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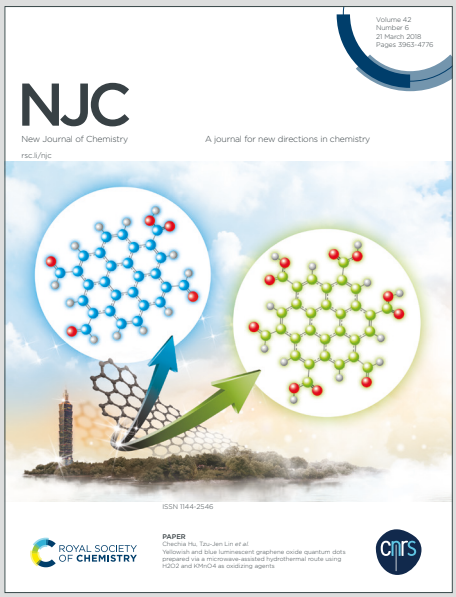

Mechanistic insights into the *in vitro* metal-promoted oxidation of (di)azine hydroxamic acids: evidence of HNO release and N,O-di(di)azinoyl hydroxylamine intermediate

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Abstract

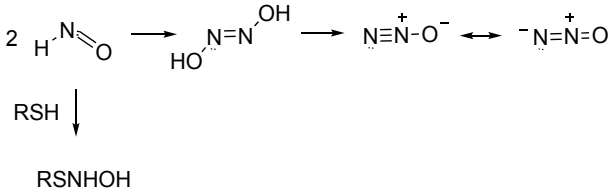
The oxidant-dependent ability of hydroxamic acids to release nitroxyl (HNO), a small inorganic molecule endowed with various biological properties, is addressed from a mechanistic standpoint. Indeed, the exact mechanism of the hydroxamic acid oxidation in physiological conditions and the direct or indirect characterization of the intermediates remain elusive. In this work, intermolecular oxidation of isonicotino-, nicotino- and pyrazino-hydroxamic acids with $K_3[Fe^{III}(CN)_6]$ at physiological pH (7.4), was monitored by ^1H NMR, MS, EPR and UV-vis techniques. While nitrosocarbonyl (di)azine intermediates, (di)Az-C(O)–NO, could be a priori envisaged, it was in fact the corresponding *N,O*-di(di)azinoylhydroxylamines (AzC(O)NHOC(O)Az) and HNO that were identified, the first by NMR ^1H and the second on the basis of EPR and UV-vis experiments using the [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3 oxide] (cPTIO) spin trap. The decomposition of the unstable *N,O*-di(di)azinoylhydroxylamine intermediates in aqueous buffer media was shown to generate the corresponding carboxylic acids as final organic products, envisaged as possible *in vivo* metabolites. The same oxidation experiments performed in the presence of methylamine led to the formation of the corresponding *N*-methyl amides suggesting that, unlike hydroxamic acids, *N,O*-di(di)azinoylhydroxylamines act as acylating agents in physiological pH conditions.

Key-words: nitroxyl donors;(di)azine hydroxamic acids; Fe(III) oxidation; *N,O*-diaroylhydroxylamine intermediate; physiological pH conditions.

Introduction:

Nitroxyl or azanone (HNO) is an unstable inorganic weak acid (pKa = 11.4),[1,2] which can also be considered as the protonated form of the one-electron reduction product of the nitric oxide radical ($NO^{\cdot}$). Nitroxyl displays several important biological activities,[3] remaining however, poorly understood. One of the main pharmacological properties of HNO is its beneficial

influence on the cardiovascular system. HNO, which is indeed known to induce vasodilation,[4,5] exhibit positive inotropic/lusitropic effects[6,7] and inhibit platelet aggregation,[8] is thus envisaged to play a key role for the treatment of patients with heart failure. HNO also exhibits an anti-angiogenic activity, as opposed to its sibling NO that promotes angiogenesis. This HNO property has opened many potential pharmacological applications including in cancer therapy.[9] Since HNO cannot be directly employed *in vivo* due its high irreversible instability in aqueous media (dimerization and dehydration giving nitrous oxide, N_2O, Scheme 1)[1,2] and high reactivity towards nucleophilic biomolecules (mainly with sulfur compounds),[3,10] all studies on biological properties of HNO or its use as a therapeutic agent require the design of HNO donor compounds. Hence research into the development of different classes of pro-drugs, with the ability to release HNO under physiological conditions and in a tunable manner, attracts the attention of the scientific community. Among the different classes of HNO donors,[11] one can mention the hydroxamic acid function -C(O)NHOH, which is prone to generate HNO upon reaction with various oxidizing agents ($NaIO_4$, metalloenzymes, $Na_3[Fe^{II}(CN)_5NH_3]$/oxidant, $K_3[Fe^{III}(CN)_6]$, radicals generated by radiolysis such as $^{\cdot}OH$, $^{\cdot}N_3$, etc.).[12] However, the exact mechanism of the hydroxamic acid oxidation in physiological conditions and the direct or indirect characterization of the transient intermediates, generated by an oxidative process in aqueous media, still constitute a matter of study.[12-14]



Scheme 1. Reactions of HNO in aqueous or biological media.

Taking into account the potential of hydroxamic acids to act as HNO donors for therapeutic treatments, the elucidation of their oxidation mechanism in physiological pH conditions is crucial to improve their properties as possible pro-drugs able to release HNO in a controllable way. Recently, we have developed a pentacyanoferrate(II) complex of pyrazinohydroxamic acid and have

