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Original article

Scavenging activity of aminoantipyrines against hydroxyl radical

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ABSTRACT

The pyrazolone derivatives antipyrine and 4-(*N*,*N*-dimethyl)-aminoantipyrine (aminopyrine) have long been used as analgesic, antipyretic and anti-inflammatory drugs. However, in spite of its recognized therapeutic benefits, the use of pyrazolones has been associated with agranulocytosis. Though the oxidation of aminopyrine by neutrophil-generated hypochlorous acid (HOCl), leading to the formation of a cation radical, has been considered responsible for the potential bone marrow toxicity, the reaction mechanisms of pyrazolones against other reactive oxygen species (ROS) remains elusive. Thus, the reactions of 4-aminoantipyrine and methylated derivatives with hydroxyl radicals (HO$^{\bullet}$) were studied as a model of their reactivity against ROS. The results show that 4-(*N*,*N*-dimethyl)-aminoantipyrine (aminopyrine) undergoes demethylation when reacting with HO$^{\cdot}$ radical, leading to 4-(*N*-methyl)-aminoantipyrine, which is further demethylated to 4-aminoantipyrine. In addition, it was also observed that another favorable reaction of 4-aminoantipyrines in these conditions is the hydroxylation on the aromatic ring, a reaction that is common to aminopyrine, 4-(*N*-methyl)-aminoantipyrine, and 4-aminoantipyrine. Whether these reaction mechanisms give rise to harmful reactive intermediates requires further chemico-biological evaluation.

1. Introduction

The pyrazolone derivatives antipyrine (**1**) and 4-(*N*,*N*-dimethyl)-aminoantipyrine (aminopyrine; **4**) (Fig. 1) have been used as analgesic, antipyretic and anti-inflammatory drugs, since the end of the 19th century [1]. These compounds are strong inhibitors of cycloxygenase isoenzymes, platelet tromboxane synthesis, and prostanoids synthesis [1]. The biological activity of these compounds has also been attributed to its scavenging activity against reactive oxygen and nitrogen species (ROS and RNS), as well as to the inhibition of neutrophil's oxidative burst. Indeed, aminopyrine was demonstrated to be a highly efficient scavenger of the ROS hydroxyl radical (HO$^{\bullet}$) [1], hypochlorous acid (HOCl) [1–3], peroxyl radical (ROO$^{\bullet}$) [1], and singlet oxygen ($^1O_2$) [4] and of the RNS nitric oxide ($^{\bullet}$NO) and peroxynitrite ($ONOO^-$) [5], as well as to effectively prevent the phorbol-12-myristate-13-acetate-induced neutrophil oxidative burst [1]. Antipyrine was shown to be the most effective pyrazolone in scavenging ROO$^{\bullet}$, though it was inefficient against the other ROS and RNS mentioned above, or to the neutrophil's oxidative burst [1,4,5].

In spite of its recognized therapeutic benefits, the use of pyrazolones has been associated with potential adverse effects characterized by leukopenia, most commonly of neutrophils, causing neutropenia in the circulating blood (agranulocytosis) [6]. Noteworthy, the oxidation of aminopyrine by neutrophil-generated HOCl was previously reported, leading to the formation of a blue cation radical, to which has been attributed the potentially fatal bone marrow toxicity, leading to agranulocytosis [2,3]. From the studies performed so far, it has been proposed that the oxidation of aminopyrine to a radical cation, by HOCl, involves an *N*-chlorination followed by loss of a chlorine radical [3]. Another possible mechanism is the formation of a dication by the loss of chloride ion from *N*-chloroaminopyrine [6]. Considering the high reactivity of aminopyrine with other ROS besides HOCl, as well as with RNS, it may be considered feasible that potentially harmful reactive intermediates may also rise from aminopyrine scavenging effects. However, the reaction mechanisms of pyrazolones against the other ROS and RNS remains elusive. In addition, the relative potency of the different pyrazolones concerning their reaction with ROS and RNS is also hitherto unknown.

