β-cyclodextrin solvent system instead of the mixture water/CH_3CN and treated with base it seemed protected from oxidation and its conversion to **5** markedly decreased (compare entry 11 with 7 and entry 14 with 12), even if the

240 presence of other side-products became not negligible (>5%). Starting from **1**, in the presence of the cyclodextrin additive the amounts of formed **3** and the related compound **5** were both significantly reduced (compare entry 5 with 4).

245 **4. Conclusions**

A study on the degradation profile of nepafenac was carried out in water solutions at different values of pH and the highest stability of the drug was observed at pH 7.6, with about 97% of the initial concentration recovered after 10 days under thermal stress conditions. Besides the known nepafenac-related compouns amfenac **2** and indolinone **3**, compound **5** was detected as degradation product and
250 structurally characterized as (2-amino-3-benzoyl)-oxoacetic acid. It was shown that the formation of this α-ketoacid **5** in alkaline water/organic cosolvent involves compound **3** as key intermediate, which undergoes aerobical oxidation on its benzylic position and hydrolysis of the lactam ring. The presence of hydroxylpropyl-β-cyclodextrin in water solution seemed to exerts in some extent a protective action from oxidation and further studies are in progress to elucidate the stabilization mechanism.

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260 **Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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