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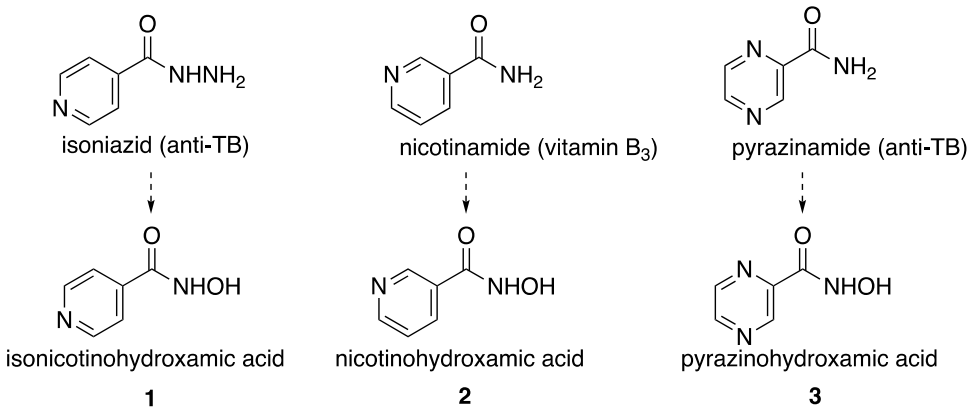
investigated *in vitro* its oxidation in the presence of H_2O_2 as well as its ability to release simultaneously HNO and the corresponding carboxylic acid (pyrazinoic acid) as the antituberculosis active metabolites of the delamanid and pyrazinamide drugs, respectively.[15] It has been proposed that oxidation of the pentacyanoferrate(II) complex of pyrazinohydroxamic acid proceeds *via* intramolecular (Fe^{III})-mediated oxidation of the C(O)NHOH function after initial oxidation of the Fe^{II} center into Fe^{III} center by H_2O_2.

In the present study, we examine if the direct intermolecular oxidation of isonicotino- (**1**), nicotino- (**2**), and pyrazino- (**3**) hydroxamic acids promoted by potassium ferricyanide ($K_3[Fe^{III}(CN)_6]$) at pH = 7.4 (Scheme 2) occurs and leads to the same final metabolites.



Scheme 2. Hydroxamic derivatives of isonicotinic hydrazine (isoniazid), nicotinamide, and pyrazinamide.

2. Results and Discussion

The hexacyanoferrate(III) ion is a mild oxidizing species, which operates by abstracting one electron from a substrate through an outer sphere (intermolecular) pathway. This reagent allows oxidative processes[16] without atom transfer. Its use is relevant for a mechanistic study that could corroborate our previous results on the oxidation of the pyrazinohydroxamic acid acting as a N-ligand at a $Fe^{II}(CN)_5^{3-}$ center, for which an oxidation of the hydroxamic portion is proposed *via* an action of a Fe^{III} center generated by oxidation of the Fe^{II} center by H_2O_2.

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It has been reported that one-electron oxidation of hydroxamic acids leads to the unstable nitroxide radical RCONHO$^{\cdot}$.[13,14,17] The fate of this radical is, however, uncertain: several alternative theoretical routes have been proposed,[14,16] and conceivable intermediates are a priori too unstable to be fully characterized (Scheme 3). On the one hand, the nitroxide radical intermediate could undergo a homolytic cleavage to form HNO and the aroyl radical (pathway a), or quickly dimerize, then rearrange to a *N,O*-diacylhydroxylamine intermediate (pathway e and f, the latter proposed by W. A. Waters).[12] On the other hand, it is proposed that the nitroxide radical readily undergoes a second oxidation to give the nitrosocarbonyl derivative, for which, however, direct evidence and complete spectral characterization in aqueous solution are lacking.[18,19] Such species are highly reactive and can decompose by either nucleophilic addition/HNO elimination (pathway c and d), or homolytic cleavage releasing NO$^{\cdot}$ and an aroyl radical (pathway b) (Scheme 3). In the first issue, it must be reminded that direct identification of HNO is still a real challenge. In the following are reported mechanistic investigations of the oxidation of isonicotino- (**1**), nicotino- (**2**) and pyrazino- (**3**) hydroxamic acids by $K_3[Fe(CN)_6]$ in aqueous buffer solution using ^{1}H NMR, MS, EPR and UV-

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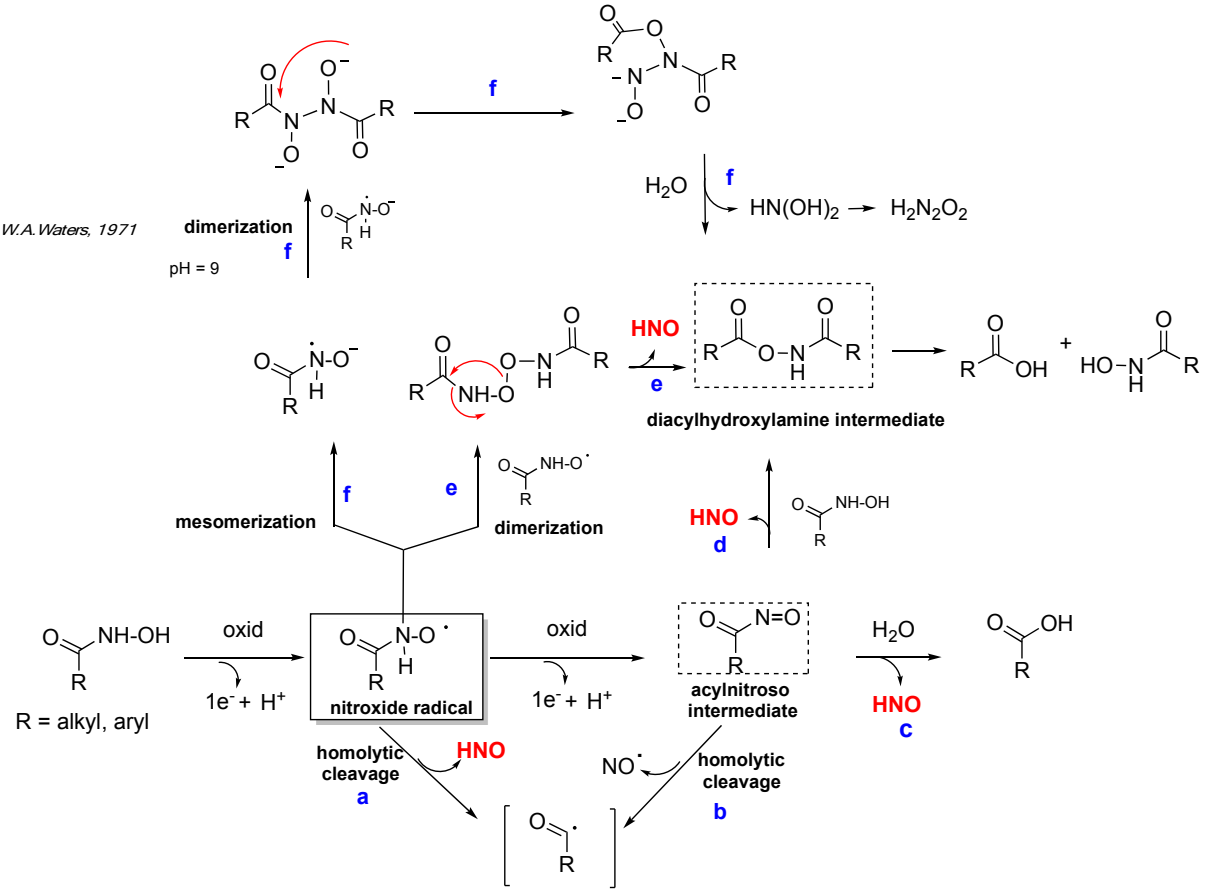
vis techniques.