The aim of the present study is to evaluate the postulate that the much higher antioxidant activity of **4** as compared to **1** may be attributed to the capacity of the former to undergo oxidation followed by demethylation in the reaction with free radicals, giving

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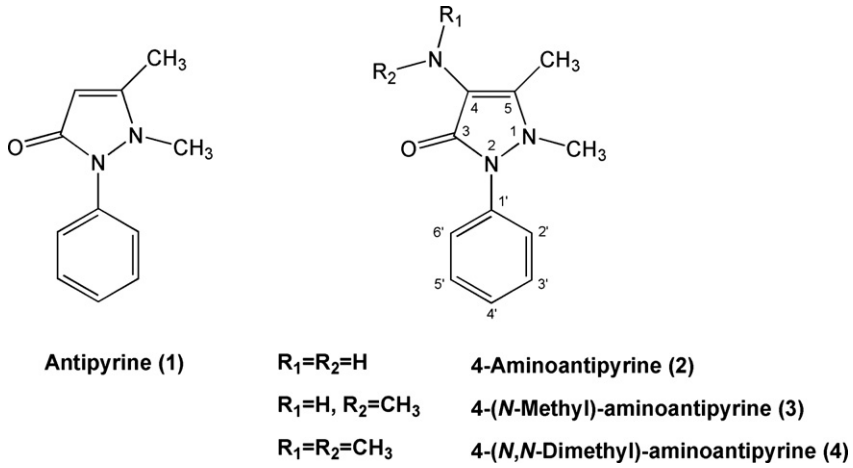


Fig. 1. Structures of studied antipyrine derivatives.

rise to 4-(*N*-methyl)-aminoantipyrine (**3**), which can be further demethylated to 4-aminoantipyrine (**2**) (Scheme 1).

With this objective, the reactions of 4-aminoantipyrine and methylated derivatives with HO• were studied as a model of their antioxidant activity against reactive oxygen species (ROS). For this purpose 4-(*N*-methyl)-aminoantipyrine (**3**) was synthesized to be used as standard. Oxidation of aminoantipyrine derivatives by HO• was then carried out and the reaction products analyzed.

2. Results and discussion

2.1. Synthesis of 4-(N-methyl)-aminoantipyrine (3)

In order to prove that **3** stemmed from the reaction of **4** with oxidizing radicals, compound **3** was synthesized to be used as a standard. This compound was prepared by an adaptation of a published methodology [7] involving initial formation of 4-phthalimidoantipyrine, followed by semi-hydrolysis with sodium hydroxide and subsequent methylation of the sodium salt of 4-(*N*-phtalyl)-aminoantipyrine with dimethylsulfate. Acid hydrolysis of 4-(*N*-methyl-*N*-phtalyl)-aminoantipyrine gave **3** in 4% overall yield. The product was fully characterized by mass spectrometry and ^{1}H and ^{13}C NMR, being the assignment of all signals based on correlations observed in HMQC and HMBC spectra (see Experimental section).

2.2. Oxidation of aminoantipyrine derivatives by the hydroxyl radical

The reaction of 4-(*N*,*N*-dimethyl)-aminoantipyrine (**4**), 4-(*N*-methyl)-aminoantipyrine (**3**) and 4-aminoantipyrine (**2**) with the HO• radical (generated by Fenton reaction) was performed in aqueous solution, at different pH conditions. The reaction mixtures were analyzed by HPLC-MS, with UV–vis (photodiode array) detection. In some instances, reaction products isolation by semi-preparative HPLC was used for further spectroscopic characterization by NMR and MS.

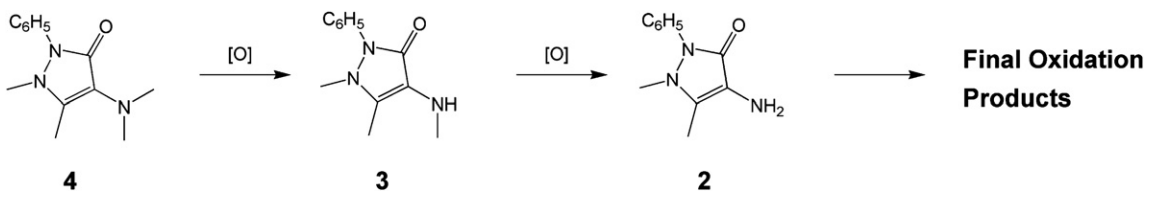
Representative HPLC chromatograms, recorded with an isocratic elution (methanol/ammonium acetate buffer 50 mM; 40:60), of the reaction mixtures of **4**, **3** and **2** are displayed in Fig. 2.

In the case of the oxidation of compound **4** (see Fig. 2a), it was observed that changing the pH of the Fenton reaction mixture from 1 (data not shown) to 7 didn't affect significantly the HPLC profile. The only noticeable difference was that at pH 1 (HCl 0.1 N aqueous medium) products **2** and **3** were formed after long reaction times (about one day), while at pH 7 (phosphate buffered solution) these products could be detected after 5 min of reaction.

This result can be explained on the basis of the degree of protonation of the molecule: at pH 1, the exocyclic amino group is protonated ($pK_a = 5.0$) [8], which will difficult the oxidation on this part of the molecule.