Scheme 3. Possible pathways and intermediates for the oxidation of hydroxamic acids (adapted from ref. 13).

2.1 Monitoring of Fe^{III}-mediated oxidation of heteroarylhydroxamic acids by ^1H NMR.

Oxidation of isonicotino-, nicotino-, and pyrazino-hydroxamic acids (1 eq) by $K_3[Fe(CN)_6]$ (2.5 eq) carried out in a sodium phosphate D_2O buffer solution (pH = 7.4), was monitored by ^1H NMR spectroscopy for 312 h (Figure 1). ^1H NMR spectroscopy was also used to determine the *in situ* relative ratio of various products.

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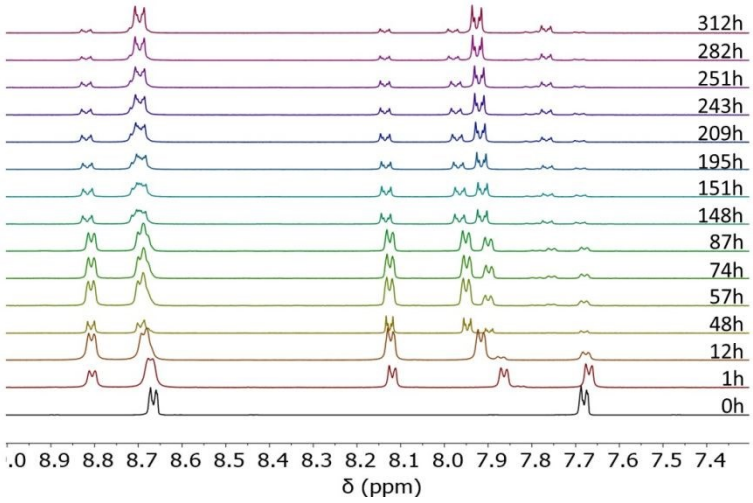

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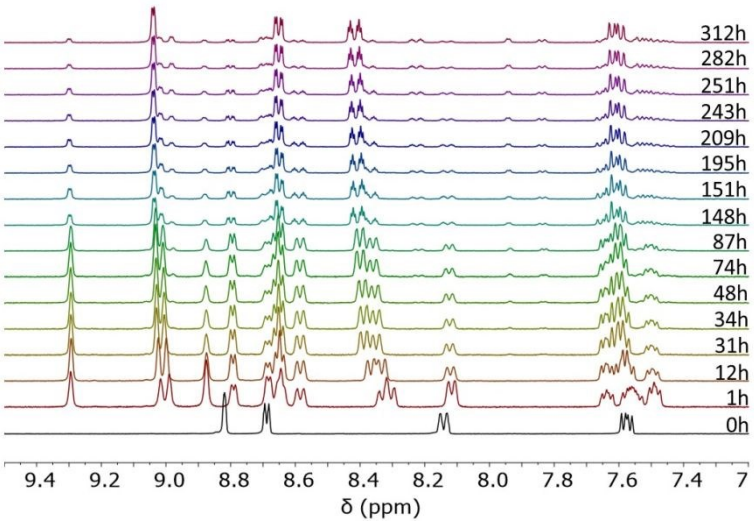

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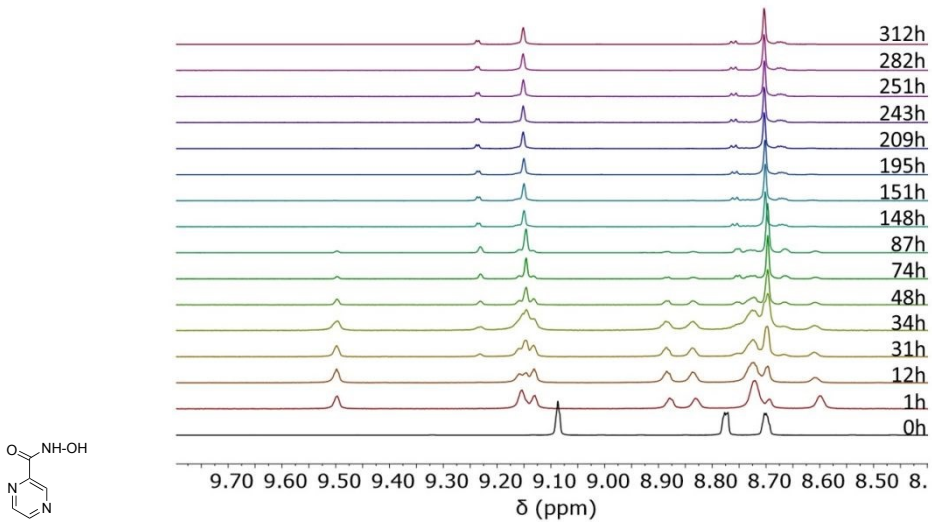

Figure 1. ^{1}H NMR monitoring of $K_3[Fe^{III}(CN)_6]$-mediated oxidation of isonicotinohydroxamic acid **1** (24 mM, a) nicotinohydroxamic acid **2** (24 mM, b) and pyrazinohydroxamic acid **3** (24 mM, c) by $K_3[Fe^{III}(CN)_6]$ (60 mM) in a sodium phosphate D_2O buffer solution (pH = 7.4) at room temperature. The spectrum at 0 h corresponds to the reaction medium before addition of $K_3[Fe^{III}(CN)_6]$.

After 1 h, ^{1}H NMR analysis indicated a partial conversion of the starting material (50%, 56% and 38% for **1**, **2**, and **3**, respectively), mainly into a single primary product evidenced through a duplication of all aromatic signals (Figure 1). Over time, the primary product tends to disappear (not completely), while giving another product characterized by new ^{1}H NMR data: 7.61-8.70 and 7.91 ppm from **1**, 8.42 and 8.68 ppm from **2**, 8.70-9.04 and 9.15 ppm from **3** (Figure 1). Even though these data are slightly shifted with respect to authentic samples of the corresponding carboxylates (Figure 2), signal patterns remain the same, in particular showing an overlap for the two vicinal aromatic protons of the pyrazine ring (Figure 1). These oxidative protocols were repeated using pure D_2O as solvent: in these conditions, the reactions were found to proceed at slower rate, and the ^{1}H NMR chemical shifts measured at t = 226 h were in agreement with those of the corresponding carboxylate form (Figure S1). These results support our proposal that the carboxylic acid is the ultimate oxidative product - potential metabolite - of the hydroxamic acid (Figure 2).

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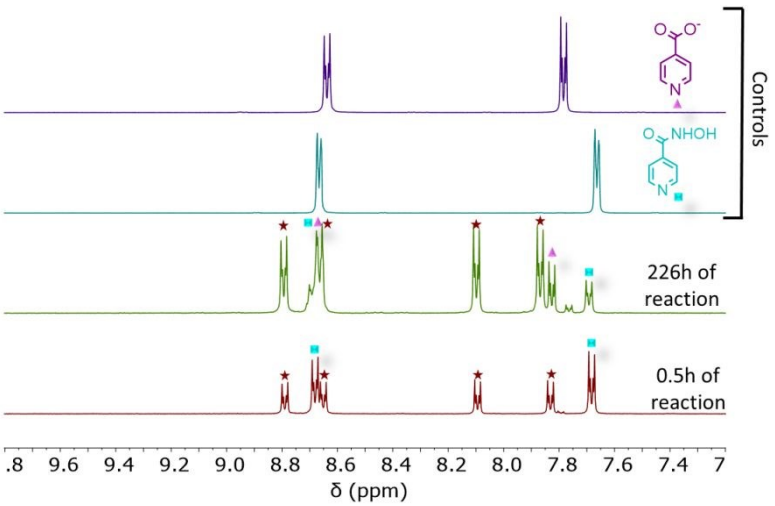

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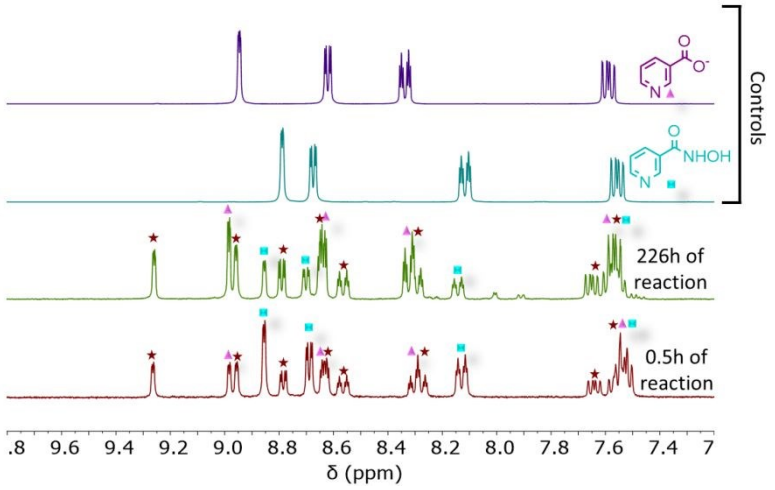

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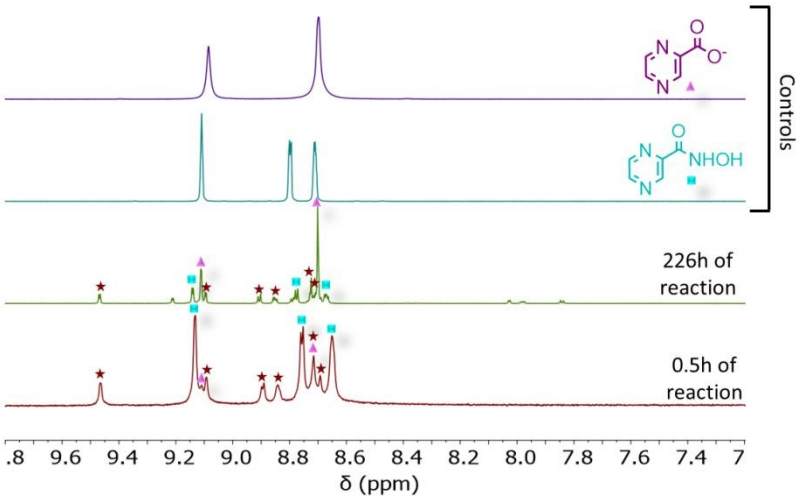

Figure 2: ^{1}H NMR spectra of the $K_3[Fe^{III}(CN)_6]$-mediated oxidation of a) isonicotinohydroxamic acid b) nicotinohydroxamic acid and c) pyrazinohydroxamic acid at 0.5 h and 226 h using $K_3[Fe^{III}(CN)_6]$ in D_2O,