Compounds **2** and **3** were identified as 4-aminoantipyrine and 4-(*N*-methyl)-aminoantipyrine, respectively, on the basis of indistinguishable UV spectra, as well as identical retention times of the corresponding commercially available and synthesized standards, when recorded under analogous conditions. Due to low chromatographic resolution of peak of compound **2** and instability of compound **3**, it was not possible to isolate these compounds to perform further analysis by ^{1}H NMR and MS. However, it was possible to confirm their structures by HPLC-MS analysis (Fig. 3).

As can be seen in Fig. 2a, apart the demethylation products three other main products are formed on the reaction of **4** with HO• (**A**, **B** and **C**). The respective mass spectra (Fig. 3) show equal molecular ions compatible with an additional OH group. Separation of this reaction mixture by semi-preparative HPLC allowed isolation of these products and their further analysis by spectroscopic methods. Compounds **B** and **C** showed a molecular ion at *m*/*z* 247 in the TOF-MS(EI) spectra and compound **A** showed a pseudo-molecular ion at



Scheme 1. Oxidative demethylation of 4-(*N*,*N*-dimethyl)-aminoantipyrine.

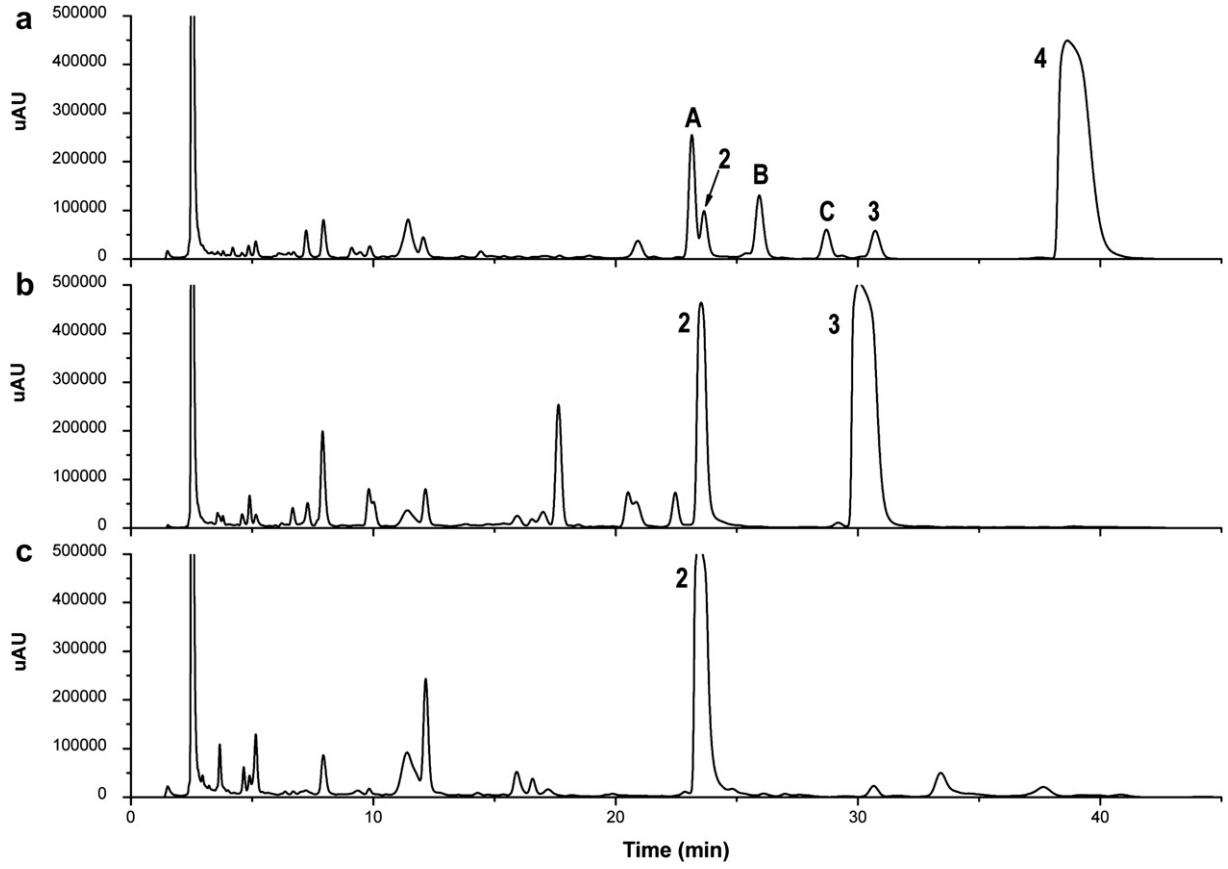


Fig. 2. HPLC-DAD chromatograms of the reaction mixtures of **4** (a), **3** (b) and **2** (c) with HO• radical at pH 7, performed with a RP18e Purospher® column (250–4 mm, 5 μm, Merck) with an isocratic elution (methanol/ammonium acetate buffer 50 mM; 40:60) and 1.00 mL min^{-1} flow rate.

m/*z* 246 when analyzed by MS(ESI-). These data are completely consistent with the formation of derivatives that resulted from the insertion of a hydroxyl group in the molecule of **4**.