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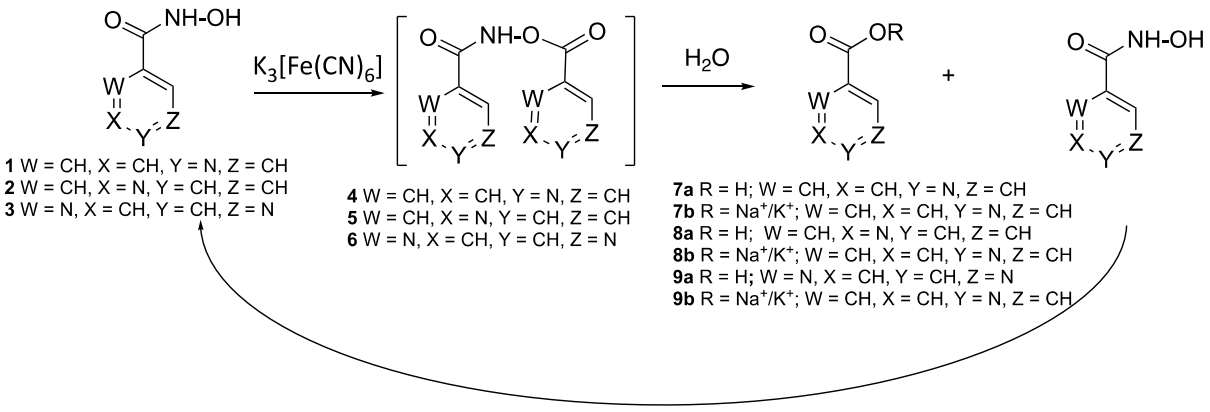
at room temperature and authentic samples of a) isonicotinohydroxamic acid and sodium isonicotinoate, b) nicotinohydroxamic acid and sodium nicotinoate and c) pyrazinohydroxamic acid and sodium pyrazinoate. Symbols: blue squares: starting hydroxamic acid, red stars: intermediate product, pink triangle: final carboxylic acid under carboxylate salt form.

Thus, we propose that the intermediate product, tentatively assigned to the di(di)azinoylhydroxylamine structure of Scheme 4, is hydrolyzed to the corresponding carboxylic acid and the starting hydroxamic acid after a nucleophilic attack at the most electrophilic carbonyl group (Scheme 4). As indicated on the ^{1}H NMR spectra, the amount of hydroxamic acid remains lower than that of the carboxylic acid because the former is oxidized again by the four-fold excess of $K_3[Fe(CN)_6]$, yielding *de novo* the intermediate di(di)azinoylhydroxylamine product.



Scheme 4. Products suggested to appear during oxidation of (di)azine carbohydroxamic acids with a five-fold excess of potassium ferricyanide.

After 312 h in buffer solution (Figure 1), ^{1}H NMR spectra showed essentially peaks according to the carboxylate structure (79%, 80% and 76% yield for isonicotinoic, nicotinoic and pyrazinoic acid from **1**, **2** and **3**, respectively).

On the basis of these results, we postulate that oxidation of (di)azine hydroxamic acids under such mild conditions occurs by first giving an unstable nitroxide radical, which is converted, after

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dimerization, to HNO and a *N,O*-di(di)azinoylhydroxylamine (Scheme 3, pathway e). The formation of the latter species could explain the duplication of the aromatic signals observed on ^{1}H NMR spectra.

As already mentioned, it could also be envisaged that the nitroxide radical undergoes a second oxidation leading to a transient nitrosocarbonyl (di)azine, which would readily react with any available nucleophile occurring in the medium. Thereby, reaction of the nitrosocarbonyl (di)azine with remaining hydroxamic acid would also lead to *N,O*-di(di)azinoylhydroxylamine and HNO (pathway d). In these conditions, one would also expect the nitrosocarbonyl (di)azine to react with H_2O, breaking down into the corresponding carboxylic acid and HNO (pathway c). However, ^{1}H NMR analysis of the reaction after 1 h showed that the conversion of the starting material (see e.g. **1,** Figure 1 and 2) leads initially only to the putative *N,O*-di(di)azinoylhydroxylamine intermediate **4** whereas the corresponding carboxylic acid product had not yet begun to be produced. All together, these results suggest that no nitrosocarbonyl (di)azine is involved in the formation of the *N,O*-di(di)azinoylhydroxylamine and carboxylic acid. This scenario is also in total agreement with the work carried out by A. Cerami *et al*. from benzene hydroxamic acid.[14]

2.2 Identification of intermediates in Fe^{III}-mediated oxidation of (di)azine hydroxamic acids

In an attempt to characterize the proposed intermediates, the reaction of isonicotinohydroxamic acid was repeated at a preparative scale (0.72 mmol, using 2.5 eq $Fe^{III}(CN)_6$) and stopped after 24 h. The new product was isolated and then characterized by ^{1}H NMR, ^{13}C NMR and MS techniques. For the isonicotinic derivative, ^{1}H NMR analysis showed a product exhibiting four doublets at 8.91 (d, 1H), 8.81 (d, 1H), 7.99 (d, 1H) and 7.79 (d, 1H) ppm, confirming the results of the ^{1}H NMR monitoring experiment. Moreover, the ^{13}C NMR spectrum revealed indeed the presence of two different carbonyl groups (167.7 and 166.0 ppm), four CH (149.7, 147.8, 123.4 and 122.7 ppm) and two Cq (146.0 and 137.5 ppm) centers, which correlate with the dissymmetric *N,O*-diisonicotinoylhydroxylamine structure (**4**). Analysis by mass spectrometry (MS, DCI-CH_4) did not allow the identification of the molecular ion peak but allowed the detection of three peaks at *m/z* =

199, *m/z* = 124 and *m/z* = 123 assigned to the amide **13** [M^+], acid **7a** [$M^+ + 1$] and amide **10** [$M^+ + 1$] (Figure 3), respectively. The amide **13** would result from a decarboxylation of **4**, while the acid **7a** and amide **10** would result from a cleavage of the hydroxamic N-O bond (Figure S2). By HRMS (ESI-positive mode), a small peak at *m/z* = 244.0726 corresponding to **4** ([$M^+ + 1$]) was detected. In the nicotinic or pyrazinic series, a mixture of compounds was obtained, containing the intermediate **5** or **6** along with the starting material **2** or **3** and the corresponding carboxylic acids. The ^{1}H NMR spectrum of the nicotinic intermediate showed signals clearly attributable to all the non-equivalent aromatic CH groups of **5** (9.18, 9.00, 8.86, 8.68, 8.39, 8.18, 7.62, 7.49 ppm), while that of the pyrazinic product **6** was slightly more speculative because of signal overlaps. The mass spectrum (DCI-CH_4) of the nicotinic products (from **2**) showed, besides peaks relative to the nicotinic acid impurity, peaks at *m/z* = 215, *m/z* = 200 and *m/z* = 123, attributable to the secondary amide **14** ([M + $CH_4^{+\cdot}$], [M + H^+]) and primary amide **11** ([M + H^+]), respectively. As observed in the isonicotinic series (from **1**), in the nicotinic series (from **2**) HRMS (ESI-positive mode) allowed detection of a peak consistent with the structure **5** at *m/z* = 244.0726 ([M + H^+]). In the pyrazinic series (from **3**), MS (DCI-CH_4) revealed peaks at *m/z* = 230, 217 and 202 assignable to the secondary amide **15** ([M + $C_2H_5^+$], [M + CH_3^+] and [M+ H^+], respectively), and at *m/z* = 124 assignable to the primary amide **12** ([M + H^+]). Oxidation of **3** in the presence of $K_3[Fe^{III}(CN)_6]$ and H_2O_2 (conditions already used for oxidation of the pyrazinohydroxamic acid-[$Fe^{II}(CN)_5$ complex])[15] allowed detection of the molecular ion peak of *N,O*-dipyrazinoylhydroxylamine **6** by HRMS-ESI$^+$ at *m/z* = 246.0619 ([M + H^+]), and this in spite of the harsher conditions. In summary, the presence of the molecular ion peaks for the di(di)azinoylhydroxylamines **4**-**6** and secondary carboxamides **13**-**15** supports the proposed mechanism.

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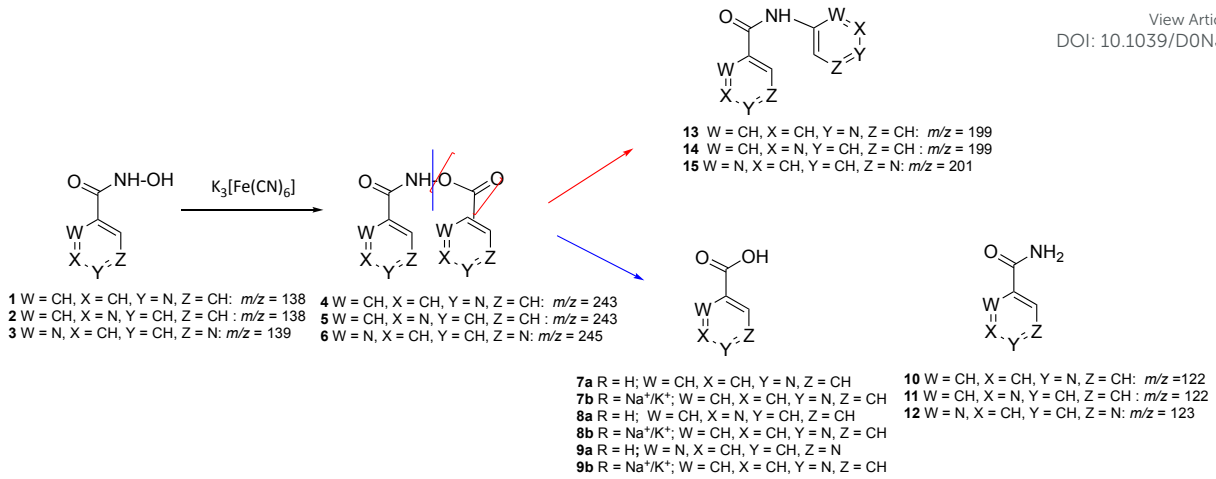
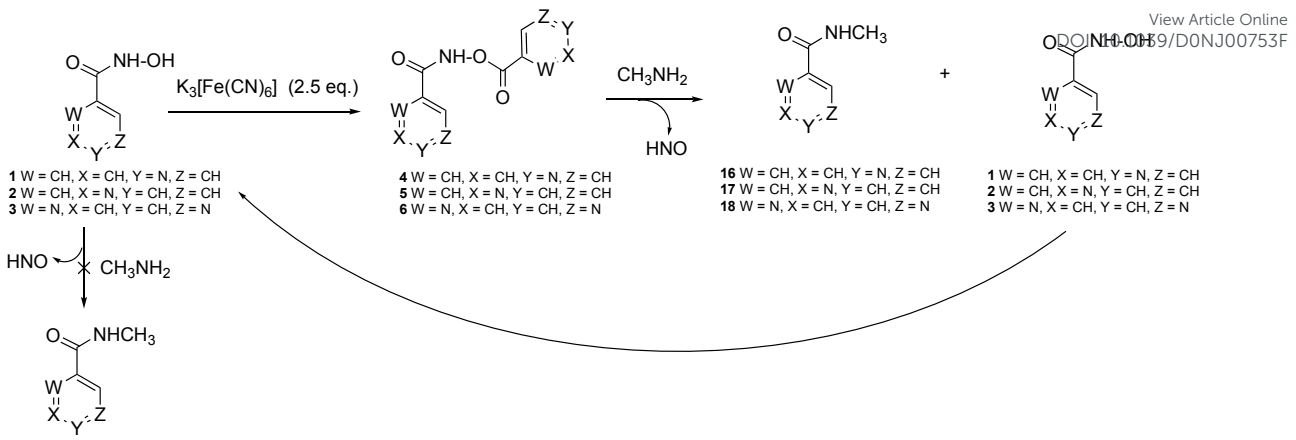


Figure 3. MS fragmentation of the proposed *N,O*-di(di)azinoylhydroxylamine intermediates in the oxidation of (di)azine hydroxamic acids.