The position of the hydroxyl substitution was established based on ^{1}H NMR spectra, where the three compounds showed very similar spectral profiles in the aliphatic region but very distinct aromatic signals. The ^{1}H NMR spectra of these three products (see Experimental section) presented three singlets (from 3.5 to 2.0 ppm) compatible with the presence of twelve protons from four methyl groups, indicating that no modification occurred in this part of the molecule. Thus, structures of these three products were discriminated on basis of the pattern of ^{1}H NMR signals from aromatic protons. Product **A** presented in the ^{1}H NMR spectrum two doublets (at 6.93 and 6.65 ppm) each one integrating for two protons which is evidence for *para* substitution on the aromatic moiety of **4**. The ^{1}H NMR spectrum of product **B** presented one triplet at 7.32 ppm (4′-H), a doublet at 7.17 ppm (6′-H) and a multiplet at 7.02–6.94 ppm (integrating for two protons) that was simplified to one singlet and a doublet when the triplet at 7.32 ppm was selectively irradiated. Taking together, these data are evidence of a substitution in the *ortho* position of the aromatic ring. Therefore, **C**, which presents only two downfield ^{1}H NMR signals at 7.29 ppm (triplet) and at 6.80–6.78 ppm (a multiplet correspondent to three protons), results from hydroxylation in the *meta* position. The formation of phenolic derivatives was also observed when aqueous solutions of antipyrine were submitted to ^{60}Co irradiation [9].

These results suggest that the most favorable reaction between **4** and the HO• radical is the hydroxylation on the aromatic ring. There is also evidence that **4** undergoes demethylation leading to **3**, which is further demethylated to **2**. The fact that **2** is detected by HPLC with UV–vis (photodiode array, Fig. 2b) and mass detection analysis (Fig. 3) when **3** was reacted with the radical HO• is a support of the successive demethylation process.

The hydroxylation reaction is common to all compounds **2**, **3** and **4**. In the chromatograms of Fig. 2b and c some peaks could be attributed to the corresponding hydroxylation products (assignment not shown). In addition, poly-hydroxylation is also possible; in the chromatograms 2 a, b and c peaks at retention times lower than that of compound **A** correspond to products di- and tri-hydroxylated and some of them are common to all reaction mixtures studied.

A possible mechanism for the formation of the demethylated products is the one electron oxidation of the starting compounds leading to the respective radical cations (Scheme 2). Deprotonation of this species, with the formation of an aminomethyl radical, followed by further oxidation gives the demethylated derivative with loss of formaldehyde. The ESR spectrum of the radical cation of **4** (Fig. 4) has already been referred in the literature [10–12] and the loss of a methyl group from a nitrogen atom, in the form of formaldehyde, is also a known process that occurs with this compound under enzymatic oxidation in the presence of hydrogen peroxide [11–13]. This suggests that HO• radical is the primary oxidizing species involved, which is confirmed by the results obtained by gamma radiolysis. Irradiation of N_2O saturated aqueous solutions of **4** in a ^{60}Co source (data not shown) gave results very similar to those obtained with the Fenton reaction described above.

2.3. Conclusions

From the obtained results, it may be concluded that one of the possible pathways for the reaction between 4-(*N*,*N*-dimethyl)-