2.3 Fe^{III}-mediated oxidation of (di)azine hydroxamic acids in the presence of methylamine.

With the view to controlling the apparent electrophilic trans-acylation reactivity of the postulated *N,O*-di(di)azinoylhydroxylamine intermediates, the reaction with $K_3[Fe^{III}(CN)_6]$ was performed in the presence of an excess of methylamine (10 eq) as a potential nucleophile. From the three hydroxamic acids **1**-**3**, a selective reaction was observed, allowing isolation of the *N*-methyl carboxamides **16**-**18** with c.a. 70% yield, as confirmed by ^{1}H NMR, ^{13}C NMR, IR and HRMS analyses (Scheme 5). It is noteworthy that control experiment of the hydroxamic acids **1**-**3** with methylamine in the absence of $K_3[Fe^{III}(CN)_6]$ did not produce the corresponding carboxamides by elimination of NH_2OH. The requirement of $K_3[Fe^{III}(CN)_6]$ suggests the occurrence of the intermediates **4**-**6**, as mentioned above, which could react as an acyl donor towards methylamine.



Scheme 5. Oxidation of (di)azine carbohydroxamic acids in the presence of methyl amine.

2.4 Monitoring of Fe^{III}-mediated oxidation of (di)azine carbohydroxamic acids by EPR

In order to test whether the oxidation of hydroxamic acids promoted by $K_3[Fe^{III}(CN)_6]$, in physiological pH condition, is accompanied by HNO release, the nitronyl nitroxide radical [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3 oxide] (cPTIO) was employed. This spin trap probe is currently used to discriminate between the $NO^{\cdot}$ radical and HNO using electronic paramagnetic resonance (EPR).[20] The $NO^{\cdot}$ radical reacts with cPTIO by deoxygenation, giving $NO_2^{\cdot}$ and the cPTI radical (Figure S3), which exhibits a more complex EPR signal (septet pattern). In contrast, HNO readily reduces cPTIO into the corresponding nitronyl hydroxylamine (cPTIO-H) that is an EPR-silent diamagnetic species, inducing a decrease of the initial cPTIO EPR signal (Figure S3).

Reactions of the hydroxamic acids **1**, **2** and **3** (5 mM) with $K_3[Fe^{III}(CN)_6]$ (15 mM), in phosphate buffer (40 mM, pH = 7.4) and in the presence cPTIO (200 μM), were carried out for 5.5 h and analyzed by EPR. The stability of cPTIO in the reaction conditions was first checked by EPR: alone or in the presence of hydroxamic acid, cPTIO was found to be stable without modification of the signal (Figure S4a). In the presence of $[Fe^{III}(CN)_6]^{3-}$ or $[Fe^{II}(CN)_6]^{4-}$, two complex ions expected to occur during the redox reaction, a decrease of the cPTIO EPR signal by 65% or 30%, respectively, was observed (Figure S4a). In the reaction mixture, where hydroxamic acid, $[Fe^{III}(CN)_6]^{3-}/[Fe^{II}(CN)_6]^{4-}$ and cPTIO co-exist, the EPR signal corresponding to the cPTIO radical was almost completely abolished

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(85% of reduction) after 5.5 h (Figure 4). This result disclosed that the cPTIO signal decrease is accelerated with the concomitant presence of hydroxamic acid and $K_3[Fe^{III}(CN)_6]$, and suggesting that HNO is indeed released during oxidation of hydroxamic acids promoted by $K_3[Fe^{III}(CN)_6]$.

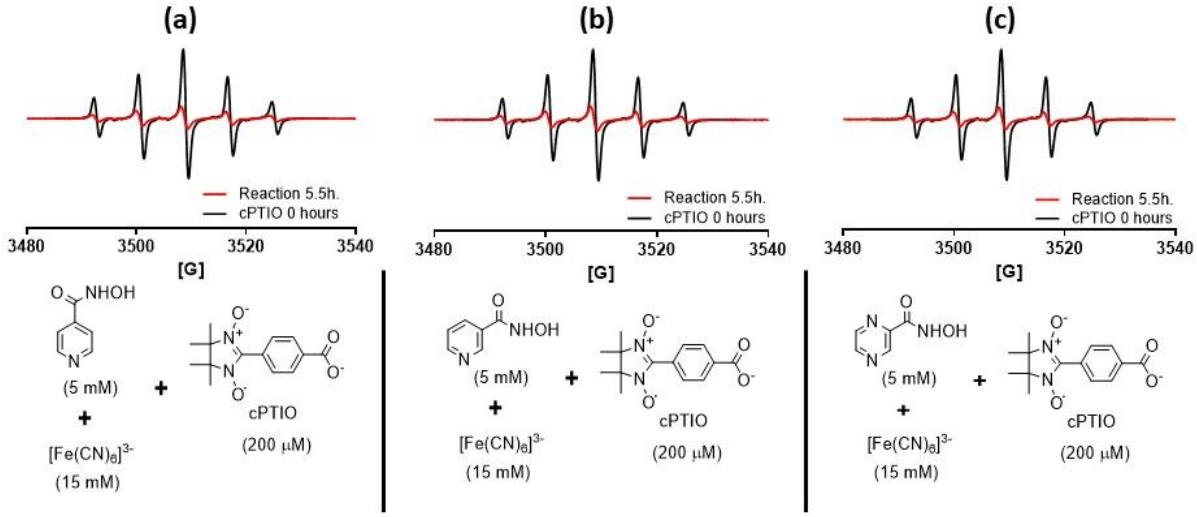

Figure 4. EPR spectra of the reaction of a) isonicotinohydroxamic acid, b) nicotinohydroxamic acid and c) pyrazinohydroxamic acid with $K_3[Fe^{III}(CN)_6]$ (15mM) in the presence of cPTIO (200 μM) for 5.5 h in 40 mM phosphate buffer (pH 7.4), at room temperature.

Monitoring of Fe^{III}-mediated oxidation of (di)azine carbohydroxamic acids by UV-vis

The release of HNO during the oxidation of the (di)azine hydroxamic acids **1**-**3** promoted by $K_3[Fe^{III}(CN)_6]$, in the presence of cPTIO was also studied by UV-vis spectroscopic technique. cPTIO exhibits characteristic electronic absorptions at 360 and 560 nm. Since no other reaction component absorbs around 560 nm (in contrast to the 360 nm region), this wavelength was chosen for monitoring. The evolution of this band was first followed in the sole presence of $K_3[Fe^{III}(CN)_6]$ or hydroxamic acids. No significant interaction was evidenced between $[Fe^{III}(CN)_6]^{3-}$and cPTIO (Figure S5), or hydroxamic acids and cPTIO (Figures 5D-F). In the oxidative reaction conditions (hydroxamic acid + $K_3[Fe^{III}(CN)_6]$ + cPTIO), monitoring over 24 h showed a decline of the absorption band at 560 nm for the three substrates **1**-**3** (Figures 5 A-C). These observations confirm the consumption of

cPTIO under the given conditions, while the disappearance of the EPR signal indicated that it is accompanied by the generation of HNO (see above section).