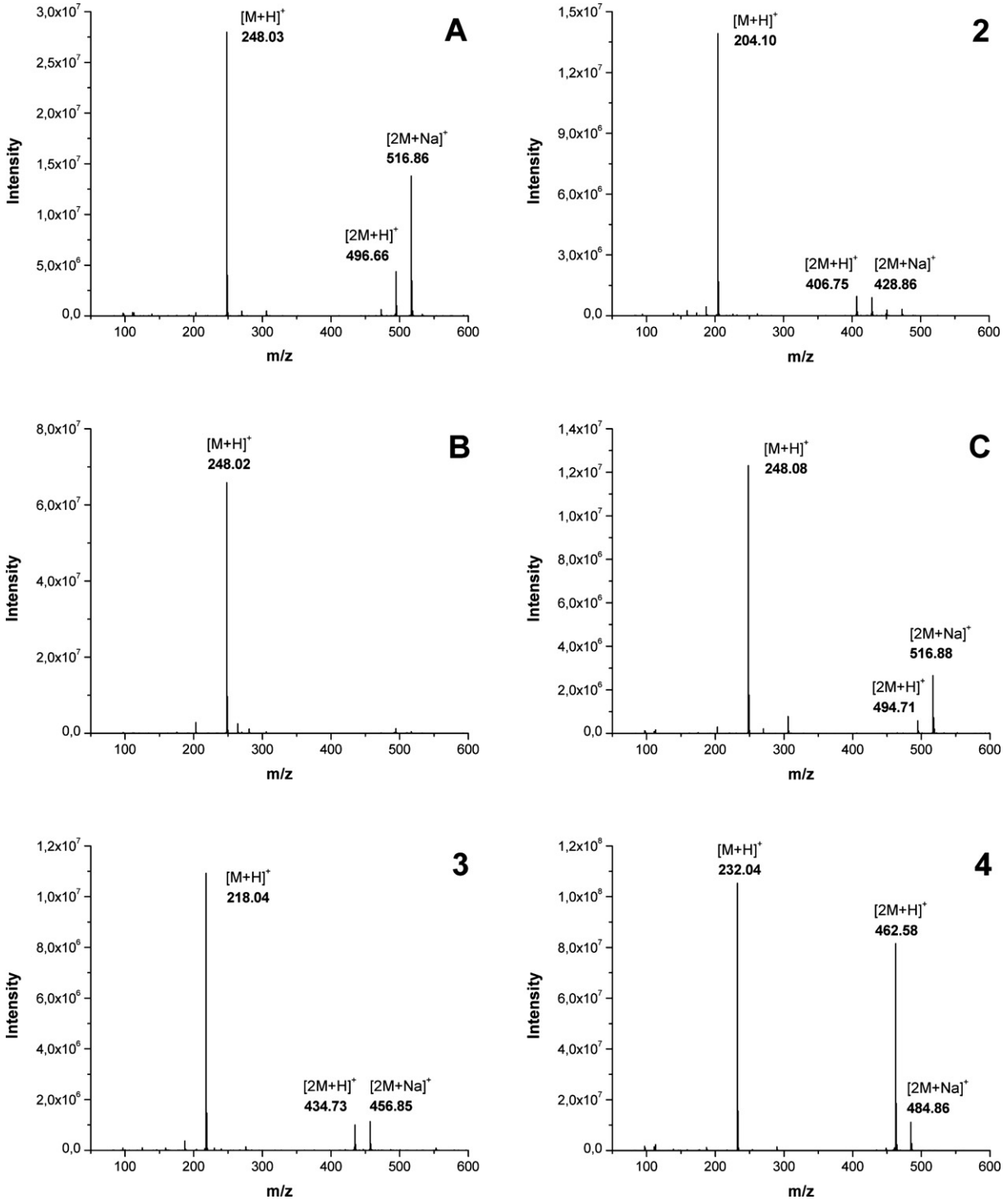
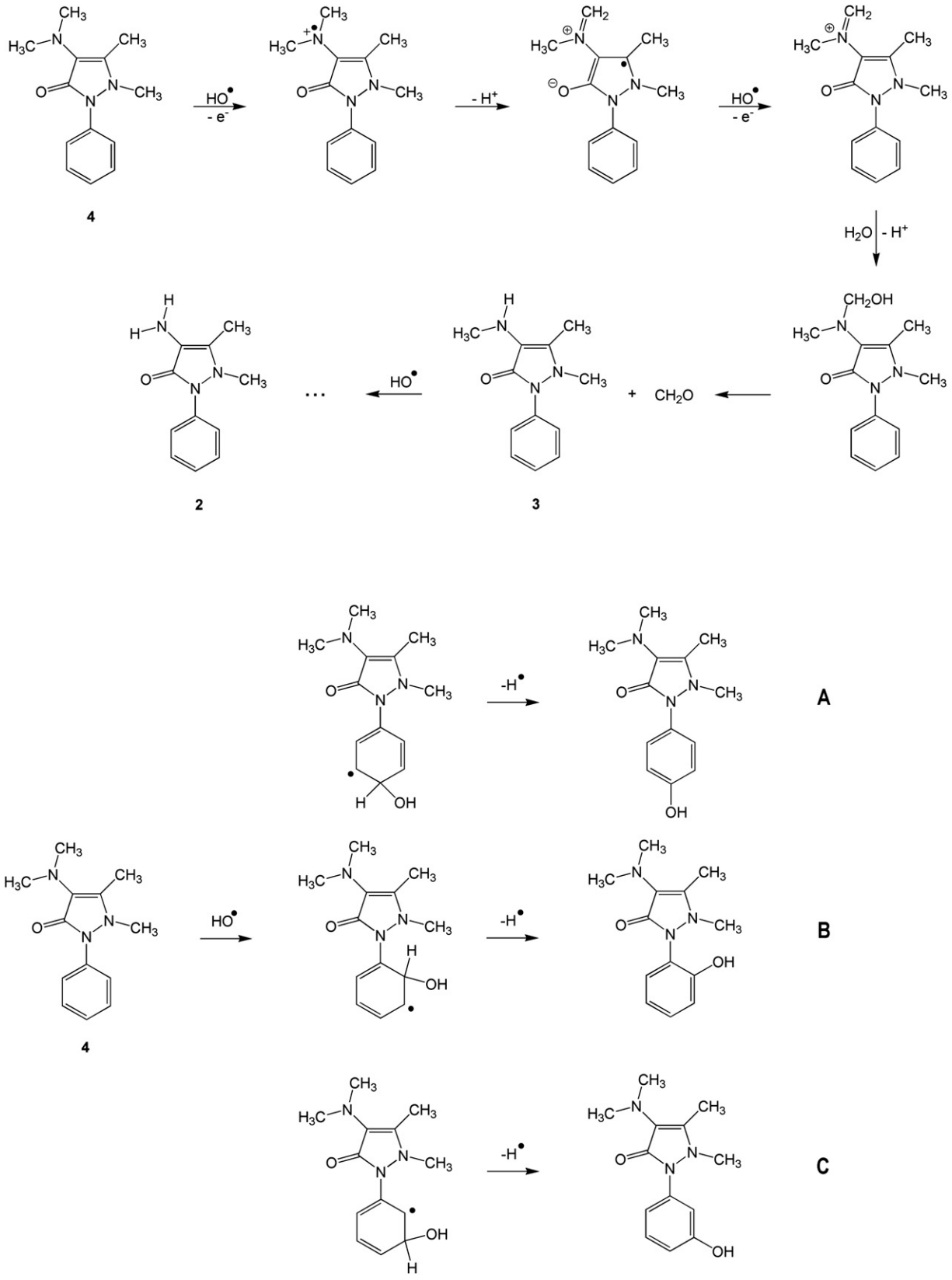


Fig. 3. Mass spectra (ESI+) of demethylation products (**2**, **3**) and hydroxylation products (**A**, **B**, **C**) from reaction of **4** with hydroxyl radical at pH 7 (HPLC profile shown in Fig. 2a).

aminoantipyrine (**4**) and the HO• radical is the hydroxylation on the aromatic ring. This reaction, common to compounds **2**, **3** and **4**, has been observed for aromatic iso- and heterocyclic systems, namely from biologically relevant compounds [14–16].

There is also evidence that compound **4** undergoes a new reaction consisting in a demethylation leading to 4-(*N*-methyl)-aminoantipyrine (**3**), which is further demethylated to 4-aminoantipyrine (**2**).

The pathophysiological importance of the observed reaction products and possible reactive intermediates is hitherto unreported, though these results add a significant amount of new information about the putative reactivity of the studied pyrazolones and HO•. Thus, in the event of oxidative stress-related inflammatory processes, where HO• may be involved, the therapy with pyrazolone NSAIDs implies the hydroxylation of the aromatic ring, as well as the demethylation reactions. These byproducts should be taken into account, either for their putative toxicity or the contribution to healing effects. The demethylation reactions can probably play a major biological role, as compared to the hydroxylation reaction, since it may configure an antioxidizing cascade in which compounds **4**, **3** and **2** all behave as antioxidants.



Scheme 2. Reaction mechanism of successive demethylation of **4** and formation of hydroxylated derivatives.