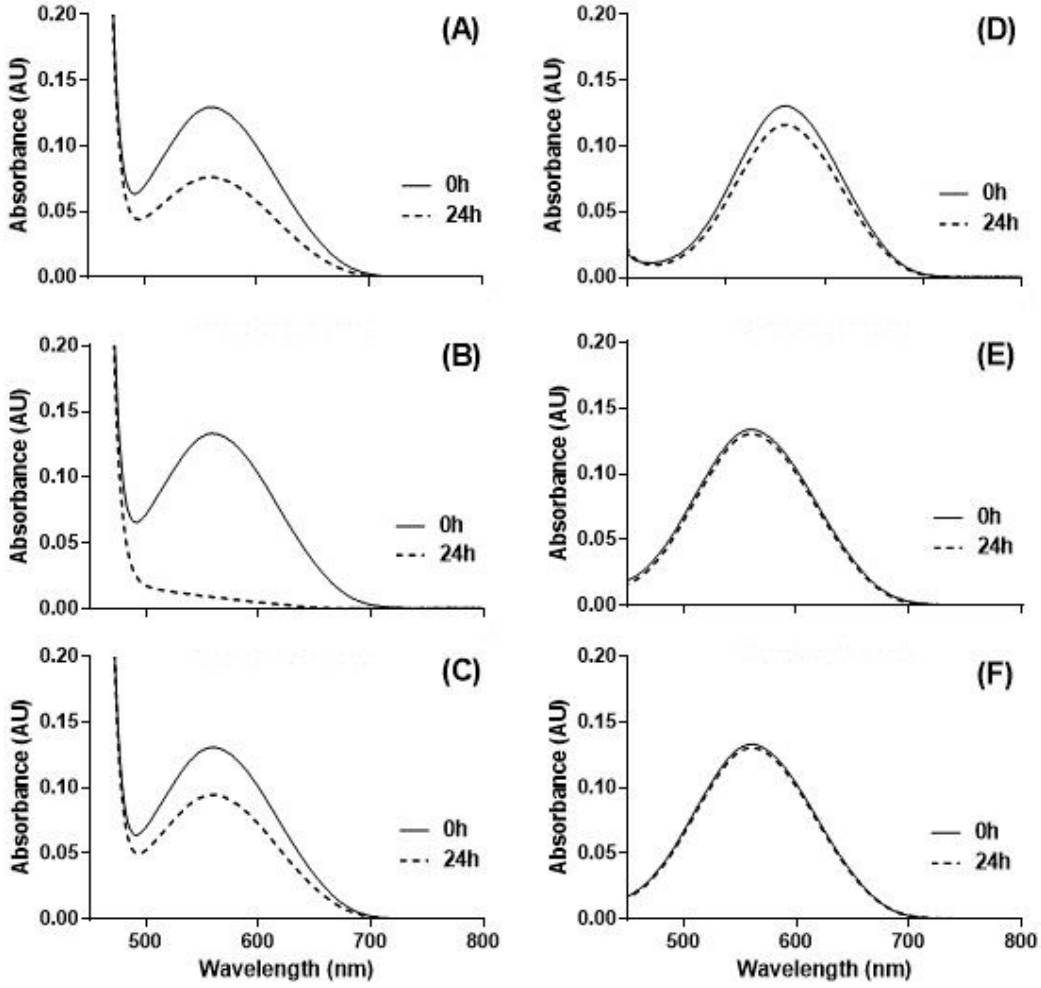

Figure 5. Electronic absorption spectra of cPTIO in oxidative reaction mixtures or with hydroxamic acids alone. Panels on the left show spectra of the reaction between isonicotino- (A), nicotino- (B) and pyrazino- (C) hydroxamic acids (750 µM) with $K_3[Fe^{III}(CN)_6]$ (15 mM) in the presence of cPTIO (150 µM). Panels on the right show the spectra of cPTIO (150 µM) in the presence of isonicotino- (D), nicotino- (E) and pyrazino- (F) hydroxamic acids (750 µM). All reactions were monitored at 0 and 24 h in 40 mM phosphate buffer (pH 7.4), at room temperature.

3. Conclusion

In this work, it was shown that (di)azine carbohydroxamic acids generate HNO at physiological pH upon oxidation by $K_3[Fe^{III}(CN)_6]$ through an intermolecular/outer-sphere process, as it was previously shown upon oxidation by H_2O_2 through an intramolecular/inner-sphere process. However, in contrast to what is often reported, HNO was not generated from the putative nitrosocarbonyl (di)azine, which would be formed after two oxidation steps of hydroxamic acid. Instead, HNO is released through a one-electron oxidation of the hydroxamic acid along with the *N,O*-di(di)azinoylhydroxylamine intermediate. This intermediate was characterized by NMR, MS, UV techniques and proved to be quite reactive, breaking down into the corresponding carboxylic acid and hydroxamic acid, which is then re-oxidized. So, globally, one molecule of hydroxamic acid generates one molecule of HNO. Since oxidation processes of arene hydroxamic acids going through either nitrosocarbonyl or *N,O*-di(di)aroylhydroxylamine intermediates, can have similar features in terms of (i) stoichiometry of HNO release, (ii) electrophilic reactivity of the intermediate and (iii) chemical nature of the final carboxylic products, a particular attention should be addressed to such mechanistic proposals. Computational studies would deserve to be considered to discriminate between the intrinsically highly reactive nitrosocarbonyl and *N,O*-diaroylhydroxylamine intermediates.

Supplementary Information

Experimental synthesis protocols and ^{1}H NMR, MS, UV-vis and EPR spectra of relevant species.

Conflicts of Interest

There are no conflicts to declare

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