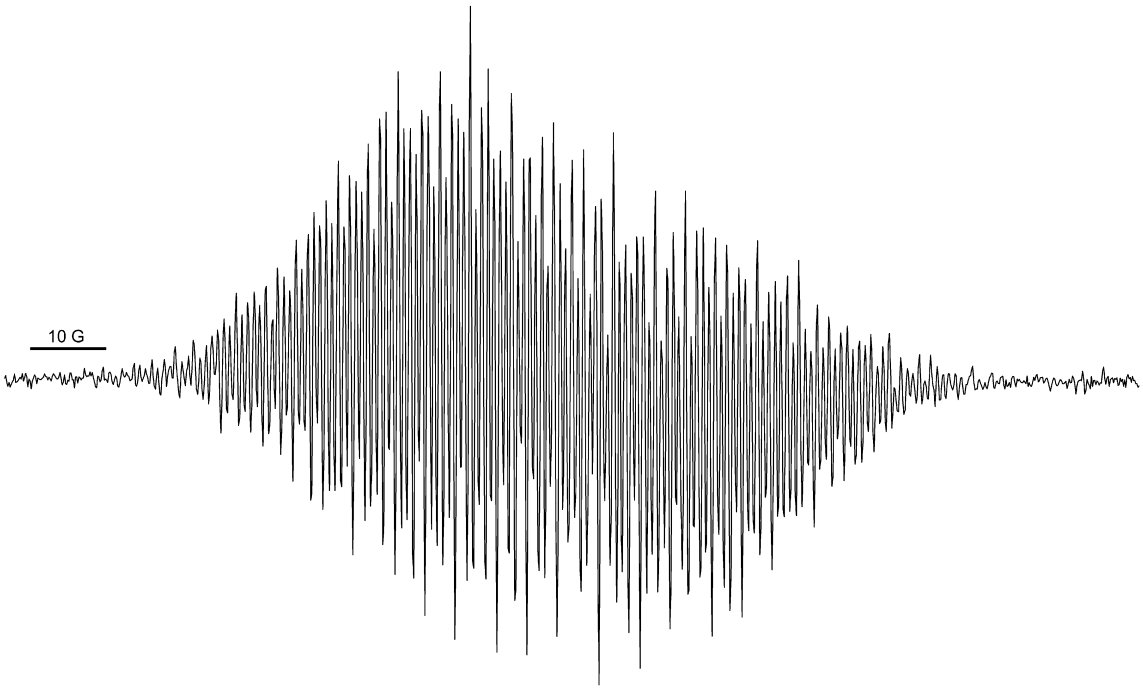


Fig. 4. ESR spectrum of 4-(*N*,*N*-dimethyl)-aminoantipyrine radical cation obtained by Fenton reaction (**4** 30 mM, H_2O_2 30 mM, $FeSO_4$ 0.5 mM, pH 1).

3. Experimental section

3.1. Separation and spectroscopic techniques

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker ARX 400, operating at 400 and 100.62 MHz, respectively. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane, and coupling constants (J) are reported in Hz.

Infrared (IR) spectra were recorded on a Perkin Elmer 683 FT-IR spectrometer; group frequencies are reported in cm^{-1}.

Reverse phase chromatography RP18 (LiChrospher®, column 250–4 mm, 5 μm, Merck) of reaction mixtures was performed with DAD (Diode Array Detector) detection from 200 to 600 nm with a 1.0 mL/min flow, using a well degassed mobile phase with methanol (20–80%)/ammonium acetate buffer 50 mM (80–20%) with a gradient elution. HPLC-DAD-ESI-MS were carried out in a HPLC system (Thermo Surveyor) and ESI-ion trap MS system with a LCQ ion trap mass spectrometer (Thermofinnigan) equipped with electrospray source and run by Xcalibur (Thermofinnigan) version 1.3 software. The spectral mass/charge range used was 50–1000 Da.

The sample analyzed by mass spectroscopy was dissolved in methanol and delivered by an infusion pump (Harvard Apparatus) at 0.6 mL/h to a quadrupole VG Platform (Micromass, UK Ltd) spectrometer equipped with an electrospray ionization source operating in negative mode. Capillary temperature was kept between 100 °C and 120 °C, using a cone voltage of 140 V and capillary voltage of 3.5 kV. Nitrogen was used as drying and nebulizing gas at 300 L/h and 10 L/h, respectively. Spectral mass/charge range used was 150–300 Da.

Samples analyzed by mass spectroscopy (electron impact) were delivered by direct probe to a GCT-MS(TOF) (Micromass, UK Ltd) spectrometer.

For detection of MSTFA derivatized antipyrines by GC/MS the derivatization was carried out in vials with 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) for 30 min at 60 °C. The separation and detection of the trimethylsilyl derivative of the antipyrine were achieved using a GC/MS system: GC 6000 (Carlo Erba Vega Series 2) and MS, QMD1000 Carlo Erba. The gas chromatograph was equipped with a silica capillary column (30 m × 0.32 mm i.d.; d_f: 0.25 μm) covered with 5% phenyl-polisiloxane/95% dimethylpolisiloxane (DB-5ms, Agilent-J&W Scientific). GC temperatures: 200 °C isothermal 3 min, 3 °C/min–250 °C. The Helium flow was 1.5 mL/min and the head pressure was 85 kPa. The ion source and the transference line were kept at 150 and 220 °C respectively. The MS spectra were obtained by Electronic Impact (EI) at 70 eV by using MassLab™ software (Micromass). The spectral mass/charge range used was 50–350 Da.

3.2. Preparation of 4-(N-methyl)-aminoantipyrine

4-(*N*-Methyl)-aminoantipyrine (4% overall yield) was prepared by adaptation of a published technique [7] as a yellow oil. IR ν_{max}(NaCl)/cm^{-1} 1651 (C=O). NMR δ_H($CDCl_3$) 7.38–7.32 (4 H, m, Ph), 7.15 (1 H, t, *J* 6.9, 4′-H), 2.77 (3 H, s, 1-Me), 2.73 (3 H, s, N^4-Me), 2.16 (3 H, s, 5-Me); δ_C($CDCl_3$) 162.7 (C3), 140.2 (C5), 135.7 (C1′), 129.5 (C3′ + C5′), 124.4 (C4′), 123.4 (C2′ + C6′), 38.1 (1-Me), 35.1 (N^4-Me), 11.3 (5-Me). GC–MS *m*/*z* (EI) 217 (M^+, 7%), 202 (0.5, M − CH_3), 98 (4, M − C_6H_5NCO), 83 (28, M − C_6H_5NCO–CH_3), 77 (7, C_6H_5), 56 (100, CH_3CNCH_3).

3.3. Oxidation of 4-(N,N-dimethyl)-aminoantipyrine by HO• (Fenton reaction)

Reaction mixtures at pH 7 were prepared by using the antipyrine derivative 10 mM, H_2O_2 10 mM, FeEDTA 2 mM and ascorbic acid 1 mM buffered with Na_2HPO_4 and KH_2PO_4. At pH 1, solutions were prepared in an HCl 1 M medium with $FeSO_4$ 1 mM as catalyst. Samples were collected at different reaction times.

3.4. Gamma radiolysis

Solutions were prepared in MilliQ water saturated with N_2O typically containing the antipyrine derivative 10 mM at pH 7 or pH 1 and were irradiated in a ^{60}Co gamma source with doses ranging from 0.9 to 2.7 kGy.

3.5. ESR spectroscopy

Solutions for ESR spectroscopy (30 mM in 4-(*N*,*N*-dimethyl)-aminoantipyrine, 30 mM in H_2O_2 and 0.5 mM in $FeSO_4$ at pH 1) were placed into a quartz flat cell located in the ESR cavity. The X-band ESR spectra were recorded in a Bruker ESP300E spectrometer. In some cases a computer smoothing of the spectrum (available at the Bruker spectrometer) was applied.

3.6. Characterization of products isolated by semi-preparative HPLC

3.6.1. 4-(N,N-dimethyl)-4′-hydroxyaminoantipyrine (0.8 mg, 7%)

NMR $\delta_H(CDCl_3)$ 6.93 (2H, d, *J* 8.6, Ph), 6.65 (2H, d, *J* 8.6, Ph), 3.26 (6H, s, N^4-$(Me)_2$), 3.14 (3H, s, 1-Me), 2.75 (3H, s, 5-Me); MS *m*/*z* (ESI-) 246 ($M - H^+$), 278 ($M - H^+ + CH_3OH$).

3.6.2. 4-(N,N-dimethyl)-2′-hydroxyaminoantipyrine (1.5 mg, 12%)

NMR $\delta_H(CD_3OD)$ 7.31 (1H, t, *J* 7.1, 4′-H), 7.17 (1H, d, *J* 7.7, 6′-H), 7.02–6.95 (2H, m, 3′-H and 5′-H), 3.11 (3H, s, 1-Me), 2.75 (6H, s, N^4-$(Me)_2$), 2.25 (3H, s, 5-Me); TOF-MS *m*/*z* (EI) 247.130 (M^+), 191.081 ($M - CH_3CNCH_3$), 56.048 (100, CH_3CNCH_3).

3.6.3. 4-(N,N-dimethyl)-3′-hydroxyaminoantipyrine (1.3 mg, 10%)

NMR $\delta_H(CD_3OD)$ 7.29 (1H, t, *J* 8.4, 4′-H), 6.80-6,78 (3H, m, Ph), 3.06 (3H, s, 1-Me), 2.74 (6H, s, N^4-$(Me)_2$), 2.25 (3H, s, 5-Me); TOF-MS *m*/*z* (EI) 247.129 (M^+), 56.048 (100, CH_3CNCH_3).

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2010.01.071.

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